MACROLIDES, FLUOROQUINOLONES AND SULFONAMIDES RESIDUES SURVEILLANCE IN POULTRY MEAT AND EGGS AND THEIR ROLE IN EMERGENCE OF MICROBIAL RESISTANCE

BY

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DEPARTMENT OF MICROBIOLOGY

QUAID-I-AZAM UNIVERSITY

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بسم الله الرحمن الرحيم

Dedicated to

The Holy Prophet

Hazrat Muhammad (P.B.U.H)

who exhorted his followers to seek knowledge

from cradle to grave

MACROLIDES, FLUOROQUINOLONES AND SULFONAMIDES RESIDUES SURVEILLANCE IN POULTRY MEAT AND EGGS AND THEIR ROLE IN EMERGENCE OF MICROBIAL RESISTANCE

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CERTIFICATE

This thesis, submitted by Mr. Muhammad Saleem is accepted in its present form by the Department of Microbiology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the thesis requirement for the thesis requirement for the degree of Doctor of Philosophy (Ph.D) in Microbiology.

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LIST OF ABBREVIATIONS

ADI	Acceptable Daily Intake.
BAA	Bile Aesculin Agar.
BHI	Brain Heart Infusion.
CAST	Calf Antibiotic & Sulfa Test.
CRL	Community Reference Laboratory
cfu	Colony Forming Unit.
CLSI	Clinical Laboratory Standards Institute.
CPMA	Combined Plate Microbial Assay.
EIA	Enzyme Immunoassays.
FAST	The Fast Antibiotic Screen Test.
EU4Pt	European Union Four Plate method.
I	Intermediate.
KIS	Kidney Inhibition Swab.
LAST	Live Animal Swab Test.
LOD	Limits of Detection.
MCSTAR	Microbiological & Chemical Screening Test for Antimicrobial Residues.
MH	Mueller-Hinton.
MIC	Minimum Inhibitory Concentration.
MRL	Maximum Residue Limit.
MRSA	Methicillin-resistant *Staphylococcus aureus.*
MRSE	Methicillin-resistant *Staphylococcus epidermidis.*
NAT	Nows Antibiotic Test.
PABA	Para Amino Benzoic Acid.
R	Resistant.
S	Sensitive.
SRC	Standard Reference Concentration.
STAR	Screening Test for Antibiotic Residues.
STOP	Swab Test on Premises.
THF	Tetra Hydro Folate.
TLC	Thin Layer Chromatography.
Rf	Retention factor.

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ABSTRACT

In both veterinary and human drugs have therapeutic, prophylactic, metaphylactic and nutritive applications. In farm animals using growth promoter antibacterials in feed gives producers an economic advantage in raising animals to market weight faster and cheaper. The use of veterinary drugs in food-producing animals may result in trace quantities of the drugs or their metabolites being present as residues in food. The residues of veterinary drugs in food may pose a risk of zoonotic resistance due to their minute antibiotic quantities. The present study aimed to quantitatively estimate the general contamination level of Pakistani poultry meat and eggs with some antibacterial residues.

Validation of a microbiological method for the screening of antibiotic residues in poultry meat, liver, bone marrow and egg yolks were modified and augmented with spot test, TLC, UV spectrophotometeric and chemical tests for identification of unknown residues. Microbiological and Chemical Screening Test for Antibiotic Residues (MCSTAR), was established by the analysis of poultry meat, liver, bone marrow and egg yolks samples for 12 antibacterials. Macrolides, fluoroquinolones and sulfonamides were screened for residues. Twelve antibacterials were detected by the MCSTAR protocol at or below the maximum residue limit (MRL). The sensitivity of the MCSTAR protocol was at or below the MRL for three macrolides, four sulfonamides and five fluoroquinolones. Among three plate test method, each plate was preferentially sensitive for one or two families of antibacterials: the plate of *Escherichia coli* for quinolones, the plate *Bacillus subtilis* ATCC 6633 and *Bacillus cereus* ATCC 11778 for macrolides the plate *Bacillus stearothermophilus* ATCC 12980 for sulfonamides. This method has been used for direct the physicochemical confirmation towards one or two families of antibacterials. Considering the high cost of liquid chromatography coupled with tandem mass spectrometry detection analysis, the reduction of the range of antibacterials to test for confirmation is a significant gain in time and money through MCSTAR.

During the three years (2006-2008) period 21383 samples were collected and screened for the presence of antimicrobials residues. Out of total 2752 positive samples, 2338 were of poultry meat, liver and bone marrow whereas, 364 samples were of egg yolks. Maximum residues (12.9 %) were detected during the year 2008 followed by 2006 (12.9 %) and 2007 (12.8 %). Among macrolides erythromycin, tylosin and tilmicosin were detected in breast meat, liver bone marrow and poultry eggs. During three years fluoroquinolones residues of norfloxacin, enrofloxacin, ciprofloxacin, sarafloxacin and flumequine were detected. Residues of sulfonamides including sulfadiazine, sulfaquinoxaline, sulfachlorpyridazine and sulfadimidine were detected.

A total 4652 isolates were screened comprising 2156 of poultry and 2496 of human origin. *E. coli*, *Salmonella*, *Clostridium* and *Staphylococcus* were opted as the most prevalent representative organisms for resistance studies. Among total 2156 isolates of poultry origin screened during the three years (2006-2008), total 699 (32.4 %) were antibiographed during the 2006 and 717 (33.3 %) were tested during 2007 whereas, 740 (34.3 %) were tested during 2008. The tylosin tartrate, erythromycin, tilmicosin, norfloxacin, enrofloxacin, ciprofloxacin, sarafloxacin, flumequine, sulfadiazine, sulfaquinoxaline, sulfachlorpyridazine and sulfadimidine were included in the

antibiography. During the year 2006 among the total 699 poultry isolates screened 37.5 percent were resistant to these antimicrobials. The maximum resistance (61.4 %) was observed against norfloxacin, followed by enrofloxacin (51.7 %) and erythromycin (48.5 %), whereas among sulfonamides highest resistance (36.5 %) was observed against sulfaquinoxaline. An overall resistance was 37.5 percent in 2006 which remain in increasing trend in 2007 and touches the level of 39.8 percent which turned the situation gloomier in 2008 where peaked to the level of 41.4 percent. Trends in intermediate susceptible organisms were high temporally. The ultimate target of resistant organisms is human beings; therefore, during the three years of study a total of 2494 isolates were screened. An overall resistance 23.0 percent was observed in *E. coli*, *Salmonella* and *Staphylococcus* during 2006 and increased up to 34.9 percent in 2007 whereas; the level of resistance was 39.4 percent in 2008.

Testing methods/techniques of tylosin tartrate, erythromycin thiocyanate, tilmicosin, norfloxacin, enrofloxacin, ciprofloxacin, sarafloxacin, flumequine, sulfadiazine, sulfaquinoxaline, sulfachlorpyridazine and sulfadimidine were microbiologically, chemically and spectrophotometerically established (MCSTAR). A sampling plan was designed according to statistical considerations. Current investigation throw light on the intensity of the issue that larger based residue monitoring plans ensure better protection levels for consumers, than control systems designed simply as a proportion of production units. The occurrences of veterinary drug residues in poultry products in and around Rawalpindi-Islamabad Pakistan were determined. Drug residues surveillance studies gave an insight into the aggravated residues status of macrolides, fluoroquinolones and sulfonamides in poultry meat and eggs. Drug resistance was menace of drug residues, aspect generally ignored by the technical personals and farmers needs due consideration.

Final goal of the study was to furnish preliminary local data to drag the attention of the concerned authorities regarding the withdrawal periods and maximum residual limits. Although the current study furnished a peanut data as about 68000 local and imported registered products are irrationally used in poultry production needs due consideration. Local population is using poultry meat and eggs as cheaper protein source. Threat of resistance is not restricted only to Pakistani population it is global. As poultry industry of Pakistan is planning export of poultry meat and eggs to other countries therefore; establishment of monitoring system, fixation of withdrawal periods and MRLs is time demand to check the transmission of drug residues globally. A cheaper and practicable testing method/technique MCSTAR will be helpful for future studies of other drugs.

INTRODUCTION

CHAPTER 1

INTRODUCTION

With increased industrialization and urbanization the fast food industries have picked up throughout the world. As this involves processing and cooking of food by restaurants and food manufacturers, their practices are different from the domestic preparations (Love, *et al.*, 2012). These producers tend to buy raw materials in bulk from large poultry producers (Narahari and Amutha, 2007; Hilbert and Smulder, 2012). The greed to fulfill the demands of the hoteliers leads to the malpractices by poultry producers. Therefore, currently all food products are at risk of residual contamination directly or indirectly particularly poultry meat and eggs which was major concern for consumer health (Narahari and Amutha, 2007).

Poultry meat and eggs are an important source of animal protein and also contribute to everyday needs for minerals, vitamins and fatty acids. The consumption of poultry meat and eggs has increased in comparison to other edibles (Jimenez *et al.*, 2011). In 2010, per capita annual consumption was 375 eggs in Mexico compared to Japan (347 eggs), USA (258), Brazil (132) and 30 eggs in Pakistan (Quevedo, 2011). Brazil was seventh in the world ranking of egg producers, only behind China, USA, India, Japan, Mexico and Russia (FAO, 2010). Export sector of poultry products has increased considerably since 2004. Participation in the strictest markets, such as European Union, needs to monitor traceability, wholesomeness, quality, animal welfare principles and manage contaminants and residues (Quevedo, 2011). Poultry meat consumption per capita in USA was 43.30 kg followed by Brazil 40.20 kg, Canada 29.40 kg, France 28.50 kg, Europe on average 20.80 kg and Pakistan 6.00-7.00 kg. World poultry meat production in 2010 stands at 94.20 million tones marginally above the previous year. Poultry production has been affected by the high cost of feeds. In Europe, production growth has been revised upwards and now stands at 12 percent higher (FAO, 2012).

Achievement of higher productivity results from lesser numbers lead to abuse of growth promoters. About 80 percent of veterinary drugs are used in food animals, which are ultimate causes of residues in milk, meat and eggs (Mitchell *et al.*, 1998). Drugs are administered to shorten feeding time and to abate the risk of losses (Lee *et al.*, 2001). Conventionally, antibacterial drugs are used in treatment (Johnston, 1998), prevention and control of diseases (Teuber, 2001) and to improve feed conversion efficiency (Tollefson and Miller, 2000). Antimicrobials (Pikkemaat *et al.*, 2011), anti inflammatory drugs (Niewold, 2007) and hormones (Bovee, 2009) are most commonly used for treatment purposes (Wierup, 2001; Lee *et al.*, 2001). In some countries such as US (Kennedy *et al.*, 2000), Canada (Bedford, 2000) and Australia (Schneider *et al.*, 2009), the addition of controlled medicinal compounds in animal feeds was a lawful and low cost tool to enhance yields (Dibner and Richard, 2005), but an illegal or unsuitable use increases the risk of introducing harmful residues into the human food chain (Casewell *et al.*, 2003). Adverse effects for consumer health are connected with the intrinsic toxicity of a drug and its metabolites (Gallo *et al.*, 2008). The main concern regarding the ingestion of antibiotic residues was the development of resistant bacterial strains (Guardabassi and Dalsgaard, 2004), while anti-inflammatory drugs can induce gastric intestinal disturbances (Gorbach, 1993; Perrin *et al.*, 2001). The suspicious carcinogenicity of growth promoting agents has prompted the European Union to ban these compounds since 1988. Import of meat from third world countries that authorize use of growth promoter for fattening purposes was forbidden (Companyo *et al.*, 2011).

The chemotherapy has significant role in meeting the ever growing demands of animal origin protein (Guardabassi and Dalsgaard, 2004). Initially researchers, did not fully understand the mechanism and mode that how antibacterial enhance and improve animal growth and performance (Adams, 1995). Later on, it became evident that immune system of sick animals was responsible for the depression in growth (Brown, 1996). Infected animals release cytokines, which push the gut to produce peptides resulting in anorexia (Choi *et al.*, 1999). Five major classes of drugs used in food animals are topical antiseptics (Dubois *et al.*, 2004), ionophores (Matabudul *et al.*, 2001), hormone (Bovee, 2009) and hormone like products (Dubois *et al.*, 2004), antiparasitic drugs (Dubois *et al.*, 2004) and antibacterials to promote growth (Pikkemaat *et al.*, 2007). Until recently, the so called "zero tolerance" approach had to be applied to group A substances but this criterion can only be considered valid for synthetic compounds, while

prohibited substances such as hormones are still present in animal food products. In order to avoid ambiguity, the minimum required performance limit (MRPL) has now been introduced (Kennedy *et al.*, 2000). In EU, the food safety problem has been tackled by defining a series of legislative measures in order to assure a high protection level for consumers. According to the recommendations substances are divided in two major groups A and B. Compounds in group A comprise prohibited substances (EC, 2003), whereas group B compounds enlists substances with MRLs (Kennedy *et al.*, 2000). The animal was, in effect, acting as a further safety buffer and few of the above chemicals produce residues in animal tissues (Companyo *et al.*, 2011). Failure to observe withdrawal periods of drugs recently employed to treat animal appears to be main cause of drug residues in milk, meat and eggs (Jimenez *et al.*, 2011).

Broadly among total of collected targeted samples, 40.90 percent were anabolic and prohibited substances (group A) and 63.10 percent were veterinary drugs and contaminants (group B). During 2008, the most common detected anabolic steroids were dexamethasone, prednisolone, boldenone, nitroimidazoles and nitrofurans. There were 0.26 percent disobedient samples of antimicrobials such as chloramphenicol. Among 0.14 percent bovines samples identification of anthelmintics were report (EC, 2003).

Consumer requirement for natural and untreated foodstuffs has amplified, somewhat owing to an observation that such goods are better and attached smaller amount of harmful drug residues, (Diebold and Eidelsburger, 2006). UPLC-MS methods were employed for the tylosin, erythromycin, Tilmicosin, ciprofloxacin, danofloxacin, sulfadimethoxine, sulfamethazine, and florfenicol. Drug residues above their relevant US maximum residual limits were detected among six ground beef samples (Bowling, 2011). Ractopamine and ampicillin residues were identified in USDA certified crude samples. Ampicillin, phenylbutazone and sulfadimethoxine beyond the US tolerance limit in one sample of bull and cow category was found positive (Bowling, 2011). Administration of sulfonamides in production of layers was a public health risk since it certainly consequences in sulfonamide residues in preovulatory yolks (Donoghue *et al.*, 2005) and eggs (Sasanya *et al.*, 2005). Pharmacologically active substances and non steroidal anti inflammatory drugs were reported in disobedient samples in beef, poultry products and milk. The drug residue status during 2009 was comparable to the two preceding years for the entire categories (Chico *et al.*, 2008).

Tests used for residual drug detection emphasized mostly on immunoassay techniques (Stead *et al.*, 2007) initially, technical concerns expressed by the dairy industry to the present public health and international trade implications, and there has been an ongoing need for reliable, sensitive, and economical methods for the detection of antimicrobial residues in food animal products such as milk, meat and eggs. Initially there were microbial growth inhibition tests (Pikkemaat *et al.*, 2011) followed by more sensitive and specific methods based on receptor binding, immunochemical, and chromatographic principle (Stead, *et al.*, 2004). Inhibition of the growth of various bacterial strains was employed as the preliminary test for detection and screening methods used for determining antibacterial residues in animal origin foods and milk (Mitchell *et al.*, 2011). Such methods were based on microbial agar diffusion tests and/or on the inhibition of acid production by test organisms (Mitchell *et al.*, 1998). In 1950s, primitive type assay was developed for the screening of residues in the tissues and milk (Huber *et al.*, 1969; Tak and Kov, 1969). The new procedure replaced and modified the previous cumbersome methods and procedures (Rosdahl *et al.*, 1979). A microbiological method was optimized in 1970 both for detection and identification of antimicrobials in meat and milk (Van-Schothorst and Peelen, 1970). Chemical, spectrophotometric, ELISA, HPLC and gas chromatography methods were more commonly in practice for such qualitative analysis (Okerman *et al.*, 2004).

Today's purchaser stress that producers that make foodstuffs be open and liable in their methods. Failure to do so hoists apprehension and doubts about the practices that involve public health (Casewell *et al.*, 2003). With a huge magnitude of facts and fast entrance to it, several customers and medics are soliciting queries regarding matters that sort from ecological contamination to microwave radiation from entertaining electronic devices (EPA, 2012). Customers desire an ample range of foodstuffs at rational costs and they also insist natural, nutritious and protected foodstuffs. Clients are also alarmed regarding the effects of residues in the food they eat. Drug residues in animal origin food exceeding the MRLs are hazard to human health (Hilbert and Smulder, 2012). The significance of food security through decline of residues in our food chain cannot be exaggerated. It was a time demand for the administration and related authorities to guarantee suitable utilization of antimicrobials in food animals and launch a general catalog to supervise microbial infections in human beings and modes of drug resistance that might consequence from drug application in animals (Lorenza *et al.*, 2012).

Macrolides, lincosamides, ionophores and antimicrobials were administered *via* feed or drinking water which leads to potentially harmful levels of residues in commercial eggs was prohibited in large animals (ASM, 2009). Lincomycin and tylosin were banned in chicken and tylosin in laying hens if used; eggs must be discarded 10 days after the last administration (Brasil, 2008; Compendia de Produtos Veterinarians, 2009). Residues of tylosin were found in eggs from Spain in 2008. European Union MRLs of 150, 200 and 50 µg/kg were for erythrocin, tylosin and lincomycin, respectively (US CFR, 2009). In Brazil, the MRL of 300 µg/kg of tylosin in eggs was proposed by Commission (Mortier *et al.*, 2005). Sulfonamides have directed allergic or toxic reactions (Dayan, 1993) even after administration of therapeutic doses to humans have been described (Riedl and Casillas, 2003). Hypersensitivity and antibacterial resistance was an outcome of the residues of sulfonamides accumulated in the human body after intensive consumption of food of animal origin (Booth, 1988; Spoo and Riviere, 1995). Sulfonamides due to histotropism target thyroid gland with their harmful effects and consequently may result in development of thyroid gland tumors (Ahmed and Ahmed, 1989).

Fluoroquinolones production and its usage in EU, USA, Japan and South Korea are estimated to be 30 to 50 tons for proprietary products, whereas due to their lower prices about 70 tones of generic fluoroquinolones are consumed in livestock (Hermo *et al.*, 2010). However, available usage data, particularly for non proprietary fluoroquinolones, are known to be grossly incomplete. Fluoroquinolones consumption in animals in China estimated to be annually 470 tones and consumption in human medicine was about 1,350 tones. Hence, excessive use of fluoroquinolones in animals was creating resistance in animal bacteria (Guardabassi and Dalsgaard, 2004; Hordijik *et al.*, 2012).

Multiple drug resistance was a sequel attributed to the direct excessive use of antimicrobials or through the contaminated food (Perrin *et al.*, 2001; McGlinchey *et al.*, 2008). Exposure of pathogens with low dose of antibacterials may lead to emergence of resistance against specific drug (Tancrede and Barakat, 1989; Vermoote *et al.*, 2011). A strong correlation has been observed in streptomycin, gentamicin, tetracycline and chloramphenicol residues and the development of resistance in pathogens. Microbes in animals exposed to low inhibitory concentrations of antimicrobials exert a positive pressure towards the selection and expression of resistance in pathogens (Vazquez *et al.*, 1990; Szmolka *et al.*, 2012).

Several queries establish whether this resistance will consequence in an amplified danger for humans; Firstly, is the pathogen a zoonotic and can result a human infection by touching from the animal to human (Benancora *et al.*, 2012). Secondly, are there errors in ordinary security measures for meting out and management animal origin groceries that are anticipated to decrease the threat of spread of zoonotic bacteria to humans, whether they are resistant to antimicrobials or not (Agers *et al.*, 2012). Third, if transferred to humans from an animal source, is the germ more dangerous than in its less antimicrobial resistant type (Gharsa *et al.*, 2012). Fourthly, if the bacterium is zoonotic, is zoonosis curable with other antimicrobials (Szmolka *et al.*, 2012). Finally, are there adequate new antimicrobials under progress to alternate for drugs to which microbes have developed resistance (Borel *et al.*, 2012). How these issues are responded conclude the degree of risk to humans.

Medics are facing problems which are limiting their ability to treat infections in human due to increased number of antibacterials and chemotherapeutic resistant strains (Rattan, 1999). A major controversy exists in opinion on the antibacterial resistance aspect *i.e.* whether the conventional use of antibacterials in livestock animals contributes to enhance the multiple drug resistant strains. Ban has been imposed in European countries on the conventional use of antibacterials in the poultry rations. However, the restriction on the antimicrobials use in the poultry production and feeds will dramatically increase the cost of food of animal origin in general and poultry, in particular, leaving farmers with limited approaches to control diseases and it will enhance the inputs in term of investment and limited options to prevent pathogens (Okerman *et al.*, 1998).

All pathogens have an intrinsic blueprint of sensitivity and resistance to definite antimicrobials (Vanni *et al.*, 2012). Antimicrobial resistance may also be acquired (Neinhoff *et al.*, 2011). This can happen in the course of mutation and selection and cross resistance (Clothier *et al.*, 2012). Antimicrobials are selecting an ever increasing number of resistant microbes and this is being potentiated by gene transfer (Szmolka *et al.*, 2012). Allergy to sulfa drugs and penicillins steadily expand serious aplastic anemia in people susceptible to chloramphenicol (Lorenza *et al.*, 2012), attainment of sensitivity or allergy to the drug or acquired resistance by human pathogens. Chloramphenicol and furazolidone was potentially carcinogenic (Snyder, 2004).

Drug resistance was an increasing trouble in human medication and distress has been uttered that application of antimicrobials in animals may be a causative aspect in this regard. Even though greater portion of human pathogens exhibiting antimicrobial resistance have no linkage with animals, the subject of animal consumption of antimicrobials remains debatable, chiefly with reverence to antimicrobial growth promoters (AGP). Microbial threat evaluations were vital in judging qualitatively or quantitatively whether the risk of using a definite AGP was suitable in terms of probable danger to human fitness. Examination of resistance development was a crucial part of such microbial hazard estimations, but such observations should be vigilantly premeditated to shun puzzling aspects that could nullify any conclusions (Bywater, 2005).

There has also been amplified importance from the regulatory organizations to observe and avoid residues (Kumar and Companyo, 2011). It was very important that farmers exploit this agenda to pick up their command of on application of antimicrobials (EC, 2010). In this they will be able to avert any wearing of assurance in the milk, meat and eggs they produce and put up for sale (Jimenez *et al.*, 2009). No troubles should happen for humans or animals if the management of any disease was conducted under the direction of a veterinarian and mostly as such treatments will be intermittent fairly than constant. However, residues can emerge in animal foodstuffs when vast amounts were directed instantly preceding to slaughter, or in the milk of animals experiencing therapy (Le Breton *et al.*, 2007).

This study aims at development of preliminary data of drug residues of macrolides, fluoroquinolones and sulfonamides in poultry meat and egg yolks. Objectives of these investigations are to monitor the flow of residues in edible broiler meat and layer eggs and possibility of the bacterial resistance in human pathogens through residual exposure. Establishment of screening of positive samples, standardization of extraction and testing procedures with local resources will follow identification and quantification of macrolides, fluoroquinolones and sulfonamides residues in poultry meat and eggs. Microbiological, chemical and spectrophotometric methods/techniques will be employed to detect tylosin tartrate, erythromycin thiocyanate, tilmicosin, norfloxacin, enrofloxacin, ciprofloxacin, sarafloxacin, flumequine, sulfadiazine, sulfaquinoxaline, sulfachlorpyridazine and sulfadimidine. The outcome of this study would be the generation of preliminary local data regarding microbial drug resistance and determination of residual contents of selected drugs to draw the attention of authorities for setting up standards regarding the withdrawal periods of various drugs and maximum residual limits.

REVIEW OF LITERATURE

CHAPTER 2

REVIEW OF LITERATURE

Population of Pakistan was increasing more than three percent annually. In order to meet food requirements, the agriculture and livestock sectors were striving in all avenues. As an agricultural country, shortage of grains was a serious threat due to compromised production potentials. Hence, for compensating the protein shortage, poultry industry was playing vital role in fulfilling the requirements of its ever increasing population. Poultry plays a significant role in bridging the gap of animal origin protein through eggs and meat. As the fastest and the cheapest protein source poultry produces 450000 metric tons of meat, which contributes 27 percent of total meat consumption, whereas 4500 million table eggs were further facilitation (PPA, 2005). However, high prevalence of viral, bacterial, protozoanal and fungal diseases was a serious threat to fast growing poultry industry. Therefore, control overwhelming diseases, antibacterials were widely used in the treatment and prophylaxis (Javed, 1992). Farmers were enhancing production performance by addition of growth promoters in the poultry feed. Although these practices give edge in getting maximum production efficiency but inappropriate usage of antibacterials results in drug residues at some level in edible products.

The medication of antibacterials to beef and milk animals end up in potent source of drug residues contaminating the human food chain and consequently consumed by innocent people. The data regarding chemical nature of drug, microbial resistance, pharmacological information, indications and toxicology of the drug may be considered at the time of registration by the regulatory and registration authorities to ensure the safety of the consumer. In Europe, Australia and America, veterinary drug registration was conditional with the provision of the animal product safety services and data of safety, animal poison control data and safety assessment of microbiologic hazards by consuming the antimicrobial residues which was thoroughly considered as well. Effect of the residue on the intestinal flora was well considered as to make safer the microbiota of the GIT. The residue monitoring implementation differs nation to nation and

country to country according to the eating pattern of the respective nation. Test protocols and methods for testing were being designed to ascertain the safety of the environment by microbiologically monitoring drug residues in animal origin foods. The regulatory institutions were making their efforts by limiting the residues transfer from animals to innocent food consumers by covenanting *in vitro* and *in vivo* testing methods to fix permissible daily allowance of residues in foods ingested from animals getting antibacterial therapy. At the time of registration of veterinary medicine in America, Europe and Australia, data of product safety was always asked to make sure that the residues of these products if passed into the animal food will not hurt the intestinal flora of the consumer. In this regard in most of the developed countries drug application and drug evaluation centers have been developed. In the advanced countries, instead of drug administration the agency was called Food and Drug Administration. The major issue of safety in the registration process was to secure the transfer of bacterial resistance and saving the intestinal flora, which provides a barrier and competitively exclude the pathogens that invade the intestine (Cerniglia and Kotarski, 2005).

Practically, production of residue free food was an unachievable and basically difficult goal. Most of human health hazards linked with the products consumption of animal origin were observed. The quantum of the drug residue risks was very difficult to judge, as the issue was an iceberg. Tracing the origin of the disease in human being goes back to the animals from where the disease comes in farms and track down to the retailer market and consequently to the end consumer who suffer, the disease by consuming the animal food with residues. Many of the aspects of risks to humans due to consumption of contaminated animal sourced foods were well known and limiting the risk was manageable by which the menace of drug residues may be controllable. Therefore, to curtail the risks a logical and prolonged action was needed to check the food contaminated with residues of drugs. The threat and risks, the consumers health was directly faced with due to use of antibacterial drugs in animal origin food and products, was a very serious issue. Practically, what approaches were commonly in practice in general to control and manage the situation and need to be improved, was a more serious issue to rule out the flow of residues, which was still not known (Sasanya *et al.*, 2005).

FLUOROQUINOLONES

Fluoroquinolones are synthetic, bactericidal antibacterial agents with broad-spectrum activity. They inhibit the enzyme *topoisomerase II*, a DNA *gyrase* that was necessary for the replication of the microorganism. *Topoisomerase II* enzyme produces a negative supercoil on DNA, permitting transcription or replication so by inhibiting this enzyme, DNA replication and transcription was blocked. As a general rule, gram-negative bactericidal activity correlates with inhibition of DNA *gyrase*, and gram-positive bactericidal activity corresponds with inhibition of DNA type IV *topoisomerase*. The first quinolone, nalidixic acid was introduced in 1962. Since then, structural modifications have resulted in second, third, and fourth generation fluoroquinolones, which have improved coverage of gram-positive organisms (Okerman *et al.*, 2007; Maraschiello *et al.*, 2001).

ANTIMICROBIAL ACTIVITY

The fluoroquinolones can be classified into four generations based on antimicrobial activity. First-generation agents, which were used less often today, have moderate gram-negative activity and minimal systemic distribution. Second generation fluoroquinolones have expanded gram-negative activity and atypical pathogen coverage, but limited gram-positive activity. These agents were most active against aerobic gram-negative bacilli. Ciprofloxacin remains the quinolone most active against *Pseudomonas aeruginosa* (De-Baere *et al.*, 2000). Third-generation quinolones retain expanded gram-negative and atypical intracellular activity but have improved gram-positive coverage. Finally, fourth-generation agents improve gram-positive coverage, maintain gram-negative coverage, and gain anaerobic coverage (Okerman *et al.*, 2007). First generations of quinolones most commonly have nalidixic acid and cinoxacin which were effective against *Enterobacteriaceae*. Second generation (class I) contains eomefloxacin, norfloxacin and enoxacin these products were effective against *Enterobacteriaceae* too. Whereas, class II ofloxacin, ciprofloxacin were sensitive to *Pseudomonas aeruginosa*, *Enterobacteriaceae*. Third generation comprised of levofloxacin, sparfloxacin, gatifloxacin and moxifloxacin were drug of choice against *Enterobacteriaceae*, atypical pathogens and *Streptococci*. Fourth generation contains trovafloxacin which was best choice for *Enterobacteriaceae*, *P. aeruginosa* (reduced or absent), atypical pathogens, methicillin-susceptible *Staphylococcus aureus*, *Streptococci* and anaerobes (Okerman *et al.*, 2007).

In USA, EU, Japan and South Korea considerable variations in the usage and regulatory processes for drugs exist. In some countries, there was no legal framework for prescription of veterinary pharmaceuticals (Shims *et al.*, 2003). Antibacterial resistant animal origin pathogens were prevalent in food stuff and resistance was an increasing trend in consumers of particularly of *Salmonella* and *Campylobacter* and was well documented culprits with high intensity risk. Although, the issue was very serious and needs due consideration of the problems and ignored areas for monitoring and the flow of residues of fluoroquinolones and their sources on incorporation in the edible diet must be checked. This exercise will be a helpful step in controlling the disease and increasing the effectiveness of the treatment in human being. A consultation group was organized to monitor some of the above mentioned ignored areas by W.H.O. Identification of strong relationship has been ruled out between the drug residues of fluoroquinolones and fluoroquinolones resistance from food borne bacteria and human zoonotic infection needs attention (Maraschiello *et al.*, 2001).

In a residues monitoring control study, selected antibacterials were given orally in the commercial layers for 7 days or 9 days in drinking water sulfadimethoxine (0.05 %), enrofloxacin (0.005 %) and tylosin tartrate (0.05 %). On day 1, 3 and 7 post antibacterials administration fluoroquinolones were detected. Eggs were collected from the treated layers from each experimental group, corresponding to days 2-6 of antibacterials administration, days 4-6 post antibacterials administration and days 14-16 post antibacterials administration were grouped for incubation and day old chicks hatched from these residue containing eggs have no or undetectable antibacterials residues (McReynolds *et al.*, 2000).

Detection of drug residues in animal meat and eggs has been established by fixing the detection level 0.02 µg/g. Danofloxacin, enrofloxacin and ofloxacin were detected around 0.01 µg/g, whereas benofloxacin was detected at 0.02 µg/g gave good monitoring data. In all the compounds tested range was in linear order at a residue concentration of 0.02 µg/g, whereas the lowest detection was conducted at a level of 0.30 µg/g in more than 92 percent in the tested samples (Sunderland *et al.*, 2004). The fluoroquinolones in general and ofloxacin in particularly has been detected in the poultry kidneys, liver, muscles, and adipose tissues and even in the skin was well cited in literature. As the preliminary step extraction of the drug residues was conducted with 0.15 M hydrochloric acid and final elution and solid phase

columns made extraction. The collected suspected samples were positive for drug residues at a frequency level ranged from 80 to 100 percent. By fixing the detection limit at 60 µg/kg for visceral organs including kidneys, and liver whereas adipose tissues and skin was fixed at 25 µg/kg in acidic pH ranges (Maraschiello *et al.*, 2001).

Drug residues of sarafloxacin were detected with high accuracy and frequency followed by difloxacin in the muscles of commercial poultry chickens. Clean up procedures for the drug residues pre levels of drugs in commercial poultry muscle samples may be affected due to retention in the solid phase column during extraction procedures. Limit of detection of the drug residues of sarafloxacin was 25 µg/kg whereas, for difloxacin it was around 10 µg/kg these ranges also encircle the MRL and gave the data within the permissible ranges fixed and were conventional under the Act of EU (Barron *et al.*, 2002). Enrofloxacin and ciprofloxacin drug residues were ranged from 10-300 µg/kg, however, enrofloxacin was resulted from 74 percent and ciprofloxacin was present in 54 percent in the collected suspected samples. The limits of drug residues detection for both fluoroquinolones was below 25 µg/kg, which facilitated, the monitoring of the fluoroquinolones in the muscles, within the range of mentioned detection limits of positive muscle samples, at maximum drug residue limits (Barron *et al.*, 2001).

Monitoring of drug residues of fluoroquinolones for regulatory purposes as far as the majority of the antibacterials was concerned were estimated and monitored with the modern techniques, though it cost very high but precision was the highest. In comparison with very costly techniques, a simple, cheaper microbiological assay has the tendency to detect fluoroquinolones drug residues in the poultry meat up to 300 ppb. Birds were given three divided doses each day for the 3 days of the trial. Samples were collected at the end of each experimental or after completion of the three doses of the respected day and in a similar pattern for 3 days post dosing trials chicken under treatment were slaughtered to get the thigh and breast musculature for analysis. The level of drug residues was 26 ppb in the thigh and breast muscles. Enrofloxacin was detected in the range of 60.10 ppb whereas ciprofloxacin were detected in the range of 0.50 ppb. Samples that had more MRL values of the drug residues were monitored by the microbiological assay (Schneider and Donoghue, 2004).

Ciprofloxacin, danofloxacin, enrofloxacin and sarafloxacin residues in edible chicken tissues were surveyed. The analytes were extracted from chicken muscle, skin, fat, liver and kidney by aqueous potassium dihydrogen phosphate of different pH values through homogenization. The four fluoroquinolones were analyzed in spiked samples of four chicken tissues with mean recoveries in the range of 53.90 to 93.40 percent at spiked levels 20 to 300 µg/kg. The detection limits of quantification were 20 µg/kg for ciprofloxacin, enrofloxacin and sarafloxacin and 4 µg/kg for danofloxacin (Dong *et al.*, 2005; Yorke and Froc, 2000).

MACROLIDES

Spiramycin, tilmicosin and tylosin were macrolides of an efficacious group of antibacterials used frequently in human and animal treatment. These products had high and fast absorption rate after oral administration and quick pharmacokinetics and pharmacodynamics (Salisbury *et al.*, 1995). In addition, macrolides in general had low toxicity in animal tissues particularly in the lungs, kidneys and liver as compared with other antibacterials. Macrolides with this character come up as a drug of choice for the treatment of variety of infectious diseases regarding liver, kidneys and lungs. This was the reason why spiramycin, tilmicosin and tylosin were frequently used in animal and poultry macrolides residues were transferred in meat, kidneys, lungs and poultry eggs. Observing withdrawal period of macrolides was one of the safest tools to get rid of residues, otherwise egg discard times were relatively longer (Aiello and Mays, 1998).

Macrolides are a class of antibiotics, the basis of the chemical structure of which was macrocyclic lactone ring. Depending on the number of carbon atoms in the ring, macrolides are divided into 14-membered (erythromycin, roxithromycin, clarithromycin), 15-membered (azithromycin) and 16-membered (midecamycin, spiramycin, josamycin). The main clinical importance has the activity of macrolides against gram-positive *cocci* and intracellular pathogens (*mycoplasma*, *chlamydia*, *campylobacter*, *legionella*). Macrolides were considered to be the safe and well tolerable antibiotics. Macrolides include, erythromycin, spiramycin, josamycin, midecamycin whereas, among semi synthetic, clarithromycin, roxithromycin, azithromycin and midecamycin acetate were important (Sakai *et al.*, 2004)

The antimicrobial effect was expressed due to violation of the protein synthesis in ribosomes of the microbial cells. Typically, macrolides exert bacteriostatic effect, but in high concentrations can act bactericidal on beta-hemolytic *Streptococcus* group A, *pneumococcus*, causative agents of whooping cough and diphtheria. Macrolides exhibit antibacterial effect against gram-positive *cocci*. In addition to the antibacterial action, macrolides possess immunomodulatory and moderate anti-inflammatory activity (Bassissi *et al.*, 2004).

Macrolides are active against gram-positive *cocci*, such as, *S. pyogenes, S. pneumoniae, S. aureus* except MRSA. Macrolides act on the causing agents of whooping cough and diphtheria, *moraxella*, *Legionella*, *Campylobacter*, *Listeria*, *Spirochete*, *Chlamydia*, *Mycoplasma*, *Ureaplasma*, anaerobes (excluding *B. fragilis*). Azithromycin was superior to other macrolide antibiotics in activity against *H. influenzae*, and clarithromycin against *H. pylori* and atypical *Mycobacteria* (*M. avium*, etc.). Spiramycin, azithromycin and roxithromycin were active against some protozoa (*T. gondii*, *Cryptosporidium spp.*). Macrolides belong to the fabric antibiotics, because their concentrations in serum were significantly lower than in the tissue and vary in different preparations. Macrolides in varying degrees were bound to plasma proteins. They were well distributed in the body, creating high concentrations in various tissues and organs (including prostate), especially during inflammation. Poor passing through the blood-brain barrier and blood aqueous barrier were important characteristics of this antimicrobial class. On the other hand the drugs pass normally through the placental barrier and pass into breast milk. Macrolides were metabolized in the liver, with the participation of microsomal cytochrome P-450, metabolites were excreted primarily with bile. One of the metabolites of clarithromycin possesses antimicrobial activity (Bermudez and Yamazaki, 2004).

Macrolide antibiotics have been used to treat acute and chronic inflammation in the respiratory tract based on their antibacterial activity. Now it was well known that macrolides down-regulate damaging prolonged inflammation as well as increase mucus clearance and decrease bacterial virulence. Some drug interactions with macrolides have been documented in clinical trials mainly connected with their influence on liver function. Adverse reactions were few and self-limited when used at the recommended dosage. Seven new macrolides having a 12-membered

ring, which we termed pladienolides, were isolated from the fermentation broth of *Streptomyces platensis* Mer-11107. Six of the seven pladienolides inhibited hypoxia-induced reporter gene expression controlled by human VEGF promoter with IC50 values of 0.0018-2.89 μM. They also demonstrated growth-inhibitory activity against U251 human glioma cells *in vitro*. Pladienolides were highly potent inhibitors of both hypoxia signals and cancer cell proliferation, and thus may be useful as antitumor agents (Sanders *et al.*, 2006).

New macrolides, such as clarithromycin and azithromycin, were active agents to *Mycobacterium avium* complex (MAC). Both clarithromycin and azithromycin were well-known for the ability to improve the prognosis of AIDS patients with disseminated MAC infection. However, the administration of monotherapy with a macrolide was usually associated with the emergence of drug resistance after a few months of use. Therefore, the recommended treatment for MAC infection involved the use of at least two antibiotics, which includes a macrolide in combination with rifabutin, moxifloxacin and/or ethambutol. When used as prophylactic therapy in AIDS patients, azithromycin was more convenient (1200 mg, once a week) than clarithromycin (500 mg, twice a day). Ketolides were a semi synthetic derivative of erythromycin A, which differs from erythromycin A by substitution of a 3-keto group for L-cladinose. Telithromycin has a carbamate group linked to an imidazolium and pyridium nucleus at C11-C12. In mice model, both telithromycin and ABT-733 were active *in vivo* against MAC (Lauby *et al.*, 2004).

Tylosin, erythromycin and tilmicosin were major macrolides antibacterials used in livestock production. The bi dimensional TLC techniques were conducted for the determination of the tylosin in the commercial poultry meat and eggs and milk of dairy animals. A limit of detection for tylosin was 0.10 ppm in the meat, 0.05 ppm in poultry eggs and 0.01 ppm in diary milk. These limits were 0.25 ppm in the meat of poultry for erythromycin whereas, tylosin was in milk. Liquid chromatography was found to be suitable technique for residues detection in serum and tissues of animals. Through LC technique tylosin may be detected in a range of 0.10 ppm in tissues whereas detection sensitivity of milk residues may be detectable at lower level then in milk samples (Moats, 1985).

Data of 10 layer flocks was analyzed regarding the use of antibacterial drugs for 10 weeks. Sensi disk diffusion bacterial inhibition method by the American type culture collection organism *Micrococcus luteus* (9341) and American type culture collection organism *Bacillus cereus* (11778) was used for survey of 200 poultry eggs and 378 feaces samples at the time of slaughtering from poultry carcasses. The data gave a fair indication that all the 10 farms included in the study used at least one time antibacterial, whereas 9 farmers used antibiotic drugs for prevention, treatment or both. Among these farms, none of the farmers observed withdrawal period of the used therapy. One percent poultry eggs, whereas 21.80 percent of the chicken feaces samples of slaughtered poultry were positive for drug residues. At the time of slaughtering relatively higher percentage (33.10 %) of antimicrobials were positive in broilers as compared with layers (23.60 %) and 4.80 percent in local chicken (Kabir *et al.*, 2004).

In the analysis of animal antimicrobials drugs and growth promotants, two major methods were used to detect these principles. Selection of samples for monitoring was the first criteria used in the veterinary therapeutics. Commonly, liver, kidneys and meat samples were only analyzed for monitoring studies. Due to the high frequency of animal meat and the products in which eggs and meat was used most of the time involved in food scandals therefore the consumers opted vegetable food sources and product. This was the reason, why importance has been given to the alternative products like eggs and fish for residue monitoring programmes. Most of the food scandals were initiated due to the surveillance of the animal feeds contaminated with drug residues, therefore, interests in the feed monitoring surveillance got due consideration (Stolker *et al.*, 2007).

Residues of tylosin were detected using *B. stearothermophilus var. calidolactis* C953. The recovery from spiked poultry meat samples ranged from 66.70 percent (1 mg/g) to 77.60 percent (0.10 mg/g). HPLC method tylosin residues were detected from 0.025 to. 0.01 mg/kg after 24 hours post administration. The limits of detection of these tests was around 0.01 µg/g in muscle whereas 0.025 µg/g of kidneys and liver. Average recoveries ranged from 80.40 to 88.30 percent. Relative standard deviation values ranged from 5.20 to 12.10 percent. Residue depletion of tilmicosin in broiler chickens was examined after dosing over a 5 day period by incorporation of the drug into drinking water at 37.50 and 75.00 mg/l. Tilmicosin concentrations in liver and kidney were highest on day 3 of medication and on day 5 in muscle, in both low and high dose groups. The residue levels in both groups were significantly higher in liver than in

kidney or muscle. A minimum withdrawal time of 9 days was indicated for residue levels in muscle, liver and kidney tissues below the maximum residue level (Zhang *et al.*, 2004).

Tilmicosin residues were quantified in cattle and sheep edible tissues, as well as in chicken fat, skin and muscle over a concentration range of 0.025 to 0.250 µg/g. The chicken kidney and liver had residues over a range 0.060 to 20 µg/g. The quantification limit of testing method was about 0.025 µg/g for all tested tissues except chicken kidney and liver, for which the limits of quantification was 0.06 µg/g. Average recoveries for tissue samples ranged from 73 to 98 percent (Wiley *et al.*, 2000).

Studies had been reported regarding the monitoring of metabolites and residues of 130 veterinary pharmaceuticals in livestock animals and poultry meat. Using methanol and acetonitrile (5:95) volume/volume extraction was conducted with solution of the saturated n-hexane with acetonitrile was used for the dilipidation of the samples. Procedures of extraction of drug residues in muscles were made through a mixture of acetonitrile & methanol (95:5) volume/volume ratio. The extractions were developed with n-hexane solution saturated with acetonitrile. Extracts were dried by evaporation and again dissolved in methanol to fix the total volume. Samples were analyzed by liquid chromatography and the elution was done using C18 column and residues were determined by electro spray ionization tandem mass spectrometry. The drug residues were detected in a range of 0.03 to 3.00 ng/g. The quantification limits were set in a range of 0.10 to 10.00 ng/g. One hundred eleven, 122 and 123 drugs from bovine and chicken muscle respectively showed recoveries between 70 and 110 percent (Yamada *et al.*, 2006).

Immunochemical, microbiological and physicochemical methods were opted for the detection of drug residues in the food products of animal origin. Considering the complexity of the biological materials such as meat of commercial chicken and poultry eggs and milk of dairy animals the sensitivity of the test was more demanding to get the results with sophistication in process and after process. To gain the speed in analyzing the samples, automation with precision was the current area which needs due consideration (Aerts *et al.*, 1995).

SULFONAMIDES

Sulfonamides were the oldest group of chemotherapies still used for the treatment of human beings and animals. Sulfonamides were an effective remedy for the treatment of coccidiosis in the poultry in general and layers in particular. The sulfonamides pass in the eggs and drug residues, which was serious threat and risk for human health (Ivona *et al.*, 2001). Allergic and toxic reactions have also been reported and well known by the direct administration of sulfonamides in humans by consumption of food containing drug residues for longer time have deleterious effect on human health (Spoo and Riviere, 1995). The presence of these metabolites in animal origin food products leads to resistance development of sulfonamides (Agarwal, 1992).

Sulfonamide was an organic sulfur compounds containing the radical -SO2NH2 (the amides of sulfonic acids). Its molecular structure was similar to p-aminobenzoic acid (PABA) which was needed in bacteria organisms as a substrate of the enzyme *dihydropteroate synthetase* for the synthesis of tetrahydrofolic acid (THF). Sulfonamides, derived from chiefly sulfanilamide, were capable of interfering with the metabolic processes in bacteria that require PABA. They act as antimicrobial agents by inhibiting bacterial growth and activity and called sulfa drugs. They were used in the prevention and treatment of bacterial infections, diabetes mellitus, edema, hypertension, and gout. Sulfa drug includes; sulfadiazine was a diazine; 4-amino-N-2-pyrimidinyl-benzenesulfonamide; diazolone; N1-(2-pyrimidinyl)-sulfanilamide; sulfadiazine; sulfacombin; sulfapyrimidine; sulfolex; theradiazine; topical sulfonamides: sulfacetamide; silver sulfadiazine; mafenide; mafenide acetate. Rapidly absorbed and eliminated sulfonamides: sulfisoxazole diolamine; sulfadiazine; sulfamethoxazole. Hydrophylic sulfonamides: phtalylsulfacetamide; phtalylsulfathiazole; sulfasalazine; sulfaguanidine. Long lasting sulfonamides: sulfadoxine, others: sulfamazole; sulfamazone; sulfametopirazine; sulfametoxypiridazine; sulfametrol; succinylsulfathiazole. Sulfonamides or "sulfa drugs" was the first class of antibacterial preparations approved for widespread use. For the recent years the use of sulfonamides in medical practice became limited because of lower bacteriostatic activity and higher toxicity in comparison with other up-to-date antibacterial remedies. Due to the fact that sulfonamides were used in medical practice for a long period of time, many of microorganisms developed resistance to the majority of sulfa drugs (Agarwal, 1992; Furusawa and Mukai, 1994).

SPECTRUM OF ACTIVITY

Originally sulfonamides were active against a broad spectrum of gram-positive (*S. aureus, S. pneumoniae*, etc.) and gram-negative (*gonococci, meningococci, H. influenzae, E. coli, Proteus spp., Salmonella, Shigella*, etc.) bacteria. Moreover, they act on chlamydia, nocardia, pneumocystis, actinomycetes, malaria parasite, toxoplasma. Later on, many strains of *staphylococci, streptococci, pneumococci, gonococci, meningococci, enterobacteria* were characterized by high levels of acquired resistance. *Enterococci, Pseudomonas aeruginosa* and most anaerobes have natural resistance to sulfa drugs. Preparations containing silver were active against many pathogens of wound infections-*Staphylococcus spp., P. aeruginosa, E. coli, Proteus spp., Klebsiella spp.* and *Candid*a strains. Sulfonamides were contraindicated for use in the following conditions: allergic reactions to sulfonamides, furosemide, thiazide diuretics, and sulfonylurea drugs. It was not recommended to use sulfa drugs in children for the exception of inborn toxoplasmosis (De-Baere *et al.*, 2000).

In order to decrease detrimental threats to human being and ensuring safety of the consumers by limiting the sulfonamides residues in beef, mutton, poultry meat and eggs to tolerable level, withdrawal of the veterinary products must be observed and drug must be used in recommended indications. Withdrawal period was seven days since 1980, which had been increased more than 2 weeks (Augsburg, 1989). MRL fixed by legislation was 0.10 mg/kg for sulfonamides animal origin foods. Sulfadimidine administered orally at the dose of 2 g/l *via* the drinking water. Residue analysis was performed for evaluation of sulfonamides residues in the total eggs of commercial layers. Drug residues were monitored in the eggs during, pre and post administration after 15 days of the withdrawal period (Furusawa and Mukai, 1994).

The sulfonamides therapies were generally used since 1940 in domestic animals (Bevill, 1989). Growth of the chicken stimulated after the addition of the streptomycin in the diet in a similar way as growth promotion was achieved by the fermentations of chlortetracycline (Moore *et al.*, 1946). The list of other products increased with the passage of time on the merit of growth promotion poultry production performances (Stokstad *et al.*, 1949). Getting benefit from the drugs for growth promotion coincided with the handling of the larger size herds and achieving maximum outputs with minimum inputs producing animals in large drugs were used as efficiency modifiers (Gustafson and Bowen, 1997). In the historical events, scientists observed that all or similar antibacterials have the potential of growth promotion and laying efficiency in

layers. The performance had been observed in similar pattern in the dairy herds and meat type livestock. During the year, 1950 to 1960, the antibacterials were specialized according to the rout of administration and indication. The use as growth promoters assigned only to few drugs considered as growth promoters. The quantification limits were 50 ng/g for sulfadiazine and 25 ng/g for trimethoprim in samples of kidney, liver; muscle and fat of animals that received a commercial sulfadiazine trimethoprim preparation in a ratio of 5:1 with the feed for five consecutive days, 30 mg/kg body weight per day were analyzed. The quantitative results were used to calculate a withdrawal time (12 days) to reach residue levels below the respective MRLs. Monitoring recommendations set by the European agencies for the drug residues evaluation were imposed for the drug manufacturing and sale (De-Baere *et al.*, 2000).

Sulfonamides were augmented with trimethoprim as a therapy of the diseases of respiratory system and GIT tract infections (Boison *et al.*, 1996). In Europe and particularly in Finland, almost 65 percent of the antimicrobials prescribed by veterinarians were tetracyclines, amoxicillin and their derivatives were most commonly used. Addition of the substances called feed additives usually enhanced the efficiency and production of poultry and livestock. Only four antibacterials were relaxed by EU for use in animal feeding stuffs as growth promotants; avilamycin, salinomycin, flavophospholipol and monensin gradually phased out as production modifier up to the year 2006. Zootechnical growth promoter additives of the feed do not have MRLs; except for those used in veterinary medicine has a dual authorization. As a tool of disease prevention in USA in feedlot livestock producers consume huge amounts of drugs with doses of antibiotics, which were not therapeutic doses (Lathers, 2001).

Violating the MRLs still a greater incidences of certain drugs may encounter during monitoring of the residues in general and sulfonamides in particular. Almost six percent of pork samples and pig liver were positive for the presence of sulfonamides during 1985, though the intensity of the residues in swine varies from place to place and country to country. More than 2.50 percent of pork in market had above MRL drug residues of sulfamethazine. The Food Safety and Inspection Service were taking immense measures to monitor the residues with its traceability. Further restoration measure had been taken to increase the testing capability and facility at a faster rate to grasp the contaminated foods abruptly (Cordle, 1988).

Results gave fair indication that administration of sulfonamides in layer produce sharp and rapid persistent increase sulfadimidine drug residues in their eggs. The highest drug residues were around 6th day of post treatment or administration of the sulfadimidine. Following the withdrawal period of the drug residues although concentration falls very rapidly, but amounts were still at detectable range on the 10th day post withdrawal period. On 11th day, the residues were below the established MRL. Fixing the detection limit at 0.09 mg/kg and the quantification limit of sulfadimidine was 0.30 mg/kg, recovery of residues ranged from 91 to 98 percent samples (Ivona *et al.*, 2004).

Daily oral administration of two dose levels of 1 and 2 mg/kg body weight of ampicillin (groups A1 and A2), 50 and 100 µg/kg body weight of oxytetracycline (groups O1 and O2) and 50 and 100 mg/kg body weight sulfadimidine (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 ampicillin, oxytetracycline and sulfadimidine, 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 sulfadimidine and day 6 for ampicillin (Alhendi *et al.*, 2000). Getting growth promotion and increasing the efficiency of the livestock came up within the form of contamination of the human food with used drug metabolites or with their direct residues. Another area where these residues of antibacterial hurt the human beings was the damaging or depletion of the microbiota of the intestine. The decrease in number of the intestinal microflora leads to the digestion problems in human being (Cerniglia and Kotarski, 1999).

Class of antimicrobials especially sulfonamides when used in the laying hens was a serious public health concern in term of health risk as these residues pass through the eggs to the innocent peoples. Farmers may influence by knowledge, attitudes and practices of farming regarding use of sulfonamides and other antibacterials in livestock and poultry. A descriptive thorough study was conducted in five divisions of Kampala. During the study 60 farmers were schematically sampled from a list of poultry farmers in Kampala and a semi-structured questionnaire performa was completed. Sixty eggs for each layer poultry farm was collected for the monitoring of residues for the analysis of sulfadiazine and sulfamethazine residues. Total

homogenized egg was analyzed using HPLC. Detectable residue levels of the sulfonamides were confirmed in ninety eight percent of the samples. Approximately 98.30 percent of the egg samples had detectable residues of sulfonamides track down to the feed or water consumed by the layers. Consumers in Kampala district of hen eggs were at high risk of sulfonamides residues exposure due to poor farming and impractical regulatory practices (Sasanya *et al.*, 2005).

Monitoring of food products from animal origin for the presence of antimicrobial residues was preferably done using microbial screening methods because of their high cost-effectiveness. Traditionally applied methods fail to detect the MRLs which were established when EU Council Regulation came into effect. Consequently, during the last decade this has led to the development of improved microbial screening methods (Pikkemaat *et al.*, 2011).

Antibiotics were widely used in veterinary medicine and subsequently drug residues may persist in foods derived from animals, which may pose an adverse health effect for the consumer. Screening of food products from animal origin for the presence of antimicrobial residues started soon after the introduction of antibacterial therapy in veterinary medicine (Table. 1). Initially it mainly concerned process monitoring in the dairy industry to prevent problems in fermentative dairy production, but from the early 1970s regulatory residue screening in slaughter animals also became more commonly introduced (Pikkemaat *et al.*, 2009b).

An efficient screening method needs to be low-cost and high-throughput, able to effectively identify potential noncompliant samples from a large set of negative samples. Microbial inhibitions assays were the earliest methods used for the detection of antibiotic residues (Myers, 1964) and they were still widely used (Mitchell *et al.*, 1998). They were very cost-effective and in contrast to, for example, immunological or receptor-based tests, they have the potential to cover the entire antibiotic spectrum within one test. Two main test formats can be distinguished: the tube test and the (multi-) plate test. A tube (or vial, or ampoule) test consists of a growth medium inoculated with (spores of) a sensitive test bacterium, supplemented with a pH or redox indicator. At the appropriate temperature, the bacteria start to grow and produce acid, which will cause a color change. The presence of antimicrobial residues will prevent or delay bacterial growth and thus was indicated by the absence or delay of the color change (Vermunt *et al.*, 1993).

Table 1. Chronological review of residues testing globally.

S.NO	TESTING METHOD	DRUG	COUNTRY	REFERENCES
1	Survey of antibiotic residues in Canadian slaughter animals.	Antibiotic	Canada	Tittiger *et al.*, 1975
2	Antibiotic residues and their recovery from animal tissues.	Antibiotic	UK	McCracken *et al.*, 1976
3	A rapid agar-diffusion test for the detection of antibiotic residues in kidneys from slaughter animals.	Antibiotic	Scandinavia	Rosdahl *et al.*, 1979
4	Comparison of different agar diffusion methods for the detection of antimicrobial residues in slaughter animals.	Antimicrobial	Scandinavia	Korkeala *et al.*, 1982
5	A one-plate microbiological screening test for antibiotic residue testing in kidney tissue and meat: an alternative to the EC four-plate method.	Antibiotic	Belgium	Koenen *et al.*, 1995
6	Evaluation of a multiple bioassay technique for determination of antibiotic residues in meat with standard solutions of antimicrobials.	Antibiotic	Spain	Calderon *et al.*, 1996
7	Detection of inhibitors in milk by microbial tests. A review.	Inhibitors	Germany	Suhren and Heeschen, 1996.
8	Evaluation of a modified EC Four Plate Method to detect antimicrobial drugs.	Antimicrobial	UK	Currie *et al.*, 1998
9	Evaluation of the European four-plate test as a tool for screening antibiotic residues in meat samples from retail outlets.	Antibiotic	Belgium	Okerman *et al.*, 1998a
10	Detection of Antibiotic Residues in Dressed Chicken from Commercial Test and Backyard Producers Using Four Plate Test Comparison of two methods for detecting antibiotic residues in slaughter animals.	Antibiotic	Belgium	Loinda *et al.*, 1998
11	Detection of antibiotics in muscle tissue with microbiological inhibition tests: effects of the matrix.	Antibiotics	Belgium	Okerman *et al.*, 1998b
12	Detection of residues of tetracycline antibiotics in pork and chicken meat: correlation between results of screening and confirmatory tests.	Tetracycline	Belgium	De Wasch *et al.*, 1998
13	Determination of fluoroquinolones residues in animal tissues using *E. coli* as indicator organism.	Fluoroquinolone	Canada	Choi *et al.*, 1999

Table 1 (Continue)

S.NO	TESTING METHOD	DRUG	COUNTRY	REFERENCES
14	Microbiological and chemical identification of antimicrobial drugs in kidney and muscle samples of bovine cattle and pigs	Antimicrobial	Finland	Myllyniemi *et al.*, 1999
15	Presumptive identification of sulfonamide and antibiotic residues in milk by microbial inhibitor tests.	Sulfonamide and Antibiotic	Italy	Aureli *et al.*, 1999
16	Microbiological and chemical detection of incurred penicillin G, oxytetracycline, enrofloxacin and ciprofloxacin residues in bovine and porcine tissues.	Penicillin G Oxytetracycline Enrofloxacin Ciprofloxacin	Finland	Myllyniemi *et al.*, 2000
17	Inhibition tests for detection and presumptive identification of tetracyclines, β-lactam antibiotics and quinolones in poultry meat.	Tetracyclines, β-lactam Antibiotics Quinolones	Belgium	Okerman *et al.*, 2001
18	Improved agar diffusion method for detecting residual antimicrobial agents.	Antimicrobial	Taiwan China	Tsai and Kondo, 2001
19	A microbiological six-plate method for the identification of certain antibiotic groups in incurred kidney and muscle samples.	Antimicrobial	Finland	Myllyniemi *et al.*, 2001
20	Comparison between a bioassay and liquid chromatography fluorescence mass spectrometry (n) for the determination of incurred enrofloxacin in whole eggs.	Enrofloxacin	USA	Donoghue and Schneider, 2003
21	Meeting maximum residue limits: an improved screening technique for the rapid detection of antimicrobial residues in animal food products.	Antimicrobial	UK	Stead *et al.*, 2004
22	Comparison of a bioassay and a liquid chromatography fluorescence mass spectrometry method for the detection of incurred enrofloxacin residues in chicken tissues.	Enrofloxacin	USA	Schneider and Donoghue, 2004
23	Evaluation and establishing the performance of different screening tests for tetracycline residues in animal tissues.	Tetracycline	Belgium	Okerman *et al.*, 2004
24	Validation of a microbiological method: the STAR protocol, a five-plate test, for the screening of antibiotic residues in milk.	Antibiotics	France	Gaudin *et al.*, 2004

Table 1 (Continue)

S.NO	TESTING METHOD	DRUG	COUNTRY	REFERENCES
25	Calf antibiotic and sulfonamide test (CAST) for screening antibiotic and sulfonamide residues in calf carcasses.	Antibiotic Sulfonamide	USA	Dey *et al.* 2005a
26	Fast antimicrobial screen test (FAST): improved screen test for detecting antimicrobial residues in meat tissue.	Antimicrobial	USA	Dey *et al.*, 2005b
27	Comparison of various assays used for detection of β-lactam antibiotics in poultry meat.	β-lactam Antibiotics	Slovak Republic	Popelka *et al.*, 2005
28	Evaluation of the Premi Test and comparison with the One-Plate Test for the detection of antimicrobials in kidney.	Antimicrobial	Ireland	Cantwell and O'Keeffe, 2006
29	Simultaneous determination of residual veterinary drugs in bovine, porcine, and chicken muscle using liquid chromatography coupled with electro spray ionization tandem mass spectrometry.	Veterinary drugs	Japan	Yamada *et al.*, 2006
30	Validation and comparison of the Copan Milk Test and Delvotest SP-NT for the detection of antimicrobials in milk.	Antimicrobial	Switzerland	Le Breton *et al.*, 2007
31	Combined Plate Microbial Assay (CPMA) a 6-plate-method for simultaneous first and second level screening of antibacterial residues in meat.	Antibacterial	Italy	Ferrini *et al.*, 2006
32	Improved microbial screening assay for the detection of residues in poultry and eggs.	Quinolones	Netherland	Pikkemaat *et al.*, 2007
33	Rapid multi-residue screening of antibiotics in muscle and kidney by liquid chromatography electro spray ionization-tandem mass spectrometry.	Antibiotics	Sweden	Granelli and Branzell, 2006
34	Microbiological detection of 10 Quinolone antibiotic residues and its application to artificially contaminated poultry samples.	Quinolone	Ghent	Okerman *et al.*, 2007
35	New method for the rapid identification of tetracycline residues in foods of animal origin - using the Premi Test in combination with a metal ion chelation assay.	Tetracycline	UK	Stead *et al.*, 2007

Table 1 (Continue)

S.NO	TESTING METHOD	DRUG	COUNTRY	REFERENCES
36	A comparison of the FAST, Premi and KIS tests for screening antibiotic residues in beef kidney juice and serum.	Antibiotic	USA	Schneider and Lehotay, 2008
37	Comparison of three microbial screening methods for antibiotics using routine monitoring samples.	Antibiotic	Netherland	Pikkemaat *et al.*, 2009a
38	Comparison of screening methods for antibiotics in beef kidney juice and serum.	Antibiotics	USA.	Schneider *et al.*, 2009
39	Screening methods for the detection of antibiotic residues in slaughter animals: comparison of the European Union Four-Plate Test, the Nouws Antibiotic Test and the Premi Test (applied to muscle and kidney).	Antibiotic	European Union	Pikkemaat *et al.*, 2011
40	Microbial screening methods for detection of antibiotic residues in slaughter animals.	Antibiotic	Netherland	Pikkemaat, 2009
41	Nouws antibiotic test: Validation of a post-screening method for antibiotic residues in kidney.	Antibiotic	Netherland	Pikkemaat *et al.*, 2009b
42	Detection of 4-quinolone residues in rainbow trout (*Oncorhynchus mykisswalbaum*) muscle using a bioassay.	Quinolone	UK	Barker, 1994.
43	Evaluation and validation according to international standards of the Delvotest SP-NT screening assay for antimicrobial drugs in milk	Antimicrobial	UK	Stead *et al.*, 2008
44	Antimicrobial drug residues in milk and meat: causes, concerns, prevalence, regulations, tests, and test performance.	Antimicrobial	Canada	Mitchell *et al.*, 2011
45	A new microbial screening method for the detection of antimicrobial residues in slaughter animals: The Nouws antibiotic test (NAT-screening).	Antimicrobial	Netherland	Pikkemaat *et al.*, 2009c

This format was commonly applied in routine screening of milk (Suhren and Heeschen, 1996) but it was also increasingly used for analysis of other matrices (Stead *et al.*, 2004). A plate test consists of a layer of inoculated nutrient agar, with samples applied on top of the layer (Cantwell and O'Keeffe, 2006), or in wells in the agar (Kilnic *et al.*, 2007). Bacterial growth will turn the agar into an opaque layer, which yields a clear growth-inhibited area around the sample if it contains antimicrobial substances. In Europe this has been the main test format (Nouws *et al.*, 1979) since screening of slaughter animals for the presence of antibiotics started (Bogaerts and Wolf, 1980).

Inherent sensitivity of the test organism used in bioassay will determine the LOD for specific antimicrobial. In place of vegetative organisms, spore suspensions were used to achieve repeatable outcomes (Cooper, 1972). *B. subtilis* was extensively employed as test organism due to commercial availability spore suspensions as well as its broad range of antibacterial activity (Koenen *et al.*, 1995). In calf antibiotic and sulfa test (CAST), *B. megaterium* was the test organism (USDA, 1984). Tetracycline residues have been screened employing *B. cereus* strains as test organism (Okerman *et al.*, 2004). Although, *B. stearothermophilus* was commonly used test organism, it was less suitable for screening kidney samples, due to its growth inhibition with Lysozyme (Braham *et al.*, 2001). Non spore forming microorganisms were also employed as test organism. *Micrococcus luteus* was especially sensitive to β-lactams and macrolides (Bogaerts and Wolf, 1980; Okerman *et al.*, 1998a). *E. coli* strains were used to detect fluoroquinolones residues (Okerman *et al.*, 2001). Oxolinic acid and chloramphenicol had been screened with bioluminescent test organism *Photobacterium phosporeum* (Tsai and Kondo, 2001).

One of the first official methods was the *Sarcina lutea* kidney test of van Schothorst (1969), which became the statutorily prescribed method in the Netherlands in 1973. At approximately the same time, Germany introduced a *Bacillus subtilis* BGA test and other countries adopted similar test methods (Nouws *et al.*, 1979). In 1980 a standardized method for the detection of antibacterial substances was proposed by a working group of the Scientific Veterinary Commission of the European Commission (Bogaerts and Wolf, 1980). EU four-plate test (EU4pt) comprises three plates of agar medium inoculated with *B. subtilis* BGA spores at pH 6,

7.2, and 8, and a *Kocuria rhizophila* formerly known as *Micrococcus luteus* (Tang and Gillevet, 2003) ATCC 9341 plate at pH 8. The pH 7.2 medium was supplemented with trimethoprim (TMP) to increase the sensitivity for sulfonamides. For a long time the result of this test was used as an unofficial tolerance level: meat testing negative on all four test plates was considered compliant (Table. 2).

The EU4pt was developed for detection of residues in meat and was considered less suitable for analysis of kidney because it caused too many false-positive results with this matrix. Also a test comprising four plates were considered rather laborious, so in several countries one-plate alternatives were introduced (Koenen *et al.*, 1995). These tests, based on *B. subtilis*, used renal pelvis fluid or kidney as a test matrix, since residue levels in this organ were generally higher than in meat, allowing a somewhat reduced sensitivity of the test, while the results were still comparable with the EU4pt results for meat (Nouws *et al.*, 1988). Introduction of a membrane between the kidney sample and the test plate was used to prevent problems with natural growth-inhibiting compounds (Nolan *et al.*, 2000).

Ongoing harmonization of European legislation has led to a collective approach with respect to the approval of veterinary drugs (EC, 1990) and monitoring programs (EC, 1996). Before a veterinary medicinal product was allowed on the market, it has to undergo a safety and residue evaluation, after which MRLs can be defined. This process started in 1992 and currently the list of antibacterial substances (category B1 substances) for which an MRL has been established comprises over 50 antimicrobial compounds mandatory screening of a fixed percentage of all animal products for the presence of residues of antimicrobial drugs. The vast majority of the screening methods used for monitoring the presence of antimicrobial compounds were still microbial inhibition tests (Sanders *et al.*, 2006). However, the establishment of MRLs has made Scientists to reconsider the original microbial screening methods, such as the EU4pt and one-plate *B. subtilis* assays (Currie *et al.*, 1998), employed in the pre-MRL era, as it should be concluded that for many residues these tests were insufficiently sensitive (Okerman *et al.*,1998a). In the overview of EU MRLs µg/kg were established until 1 January 2009.

Table. 2. Global overview of the residues screening microbiological methods and test organisms

Type of Assay	Microorganism	Intended Matrix	Reference
Tube test	*B. stearothermophilus*	Milk	Vermunt *et al.*, 2004
Premi Test	*B. stearothermophilus*	Multiple matrices	Stead *et al.*, 2004
Premi Test	*B. stearothermophilus*	Poultry tissue fluid	Stead *et al.*, 2007
Delvotest SP-NT and Copan	*B. stearothermophilus*	Milk	Le Breton *et al.*, 2007
Delvotest SP-NT and Copan	*B. stearothermophilus*	Milk	Stead *et al.*, 2007
Single-plate assay			
New Dutch kidney test	*B. subtilis*	Renal pelvis fluid absorbed on paper disk	Nouws *et al.*, 1979
	Belgian kidney test (*B. subtilis*)	Renal pelvis fluid/kidney cortex	Koenen *et al.*, 1995
	B. subtilis plate test	Kidney tissue	Nolan *et al.*, 2000
	STOP (*B. subtilis*)	Muscle fluid absorbed with swab	Johnston *et al.*, 1981
	CAST (*B. megaterium*)	Kidney fluid absorbed with swab	Dey *et al.*, 2005a
	FAST (*B. megaterium*) bromocresol purple indicator for reduced assay time	Kidney fluid absorbed with swab	Dey *et al.*, 2005b
	Escherichia coli plate test (specific detection of quinolones)	Muscle	Ellerbroek, 1991
	Yersinia ruckeri plate test (specific detection of quinolones)	Fish	Barker, 1994
	E. coli plate test (specific detection of quinolones)	Not specified	Choi *et al.*, 1999
	Klebsiella pneumoniae plate test (specific detection of quinolones)	Egg	Donoghue and Schneider, 2003
	K. pneumoniae plate test (specific detection of quinolones)	Poultry tissue	Schneider and Donoghue, 2004

Table. 2 (Continue)

Type of Assay	Microorganism	Intended Matrix	Reference
Multiplate Methods	EU 4-plate test (*Kocuria rhizophila* and *B. subtilis* plates at pH 6, 7.2 + trimethoprim, and pH 8).	Muscle	Bogaerts and Wolf, 1980
	EU 4-plate test.	Muscle	Currie *et al.*, 1998
	EU 4-plate test.	Muscle	Okerman *et al.*, 1998a
	3-plate test (*K. rhizophila, B. cereus*, and *E. coli*) for detection and presumptive identification of tetracyclines, β-lactam, and quinolones.	Poultry muscle	Okerman *et al.*, 2001
	Combination of 7 organisms and 5 media.	Not specified	Tsai and Kondo, 2001
	3-plate test (*B. subtilis* pH 6, 7.2, and 8).	Muscle and kidney tissue	Myllyniemi *et al.*, 2000
	18 combinations of 8 test bacteria, different pH, and additions reversing the action of specific antibiotics.	Muscle and kidney tissue	Myllyniemi *et al.*, 1999
	6-plate method (*B. subtilis* pH 6 and 7.2, *K. rhizophila* pH 6 and 8, *B. cereus*, *E. coli*).	Muscle and kidney tissue	Myllyniemi *et al.*, 2001
	7-plate USDA-FSIS method (*B. cereus*, *B. subtilis*, *Staphylococcus epidermidis*, 2 *K. rhizophila* spp.).	Muscle and poultry tissue	USDA-FSIS, 2007
	7-plate USDA-FSIS method	Muscle	Calderon *et al.*, 1996
	5-plate test STAR (*B. subtilis, B. stearothermophilus, B. cereus, K. rhizophila*, and *E. coli*).	Milk	Gaudin *et al.*, 2004
	6-plate method CPMA (*B. subtilis* pH 6, 7.2 and 8, *B. cereus*, *K. rhizophila*, and *E. coli*) including confirmatory solutions.	Muscle and kidney tissue	Ferrini *et al.*, 2006

Table. 2 (Continue)

Type of Assay	Microorganism	Intended Matrix	Reference
Multiplate Methods	Multiplate assay (*B. subtilis* pH 7.2 and 8, *B. stearothermophilus*, additional plates for presumptive identification).	Milk	Aureli *et al.*, 1996
	5-plate test NAT (*B. subtilis, B. cereus, B. pumilus, K. rhizophila*, and *Y. ruckeri*).	Renal pelvis fluid absorbed on paper disk	Pikkemaat *et al.*, 2007
	4-plate method (*B. subtilis, B. pumilus, K. rhizophila, Y. ruckeri*).	Kidney fluid	Pikkemaat *et al.*, 2011
Method comparison	One-plate test (*B. subtilis* pH 7) and Premi Test comparison	Kidney fluid	Cantwell and O'Keeffe, 2006
	EU 4-plate test and Premi Test comparison.	Trout	Kilnic *et al.*, 2007
	NAT, STAR, and Premi Test comparative study.	Kidney, muscle	Pikkemaat *et al.*, 2009a
	Plate assays based on *B. subtilis, B. cereus, and E. coli* (specific detection of quinolones).	Poultry muscle	Okerman *et al.*, 2007
	Plate assay based on *Y. ruckeri* and Premi Test (specific detection of quinolones).	Poultry muscle and egg	Pikkemaat *et al.*, 2007
	EU 4-plate test, *B. stearothermophilus* disk assay and Premi Test comparison.	Poultry muscle fluid	Popelka *et al.*, 2005
	EU 4-plate test and Premi Test comparison (AFNOR validation).	Muscle fluid	Pikkemaat *et al.*, 2007
	Premi Test and *B. subtilis and B. cereus* based plate methods (specific detection of tetracyclines).	Muscle(fluid)	Okerman *et al.*, 2004
	FAST, Premi Test and KIS (*B. stearothermophilus* tube test) comparative study.	Kidney fluid and serum	Schneider and Lehotay, 2008
	FAST, Premi Test and KIS (*B. stearothermophilus* tube test) comparative study.	Kidney fluid and serum	Schneider *et al.*, 2009

METHOD DEVELOPMENT

BROAD SPECTUM METHODS

The most important trend that can be observed in the development of microbial detection methods for antibiotics was acknowledgement of the fact that adequate detection of a broad spectrum of antibiotics was only possible using multiplate assays based on a combination of different test bacteria. Okerman *et al.* (2001) presented an inhibition test for detection and presumptive identification of tetracyclines, β-lactams, and quinolones in poultry. The method comprises three pH 6 plates, inoculated with *B. cereus*, *K. rhizophila* and *E. coli*. The detection limits of a limited number of residues were compared with those of a *B. subtilis* pH 6 tests and were found to be lower for all compounds, although the differences between *B. subtilis* and *B. cereus* sensitivity for tetracyclines were remarkably small. As the authors mentioned, this method should be considered a limited-spectrum method, because aminoglycosides and sulfonamides will not be detected. Also adequate detection of macrolides would probably require a higher pH.

Tsai and Kondo, (2001) evaluated the detection levels of 31 antimicrobial agents on various combinations of seven bacteria and five media. These included the somewhat uncommon test organisms *Clostridium perfringens* and *Photobacterium phosporeum*. On the basis of the results a method comprising *B. stearothermophilus*, *B. subtilis*, *K. rhizophila* and *E. coli* was proposed. *B. cereus* was included in the evaluation but was not found to be essential as *B. subtilis* grown on minimum medium showed better sensitivity to the tetracyclines tested (oxytetracycline and chlortetracycline).

An interesting study was presented by Myllyniemi *et al.* (2000), who evaluated the regulatory prescribed Finnish two-plate test, supplemented with a *B. subtilis* pH 7.2 + trimethoprim plate. Kidney and muscle were taken from animals that were emergency-slaughtered during the withdrawal period of an antibiotic treatment. The samples were chemically confirmed and the 68 out of 89 animals that contained residues showed a wide range of penicillin, oxytetracycline, and enrofloxacin concentrations below and above the MRL. This study provided valuable data on the correlation between the concentrations found in kidney and muscle in the same carcass. It was concluded that the *B. subtilis* assays used were not sensitive enough to allow

oxytetracycline and enrofloxacin screening in muscle; only penicillin could be screened adequately from muscle tissue.

The costs of chemical confirmation can be considerably reduced by introducing a preliminary microbial identification procedure. For this reason the activity patterns of 15 different antibiotics were assessed on 18 combinations of test bacteria, varying growth medium pH values and antagonistic compounds (Myllyniemi *et al.*, 1999). This approach generated data for these specific antibiotics on a wide range of test plates, yielding a much better view of the specificity of a test plate. Activity patterns appeared to be sufficiently specific for group identification of the antibiotics tested. Additional data were generated with incurred kidney and muscle samples containing penicillin, oxytetracycline and enrofloxacin (including the metabolite ciprofloxacin) and for these compounds the microbiological identification and the chemical identification were in good agreement. Cluster analysis on the inhibition zones caused by the different antimicrobial compounds on each of the 18 test plates revealed that the number of plates required for effective preliminary identification could be narrowed down to six. It was shown that group identification of standard solutions and incurred samples of penicillin, oxytetracycline and enrofloxacin remained correct; unfortunately no data for the other antibiotic groups was available (Myllyniemi *et al.*, 2001).

The possibility for preliminary identification from activity profiles was also explored for the US Food Safety and Inspection Service (USDA-FSIS) method (USDA-FSIS, 2007; Calderon *et al.*, 1996). This method consists of seven test plates and was used by the USDA-FSIS as a microbial confirmatory procedure for samples which tested positive in initial screening tests such as swab test on premises (STOP) (Johnston *et al.*, 1981), CAST (Dey *et al.*, 2005a), and FAST (Dey *et al.*, 2005b). The method does not use the commonly applied *K. rhizophila* ATCC 9341, but uses two erythromycin- or (dihydro) streptomycin-resistant derivatives, which may improve the identification of macrolides and aminoglycosides. The method lacks a TMP-supplemented test plate and should therefore be considered insufficient with respect to the detection of sulfonamides. The *B. subtilis* plate in this method allows adequate detection of enrofloxacin. However, the lack of a specific test plate for this antibiotic group will probably lead to a situation in which most other veterinary quinolones remain undetected (Dey *et al.*, 2005b).

Under EU Council Directive 96/23/EC, AFSSA Fougeres was designated as the Community Reference Laboratory (CRL) for (among others) the B1 substances (EC, 1996). The CRL proposed an improved method for screening of meat, the screening test for antibiotic residues (STAR) (Fuselier *et al.*, 2000; Gaudin *et al.*, 2004), which was based on five individual test plates containing *B. cereus*, *B. stearothermophilus*, *B. subtilis*, *K. rhizophila*, and *E. coli* as the indicator organisms. The results of an initial collaborative study with a small number of residues in pig muscle were mainly satisfactorily, although gentamicin at 5 times the MRL was not detected and the *B. stearothermophilus* plate showed inhibition with blank samples (Fuselier *et al.*, 2000). Additionally the STAR was validated with fortified milk samples (Gaudin *et al.*, 2004). The compounds that could not be detected at levels less than or equal to the MRL were mainly sulfonamides and β-lactams; for the latter group, however, the MRLs in milk were generally lower than MRLs for other matrices. A validation study with spiked muscle tissue samples was ongoing, but preliminary results show that the detection capability for several of the substances tested appears to exceed the MRL (Gaudin *et al.*, 2008).

Ferrini *et al.* (2006), presented a six-plate method, the combined plate microbial assay (CPMA), which essentially consists of the EU4pt, extended with additional *B. cereus* and *E. coli* plates. When the proposed strategy was applied, *i.e.*, applying samples in twofold or fourfold and supplementing them with one of the confirmatory solutions *penicillinase*, 4-aminobenzoate, or magnesium sulphate, the test allows presumptive group identification and initial screening in one step. This same approach was presented earlier for a limited range of residue groups in milk (Aureli *et al.*, 1996) and meat (Ferrini *et al.*, 1997).

Reviewing the activity profiles was a relatively simple way to achieve preliminary identification (Myllyniemi *et al.*, 1999, Calderon *et al.*, 1996). The ultimate form of it was presented in the Nouws antibiotic test (NAT) (Pikkemaat *et al.*, 2008). This method comprises five test plates, each one specific for one or two groups of antibiotics, with the plate showing the largest inhibition zone revealing the group identity of a residue. The method was based on the analysis of renal pelvis fluid. It uses a format that slightly differs from most plate tests, as it does not apply samples on top of the agar layer, but in punch holes which were supplemented with a plate-specific buffer. This approach yields good sensitivity, though the procedure becomes more

complex, which might be a disadvantage in terms of robustness. In accordance with this same principle, post screening methods for the analysis of kidney and meat (Pikkemaat *et al.*, 2011) were developed, and were used for screening of slaughter animals within the framework of the National Monitoring Program in the Netherlands (Pikkemaat *et al.*, 2009 a).

It can be concluded that the increased number of test plates, required to achieve adequate detection, has resulted in more laborious methods. However, they bring the advantage of enhancing the possibilities and the accuracy of presumptive antibiotic group identification, which may reduce confirmatory costs and efforts.

Some of the studies concerning microbial screening methods do not intend to cover the entire spectrum of antimicrobial residues, but only a specific group of antimicrobials. This was particularly true for the quinolones, a major antibiotic group that only became veterinary relevant during the last decade of the twentieth century.

Ellerbroek, (1991) compared the sensitivity of *B. subtilis* BGA and *E. coli* (Bay) 14 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 quinolones 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. A third comparative study evaluated the susceptibility of the same organisms as in (Ellerbroek, 1991) for ten different fluoroquinolones (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 nalidixic acid, flumequine, oxolinic acid and difloxacin appeared to be optimal at pH 6; for the others pH 8 was favorable. It was concluded that the addition of an *E. coli* at pH 8 test was the best option to include in existing screening methods. However, depending on the matrix it might be necessary to include a plate at pH 6 for adequate detection of flumequine, since the MRL of

flumequine in muscle differs between species (600 µg/kg in fish, 400 µg/kg in poultry and 200 µg/kg in other species). Most of the broad-spectrum multiplate methods mentioned in the previous section comprise a specific *E. coli* test plate for fluoroquinolones detection, either *E. coli* (Bay) 14 at pH 6 (Okerman *et al.*, 2001) or *E. coli* 11303 at pH 7.2 (Myllyniemi *et al.*, 1999; Myllyniemi *et al.*, 2001 & Ferrini *et al.*, 2006) or pH 8 (Gaudin *et al.*, 2004).

Two alternative bacterial species been proposed for the detection of quinolones, *Klebsiella pneumoniae* ATTC 10031 (Donoghue and Schneider, 2003; Schneider and Donoghue, 2004) and *Yersinia ruckeri* NCIMB 13282 (Barker, 1994; Pikkemaat *et al.*, 2007). For *K. pneumoniae* only data on its sensitivity towards enrofloxacin have been published (Donoghue and Schneider, 2003 & Schneider and Donoghue, 2004). The *Y. ruckeri* assay was originally developed for the detection of oxolinic acid in fish (Barker,1994); the detection capability of a *Y. ruckeri* based pH 6.5 assay for several additional quinolones in egg and poultry muscle was published later (Pikkemaat *et al.*, 2011; Donoghue and Schneider, 2003). The Nouws antibiotic test was the only multiplate method so far that has implemented this species for quinolone detection (Pikkemaat *et al.*, 2008; Pikkemaat *et al.*, 2009b). It has been claimed that the use of this organism provides a better balance between sensitivity towards enrofloxacin and oxolinic acid and flumequine (Pikkemaat *et al.*, 2007), though a straightforward comparison between the two species was lacking so far (Schneider and Donoghue, 2007).

TUBE TEST

From a practical perspective, tube tests form an attractive alternative to multiplate methods. Almost without exception these tests use *B. stearothermophilus var. calidolactis* as the indicator organism. The only equipment needed was a device (*e.g.*, garlic press) to obtain tissue fluid and an incubator or water bath at the appropriate temperature. Assay results were available within 4 hours and the use of spores instead of vegetative cells allows prolonged shelf life, which makes commercial distribution feasible. Initially, commercially available *B. stearothermophilus* tube tests were developed for the analysis of milk, but for several years tests intended for other animal matrices have also become commercially available; *e.g.*, Premi Test (DSM), Explorer (Zeu-Inmunotech) and Kidney Inhibition Swab (KIS) test (Charm Sciences). The only test for which a substantial amount of literature was available was Premi Test (Stead *et al.*, 2007).

B. stearothermophilus was widely used for detection of antibiotics in milk, because it was very sensitive to what was considered the most important group of antimicrobials for this matrix, the β-lactam antibiotics. Popelka *et al.* (2005) showed that Premi Test exhibits excellent sensitivity for penicillin, amoxicillin, ampicillin, oxacillin, and cloxacillin. The study includes results of poultry muscle samples originating from animals treated with amoxicillin. Premi Test appeared to be capable of detecting residue levels down to 21 µg/kg.

Premi Test recently received AFNOR (French Association for Normalization) certification. The AFNOR validation mark certifies the analytical effectiveness of commercial methods for a defined field of application, which should be comparable to the effectiveness of a reference method. The organization mainly certifies microbiological detection methods in food and water. Certification of antibiotic detection methods was limited so far; besides Premi Test only a receptor assay for β-lactam antibiotics, beta-STAR, has received an AFNOR certificate (Pikkemaat *et al.*, 2007).

The results of the validation study, which was performed by the CRL, had been published. The study comprised several steps. In the first step, the detection capability of the test for amoxicillin, ceftiofur, sulfamethazine, oxytetracycline, tylosin, and gentamicin in fortified meat juice samples was analyzed. Detection of amoxicillin, ceftiofur, and tylosin at their respective MRLs was satisfactorily, sulfamethazine and oxytetracycline were adequately detected at twice the MRL, but gentamicin was not. The false-positive rate was fairly high, with six "doubtful" results out of 40 measurements (Gaudin *et al.*, 2008). The second step concerned a comparison between Premi Test and the EU4pt with incurred muscle samples. Since this was the French official method for monitoring muscle samples, it was assigned as the reference method. Incurred samples containing 750 µg/kg tylosin (MRL 100 µg/kg), 270 µg/kg amoxicillin (MRL 50 µg/kg), and a combination of 760 µg/kg oxytetracycline and 150 µg/kg sulfadimethoxine (MRL for both 100 µg/kg) were compared. The method performance was evaluated in terms of relative accuracy, relative specificity, and relative sensitivity and it was concluded that the results were similar, with Premi Test yielding fewer false-negative and false-positive results. In more detail Premi Test detected all incurred samples, while the EU4pt showed a false-negative rate of 20 percent for the amoxicillin and 80 percent for the tylosin incurred tissue samples.

These results form, the major argument on which the AFNOR certification was based (Pikkemaat *et al.*, 2007).

From these results it may seem fair to conclude that Premi Test performs equally well as or better than the reference method. However, evaluating the performance of a method against a reference method that, although it was still used on a large scale, was widely recognized to be insufficiently sensitive was arguable. Moreover, the samples that were used for the comparative study contained residue concentrations that were considerably higher than "the level of interest," the MRL, so performing better than the reference method was no guarantee of performing adequately at the relevant residue concentrations. Finally, the number of different residues evaluated was very limited, which makes the outcome only of limited value in judging the application as a broad-spectrum-antibiotic screening method (Pikkemaat *et al.*, 2009c).

Additionally field samples testing positive with either Premi Test or an additional *B. cereus* test were retested by the CRL using Premi Test, the EU4pt, and the STAR method (Gaudin *et al.*, 2004). Besides a disturbingly high false positive rate for Premi Test (62 %), this part of the study also showed clearly that the STAR method was much more sensitive than the EU4pt. Field laboratories in France had been authorized to use Premi Test as a prescreening, under the condition that all positive results were reanalyzed with the EU4pt (Gaudin *et al.*, 2004).

Premi Test claims to be suitable for matrices such as kidney, fish, eggs, and feed, but literature data on these matrices was very limited so far. Residue detection in eggs has only been studied with sulfadimidine (Hussein *et al.*, 2005), and detection in fish fluid was only tested with four antibiotics (Kilnic *et al.*, 2007). A much more extensive study, including the comparison with a one-plate *B. subtilis* test, was performed with 18 different antibiotics in kidney fluid (Cantwell and O'Keeffe, 2006). Using kidney as a matrix may be an advantage, since the MRLs of several antibiotics were higher for this organ. Initially the detection capability was determined using antibiotic standard solutions. The sensitivity for most of the antibiotics tested appeared to be below the kidney MRL, except for chlortetracycline, sulfamethazine, streptomycin, and flumequine and the banned residue chloramphenicol. In particular, for β-lactam antibiotics and sulfonamides the Premi Test outperformed the one-plate test. However, when the same

comparison was repeated using fortified kidney fluid samples, a considerable matrix effect was observed. The sensitivity of Premi Test for β-lactam antibiotics remains below their MRLs, but with exception of doxycycline, all other antimicrobials were no longer detected adequately (Okerman *et al.*, 2007).

Okerman *et al.* (2004) compared several methods, including Premi Test, for the detection of tetracyclines in animal tissue. The Premi Test results of chicken muscles spiked with 100 µg/kg of all four veterinary used tetracyclines were negative. The study included the analysis of incurred samples. Unfortunately the highest doxycycline concentration (108.80 µg/kg) yielded a negative Premi Test result, so no conclusions regarding the detection limit for this compound could be made. Oxytetracycline incurred samples were available at a much wider range and with this residue Premi Test gave a positive result between 192.8 and 427 µg/kg. Using a *B. cereus* based plate test detection of oxytetracycline at half the MRL appeared feasible. It should be mentioned that all the microbial test methods evaluated in this study were outcompeted by a commercial receptor test (Tetra Sensor) (Okerman *et al.*, 2007).

A comparison between two multiplate tests and Premi Test also revealed insufficient sensitivity of Premi Test with respect to the detection of tetracyclines (Ellerbroek, 1991). Analysis of 591 slaughter animals yielded four MRL violations, of which three were tetracyclines that remained undetected by Premi Test. A study on quinolone detection in poultry and eggs by the same group showed that Premi Test was also not suitable for this group of antibiotic residues, as all compounds tested remained undetected at their MRL (Pikkemaat *et al.*, 2007).

Stead *et al.* (2004) proposed an acetonitrile/acetone extraction to enhance the sensitivity of Premi Test. Detection limits for a broad range of antibiotics and matrices were presented using this sample pretreatment. An advantage of using ampoule-based tests was the potential for objective automated processing of the results, using scanner technology as an alternative to subjective visual assessment (Stead *et al.*, 2005). Like one-plate tests, a tube test lacks the possibility for group identification. However, secondary screening for antibiotic group identification by repeating the assay supplemented with 4-aminobenzoate or *β-lactamase* can

selectively identify the presence of sulfonamides or β-lactams (Stead *et al.*, 2004). In the same way, group identification of tetracyclines can be obtained after addition of a calcium-containing buffer (Stead *et al.*, 2007).

The performances of Premi Test and a similar *B. stearothermophilus* tube test, KIS, were evaluated to assess the possibility to replace FAST (Dey *et al.*, 2005b), a *B. megatherium* one-plate test operated by the US FSIS (Schneider and Lehotay, 2008; Schneider *et al.*, 2009). In addition to kidney fluid, which was the test matrix for FAST, also serum was tested since it would allow ante mortem screening. KIS was specifically designed for the analysis of kidney and in practice employs a disposable swab format, but for this study samples were directly pipetted onto the test tube. Initially eight antibiotics (penicillin, sulfadimethoxine, oxytetracycline, tylosin, danofloxacin, streptomycin, neomycin and spectinomycin) were tested (Schneider and Lehotay, 2008). As may be expected from the fact that they exploit the same test organism, differences in the results of the two *B. stearothermophilus* tests were only minor. FAST appeared significantly more sensitive for the aminoglycosides, but for most of the other residues the *B. stearothermophilus* tests show better sensitivity. KIS showed a considerable number of false-positive responses, but this may be attributed to the fact that the kidney juice samples were not subjected to a pre incubation step at 80°C. This step was included in the Premi Test protocol when one was analyzing kidney (and egg) to inactivate natural growth-inhibiting compounds (Schneider *et al.*, 2009).

Subsequently a very thorough study was carried out on suspect carcasses obtained from a meat inspection program (Schneider *et al.*, 2009). Kidney and serum were subjected to each of the three microbial tests and were also analyzed by liquid chromatography–tandem mass spectrometry. From an analytical perspective, the range of compounds and concentrations found was somewhat disappointing. Only 39 out of 235 carcasses contained residues, mainly dihydrostreptomycin, penicillin, oxytetracycline, pirlimycin, and desfurylceftiofur cysteine disulfide, at very low levels. However, the three samples showing concentrations above US tolerance levels were effectively detected by both KIS and Premi Test, while FAST missed a sample containing 141 µg/kg sulfamethazine. It should be noted that the relatively low sensitivity of *B. stearothermophilus* for tetracyclines was not an issue in the US situation, where

tolerance levels for tetracycline in kidney were set at 12,000 µg/kg. It can be concluded that tube tests can be used as a broad-spectrum screening method, but that in many cases parallel tests covering, for example, tetracycline and quinolone residues will be required (Schneider *et al.*, 2009).

METHOD VALIDATION AND PROFICIENCY TESTING

Determining the suitability and applicability of a method for a specific matrix was obviously an important issue. It has probably become clear from the previous sections that it was difficult to compare the performances of individual methods on the basis of literature data, because factors such as the type of matrix and the specific residues investigated differ between studies. To come to more standardized procedures for method evaluation; the European Commission had issued a decision on method validation (EC, 2002), which describes how analytical methods should be validated according to common procedures and performance criteria. A method has to fulfill a defined subset of performance criteria, depending on whether it concerns a qualitative or a quantitative method, and a screening or confirmatory method (Table. 3). Qualitative screening methods, such as the microbiological antibiotic detection methods, should be validated with respect to the following parameters: detection capability (CCβ), specificity/selectivity, ruggedness, and stability. Performance characteristics should be determined in method validation according to EC (EC, 2002).

DETECTING CAPABILITY

Two main dilemmas emerge when considering the validation of the detection capability of a broad-spectrum microbial screening method; the type of sample (matrix) and the number of compounds that should be assessed. Although characterization with antibiotic standard solutions was relatively simple and provides valuable information on the bioactivity of individual compounds within a group, CCβ values obtained with such an approach cannot be considered representative for practical samples (Cantwell and O'Keeffe, 2006; Myllyniemi *et al.*, 2000; Pikkemaat *et al.*, 2007; Okerman *et al.*, 1998a). The presence of an animal matrix may affect the detection capability of a method through various factors, such as the addition of growth components, local pH change, degradation and protein binding. Validation should therefore also be performed with the matrix samples. Fortifying liquid matrices such as milk and egg was

straightforward, but validating the detection capability of a method for meat or kidney was somewhat more complicated. For methods based on the detection of meat or kidney fluid, fortification was relatively easy, although two different approaches were being employed: fortification extracted fluid (Cantwell and O'Keeffe, 2006; Okerman *et al.*, 2004) and fortification of tissue with subsequent extraction (Stead *et al.*, 2004; Pikkemaat *et al.*, 2011). Most methods for the screening of meat and kidney, however, rely on the analysis of intact pieces of tissue. Although the use of frozen pieces of fortified minced tissue, referred to as simulated tissue, has been reported, it remains difficult to find a proper fortification strategy for this type of test (Gaudin *et al.*, 2004).

It would be highly preferable, especially with tests analyzing intact tissue, to assess the detection capability using incurred samples. Some studies evaluating the performance of microbial screening methods used tissues originating from animal medication experiments (Gaudin, 2008; Okerman *et al.*, 2004; Croubels *et al.*, 1999), but the production of incurred materials for each antibiotic at the appropriate concentration was a difficult and expensive task. Alternatively, samples originating from monitoring programs had been used for method evaluation (Okerman *et al.*, 2001; Myllyniemi *et al.*, 2000; Myllyniemi, 2000; Myllyniemi *et al.*, 2001; Pikkemaat *et al.*, 2009a; Schneider *et al.*, 2009). The most fruitful approach was to use emergency-slaughtered animals for which medication information indicates they were slaughtered before the end of the withdrawal period (Myllyniemi *et al.*, 2000). In general, however, these studies yielded only limited numbers of positive samples representing only a very limited group of substances, raising the question whether other residues were not present, or were not found because the method was too insensitive (De Wasch *et al.*, 1998).

It had been proposed to limit the number of compounds to be validated for broad-spectrum methods by assigning "representative compounds" (Pikkemaat *et al.*, 2009b; Gaudin and Sanders, 2005). It was assumed that one or two compounds within an antibiotic group can act as representatives for the entire group. This may be a legitimate assumption, but it should be treated with care. The relative bioactivity of compounds within a group may differ when they were exposed to a different test bacterium. Assuming that the representative compound should be the one that was detected least sensitively with respect to its MRL, also the fact that the

MRLs vary between matrices may have consequences. So far only a few microbial screening methods claim to have been validated (Pikkemaat *et al.*, 2008; Pikkemaat *et al.*, 2009c; Stead *et al.*, 2007; Le Breton *et al.*, 2007). All of them determine CCβ using fortified concentration series. CCβ was determined as the lowest concentration for which 20 measurements (or more) give less than 5 percent false-negative results, so it would probably be more correct to state that CCβ was smaller than the established concentration (Table. 3).

OTHER VALIDATION CRITERIA

The other criteria for qualitative screening methods, specificity/selectivity, ruggedness, and stability, can be interpreted in many ways. Pikkemaat *et al.* (2008 & 2009a) determined specificity by analyzing high (2-5 times the MRL) concentrations of all residues on each of the test plates. Le Breton *et al.* (2007) claimed that a microbial inhibition tube test by definition was not specific, and only additional blank milk samples were tested. The same assay was validated according to the ISO/IDF 183 guideline as well (Stead *et al.*, 2008). Specificity was tested as the susceptibility to interfering substances (differing levels of fat, high somatic cell count, different species, *etc.*).

Table. 3 Performance characteristics that should be determined in method validation according to 2002/657/EC

Performance	Characteristics	Detection limit CCβ	Decision limit CCα	Trueness /recovery	Precision	Selectivity /specificity	Applicability / ruggedness / stability
Qualitative Methods	Screening	+	–	–	–	+	+
	Confirmatory	+	+	–	–	+	+
Quantitative Methods	Screening	+	–	–	+	+	+
	Confirmatory	+	+	+	+	+	+

CCβ is determined as the lowest concentration for which 20 measurements (or more) give less than 5 percent false-negative results, so it would probably be more correct to state that CCβ was smaller than the established concentration

CCα is decision limit of drug residues in matrix

Ruggedness was defined by Le Breton *et al.* (2007) as the reproducibility using different batches of tests, two analysts, different days and spikes from different standard solutions. Tests were found rugged under the assumption that the result was judged according to a positive control. More specifically determined ruggedness aspects concerned variation in application volume and incubation temperature (Stead *et al.*, 2008). Ruggedness can also be shown by successful inter laboratory assessment (Stead *et al.*, 2007).

Finally, the EC parameter stability, although in practice it was a very relevant aspect, forms a disputable demand. Since it was independent of the method used for the analysis, it cannot be considered a characteristic of a method. Assessing stability with a qualitative method was even more disputable, as the type of method implies that no absolute values can be assigned. Nevertheless Okerman *et al.* (2007) analyzed the stability of frozen stock solutions of several β-lactams, tetracyclines, and quinolones using a *B. subtilis* plate assay. Under the assumption that a reduction more than 25 percent was significant, it was concluded that tetracycline, oxytetracycline, ceftiofur, and cefapirin were stable for less than 6 months, while amoxicillin and penicillin already showed a significant reduction after 2 months. The stability of antibiotic residues may vary between matrices and results with other storage temperatures will also be relevant, as in practice samples were, for example, stored at 4°C for several days. This problem had been recognized by the CRL, who proposed that stability data can be extracted from other laboratories studies, performed with other analytical methods, because they do not depend on the method used for analysis (Gaudin *et al.*, 2007).

PROFICIENCY TESTING

Proficiency testing was another closely related quality control aspect, which was not in the 2002/657/EC criteria, but was prescribed in the earlier Commission Decision 98/179/EC, which states that approved laboratories must prove their competence by regular and successful participation in adequate proficiency testing schemes recognized or organized by the national or community reference laboratories (EC, 1997).

In contrast to other microbiological methods, currently there were no regular proficiency testing programs operational for microbial residue screening methods, while these were considered

highly necessary to reveal the inevitable shortcomings in this area. Proficiency testing was available for chemical analysis of antibiotics, but the samples used in these studies often combine several residues in one sample, which will yield an additional or even synergistic effect when analyzed with effect-based microbial methods. Moreover, the materials were often homogenized and therefore unsuitable for tests that operate on intact tissue (EC, 2002).

In 2005 the CRL organized a proficiency test among 22 laboratories of which 21 performed microbiological screening. Even though the residue concentrations in that study (195 µg/kg danofloxacin, 376 µg/kg tylosin, and a combination of 227 µg/kg oxytetracycline and 343 µg/kg sulfadimethoxine, along with two blank samples) were considerably above the MRLs for these compounds, only 13 laboratories correctly identified all three positive samples; additionally, five more laboratories produced false-positive results. This outcome may even be considered optimistic with respect to the situation in practice, since the laboratories involved were national reference laboratories, while in many countries the initial screening was delegated to routine field laboratories (Sanders *et al.*, 2006).

The review provides the developments in the field of microbial screening methods for antibiotic residues in slaughter animals since the early 1990s, when the establishment of MRLs at levels below the sensitivity of the established and generally applied methods reconsider the existing screening methods. Although the literature may show improved methods, the lack of validation data on incurred samples hampers an accurate evaluation of their true performance. It also remains difficult to get a clear picture of the extent to which improved methods have actually been implemented in practice. The results of a proficiency test organized among the EU national reference laboratories in 2005 showed that in an alarming number of laboratories the screening methods used were not sufficiently sensitive. The EU Standing Committee on the Food Chain and Animal Health produces a yearly report on the outcome of the national monitoring programs (EC, 2009). For B1 substances the percentage of noncompliant results remains rather stable, around 0.2-0.3 percent. Considering the shortcomings of the currently applied screening methods, this figure was likely to be a serious underestimation of the actual noncompliance rate. It should be noted, however, that these data also include results of additional control programs

for which the result of the microbial test was sufficient to reject the carcass. For some categories of animals these results represent over 50 percent of the total noncompliant results (EC, 2009).

The fact that different methods were used and also target organs differ makes it impossible to compare the results between countries. One could argue that the change from prescribing routine or reference methods to an approach in which performance criteria and procedures for the validation of detection methods were established (EC, 2002) had not made this easier. Moreover, despite the attempt to standardize validation procedures, still leaves a lot of room for interpretation and was not considered very suitable for microbial methods (EC, 2002).

Chemical methods generally were considered too specific and expensive to be applied as an initial screening. However, liquid chromatography–tandem mass spectrometry methods capable of simultaneous detection of multiple classes of antibiotics were increasingly becoming available (Schneider and Lehotay, 2008; Yamada *et al.*, 2006; Granelli and Branzell, 2006; Stolker *et al.*, 2008) and may in some situations represent a cost-effective alternative. It should certainly be considered feasible for use within a national reference laboratory, as, for example, was already effectuated in Sweden (Granelli and Branzell, 2006). However, in particular for those countries that rely upon a monitoring infrastructure including dozens of routine field laboratories, it can be concluded that there was still a strong need for the development and implementation of adequate microbial screening methods and more regular proficiency testing to reveal the shortcomings in the currently applied screening methods. It should be realized that these methods form the first line of defense in antibiotic residue monitoring, so it was essential to have accurate screening methods in place (Stolker *et al.*, 2008).

SOURCES OF DRUGS RESIDUES IN MEAT

Among the very common reasons of violation of the MRL drug residues was due to failure to observe withdrawal period of the particular drug (Paige, 1994). Another and the most common practice was that farmers were negligent of keeping record of the treated animals and the therapies used in the disease conditions. Failure to keep proper record of treatment or failure to identify treated animals frequently may lead to their omission (Sundlof, 1989). Rendering of the defecation material of the animals treated with medicines excreted in the feces and consequently

may pass in to the group of animals consuming the feed of recycled fecal materials (Bevill, 1984; McCaughey *et al.*, 1990). Extra label use of drugs and sub therapeutic dosage and use of drugs in those species for which the drug was not registered or approved by authorities may breach the way for development of drug residues in products of the animal's violative residues (Higgins *et al.*, 1999).

Neonates received milk or colostrum from the dams if the cow was receiving any therapy, the calf will receive the residues of that drug (Guest and Paige, 1991). Feedstuff may contaminate with the number of compounds passed in the animal origin foods. Unluckily, the dedicational contamination of animal feed considered being the huge source of ignored area of antimicrobial monitoring in the animal food chain. Drug residues in the treated animals may be present at stages or at various points in the production process, consequently contamination of residues remain in all batches of food of meal after these were presented as finished foods or feeds (Kennedy *et al.*, 2000).

DELETERIOUS EFFECTS OF RESIDUES

The function of living microflora in the intestines of human being was an aid to digestion, whereas residues of drug present in the food consumed by the human being the antimicrobial may kill, decrease or modifies the function of these microflora and consequently the physiological functions and digestion of the consumers (Nord and Edlund, 1990). Metabolic activity of microflora may be affected due to the functional or number change in the microflora population due to changes in microbiota population's change in function may occur (Perrin *et al.*, 2001). As the microflora was present in the intestine it provides a barrier due to colonization resistance, whereas the reduction of this resistance in the intestine may lead to the emergence of intestinal and systemic diseases. The presence of microflora in the intestine excludes the enteric pathogens on the basis of competitive exclusion. Due to the drug residues the colonization of the microbiota decreases which was a breach to the defense system and threat to the human health. Infected animals and human being shed the pathogen at high and faster rate for a longer period in comparison to those individuals, who were disease free and have normal non pathogenic flora (Van den and Stobberingh, 1999).

Hypersensitivity to any drug was always an immune system mediated response to a particular drug or chemicals exposure or ingestion by sensitive individuals. All drug allergic reactions were

illicit due to the Immunoglobulin E (Riedl and Casillas, 2003). Body took the antibacterials as non body foreign elements or compounds; weights of the compound at molecular level were too small to be considered by the immune system as immunogenic. When it combined with carrier proteins and macrophages, then immune cell considered them foreign particles and took them as immunogenic and illicit antibodies production (Dewdney *et al.,* 1991). Immunologic reactions may induce from life taking to the variable degree of anaphylactic reactions, such as rashes or urticaria. Drug allergic reaction may be started within 60 minutes were called acute reactions. Sub acutely reactions, usually takes around one day to several weeks. Acute allergic reactions and sometimes sub acute allergic response do occur, but all the times it was due to the type I allergy which was due to always IgE mediated reactions and, more rarely, due to IgG antibodies which was type II hypersensitivity. Hypersensitivity due to immune complex disorders was type III, whereas type IV called cellular response and develops relatively in slower order. Anaphylactic shock, asthma and angioneurotic oedema were the major types of disorder. Anaphylactic reaction was due to the hypersensitivity type angioneurotic oedema and the common symptoms may include difficult breathing as asthmatic condition. Hemolysis and anemia and decrease number of white blood cells as symptoms hypersensitivity type II. Serum sickness and allergic vesicle dermatitis may be seen in hypersensitivity type III. In hypersensitivity type IV only allergic skin inflammation was seen (Dayan, 1993; Riedl and Casillas, 2003).

Hypersensitivity may induce anaphylactic shock which end up to abrupt bronchospasm and constriction of the bronchi and bronchioles, most of it appeared as difficulty in breathing. Decreased blood pressure, possibly edema and rapidly progressing illness (Dayan, 1993). Drug residues of antibacterials may become the cause of adverse drug reaction in consumers of the contaminated foods. Antibacterial drug residues in previously hyposensitized individuals may elicit an allergic reaction. Primary sensitization may occur due to the minute quantities of the drug residues grasped by the immune in cases were responsible for liver injuries; caused by a cells and system. Sulfonamides, aminoglycosides and tetracyclines may also cause hypersensitivity (Paige *et al.,* 1997). Some of the macrolides have involved in allergic reaction that was their exceptional character specific allergic response to macrolides metabolite may induced pathological changes in the hepatocytes (Dewdney *et al.,* 1991). Although the residues in the animal origin foods were in abundance, but only a few cases of drug hypersensitivity have been indicated as a result of consumption of these drug residues through animal proteins (Raison *et al.,* 2001).

ACCEPTABLE DAILY INTAKE

Acceptable daily intake was a guess articulated in terms of body mass could be continually ingested over a life span devoid of considerable health danger (EC, 2001). The ADI methodology was formerly developed to elucidate consequences established on typical toxicology and it was applied to the outcomes of conventional toxicology investigations. The ADI (Table. 4) was established by dividing it with an appropriate safety factor, mostly 100, which suppose that there was a 10-fold range compassion among human inhabitants and that human beings were 10 times extra responsive than livestock (Woodward, 1998).

Different *in vitro* as well as *in vivo* techniques have been exploited to ascertain a microbiological ADI, for the assessment of consequences of drug residues on human gut microbiota. Transformations in microbiota could be evaluated by specific cultivation plus recognition of prevalent species by conventional microbial inhibition tests, by interpreting *enterobacteria* as a colonization resistance level mark, by assessing the biochemical events of microbial enzymes in human fecal specimens as well as by scrutinizing the vulnerability of inhabitant microbes to colonization by a confronting microbe. Although to ascertain the MIC by *in vitro* investigations was cheaper as well as easy but were infrequently descriptive of the pertinent microorganisms and ignore pH, hindrance and anaerobiosis like aspects (Cerniglia and Kotarski, 1999; Boisseau, 1993).

Exploitation of *in vitro* MIC statistics to determining a microbiological no effect level was acknowledged by US FDA. The human microbiota associated rodent pattern might have high significance in establishing the outcomes of minute doses of antibacterials on human microflora (Boisseau, 1993). For assessment of these upshots, an *in vitro* GIT stimulation model has also been found excellent (McConville *et al.*, 1995). Investigations in human volunteers facilitated the establishment of a no effect level in circumstances that very much imitate the conditions of operation. Human body weight, mass of gut contents and portion of ingested drug concentration presented for microbes as well as MIC50 were employed in equation used by CVMP. A model system exploited to ascertain the microbiological NOEL should estimate alterations in colonization resistance to probable pathogens plus assortment of antimicrobial resistant bacteria in addition to metabolic modifications of gut flora, in order to meet US FDA standards (Greenless, 2003).

Table. 4. Overview of EU MRLs (µg/ kg), established until 1 January 2009.

Pharmacologically active substance	Target tissues						ADI
	EU			USA			
	Muscle	Liver	Egg	Muscle	Liver	Egg	
Amoxicillin	50	50		50	0.01	0.01	0.01
Ampicillin	50	50		50	0.01	0.01	0.01
Penicillin V	50	50		50	0	0	0
Ceftiofur	1,000	2,000		1,000	30	30	30
Gentamicin	50	200		50	500	500	10
Spectinomycin	300	500		300	0.1	0.1	25
Chlortetracycline	100	300		100	2	2	25
Doxycycline	100	300		100	2	2	25
Oxytetracycline	100	300		100	2	2	25
Tetracycline	100	300		100	2	2	25
Erythromycin	200	200		200	0.125	0.125	0.125
Spiramycin	250	300		250	300	1.2	25
Tilmicosin	75	1000		75	1.2	1.2	25
Tylosin	100	100		100	0.2	0.2	0.2
Tiamulin	100	100		100	0.6	0.6	0.6
Lincomycin	100	500		100	NR	NR	25
Danofloxacin	200	400		200	0.2	0.2	2.4
Enrofloxacin	100	200		100	0.5	0.5	3
Flumequine	400	800		400	800	0.5	3
Oxolinic acid	100	150		100	0.2	0.2	2.4
Sarafloxacin	50	100		50	0.5	0.5	3
Trimethoprim	50	50		50	0.2	0.2	2.4
Baquiloprim	100	300		100	300	0.1	0.1
Chloramphenicol analog	NA	NA	NA	NA	NA	NA	NR
Florfenicol	100	2500		100	2500	10	10
Colistin	150	150		2	2	25	25
Sulfonamides	100	100		2	2	25	25

NA = Not allowed
NR = No residues

In the EU, microbiological based MRLs had been established for; tetracycline, chlortetracycline, oxytetracycline, doxycycline, enrofloxacin, cephalexin, erythromycin, gentamicin, novobiocin, kanamycin, lincomycin, pirlimycin, florfenicol, marbofloxacin, nafcillin, sarafloxacin and spiramycin (EMEA, 2004). ADI not footed on microbial effects were established only for some antibacterials; dihydrostreptomycin, streptomycin, neomycin, tiamulin, difloxacin and danofloxacin. For penicillins MRLs were based on the sensitivity of the organisms used in livestock industry and on immunological effects. Due to the difficulty to discover onset intensity for chloramphenicol in the stimulation of aplastic anaemia in human beings, the residue levels in this case matter critically. Because residues for some drugs, at whatever limits, in food from animal origin, comprise a potential hazard to consumer's health, no MRLs for such drugs could be set forth. Consequently, use of these drugs was banned for livestock production. For some drugs final MRLs have been recognized and substances for which it was not essential for public health safety to launch MRLs. Agents with interim MRLs were used in variety of treatments (EMEA, 2004).

Prior to presenting any agent for human use, a specific withdrawal period should be observed, to assure that residues have dropped to a harmless range following medication in animals. A withdrawal period determined was the time when the higher one sided acceptance limit with a specified confidence was beneath the MRLs. To evaluate the withdrawal time with a statistical means, information were frequently inadequate, for old therapeutic agents. Withdrawal period was the time where residues in all tissues of every experimental animal have fallen underneath the relevant MRLs. However, establishment of accurate withdrawal time for each animal was barely feasible with irritative formulations that intended for subcutaneous or intramuscular use (Nouws *et al.*, 1990).

It was necessary to check specified fraction of total yearly production of diverse foodstuffs of animal source for residues. The figure of specimens depends on the foodstuff manufactured throughout the following year for the animals killed. Veterinary therapeutic agents were screened for MRL observance. The directive determines the categories of agents to be restricted for foodstuff. For residues screening, specimen's assortment from a typical veterinary population was statistically randomized. Collection of samples from animals suspected to possess harmful residues was center of attention for inspection schedule (Dey *et al.*, 2003a; Sundlof *et al.*, 2000).

National residue program in Finland was established in conformity with European Union as well as national criteria. The specimens were collected from foodstuffs of animal origin as well as from live animals. Antibacterial residues in animal protein were also screened in meat inspection at slaughterhouses, in addition to national control program (MAF, 2001). Whenever there was a motivation to presume; information on medication of animal that a corpse might have drug residues; meat inspection involves microbial inhibition tests for antibacterial residues analysis. During the year 2003, a sum of 4422 alleged kidney specimens were screened with microbial inhibition tests in meat inspection and 5241 specimens according to national residue control program (NFA, 2004).

For huge level residues screening bioassays were appropriate due to their universal individuality and expediency (Haasnoot *et al.*, 1999). In a look for fast procedures for determining the relationship between organisms and antimicrobial agents, intermediates as well as final microbial metabolic products also the interaction among the organism and different energy basis have been investigated (Amsterdam, 1996). Although few methods were footed on growth inhibition in liquor media, only a minority of experiment were established on microbial inhibition tests had been described. Bioassays were unspecific, signifying just the presence of an inhibiting substance. Whereas physicochemical assays were quantitative as well as precise, but may be lengthy, if the antimicrobial to be investigated was of unknown identity prior to commence of test (Aureli *et al.*, 1996; Ferrini *et al.*, 1997).

METABOLITES AND MICROBIOLOGICAL ACTIVITY

A large un-metabolized quantity of oxytetracycline from the body was excreted in feaces as well as in bile (Riviere and Spoo, 2000). A small portion of tetracyclines was excreted in bile whereas major quantity eliminated unaffected in urine (Prescott, 2000). Cephalosporins were mostly eliminated by renal excretion whereas penicillins were almost entirely excreted in urine (Prescott, 200b, 2000c). A little amount of penicillin G was inactivated by β-lactam ring hydrolysis (Vaden and Riviere, 1995). Aminoglycosides were excreted unaffected in the urine (Sande and Mandell, 1980). Chloramphenicol was mostly eliminated as a glucuronic acid conjugate void of any microbial activity. Hence, treatment of specimens with an enzyme β-glucuronidase decrease the detection limits of chloramphenicol (Anadon, 1985). Majority of

veterinary therapeutic agent were converted in readily excrete able water soluble metabolites (Aerts *et al.*, 1995). Fluoroquinolones in elevated concentration were established in excretory organs (Walker, 2000). De-ethylation of enrofloxacin in the body mainly results in a metabolite ciprofloxacin with extraordinary microbiological activity (Anadon *et al.*, 1999).

Optimization of inoculums size was necessary as it directly affect the size of cidal zone (Cooper, 1972; Davis and Stout, 1971). An appropriate inoculums concentration produces well defined zone of inhibitions whereas very low concentrations inoculum results in rough and unsmooth cidal zone (Renard *et al.*, 1992; Davis and Stout, 1971). The inhibition zone produced in a bioassay was a result of competition between growing test organism and the spread out of the antimicrobial by diffusion. Inside the clear zone of the concentration of the antimicrobial was sufficient to prevent the growth of the test bacterium whereas outside the inhibition zone it was too low to inhibit growth (Davis and Stout, 1971). Diffusion rate of an antimicrobial drug through the agar gel depends on many factors *e.g.* incubation temperature, viscosity of the agar gel, molecular size and shape as well as concentration of the antimicrobial drug (Barry, 1976). In most of the cases the sensitivity of the bioassay was increased by pre incubation time which allows the antibacterial agent to diffuse out prior to the test bacterium growth (Koenen and De Beer, 1998). The width of inhibition zone can be recorded with a ruler or with a computerized image analysis system (Schoevers *et al.*, 1994). With sub inhibitory concentration of an antimicrobial drug, a little growth inhibition and eventual overgrowth of the test organism often results a zone of partial inhibition. Bacteriostatic drugs produce an inner zone of delayed growth (Barry, 1976). The positive functional groups on a drug molecule may be electrostatically bound to sulphate or acids groups of the agar, resulting in decreased diffusion through the agar (Acar and Goldstein, 1996).

METHODS OF DETECTION OF RESIDUES IN MEAT

A precise, susceptible and fast technique was worked out, for quantitative analysis of fluoroquinolones residues *e.g.* norfloxacin, enrofloxacin, ofloxacin and ciprofloxacin, from eggs along with edible animal tissues. Within 15 minutes and at ambient temperature, partition of residues was achieved on an Inertsil (250x4 mm) C8, 5 µm analytical column using a mixture of methanol-acetonitrile and (0.40 mol/l) citric acid (8:4;87 percent v/v). Ultraviolet recognition at

275 nm submitted limits of detection of 100 pg per 20 µl injected volume for norfloxacin as well as ciprofloxacin, 200 pg for enrofloxacin and 20 pg for ofloxacin. At enrichment levels of 40, 60 and 80 ng/g the recovery rates extend from 82.50 to 111.10 percent. The assay efficiency was ascertained by means of genuine specimens from chicks feed orally with fluoroquinolones antimicrobial agents (Samanidou *et al.*, 2005).

Using *B. subtilis* as test organism in BKT, doxycycline residues in porcine muscle and kidney tissues can be detected in concentration much less than the MRL (Croubles *et al.*, 1999; Koenen *et al.*, 1995). Comparatively chromatographic methods were extra sensitive than the microbiological STOP assessments for the identifications of oxytetracycline and chlortetracycline (Korsrud and MacNeil, 1987; Oka *et al.*, 1985; Neidert *et al.*, 1987). The stated LODs of four commercially available on-farm tests for penicillin G residues in bovine plasma were applicable also for incurred residues (Boison *et al.*, 1995). Modification and refinement of existing bacteriological methods were time demanded, as the results produced were not sensitive enough to identify the MRLs (Korsrud *et al.*, 1998). Even though microbiological inhibition tests were suitable for extensive screening, it was apparent that there was no test which detected only samples with the residue concentrations of all antimicrobials above the MRL and did not miss any of them and there will also be a number of positive test results for samples with concentrations below the MRL (Croubles *et al.*, 1999). Only a few microbiological inhibition tests were sensitive to sulfonamides (Braham *et al.*, 2001).

Microbiological inhibition tests were nonspecific as a number of substances can produce cidal zones on bioassay plates. Growth inhibition exhibited by substances other than the microbiological agents was termed as nonspecific whereas the result of such inhibition was named as false positive reaction. Nonspecific growth inhibition was commonly observed in bioassays. Semi synthetic antibiotics were developed from naturally occurring antibiotics with reduced animal toxicity and enhanced antimicrobial activity. Mechanical deterioration of feed unrelated compounds in frozen pig kidney samples may cause false positive reactions (Korkeala *et al.*, 1983). Lysozyme results false positive reactions against thermophillic spore forming gram positive bacteria (Beuchat and Golden, 1989). Microbial contamination in the media or sample can also result in false positive reactions (Okerman *et al.*, 1998b). Some bacterial processes may result in nonspecific growth inhibition such as meat chunk directly placed at bioassay plates may already possessing some natural inhibitory substances from earlier microbial activity within

the original specimen or posses an organism capable of imparting growth inhibition on bioassay plates during incubation (Smither *et al.*, 1980; Walton, 1983). Various bacterial strains produce bacteriocins which exhibits antimicrobial activity mainly against the same or other closely related species (Reeves, 1965). Nonspecific growth inhibition has also been attributed to Cadmium in horse kidneys and bile (Korkeala *et al.*, 1976; Wilson *et al.*, 1991). Addition of lysine in agar media results in reversible microbial growth inhibition (Watson and Bloom, 1952; Burger and Stahmann, 1952).

An association was also observed between changes in pH and false positive reactions (Tritschler *et al.*, 1987). The growth inhibition of *B. stearothermophilus* was observed cattle urine at pH 7.5 (Bielecka *et al.*, 1981). Inhibition of *B. subtilis* growth was found related with the high mean osmolarity and pH of urine specimens (Terhune and Upson, 1989). It was observed that although high urine pH of the specimens was correlated with the growth inhibition, actually bicarbonates were responsible for false positive reactions (Erasmuson *et al.*, 1998). Nonspecific growth inhibition was associated with certain matrix opted for assay. Contrary to muscle tissue in Charm Farm Test, no false positive reactions were observed with plasma as the test matrix (Korsrud *et al.*, 1995; Boison *et al.*, 1995). With the help of live animal swab test (LAST) false positive reactions were found associated with urine test matrix (USDA, 1983; Tritschler *et al.*, 1987; Seymour *et al.*, 1998). Specimens filtration though dialysis membrane results in separation of large molecular weight proteins from the smaller molecular weight antimicrobial molecules and this results in reduction of false positive reactions, specifically frozen horse and pig kidney specimens (Woodward and Shearer, 1995).

Although microbiological screening tests can reveal some information on the nature of the residue, a better post-screening characterization of the residue will significantly reduce efforts devoted to the identification and quantization by chemical methods. The use of a microbiological method in the post-screening stage has many advantages in terms of costs, practicability and sample throughput. Furthermore, simple or no extraction procedures were needed. Test organisms with different antimicrobial susceptibilities on media of different pH values were used in bioassays. Culture media could be supplemented with compounds enhancing or blocking the activity of some antimicrobial drugs. Antibiotics of β-lactams group were further classified in three subgroups by selective hydrolysis of β-lactam ring with enzymes *lactamase* II or *penase* TM (Medina *et al.*, 1998; Moats *et al.*, 1998).

Sulfonamides were structural analog of PABA and block the bacterial folic acid synthesis and the action of sulfonamides could be reversed with the addition of PABA (Brown, 1962). Cystein inhibits the antimicrobial activity of streptomycin (Greenstein and Winitz, 1961). Various blocking agents as well as selectively resistant bacteria could be used for identification of antimicrobial drugs. Distinction between fluoroquinolones and aminoglycosides in a specimen was difficult due to non availability of a test bacterium only resistant to fluoroquinolones. None of the microbial identification assays allow the identification of each antimicrobial when complex mixtures of drug residues were present in a specimen (Calderon *et al.*, 1996).

IMMUNOCHEMICAL ASSAYS

The ability of antibodies to bind specifically to different substances was the basis for immunochemical assays. Reversible antibodies-antigens complex formation involved weak coulomb forces, vander-waals forces as well as hydrogen bonding and hydrophobic binding (Martlbauer *et al.*, 1994). During these assays the analytes was brought into contact with the antibodies and addition of an amount of fluorescent labeled analytes or radio labeled which will compete with non-labeled analytes for available binding sites (Blake and Gould, 1984; Boison and MacNeil, 1995). In enzyme immunoassays (EIA), the enzyme substrate complex formation step was measured whether the enzymes involved in the reaction were fluorogenic or not (Boison and MacNeil, 1995). Although immunochemical methods can be employed for identification and detection of antimicrobial residues, they were rarely used during post screening for various reasons such as high cost and short shelf life of commercial EIA kits and very laborious specimen preparation step. Successful detection of β-lactams and some tetracycline from kidney specimens of porcine and bovine was achieved with solid phase extraction immunoassay (Okerman *et al.*, 2003). Immunobiosensor assays were employed as an alternative to enzyme immunoassay for drug residues screening. Use of an optical biosensor in rapid immunoassays was employed for detection of streptomycin in milk, honey and meat specimens and sulfonamides in pork muscle and bile samples (Ferguson *et al.*, 2002). The biochip comprises substrate on which separate test areas had been constructed, each representing a specific drug. Light signals emitted from each test areas were measured with the help of digital imaging technology to interpret the concentration of the drug residue (McConnell *et al.*, 2000).

In the presence of tetracycline visible blue light was emitted by the bacteria without addition of any cofactor or substrate *i.e.* the sensor was self luminescent (Chopra *et al.*, 1990). Bone

samples can be screened for tetracycline residues with a specific fluorescence assay (Buyeske *et al.*, 1960). Tetracycline residues in animal bone, kidney and meat specimens were detected by using a fluorimetric screening assay (Haagsma and Mengelers, 1989; Kuhne *et al.*, 2000). An antimicrobial drug binds to specific receptor sites present on the microbial cells. A radiolabelled analytes competes with the unlabelled drug residues for their specific receptor sites and the radioactivity of 14C or 3H of bound labeled drug was recorded with a liquid scintillation counter. Unbound labeled antimicrobial drug was washed out from the substrate before counting. Hence greater the amount of drug residues in the specimen, lesser will be the counts. The assay requires special precautionary measures and instrumentation (Nouws *et al.*, 1998). The assay can be employed for identification as well as detection of antimicrobial drug residues. A combination of microbial and chemical methods was described for the detection of tetracycline group. Screening of the residues with a bioassay was followed by extraction, purification and concentration of residues with HPLC. The net electrical charge on a molecule at a specific pH will determine the migration behavior of a majority of antimicrobial drugs. Smither and Vaughan, (1978) developed a method which separates antimicrobials electrophoretically followed by visualization with test bacteria. A reverse phase HPLC method was employed on an agar block taken from an inhibition zone produced on a bioassay plate for identification of antimicrobial residue (Kondo *et al.*, 1993).

QUANTITATIVE CONFIRMATORY ASSAYS

Gas chromatography, mass spectrometry, TLC and HPLC procedures were commonly employed in livestock drug residues surveillance (McCracken *et al.*, 2000). Removal of matrix constituents and other macromolecules, with specimen pretreatment was essential to protect and maintain the performance of the chromatographic system. GC and liquid chromatography-mass spectrometry methods were unsuitable for analysis of thermolabile and nonvolatile antimicrobial drugs. Polar, nonvolatile and sometime heat sensitive specimens were commonly analyzed by liquid chromatography (Kennedy *et al.*, 1998). The sensitivity of the assay based molecular spectrometry was enhanced with the development of coupled GC-MS method (Niessen and Tinke, 1995). No pretreatment except homogenization and dilution of the specimens was required which were subjected to purification by on line dialysis and solid phase extraction (Zurhelle *et al.*, 2000).

A combination of three plates seeded with *E. coli, M. luteus* or *B. sereus* strains could be employed for detection of residues of fluoroquinolones, β-lactams and tetracyclines groups. Each plate has optimal sensitivity for only one of these groups and has a specific pattern of inhibition typical for each antibacterial class and resulting in detection range below than the prescribed MRLs. Selection of appropriate chromatographic techniques for further identification and quantification of the residues was facilitated by preliminary screening with three plate assay. Several antimicrobial drugs belonging to tetracyclines, β-lactam and fluoroquinolones groups have been detected on a single bioassay plate seeded with *B. subtilis*, although the detection limits were higher; penicillin G 0.40 ng, amoxicillin and ampicillin 3.00 ng; doxycycline 1.00 ng, chlortetracycline 0.50 ng, tetracycline 5.00 ng, oxytetracycline 8.00 ng, ciprofloxacin 10.00 ng, enrofloxacin 4.00 ng and flumequine 4.00 ng. The assay was applied to 27 turkey thighs and 228 broiler fillets specimens collected from different poultry slaughterhouses. Nineteen broiler fillets found positive for antimicrobial activity were further confirmed with a chromatographic method (Okerman *et al.*, 2001).

RESISTANCE

Benefits and risks imposed on human health associated with antimicrobial use in livestock were major public concerns. Whereas, for veterinarians and livestock producers major concerns were favorable and unfavorable effects of antimicrobials on livestock health and the consequences of limited range of registered antimicrobials available in market. In livestock farming antimicrobial drugs were extensively used sub-therapeutically as well as therapeutically. Presence of antimicrobial residues in food chain and emergence of resistant strains of pathogen were the most important concerns among the stakeholders. It was believed that in near future, peoples from other particularly those related to genetic engineering technology would join in these issues (Sasanya *et al.*, 2005).

E. coli strains resistant to antimicrobials were frequently present in the gut microflora of poultry. Because numerous of these antimicrobial resistant *E. coli* strains were good colonizers of the gut, these strains persist long subsequent to the withdrawal of antimicrobials. Even if there was no indication that *E. coli* of animal origin cause disease incidents in man, they comprise a very much multitalented pool of transferable drug resistance (R-plasmid), which may be transmitted eventually to some infection causing microbes. Safety was an important requirement for any flock

health program. Use of any drug was only acceptable if it causes fewer troubles than it resolve. Protection concepts apply to both human being and chicks (Caldwell *et al.*, 2000).

Several fluoroquinolones were available for treatment of animals, poultry and fish in many countries in the world. Available data indicate that they were also used for disease prevention in some regions. Quinolones usage in poultry has increased in quinolones resistance in food borne pathogens and human treatment problems. Quinolones excessive use in animals was creating resistance in animal bacteria (Terry, 1994).

The outcome of another study of 1203 *E. coli* isolates from 44 hospitals in Taiwan discovered 21.70 percent isolates had condensed susceptibility moreover a further 11.30 percent isolates were resistant to fluoroquinolones. Majority of the *E. coli* isolates accountable for hospital acquired infections were established resistant. Main selective pressures appeared to be more accountable for diminished sensitivity to fluoroquinolones in *E. coli* was due to severe as well as persistent fluoroquinolones use in cancer patients (McDonald *et al.*, 2001). A large proportion of the estimated 23-25 million poultry and their products in Uganda contribute an important source of human food to the country. Therefore, control of animal diseases depends heavily on the use of vaccines as well as antimicrobials and about 10 percent of these imported drugs into Uganda were sulfonamides. Sulfonamides such as sulfadiazine, sulfaquinoxaline, sulfamethoxypyridazine and sulfamethazine have been widely used in livestock production (Tarbin *et al.*, 1999). Moreover, irrational antimicrobial usage may leads to emergence of antimicrobial resistance and a very common case of drug resistant pathogen that can infect humans was *Salmonella*, responsible for epidemics of salmonellosis (Threlfall *et al.*, 1994).

Low and extended dosage of antibacterial will influence the sensitivity of the drug and accordingly the emergence of resistance in the pathogen may occur (Tancrede and Barakat, 1989). A close association among the resistant microorganisms isolated from the specimens and chloramphenicol, streptomycin, gentamicin plus tetracycline residues was established. Existence of small levels of antibacterials may exercise an encouraging stress towards the selection as well as appearance of resistance within microorganisms inhabits live stock tissues. Antimicrobial drugs in small doses were expected to modify intestinal enzyme activity, moreover have consequences on certain hormones plus therapeutic agents (Gorbach, 1993).

Antimicrobial drugs; ampicillin, fluoroquinolones and chloramphenicol were usually included in *Salmonella enterica var. typhimurium* treatment regime. It was also reported that appearance of fluoroquinolones resistance among a previously multi drug resistant strain of *Salmonella enterica var. typhimurium* DT 104 and accordingly some infections caused by this pathogen were not easy to treat (Molbak *et al.*, 1999). It would be complicated to treat human infections caused by these challenging pathogens. Ground beef contaminated with multi drug resistant strain of *Salmonella enterica var. typhimurium* DT 104 was reported to be associated with a multi state epidemic (Dechet *et al.*, 2006).

Antibiotic resistance in *E. coli* was widespread globally and penicillin group was established loosing its susceptibility against *E. coli*. Danger of gratification by elevated rates of mutation in *E. coli* O157, subsequent interpretation that they could attain resistance determinants simply by parallel gene transmit has been observed. It was eminent that this was a probable path by means of which antibiotic resistance, from a group of ecological pathogens, might be conferred. Although antimicrobial growth supporter were indirectly besieged against the microorganisms, it remains probable that strains of this bacterium may attain resistance from the gastrointestinal flora of the livestock (Heritage *et al.*, 2001).

Vancomycin resistant *enterococci* isolated from the human and chick feaces were opted for molecular genetic study. Transmission of resistance against glycopeptides from domestic animals to human being was established associated with Tn1546 transposon (McDonald *et al.*, 1997). Van den *et al.* (1997), adopted same procedure to investigate the occurrence of vancomycin resistant *Enterococcus faecium* in feaces from turkeys as well as their farmers. They revealed that isolates from 2.13 percent turkey farms were found matching with isolates from their farmers. The genetic resemblance between human and turkey isolates was also confirmed with pulsed field gel electrophoretic investigation and polymerase chain reaction analysis, on *van* A and other cassettes.

During a study in Brazil, 91 *Salmonella enteritidis* isolates from human food, broiler carcass and chick related specimens were antibiographed. A large fraction of resistant strains, 90.10 percent was established resistant to at least one antimicrobial agent. High resistance 75.80 and 52.80 percent was found against sulfonamides and nitrofurantoin respectively. Whereas lesser levels of

resistance; 15.40 percent for tetracycline, 7.70 percent for nalidixic acid and streptomycin; 5.50 percent gentamicin, 3.30 percent for trimethoprim and norfloxacin; 2.20 percent for cefalotin and 1.10 percent for ampicillin were established, whilst non of the isolate was found resistant against ciprofloxacin. Multi drug resistance in eighteen different patterns was observed among 51.60 percent isolates of *Salmonella enteritidis*. Maximum strains isolated from poultry associated specimens were found resistant, where 100 percent strains were resistant to at least one antimicrobial drug. Phage type isolates were not interrelated to any leading resistance model. The high prevalence of antibacterial resistant *Salmonella enteritidis* in Southern Brazil signifies the requirement for sensible drug use, to circumvent emergence of antimicrobial resistance (De Oliveira *et al.*, 2005).

There may be a possibility but not a decisive sureness, that persistent use of antimicrobial drugs both to manage sickness or for growth improvement will result in selection of gut microflora in birds. Such heritable resistance was rapidly and plainly transmitted among different microbial species. Consumption of poultry eggs even contaminated with non pathogenic resistant microorganism may possibly pass on there resistance to a pathogenic bacteria of human gut microflora earlier to its revelation to a particular antimicrobial. Drug residues in foodstuff of livestock origin, could bring about selection of resistant microbes contained by the gut of consumers. This may perhaps continue in maintain residents of resistant germs although the individual have not intentionally been exposed to the antimicrobial (Cruchaga *et al.*, 1999).

Animal feed was a watch dog for the human health safety starting from farm ending at fork. Emergence of rations of pathogen has provided food for thought in the area of contamination transferred from feed to animal and consequently to the human being. Microbes contaminated animal feed was a classical vector of transfer of zoonotic diseases. In USA, animal feeds were most of the time contaminated with the non *typhi Salmonella* called *Salmonella enterica* lead to the infection and carrier stage in human beings after colonization in the gut. The infected animals contaminate the meat at slaughter house or during the transportation and spread the infection to the human beings after semi cooked or pseudo contamination. Ultimate traceability of source was very tedious work; however several out breaks were blamed to contaminated feed fed to the poultry and livestock herds (Crump *et al.*, 2002).

Retail meat was frequently established polluted with *enterococci* resistant to antimicrobial drugs, but constant intestinal carriage of these ingested contaminants was not apparent. In a randomized, double blind investigation in 18 healthy volunteers was carried out. Three groups of six volunteers were feed with a mixture of 10^7 cfu of two strains of *Enterococcus faecium* resistant to glycopeptide obtained from poultry purchased at a grocery store, 10^7 cfu of *E. faecium* strain resistant to streptogramin obtain from a pig at slaughter and 10^7 cfu of *E. faecium* strain vulnerable to both glycopeptide and streptogramin, attained from poultry acquired at a grocery store, respectively. The preparation of 250 ml suspensions in whole milk was well in the prescribed rang of Danish food regulations. Stool samples were examined for one week before and after ingestion as well as at 14 and 35 days. Specific culture techniques were employed to identify resistant *enterococci* in stool samples (White *et al.*, 2001).

During the year 1995 to 1996, fluoroquinolones resistance among 309 *Salmonella enterica* serotype *typhimurium* strains isolated from cattle, pigs, poultry and human sources were studied. Resistance against nalidixic acid, during 1995 to 1996, augmented from 8.50 to 18.50 percent. While the susceptibility statistics of drug resistant strains of *Salmonella enterica* serotype *typhimurium* have evidently indicated that 27.00 percent resistance intensity existing during 1987 amplified up to 52.40 percent in 1996. This situation reflects the irrational antimicrobial use and was demanding attention of public health establishment (Gross *et al.*, 1998).

Increasing figure of macrolides in addition to fluoroquinolones resistant *Campylobacter* strains throughout the last decade in numerous parts of world resulted in a corresponding boost in human *Campylobacter coli* and *C. jejuni* infection cases. All through this investigation frequency of fluoroquinolones and macrolides resistance in *Campylobacter* amongst human clinical isolates were evaluated and compared with their use in food animals. Susceptibility statistics advocate that owing to the appearance of resistant *Campylobacter* strains in several part of the world, task of fluoroquinolones in management of infection was greatly reduced, while erythromycin as well as other macrolides ought to stay the drug of choice by observing suitable supervision and control procedures. *C. coli* and *C. jejuni* were acknowledged as topmost GIT infection causing pathogen throughout the world since late 1970s and annually about one percent United States inhabitants were affected by these infections (Tauxe *et al.*, 1992). Polluted groceries were typical source of human infestations; accordingly, the endurance of macrolides and fluoroquinolones resistant bacteria in animal origin protein has enhanced

distress that management of human sickness will be negotiated. Normally, *C. enteritis* management with antimicrobial drugs was not mandatory because it was self-limiting; in addition to clinically mild moreover, its extent was short. On the other hand, a considerable fraction of these infections have necessitated therapy, these take account of brutal and delayed cases of septicemia, enteritis in addition to extra intestinal infections. *C. enteritis* was very frequently treated with erythromycin (Blaser *et al.*, 1995; Dryden *et al.*, 1996).

In the 1980, the initiation of a new move toward antimicrobial involvement over all the range of bacterial enteritis causing pathogens was vulnerable to fluoroquinolones. Originally thermophillic *Campylobacter spp.*, plus members of family *Enterobacteriaceae* were susceptible to fluoroquinolones (Wistrom and Norrby, 1995).

As macrolides and fluoroquinolones were usually promoted as first and second line antimicrobials intended for management of *C. enteritis*, development of multi drug resistance against these drugs was of immense concern. Supplementary resistance to additional appropriate remedial agents poses a menace whilst there was no valuable antimicrobial course of therapy intended for *Campylobacter* contamination. In recent times, it was established throughout two years supervision that entire Thai isolates were concomitantly resistant to ciprofloxacin and azithromycin (Hoge *et al.*, 1998). Additionally, the intensity of ampicillin and tetracycline resistance in Thailand was so elevated that these drugs at the moment comprise no position in the management of non cholera diarrhea plus *Campylobacter* infection (Hoge *et al.*, 1998). Simultaneous resistance tariffs; 97.00 percent tetracycline, 66.00 percent ciprofloxacin, 12.00 percent clindamycin and erythromycin moreover 2.00 percent gentamicin be presumed amongst children infected with nalidixic acid resistant *C. jejuni* (Li *et al.*, 1998). About 90.00 percent of human erythromycin resistant *C. coli* isolates likewise whole of the *C. jejuni* isolates were concomitantly resistant to clindamycin (Engberg *et al.*, 2001).

In United States, multi drug resistant *typhimurium* DT104 has happened to be a most important ailment causing organism. More efficient precautionary dealings on farm and sensible exercise of antimicrobial drugs in domestic animals were essential to shrink the spread of multi drug resistant *typhimurium* DT104 and by this way the appearance of resistance to other drugs in *Salmonella* may perhaps be restricted (Glynn *et al.*, 1998).

In Pakistan during the period from December 2007 to August 2008, the antimicrobial resistance pattern was studied for MRSA and MRSE isolated from nasal samples from patients admitted in medical and surgical intense care units. The study was conducted on 283 isolates. The results depicted that 25 percent isolates of *S. aurreus* and 29.78 percent isolates of *S. epidermidis* were multidrug-resistant especially to methicillin. The resistance rate of MRSA to various antimicrobials was found to be as follow ciprofloxacin (80%), levofloxacin (75%), tetracycline (49%) and teicoplanin 3 percent (Yameen *et al.*, 2010).

The sufficient and necessary genes for the expression of high vancomycin resistance (vanA phenotype) are present in the transposon designated Tn1546, but the so-called Tn1546-like elements or vanA elements which are often carried by conjugative plasmids are produced by several modification in different positions of the transposon causing their polymorphism. As a member of the Tn3 family, Tn1546 has a preference for insertion into plasmid DNA, but this mobile element has also been identified in chromosomal DNA. Other member of this family has shown replicative transposition. Cetinkaya *et al.* (2000), confirmed the type of vancomycin-resistance (vanA, vanB, vanC, -C2 or -C3) by polymerase chain reaction.

This study aims at development of local data of macrolides, fluoroquinolones and sulfonamides residues in poultry meat and eggs. The objectives of these investigations were to monitor the flow of residues in poultry meat and eggs and consequently the bacterial resistance in human pathogens. Establishment of screening of positive samples, standardization of extraction and testing procedures will follow identification and quantification of macrolides, fluoroquinolones and sulfonamides residues in poultry meat and eggs. Testing methods of tylosin tartrate, erythromycin thiocyanate, tilmicosin, norfloxacin, enrofloxacin, ciprofloxacin, sarafloxacin, flumequine, sulfadiazine, sulfaquinoxaline, sulfachlorpyridazine and sulfadimidine will be employed microbiologically, chemically and spectrophotometrically. Drug resistance was menace of drug residues, an aspect generally ignored by the technical personnels and farmers needs due consideration. Final goal of the study was to furnish preliminary local data for legislation regarding the withdrawal periods and MRLs.

MATERIALS AND METHODS

CHAPTER 3

MATERIALS AND METHODS

Poultry industry is a big source of white meat in Pakistan, which is not only fulfilling the local demand but also planning to export the poultry meat to Middle East and other courtiers. Currently in Pakistan there is no regulation regarding the drug residues in poultry meat and eggs. It is time demand to monitor the poultry meat and eggs for the presence of antimicrobial drug residues. Therefore, drug residues surveillance studies were conducted to monitor the frequency of more common drug residues such as macrolides, fluoroquinolones and sulfonamides in poultry meat and eggs. Screening for the positive samples was conducted. Standardization of extraction and testing procedures was followed by identification and quantification of macrolides, fluoroquinolones and sulfonamides residues in poultry meat and eggs. Testing methods of tylosin, erythromycin, tilmicosin, enrofloxacin, flumequine, ciprofloxacin, norfloxacin, sarafloxacin, sulfadiazine, sulfaquinoxaline, sulfachlorpyridazine and sulfadimidine were employed microbiologically, chemically and spectrophotometrically. Residues testing methods were developed for the quantification of each antibacterial residues status in general and macrolides, fluoroquinolones and sulfonamides in particular included in the study. The surveillance gave the status of residues in poultry meat and eggs. The microbial resistance was antibiographed temporally in poultry and human pathogens. The current surveillance will provide the ground realities to understand the deleterious effects of the residues and consequently the emergence of multi drug resistance.

SAMPLES COLLECTION

During the period of three years (2006 to 2008) a total of 21383 samples were collected for the screening of residues of macrolides, fluoroquinolones and sulfonamides. Samples of meat comprised of one lobe of liver, 50 g of breast meat and 20 g bone marrow in case of broilers and 3 eggs in case of layers per source were collected. A total of 7025 samples of poultry meat and eggs were collected during the year 2006, followed by 7128 samples during the year 2007 and 7230 samples were collected during the year 2008. Among total 21383 samples of poultry meat and eggs, 9153 samples were of liver, 7827 of poultry meat, 786 of bone marrow and 3617 of

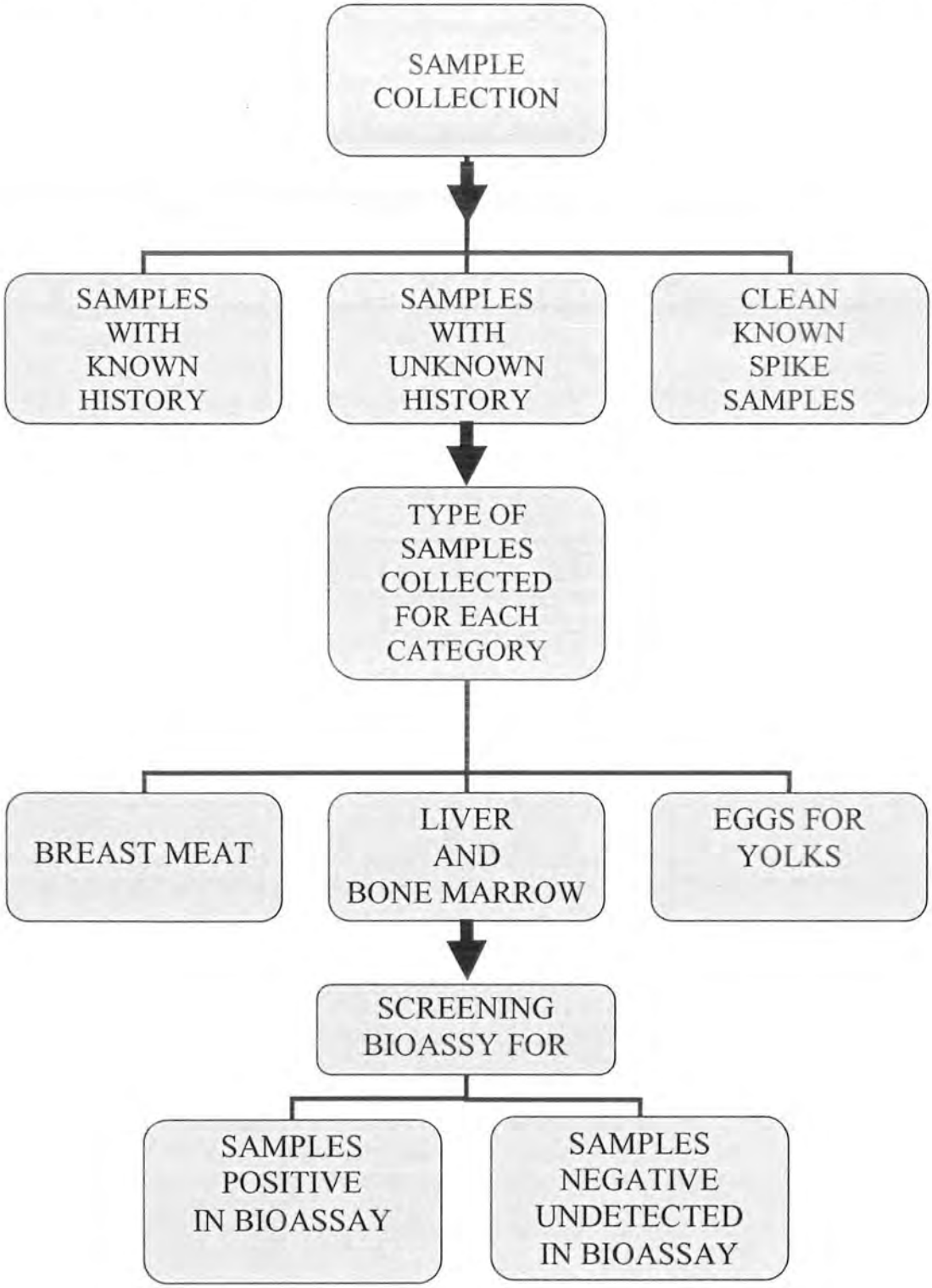
egg yolks. During the year 2006 a total of 7025 samples were collected including 3007 of liver, 2571 of poultry meat, 258 of bone marrow and 1189 of egg yolks. During the year 2007 liver comprised of 3052 samples, followed by poultry meat (2610), bone marrow (262) and 1204 samples of egg yolks. A total of 7230 samples were collected in year 2008 detailed as 3094 of liver, 2646 of poultry meat, 266 of bone marrow and 1224 of egg yolks. The samples of poultry meat, liver, bone marrow and eggs from market with unknown history of medication and samples from poultry farms with known history were collected in and around Islamabad and Rawalpindi Division of Pakistan.

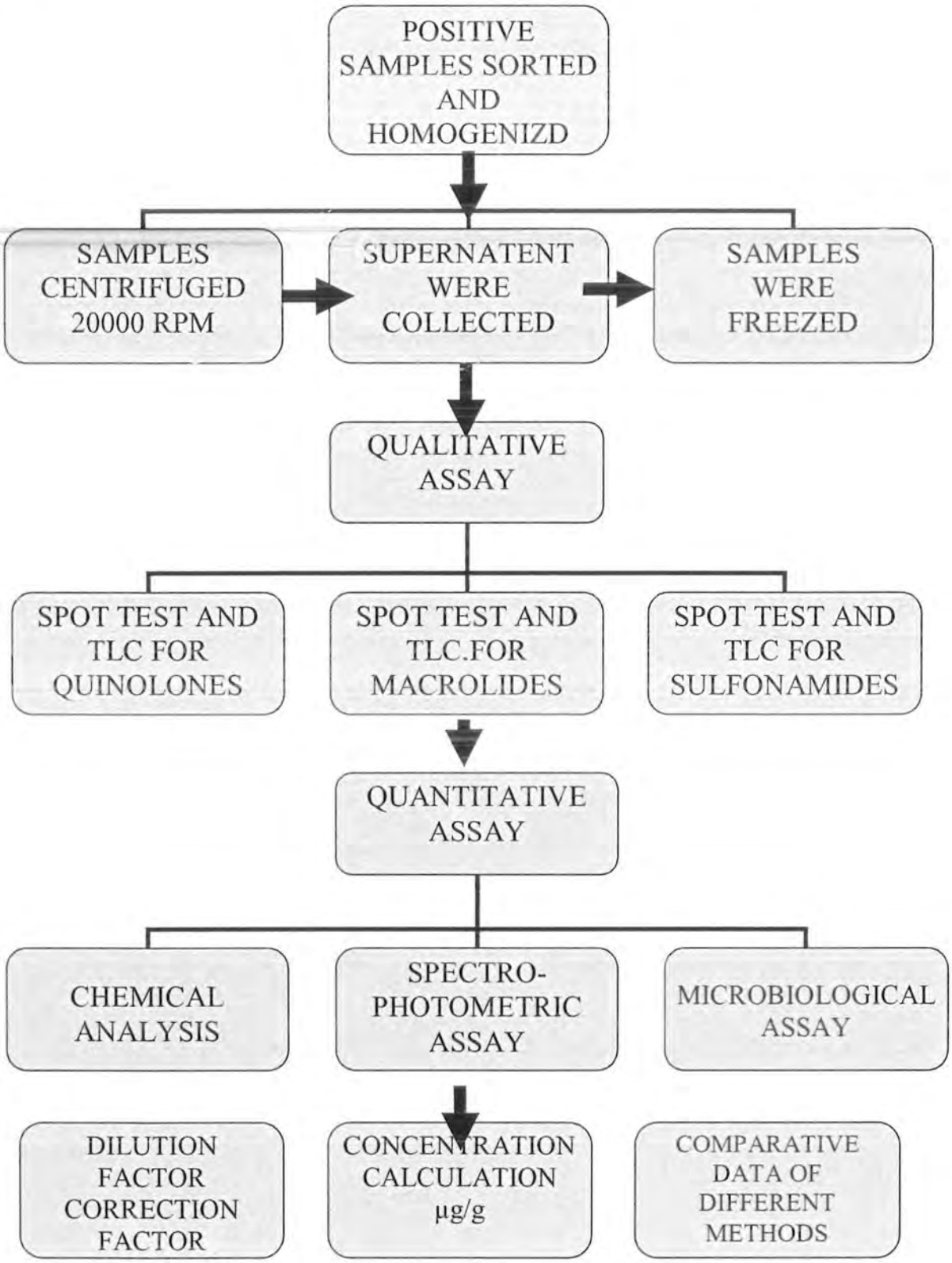
Samples were labeled and sealed with thermal sealer in sterilized polyethylene bags and stored in deep freezer before preparation. Poultry meat, bone marrow, liver and egg yolks were studied as representative parts of the edibles. Lungs, kidneys, intestine and spleen were of no importance as far as the consumption was concerned. Considering the eating habit of people the poultry meat, liver and egg yolks were included in the studies and as a fairly high percentage of people chewed the bone of chicken or used in soup, therefore, residues levels in the bone marrow were also studied. Samples were screened for residues by microbiological assay. Positive samples with antimicrobial activity were subjected to confirmation / identification through spot tests and TLC tests of macrolides, fluoroquinolones and sulfonamides. Among these samples tylosin tartrate (T), erythromycin thiocyanate (E), tilmicosin (O), norfloxacin (U), enrofloxacin (V), ciprofloxacin (X), sarafloxacin (Y), flumequine (Z), sulfadiazine (D), sulfaquinoxaline (Q), sulfachlorpyridazine (F) and sulfadimidine (A) were grouped respectively. Quantification of the residues was conducted microbiologically, spectrophotometrically and chemically (Fig. 1).

INSTRUMENTS

Centrifuge	Kokusan, Model H-251, Tokyo Japan
Centrifuge	Martin Christ Osterode, Harz, Germany
Homogenizer	National Co Japan
ICU	ICU-1 AFOS Manor Estate Hull England
Laminar flow	Flow Labs Lakewood CA, USA
Incubator	Webeco=Bed Schwartau, W Germany
Autoclave	Webeco=Bed Schwartau, W Germany
Cold Cabinet	Carnawell, Topicalized M-350 Pakistan

Fig. 1. Flow diagram showing procedure of MCSTAR of poultry meat and eggs.




POSITIVE SAMPLES SORTED AND HOMOGENIZD
SAMPLES CENTRIFUGED 20000 RPM
SUPERNATENT WERE COLLECTED
SAMPLES WERE FREEZED
QUALITATIVE ASSAY
SPOT TEST AND TLC FOR QUINOLONES
SPOT TEST AND TLC.FOR MACROLIDES
SPOT TEST AND TLC FOR SULFONAMIDES
QUANTITATIVE ASSAY
CHEMICAL ANALYSIS
SPECTRO-PHOTOMETRIC ASSAY
MICROBIOLOGICAL ASSAY
DILUTION FACTOR CORRECTION FACTOR
CONCENTRATION CALCULATION µg/g
COMPARATIVE DATA OF DIFFERENT METHODS

Deep freezer	Waves Triplet Wt118 Lahore, Pakistan
UV Spectronic 2000	Spectum UV-Visible SP-2000 UV China
Hot Air Oven	Webeco=Bed Schwartau, W Germany
Glass sterilizer	Sakura UI 50 Tokyo Japan
Micropipettes	Falcon-Beckton Dikinson California
Disc cutter	ECM Illinois, USA
Disc Vicks	Difco Kansas USA
Glass slides	Sail Brand Cat NO 7101 China
Reagents	Merck KGm A 64271 Barm Germany
Media	Difco Kansas USA
Digital vernier	Shanghai, China
Petri dishes	Pyrex France
Test tubes	ICN
pH meter	R-37 Hanna Instruments Portugal
Cuvettes	China
Pipettes	Pyrex France

CHEMICALS

Beef extract	Difco Kansas USA
Peptone	Difco Kansas USA
Agar	Difco Kansas USA
Deionized Distilled water	MediExcel Pharma Islamabad, Pakistan
Chloranilic acid	BDH
Hexane	BDH
Iodine	BDH
Ammonia	Merck
Silica gel G	Merck
Sulphuric acid	May and Baker
Chloroform	May and Baker

GLASSWARE

Media flasks	Pyrex France
TLC plates	Merck KGm 64271 Barm Germany
TLC tank	Ghani Glass Rwp, Pakistan

REFERENCE STANDARDS OF ANTIBACTERIALS

Jiangso Zhengzong Biochemistry Jiangsu, China

MICROBIOLOGICAL ASSAY

Method MCSTAR (Microbiological and Chemical Screening Test for Antimicrobial Residues) was calibrated, Minimum quantity of the reference standard produced an 8 mm zone of inhibition was the minimum inhibitory concentration (MIC) of respective antibacterial included in the study (Johnson and Case, 1995). The MIC of each microbiological assay defines detection limits for the particular antibacterial drug residues. Non pathogen with known biosafety level I and II were used for the microbiological bioassays. Using infection control unit the safest environment protection was employed with test bacteria. Precautions for personal protection was attained while using biohazard chemicals and test organisms by wearing appropriate safety clothes, latex gloves, face shields and eye goggles in practice. Infection control unit was employed to avoid the environment contamination. All glassware utilities were cleaned rinsed with deionized double distilled autoclaved water and sterilized in autoclave (Webeco) and dry heat oven. Spreaders were carefully cleaned to avoid organic, antibacterial or chemical contamination. The entire test media were prepared according to the direction of the company (Difco) and tested on the lines of quality control working manual, as mentioned in the standard operational manual (Shafique, 2007).

MEDIA

Two percent nutrient media was selected for organism, used in the studies were prepared by dissolving 20 g in 1000 ml of deionized double distilled water of the following media,

Beef extract	3 g
Peptone or Gelysate	5 g
Agar	15 g
Deionized Distilled water	1000 ml
pH of media	6.8 ± 0.1

Ingredients were dissolved at light heat and were autoclaved in wrapped flasks. Ten ml of the basic media, at 50 °C, was poured into petri dishes and left to cool and solidify. Media was poured in petri dishes in front of flame under laminar flow hood to avoid contamination. Solidified dishes were put into the incubator for 24 hours to get contamination free petri dishes for the testing of the respective antibacterial residues (Yasir, 2006).

CULTURE USED FOR THE MICROBIOLOGICAL ASSAY

The following test organisms were used for the microbiological assay according to the sensitivity of the organism to the antibacterial under investigation.

Bacillus subtilis ATCC 6633
Bacillus cereus ATCC 11778
B. stearothermophilus ATCC 12980
Escherichia coli ATCC 25922

The cultures have specificity of sensitivity to different antimicrobials to facilitate the screening on broader scale. Culture identity and purity were verified of *Bacillus subtilis* ATCC 6633 (ME) spores. Respective master seeds were inoculated in the nutrient broth and incubated 18 hours before using in the test. Optical density (OD) of 18 hours fresh broth was standardized in the spectrophotometer, using the calibrating reference ampoule of *Bacillus subtilis* ATCC 6633 and *Bacillus cereus* ATCC 11778. The 18 hours broth culture (100 µl) serially diluted and streaked on the nutrient media and incubated in the incubator for 24 hours. Colonies were counted under colony counter to establish the cell count in the known volume. The dilution streaked in the bioassay contained at least $1x10^6$ cfu/ml (Javed, 1992).

OPTIMIZATION OF BIOASSAY

Standard curve was plotted by establishing bioassay of set of antibacterial dilutions of macrolides, fluoroquinolones and sulfonamides. Standard curve was base line used for calculation and computation of the unknown quantities of antimicrobials residues present in the sample bioassayed. On fixed cfu a clear zone of inhibition developed was a minimum concentration of each standard curve dilution was considered a specific zone for the specific standard concentration. Adjustments were made to get uniform growth of bacterial and clear zone of inhibition for optimization of bioassay results. To determine the specific cfu of spores fixed for the bioassay plate was determine by using the following equation:

$$X = (TV)/C$$

Where:

X = quantity of fresh culture suspension spreaded on agar (ml)

T = cfu per plate

V = quantity of agar per plate

C = concentration of cfu per plate

For the lowest concentration of the specific antibacterial in bioassay the minimum 8 mm zone was considered positive indicator for the lowest activity of antibacterial residues. More than 8 mm zone of inhibition (S) was considered as positive whereas less than 8 mm or no zone of inhibition (R) was considered as negative (Johnson and Case, 1995).

STANDARD CURVES

For each antibacterial five concentrations were used to develop individual standard curve. Third concentration was assigned antibacterial standard reference concentration. Multiple standard reference concentration (SRC) was placed on petri dishes along with unknown samples for quantitative analysis of residues. Sterilized blank ID marked discs were placed on the surface of the streaked media. Discs were placed moving clockwise from the starting disc alternative three discs be impregnated with 40 µl of the buffered sample extract. Diameters of the zones of inhibition were read using digital caliper. Inhibition zone measuring and reading was started from the first disc from marked radial line. Diameters of each zone of inhibition were recorded for all antimicrobials concentrations on each bioassay plate. Data was recorded to achieve for the correction and averaging of inhibition zones. Zones were measured against the standards using curve. Each antibacterial dilution value was calculated from a set of three bioassay plates. Cumulative average zones for all SR concentrations on the entire set of three bioassay plates were computed.

DETECTION OF RESIDUES

Using cork borer all the samples were bored and chunk of tissues and drop of egg yolks were placed on inoculated plates for preliminary screening of antibacterial activity (Fig. 2, 3, 4 & 5). Samples showed cidal zone were processed for identification and quantification. Twenty gram of tissue homogenate of meat, liver, bone marrow and egg yolks were taken and diluted with 20 ml of phosphate buffer having pH 7.2. The mixture was stirred for 15 minutes using magnetic stirrer. Four gram of the mixture was taken in sample tube and centrifuged at 20000 rpm for 15 minutes. The final concentration of tissue/egg yolks was 500 mg/ml. Approximately 2 ml of each supernatant was transferred to test tube and extracts were stored in freezer for testing. Spiked tissues were prepared in order to validate assay (Fig. 6 & 7).

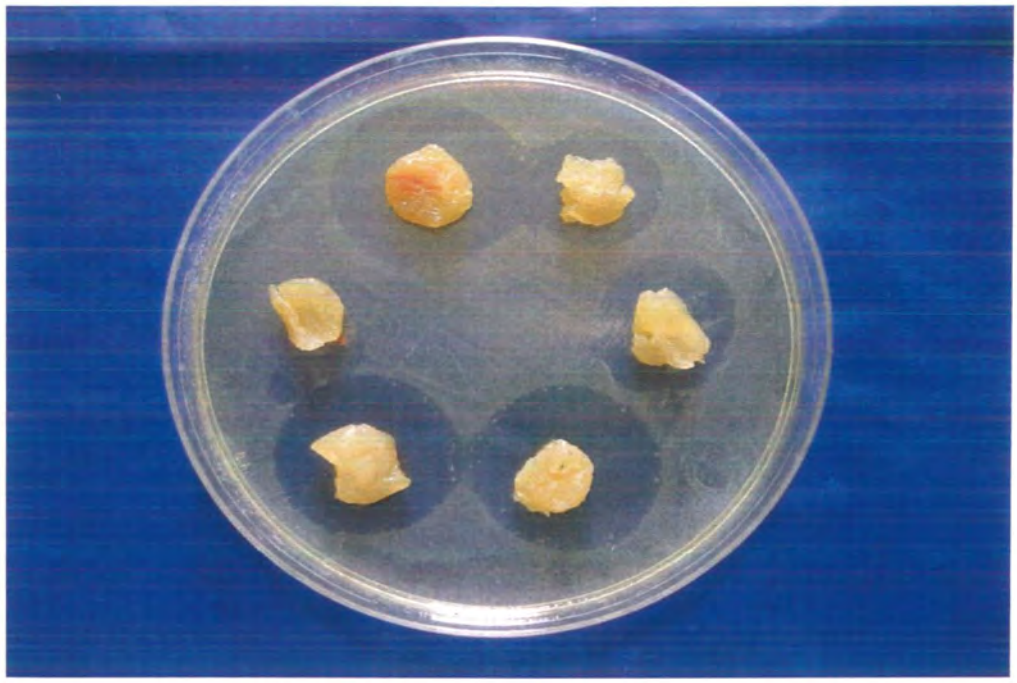


Fig. 2. Showing microbicidal zone around the sample of poultry breast meat indicating presence of antimicrobial drug residues on nutrient agar media and *Bacillus cereus* ATCC 11778.

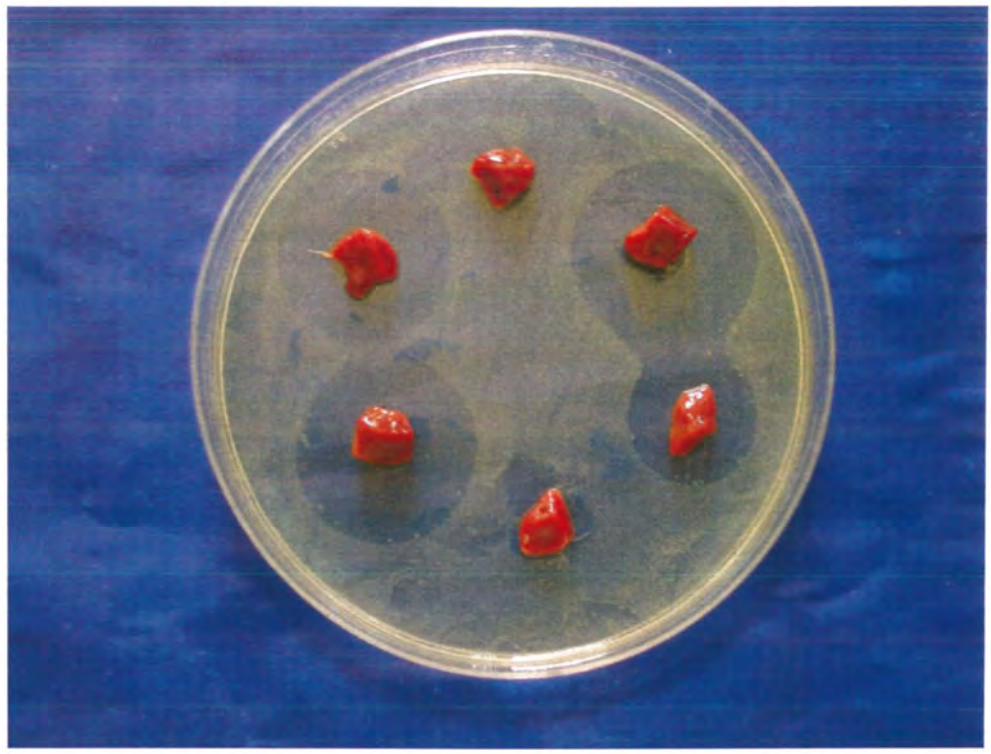


Fig. 3. Showing microbicidal zone around the sample of poultry liver indicating presence of antimicrobial drug residues on nutrient agar media and *Bacillus subtilis* ATCC 6633

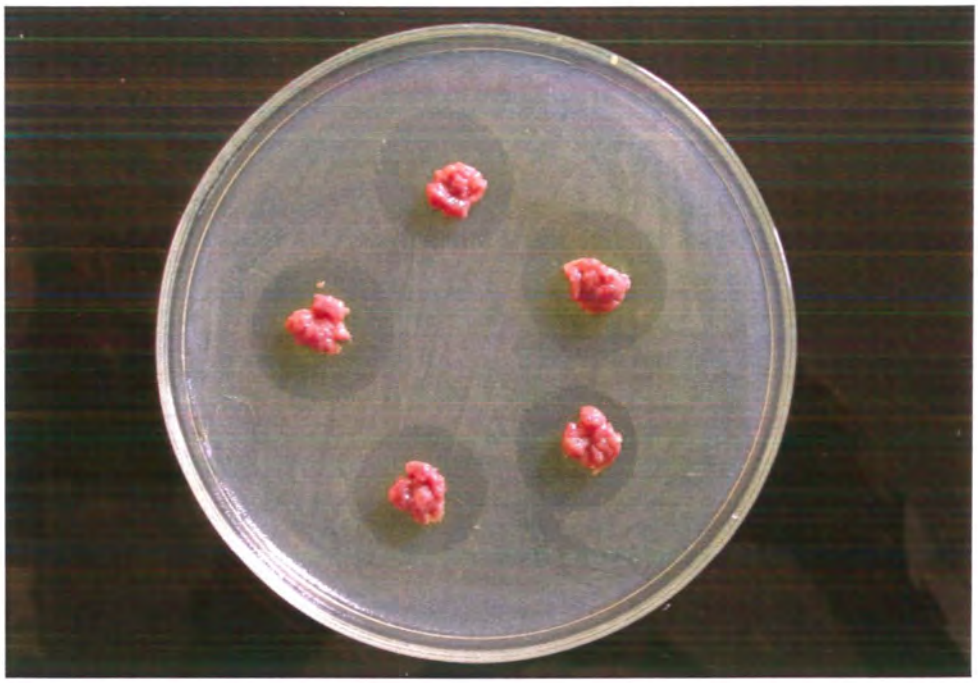


Fig. 4. Showing microbicidal zone around the sample of poultry bone marrow indicating presence of antimicrobial drug residues on nutrient agar media and *B. stearothermophilus* ATCC 12980

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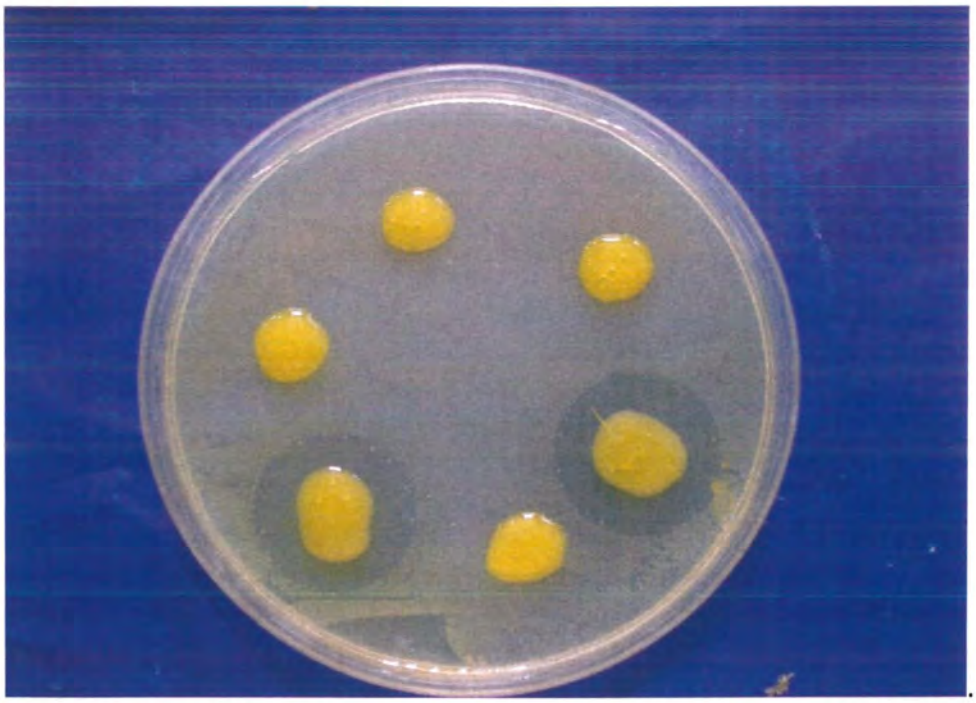

Fig. 5. Showing microbicidal zone around the sample of poultry egg yolks indicating presence of antimicrobial drug residues on nutrient agar media and *Escherichia coli* ATCC 25922

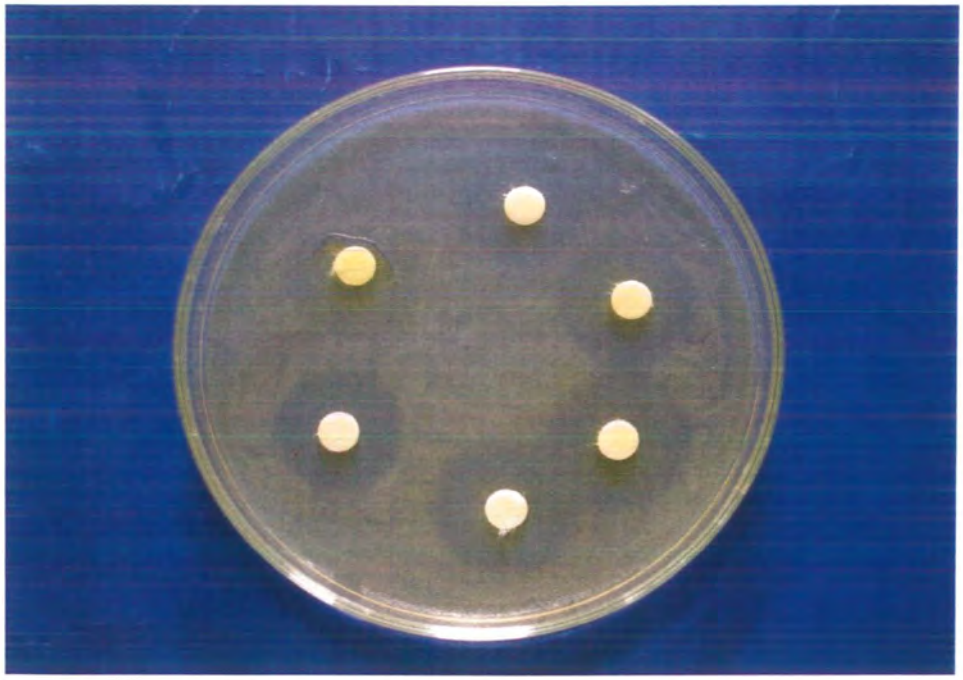

Fig. 6. Showing microbicidal zone around the sample of poultry egg yolks indicating presence of antimicrobial drug residues on nutrient agar media and *Escherichia coli* ATCC 25922

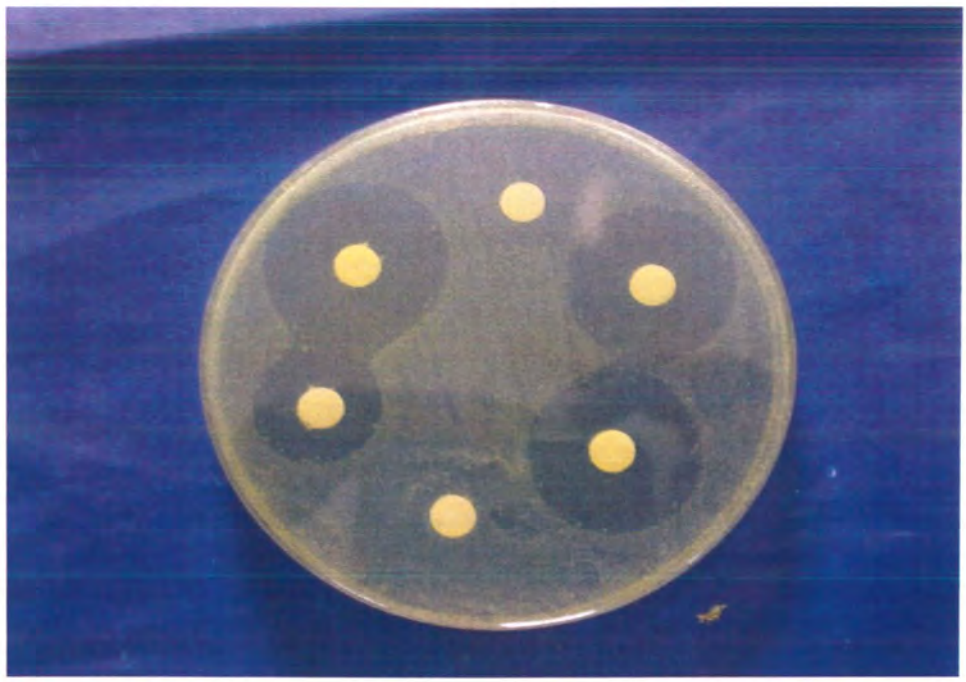

Fig. 7. Showing microbicidal zone around the sample of poultry egg yolks indicating presence of antimicrobial drug residues on nutrient agar media and *Escherichia coli* ATCC 25922

METHOD FOR IDENTIFICATION OF RESIDUES

Extracts of the samples microbiologically positive for antimicrobial activity were further processed for the identification of active principles. Identity test were applied on all positive samples using chemical and UV spectrophotometric, spot tests and TLC methods.

SPOT TEST FOR SULFONAMIDES

Half ml of homogenized breast, liver, bone marrow and egg yolks were placed on glass plate. Diluted HCl 0.5 ml was added to each sample and gently mixed. Starch Iodate paste as an indicator was added into the pre mixed sample and HCl. Half ml of 0.1 M sodium nitrite solution was added to the mixture. The brown yellow color developed as an indication that sample was positive for residue of sulfonamides (Fig. 8, 9).

SPOT TEST FOR MACROLIDES

Half ml of homogenized breast, liver, bone marrow and egg yolks were placed on glass plate. Concentrated HCl 0.5 ml was added to each sample and gently mixed. Half ml of acetone was added to each sample already mixed with HCl. Orange color developed as an indication that sample was positive for residue of macrolides for preliminary screening (Fig. 10).

SPOT TEST FOR FLUOROQUINOLONES

To the 0.5 ml homogenized breasts, liver, bone marrow and egg yolks were placed on glass plate. Half ml of ethyl acetate and 0.5 ml of chloroform was added to each sample and mixed gently. Chloranilic acid as an indicator was added into the pre mixed sample. The brownish color formation was developed as an indication that sample was positive for residue of fluoroquinolones (Fig. 10).

CONFIRMATION OF SULFONAMIDES RESIDUES BY TLC

TLC is a selective, sensitive, rapid and well reproducible densitometric method for simultaneous determination of sulfonamides. The method is suitable not only for quantitative evaluation of sulfonamides but also for checking the identity of the active substances. Method was standardized as described by Klein and Mader, (2006). Two hundred gram of Kieselgel G was

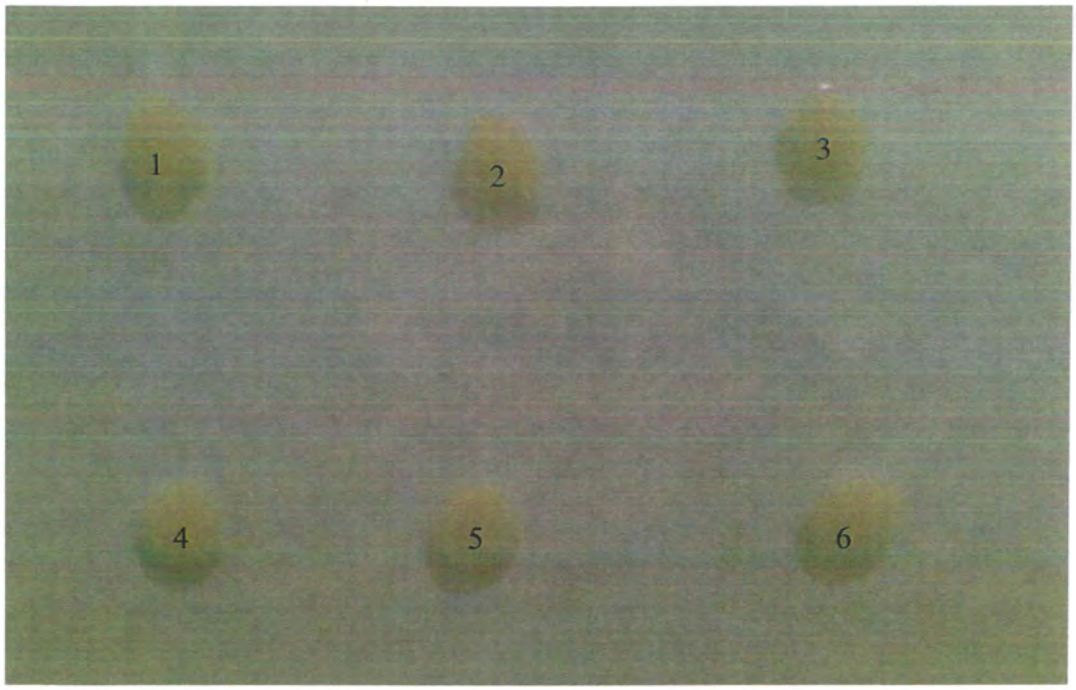

Fig. 8. Showing buffer extracts of poultry beast meat (1, 3, 4 and 6) and poultry liver (2 and 5).

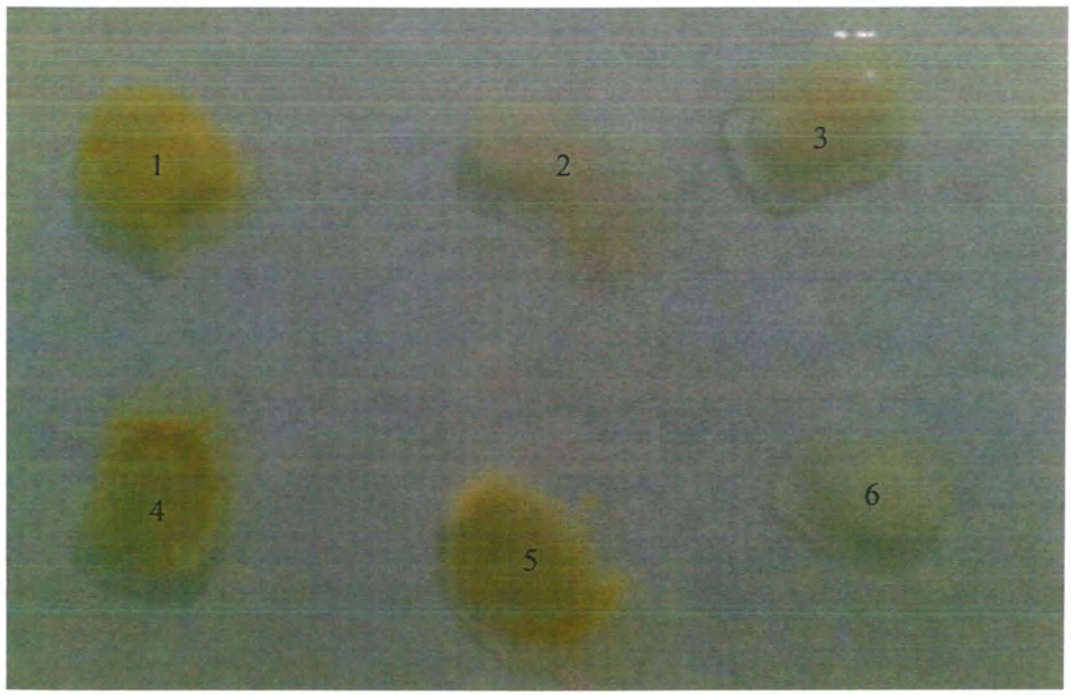

Fig. 9. Showing yellowish brown color spot test positive for sulfonamides (1, 4 and 5) and spot test negative (2, 3 and 6).

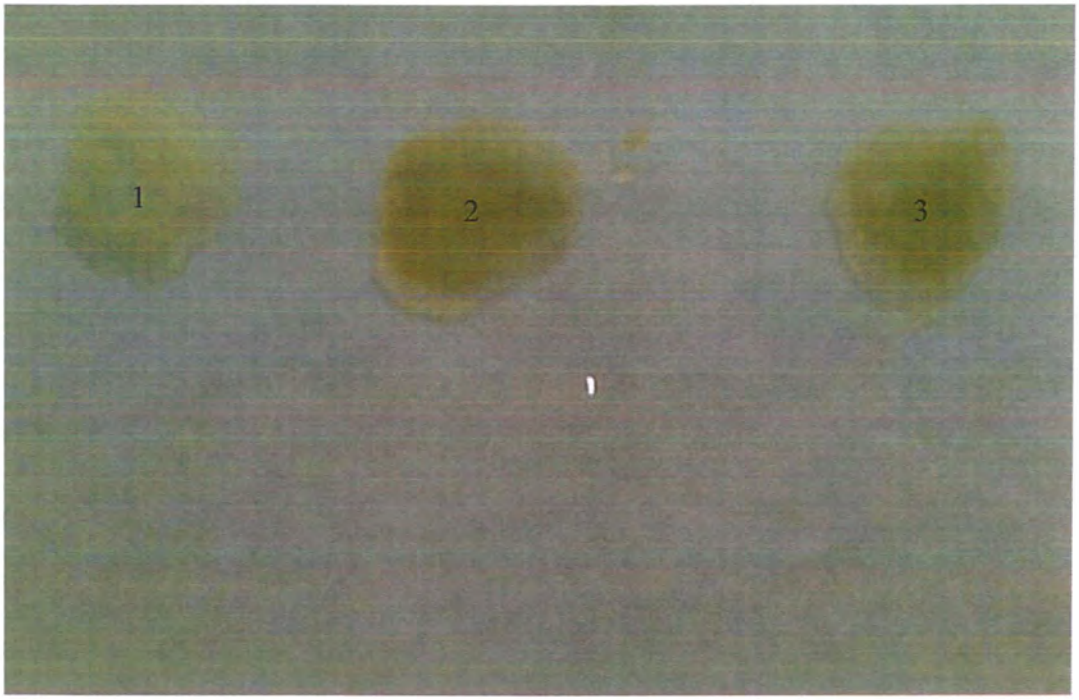

Fig. 10. Showing brownish color positive for fluoroquinolone (1) and orange color for macrolides (2 and 3)

dissolved in deionized filtered water and heated in the glass flask at 80 °C for 10 minutes. The glass plates measuring 12 × 7cm were fixed in the TLC plate coating channel. The preheated mixture of Kieselgel G pored in to the coating boat. The boat was gently moved to coat about 1mm layer of Kieselgel G on the glass plates. Coated plates were dried in hot air oven. Sulfonamide standard solutions were prepared in acetone. Each individual sulfonamide standard solution and blend of all standards was transferred to a glass capillary tube. After that, the solutions contained in the glass capillary tube were spotted on coated plate. The plate was then placed in a chromatographic chamber containing mobile phase n-butanol for about 45 minutes. When 75 percent of the mobile phase moves up the plate, the plate was removed from the chromatographic chamber and the mobile phase front was marked. Hot air was showered to dry the plate and the plate was sprayed with diazo reagent or p-dimethylaminobenzaldehyde, as little as 0.25 µg of the sulfonamide were detected. Preliminary screening of plates was visualized under short wave UV and the center of each detected spot was marked. The distances from the spots to the mobile phase front and to the center of the detected spot of each sulfonamide were measured using a ruler. The retention factor, Rf value for each sulfonamide was then calculated. Equal values of Rf was a land mark of sulfonamide identity on the density basis (Petz *et al.*, 1987).

CONFIRMATION OF MACROLIDES RESIDUES BY TLC

Samples suspected for macrolides were extracted from poultry meat, liver, bone marrow and eggs with phosphate buffer and centrifugation at 20000 rpm. TLC method was standardized as described by Petz *et al.* (1987). Two hundred gram of Kieselgel G was dissolved in deionized filtered water and heated in the glass flask at 80 °C for 10 minutes. The glass plates measuring 12 × 7cm were fixed in the TLC plate coating channel. The preheated mixture of Kieselgel G pored in to the coating boat. The boat was gently moved to coat about 1mm layer of Kieselgel G on the glass plates. Coated plates were dried in hot air oven.

Macrolides standard solutions were prepared in acetone. Each individual macrolides standard solution and blend of all standards was transferred to a glass capillary tube. After that, the solutions contained in the glass capillary tube were spotted on coated plate. Next to the standards, 20 µl of each extracted samples were spotted for the identification of macrolides. The plate was then placed in a chromatographic chamber containing mobile phase n-butanol for about 45 minutes. Plate was removed from the chromatographic chamber after the mobile phase

had travelled 75 percent of the plate and the mobile phase front was marked. Plate was dried with hot air. Preliminary screening of plates was visualized under short wave UV at 525 nm and the center of each detected spot was marked. Plates were sprayed with xanthydrol and macrolides were detected as purple spots. The retention factor, Rf value for each were calculated. Equal values of Rf was a land mark of macrolides identity on the density basis (Petz *et al.*, 1987).

CONFIRMATION OF RESIDUES OF FLUOROQUINOLONES BY TLC

Forty gram of silica gel (G1) were added to 80 ml of acetone in a clean beaker and mixed to form pourable slurry. The slurry was spread over clean dried 20×10 TLC glass plates to a thickness of 1 mm using a kenso spreader (model CJK-520). The coated plates were air dried at room temperature and stored in an oven at 110 °C for 30 minutes and allowed to cool to room temperature just before use. Each of the samples was spotted on a pair of coated plates. The plates were then placed vertically in a development tank containing a solvent system chloroform, ethyl acetate, hexane, and water in the ratio of 1:3:0.5:1 respectively and allowed to stand undisturbed until the solvent front reached 15 cm from the origin. The plates were then removed, air dried and sprayed with an appropriate locating reagent such as iodine or chloranilic acid and examined for brownish color formation. Plates were counter sprayed with dimethylformamide (DMF) reddish brown and violet color was developed respectively (Shu *et al.*, 2007).

Samples confirmed through specific identity test for the antibacterial residue were grouped specifically as known samples of antibacterial included in the studies. The confirmed samples were quantified both by the microbiological and chemical methods for the concentration of specific residues in the meat, liver, bone marrow and egg yolks accordingly.

QUANTIFICATION OF RESIDUES BY MICROBIOLOGICAL ASSAY

Microbiologically residue concentration of identified antibacterial was determined in the tissue extracts of muscle, liver, bone marrow and egg yolks. One bioassay petri plate with six sterilized plain sensitivity discs was used for each sample extract separately. Alternative three discs were impregnated with 40 µl of the buffered sample extract on the media surface. Triplicate discs were impregnated with known quantities of the reference standard solution of each antibacterial under investigation. Including the negative tissue samples and positive spiked tissues samples for bioassay to get background readings attained the precision and accuracy of bioassay (Fig.11). It was in addition to the standard reference (SR) controls that are used on

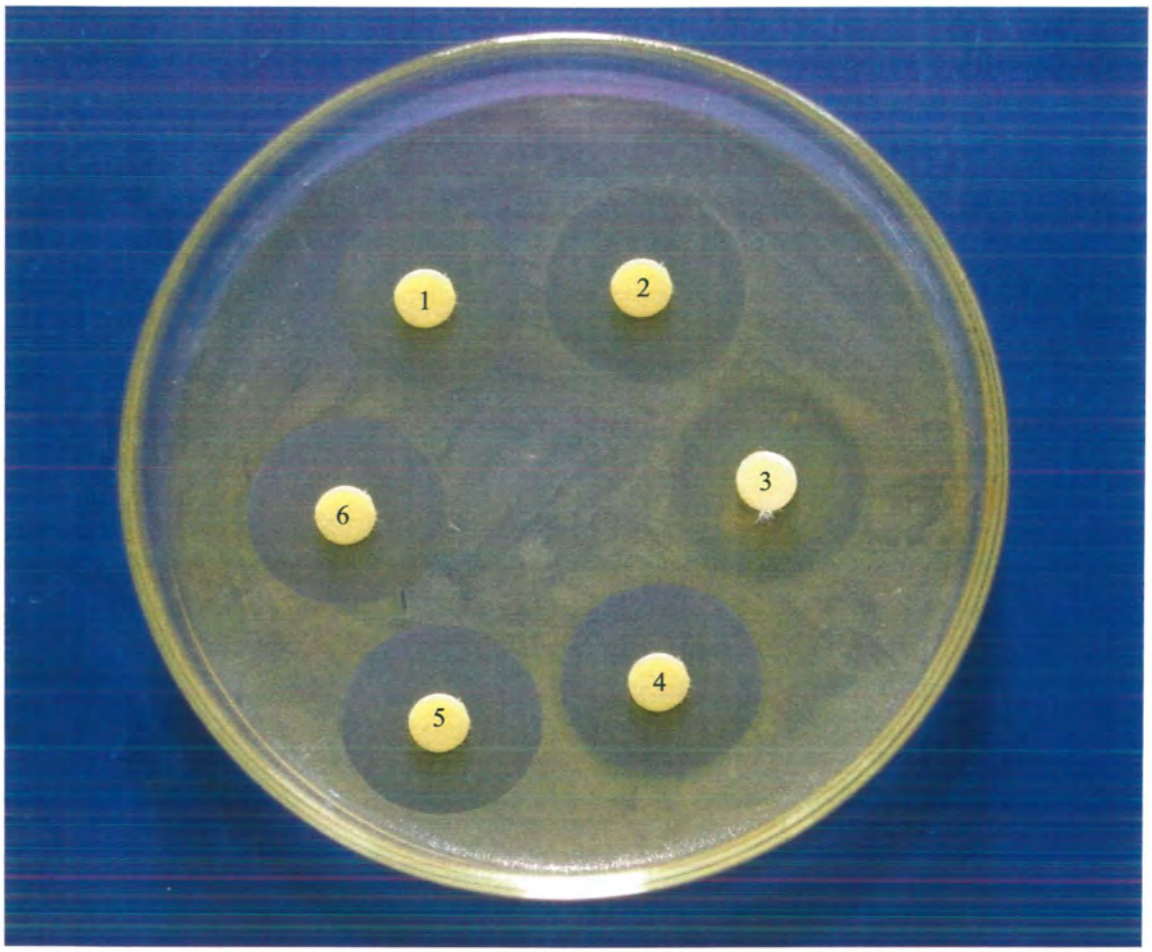

Fig. 11. Showing zone of inhibition around SR concentration solution discs (1, 3 and 5) and zone of inhibition around sample extract discs (2, 4 and 6).

each bioassay plate. Inoculated and disc bearing petri plates were put in incubator at 37 °C for 18 to 24 hours; zones were read and recorded for each of the plate. Specific antibacterial standard curve were plotted to compare and achieve the unknown quantities of the samples bioassayed in the study. Dilution and correction factors were applied to standardize the data.

UV SPECTROPHOTOMETRIC QUANTIFICATION MACROLIDES

Positive macrolides samples confirmed through identity test for tylosin tartrate, erythromycin thiocyanate and tilmicosin were quantified by spectrophotometric method (Shafique, 2007). All tests were performed in Spectum UV-Visible SP-2000 UV China having 4 Cuvettes reading system with built in instant display and printable screen showing absorbance, transmission and concentration. Older spectrophotometer has one slit and standard curve was to be plotted on graph paper and concentration of unknown was calculated (Table. 5).

Standard solution containing 2.5 and 5 µg of tylosin tartrate per ml was prepared in 0.1 N HCl solutions. Absorption of the standards was recorded at wavelength of 290 nm respectively and samples grouped for tylosin were read with these standards. Standard solution containing 2.5 and 5 µg erythromycin thiocyanate per ml was prepared in methanol. Absorption of the standards was recorded at 210 nm respectively and samples grouped for erythromycin thiocyanate were read with these standards. Standard solution containing 2.5 and 5 µg tilmicosin per ml was prepared in 0.1 N HCl solution. Absorption of the standards was recorded at 285 nm respectively and samples grouped for tilmicosin were read with these standards. Strengths of the unknown samples were calculated using following method.

CALCULATION

Absorption of Standard N1

Absorption of Standard N2

Absorption Residue Sample Replicate T1

Absorption Residue Sample Replicate T2

Absorption Residue Sample Replicate T3

Concentration was calculated both by calculation and directly printed by the spectrophotometer using software already built in the LCD. The average of samples was calculated using Microsoft Excel spread sheet along with the deviation. The results were µg/g of the tissue or egg yolk.

Table. 5. Peak absorbance of given concentration of drug at optimized wavelength for standardization of the UV spectrophotometric test for the drug residues monitoring.

S.NO	DRUG	CONC	SOLVENT	REFERENCE	λ	ABSOR
1	Tylosin	40 µg/ml	0.1N HCl	MEP Lab Spec	290 nm	0.880
		40 µg/ml	0.1N HCl	BP	278 nm	0.298
2	Erythromycin	40 µg/ml	Methanol	MEP Lab Spec	210 nm	0.156
		40 µg/ml	Methanol	BP/USP	215 nm	0.152
3	Tilmicosin	10 µg/ml	0.1N HCl	MEP Lab Spec	285 nm	0.274
		10 µg/ml	0.1N HCl	USP	280 nm	0.243
4	Enrofloxacin	10 µg/ml	0.1N NaOH	MEP Lab Spec	254 nm	0.470
		10 µg/ml	0.1N NaOH	USP	258 nm	0.470
5	Flumequine	10 µg/ml	1M H_2SO_4	MEP Lab Spec	247 nm	0.830
		10 µg/ml	1M H_2SO_4	USP/BP	247 nm	0.830
6	Ciprofloxacin	10 µg/ml	Distilled water	MEP Lab Spec	271nm	0.826
		10 µg/ml	Distilled water	USP	276 nm	0.686
7	Norfloxacin	10 µg/ml	0.1N NaOH	MEP Lab Spec	273 nm	0.940
		10 µg/ml	0.1N NaOH	USP	273 nm	0.940
8	Sarafloxacin	10 µg/ml	0.1N HCl	MEP Lab Spec	274 nm	1.197
		10 µg/ml	0.1N HCl	BP	276 nm	1.161
9	Sulfadiazine	10 µg/ml	0.1N NaOH	MEP Lab Spec	254 nm	0.815
		10 µg/ml	0.1N NaOH	USP	254 nm	0.815
10	Sulfaquinoxaline	10 µg/ml	0.1N NaOH	MEP Lab Spec	252 nm	1.130
		10 µg/ml	0.1N NaOH	USP	252 nm	1.130
11	Sulfachlorpyridazine	10 µg/ml	0.1N NaOH	MEP Lab Spec	265 nm	0.575
		10 µg/ml	0.1N NaOH	USP	265 nm	0.575
12	Sulfadimidine	10 µg/ml	0.1N NaOH	MEP Lab Spec	254 nm	0.829
		10 µg/ml	0.1N NaOH	BP	258 nm	0.820

BP = British Pharmacopoeia; USP = United States Pharmacopoeia; λ = Wave length;
MEP Lab Spec = MediExcel Pharmaceuticals Laboratory Specifications

FLUOROQUINOLONES

Fluoroquinolones positive samples confirmed through identity test for norfloxacin, enrofloxacin, ciprofloxacin, sarafloxacin and flumequine were quantified by spectrophotometric method (Yasir, 2006).

Standard solution containing 2.5 and 5 µg of norfloxacin per ml was prepared in 0.1 N sodium hydroxide solutions. Absorption of the standards was recorded at 273 nm respectively. Samples grouped for norfloxacin were read with these standards. Standard solution containing 2.5 and 5µg enrofloxacin per ml were prepared in 0.1 N NaOH solutions. Absorption of the standards was recorded at 254 nm respectively & samples grouped for enrofloxacin were read with these standards. Standard solution containing 2.5 and 5 µg ciprofloxacin per ml was prepared in distilled water. Absorption of the standards was recorded at 271 nm respectively and samples grouped for ciprofloxacin were read with these standards. Standard solution containing 2.5 and 5µg sarafloxacin per ml was prepared in 0.1 N HCl solutions. Absorption of the standards was recorded at 274 nm respectively and samples grouped for sarafloxacin were read with these standards. Standard solution containing 2.5 and 5 µg flumequine per ml was prepared in 1 M H_2SO_4 solutions. Absorption of the standards was recorded at 247 nm respectively and samples grouped for flumequine were read with these standards. Strengths of the unknown samples were calculated using following method.

CALCULATION

Absorption of Standard N1
Absorption of Standard N2
Absorption Residue Sample Replicate U1
Absorption Residue Sample Replicate U2
Absorption Residue Sample Replicate U3

Concentration was calculated both by calculation and directly printed by the spectrophotometer using software built in the LCD. The average of the samples was calculated using Microsoft Excel spread sheet along with the deviation. Results were µg/g of the tissue or egg yolk.

SULFONAMIDES

Samples positive for sulfonamides confirmed through identity test for sulfadiazine, sulfaquinoxaline, sulfachlorpyridazine and sulfadimidine were quantified by spectrophotometric method (Koenen *et al.*, 1995).

Standard solution containing 2.5 and 5 µg per ml of sulfadiazine was prepared in 0.1 M sodium hydroxide solution. Absorption of the standards was recorded at 254 nm respectively and samples grouped for sulfadiazine were read with these standards. Standard solution containing 5 and 10 µg per ml of sulfaquinoxaline sodium was prepared in 0.1 N sodium hydroxide solution. Absorption of the standards was recorded at 252 nm respectively and samples grouped for sulfaquinoxaline were read with these standards. Standard solution containing 5 and 10 µg sulfachlorpyridazine per ml was prepared in 0.1 N NaOH solutions. Absorption of the standards was recorded at 265 nm respectively and samples grouped for sulfachlorpyridazine were read with these standards. Standard solution containing 5 and 10 µg sulfadimidine per ml was prepared in 0.1 N NaOH solutions. Absorption of the standards was recorded at 254 nm respectively and samples grouped for sulfadimidine were read with these standards. Strengths of the unknown samples were calculated using following method.

CALCULATION

Absorption of Standard N1
Absorption of Standard N2
Absorption Residue Sample Replicate D1
Absorption Residue Sample Replicate D2
Absorption Residue Sample Replicate D3

Concentration was calculated both by calculation and directly printed by the spectrophotometer using software already built in the LCD. The average of samples was calculated using Microsoft Excel spread sheet along with the deviation. The results were µg/g of the tissue or egg yolk.

DRUG SENSITIVITY STUDIES

Antibiogram of the various isolates of *E. coli*, *Salmonella* and *Clostridium* of poultry origin were antibiographed. Strength of tylosin tartrate (T) 10 µg/disc, erythromycin thiocyanate (E) 10 µg/disc, tilmicosin (O) 10 µg/disc, norfloxacin (U) 10 µg/disc, enrofloxacin (V) 10 µg/disc, ciprofloxacin (X) 10 µg/disc, sarafloxacin (Y) 10 µg/disc, flumequine (Z) 10 µg/disc, sulfadiazine (D), sulfaquinoxaline (Q), sulfachlorpyridazine (F) and sulfadimidine (A) were 30 µg/disc. Sensitivity was conducted on iso-sensitivity media (Javed, 1992). Antibiogram of the various isolates of *E. coli*, *Salmonella* and *Staphylococcus* from human sources was also performed. Strength of erythromycin and ciprofloxacin were 40 µg/disc.

MUELLER-HINTON PLATE TEST

MH agar plate (one for each organism to be tested) brought to the room temperature. To prepare MH media plate ten ml of agar was dispensed in the disposable petri dishes measuring uniformity of media in the petri plate. Pouring was made on the leveled surface in the laminar flow until media hardened. Plates were dried in an incubator at 35°C or in a laminar flow hood at room temperature until dry. A radial line was drawn under the bottom of the petri dishes as a grid line of alignment for proper placement of discs. To inoculate the plates, sterile swab was dipped into the inoculum tube. Each agar solidified petri plate was inoculated with specified amount of 18 hours fresh culture isolate ($1x10^6$ cfu/ml). The specific standardized concentrations of fresh culture broth inoculum was used for each bioassay plate were standardized to get uniform bacterial growth on the surface of the media. Dried surface of a MH agar plate was streaked by the swab three times over the entire agar surface. Plate was rotated three times at approximately 60 degrees each time to ensure even distribution of the inoculum. Plate was placed at room temperature at least 3 to 5 minutes to dry the plate before proceeding to the next step. One disc for each antimicrobial was placed at equidistance and incubated for 24 hours. Discs were slightly pressed down with forceps to ensure complete contact with the agar surface to avoid irregular zone shapes may occur. Following incubation, zones were measured to the sizes to the nearest millimeter using a digital vernier caliper (Fig. 12). All measurements were made with the unaided eye while viewing the back of the Petri dish.

S = Sensitive (> 20 mm inhibition zone);

I = Intermediate (< 15 mm inhibition zone);

R = Resistant (≤ 8 mm inhibition zone) (CLSI, 2006).

STATISTICAL ANALYSIS

All the data for the experiment was analyzed by using the "M Stat c" developed by Russell D. Fred and Scott P. Eisensmith (1995). Significant differences among different treatments completely randomized design with three replicates. Level of significance was $P < 0.05$ in all analysis.

RESULTS AND DISCUSSIONS

CHAPTER 4

RESULTS AND DISCUSSIONS

Poultry meat has played a vital role in bridging the protein gap through eggs and poultry meat. To enhance the production of poultry and their products many irrational approaches are in practice. Amongst the most common are nutritional supplementation and addition of antimicrobials. Use of chemotherapeutics is a necessity in the intensive poultry farming system globally. In both veterinary and human medicine, antimicrobials have therapeutic, prophylactic, metaphylactic and/or nutritive applications. Efforts to minimize potential consumer health risk attempts in reduction of the antimicrobials residue level in poultry meat and eggs is the area need due consideration. At present there is no monitoring and legislation system to control MRLs in Pakistan therefore, study was planed to monitor the current status of antimicrobials residues, in general and macrolides, fluoroquinolones and sulfonamides in particular. Results of these investigations enlightened the flow of residues from poultry meat and eggs and consequently the bacterial resistance depicted by the zoonotic pathogens. An objective of the study was to highlight local status of macrolides, fluoroquinolones and sulfonamides residues in poultry meat and eggs. Standardization of cheaper and economical testing procedures / techniques of extraction followed by identification and quantification of macrolides, fluoroquinolones and sulfonamides residues in poultry meat and eggs were re-established after modification. Tylosin, erythromycin, tilmicosin, norfloxacin, enrofloxacin, ciprofloxacin, sarafloxacin, flumequine, sulfadiazine, sulfaquinoxaline, sulfachlorpyridazine and sulfadimidine were quantified microbiologically, chemically and spectrophotometerically with MCSTAR.

Currently Pakistan does not have the regulation to control the dug residues in food commodities. It was preference in USA and EU, but with the passage of time it was becoming a conventional approach. Lot of reports in USA (Bowling 2011) reported that most of the meat samples in the market approved USDA and FDA as organic meat were positive for many antibiotics. The Soil

Association of UK is persistently reporting the presence of drug residues in poultry meat and eggs. Results of these investigations enlightened the flow of residues from poultry meat and eggs and consequently the microbial resistance depicted by the zoonotic pathogens.

OPTIMIZATION OF MCSTAR ASSAY

OPTIMIZATION OF SAMPLE EXTRACTION

Phosphate buffer was opted as the solvent for extraction of residues of antimicrobial drugs instead of using costly organic solvents. In order to optimize extraction procedure of drug residues from different text matrices phosphate buffers of different pH were studied. Phosphate buffer of pH 7.2 was found to best for maximum extraction of antimicrobial drug residues from the poultry meat, liver, bone marrow and egg yolk samples. Twenty gram tissue homogenate of sample in 20 ml volume of the phosphate buffer was found best after several trials by using different volumes of the phosphate buffer of pH 7.2. Centrifugation of the tissue extract at 20000 rpm for 15 minutes was also opted after a series of experiments at different rpm.

OPTIMIZATION OF BIOASSAY

In preparation of bioassay plates the 10 ml volume of nutrient agar used was calibrated to achieve a satisfactory and uniform thickness of basic media as the thickness of agar layer directly affects the size of the inhibition zone formed around the sensitivity discs. The 100 µl volume and $1X10^6$ cfu/ml concentration of the 18 hours fresh culture streaked on the bioassay plates were also optimized after several trials as both of them were very critical in order to attain a confluent lawn growth on the plate and were directly affecting the size of the zone of inhibition. The volume of 40 µl of the sample extract as well as standard solutions of different antimicrobial drug was also optimized after series of experiments as it was also very important to achieve uniformity in the bioassay and avoid flow of liquids instead of diffusion mechanism.

For preparation of standard curve for each antimicrobials under study a series of 5 dilutions were used. The middle one was referred as standard reference concentration (SR) while the lowest concentration must produce a zone of inhibition of at least 8 mm on the bioassay plate. The five dilutions used for preparation of standard curves for tylosin tartrate, erythromycin,

tilmicosin, norfloxacin, flumequine and sulfadiazine were 0.8, 1.6, 3.2, 6.4, 12.8 µg/ml concentration (Fig.12). Sulfachlorpyridazine, enrofloxacin and sarafloxacin were 0.4, 0.8, 1.6, 3.2, 6.4 µg/ml concentration. Sulfadimidine, ciprofloxacin and sulfaquinoxaline were 0.1, 0.2, 0.4, 0.8, 1.6 µg/ml concentration.

SURVEILLANCE OF DRUG RESIDUES

During three years (2006 to 2008) 21383 specimens of poultry were collected for the screening of residues of macrolides, fluoroquinolones and sulfonamides. Specimens of meat comprised of one lobe of liver and/or 50 g of breast meat and/or 20 g bone marrow in case of broilers and 3 eggs in case of layers per source were collected. A total of 7025 specimens of poultry meat and eggs were collected. During 2006, followed by 7128 specimens during the year 2007 and 7230 specimens during the year 2008 (Table. 6).

These specimens were collected from the slaughter shops with unknown history of medication and from poultry farms with known history of medication in and around Islamabad and Rawalpindi division of Pakistan. Specimens positive for antimicrobial activity were subjected to conformation of macrolides, fluoroquinolones and sulfonamides. Poultry meat, bone marrow, liver and egg yolks were studied. Lungs, kidneys, intestine and spleen were of no importance as far as the consumption is concerned. Considering eating habit of people poultry meat, liver and egg yolks was included in the studies. Fairly high percentage of people chewed the bone of chicken or use in soup therefore levels in the bone marrow were included.

Using antibiotics in feed gave producers an economic advantage in raising animals to market weight quickly and at low cost. The routine use of antibiotic in growth promotion of animals is a popular technique in the competitive market to get more profit (Brown, 1996). Initially researchers did not fully understand the mechanism and mode that how antibacterials enhance and improve animal growth and performance. Use of Chemotherapeutics is a necessity in the intensive poultry farming system globally. Poultry meat has played a vital role in bridging the protein gap through eggs and poultry meat (Guardabassi and Dalsgaard, 2004). Antibiotics have been used for therapy, prophylaxis and for growth promotion in poultry. Most importantly the poorly planned, ineffective, unwarranted or unethical medication programs leads to the

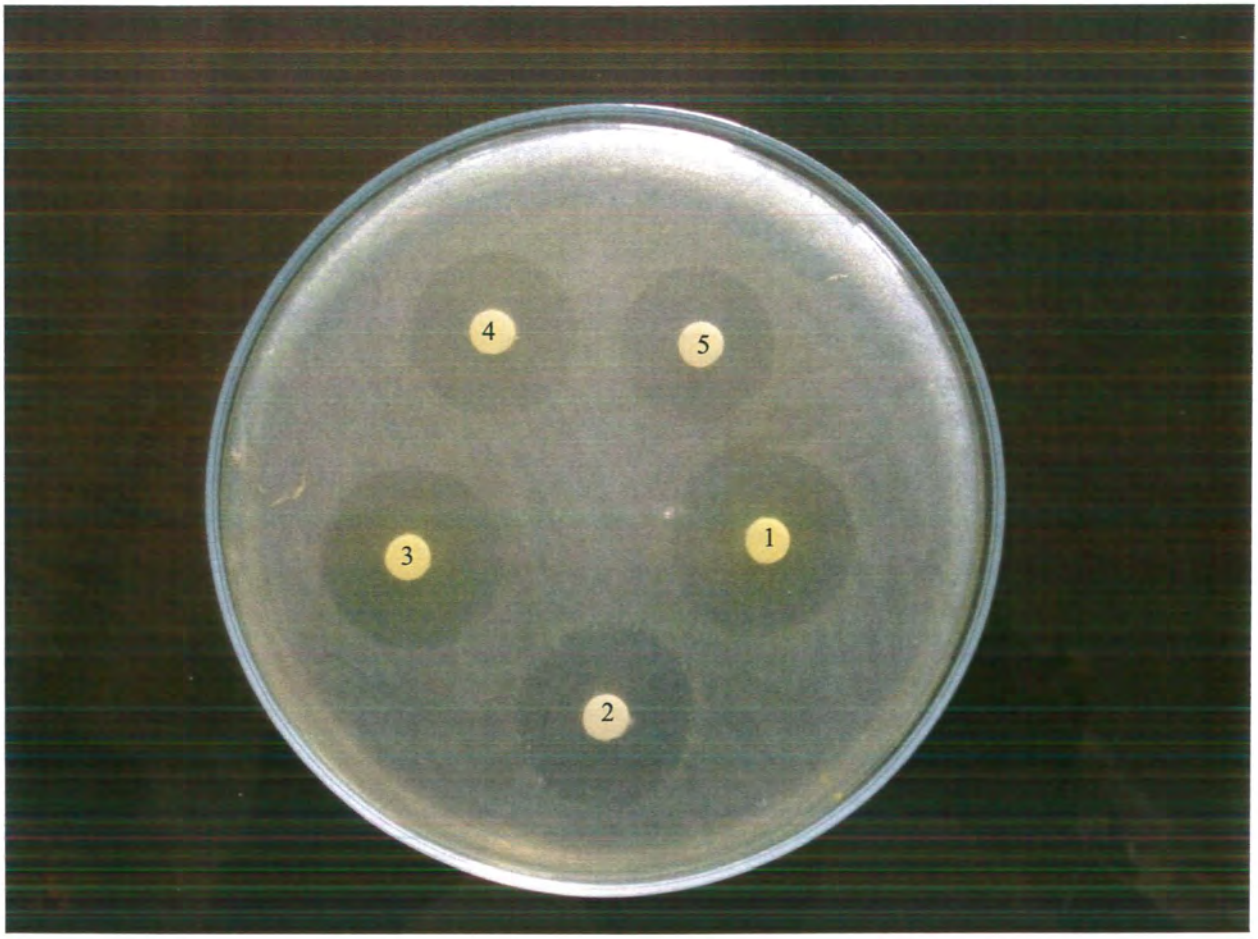

Fig. 12. Showing zone of inhibition of 12.8 µg/ml(1), 6.4 µg/ml(2), 3.2 µg/ml(3), 1.6 µg/ml(4) and 0.8 µg/ml(5) of Tilmicosin standard solutions.

Table 6. Macrolides, fluoroquinolones and sulfonamides residues distribution in poultry liver, meat, bone marrow and egg yolks through microbiological (M) assay during the years 2006-2008.

ORGAN	NO OF SAMPLES ASSAYED (%)				NO OF SAMPLES POSITIVE FOR MACROLIDES, FLUOROQUINOLONES AND SULFONAMIDES (%)			
	2006	**2007**	**2008**	**TOTAL**	**2006**	**2007**	**2008**	**TOTAL**
LIVER	3007^{A} (42.80)	3052^{A} (42.82)	3094 (42.79)	**9153 (42.80)**	452 (15.03)	460 (15.07)	464 (15.00)	**1376^{B} (15.03)**
MEAT	2571^{A} (36.60)	2610^{A} (36.62)	2646 (36.60)	**7827 (36.61)**	300 (11.67)	300 (11.49)	306 (11.56)	**906^{D} (11.57)**
BONE MARROW	258^{A} (3.67)	262^{A} (3.68)	266 (3.68)	**786 (3.68)**	33 (12.79)	35 (13.36)	38 (14.29)	**106^{C} (13.49)**
EGG YOLK	1189^{A} (16.93)	1204^{A} (16.89)	1224 (16.93)	**3617 (16.92)**	118 (9.92)	120 (9.97)	126 (10.29)	**364^{E} (10.06)**
TOTAL (%)	**7025 (100.00)**	**7128 (100.00)**	**7230 (100.00)**	**21383 (100.00)**	**903 (12.85)**	**915 (12.84)**	**934 (12.92)**	**2752 (12.87)**

Values with same superscripts (A-E) are non significantly different ($P < 0.05$) whereas, values with different superscripts (A-E) are significantly different within the same column.

development of resistance problems (Harrison and Svec, 1998). European countries have banned the routine use of antibiotics in chicken feed (Okerman *et al.*, 1998). However banning antibiotics would dramatically increase food costs, leaving animal producers with few strategies to control diseases and pathogens and greatly reduce investment in new pharmaceuticals to fight infection in both animals and people (Kennedy *et al.*, 1998).

Frequency of samples collected for monitoring comprised of 21383 specimens of poultry meat and eggs, 42.8 percent specimens were of liver, 36.6 percent of poultry meat, 3.7 percent of bone marrow and 16.9 percent of egg yolks during the year 2006. Year later in 2007 a total of 7128 specimens were comprised of 42.8 percent of liver, 36.6 percent of poultry meat, 3.7 percent of bone marrow and 16.9 percent of egg yolks. Next and the last year of study during 2008 samples collected were comprised of 42.8 percent of liver, 36.6 percent of poultry meat, 3.7 percent of bone marrow and 16.9 percent of egg yolks (Table. 6). Intensity of the surveillance samples were planed to have non significant difference in sampling numbers. The equal numerical values would be helpful in determining the variation in trends; it was noteworthy that the status of drug residues frequency remained unchanged. Due to the non legislative and regulatory approaches of the authorities to curtail the drug residues the results remained static or undisturbed.

Among total collected samples (21383) during the three years (2006-2008), a total of 12.9 percent samples were positive. Among 9153 samples 15.0 percent were positive of liver, whereas, poultry meat 11.6 percent were positive during years 2006 through 2008. Bone marrow was positive in 13.5 percent and among and egg yolks 10.1 percent were positive for antimicrobial activity (Table. 6 & Fig. 13). These samples were subjected to confirmation of macrolides, fluoroquinolones and sulfonamides. Maximum residues 12.9 percent were detected during the year 2008 followed by 12.9 percent in year 2006 and it was 12.8 percent during the year 2007. Static intensity indicated that residues were unchecked as the behavior of the farmers is fixed since long. As for as the human health was concerned liver, meat and eggs were the most contaminated edible commodities play role in resistance development in human pathogens. Threat index was too high in Pakistan where 15 percent liver and 11.5 percent poultry meat were positive for drug residues.

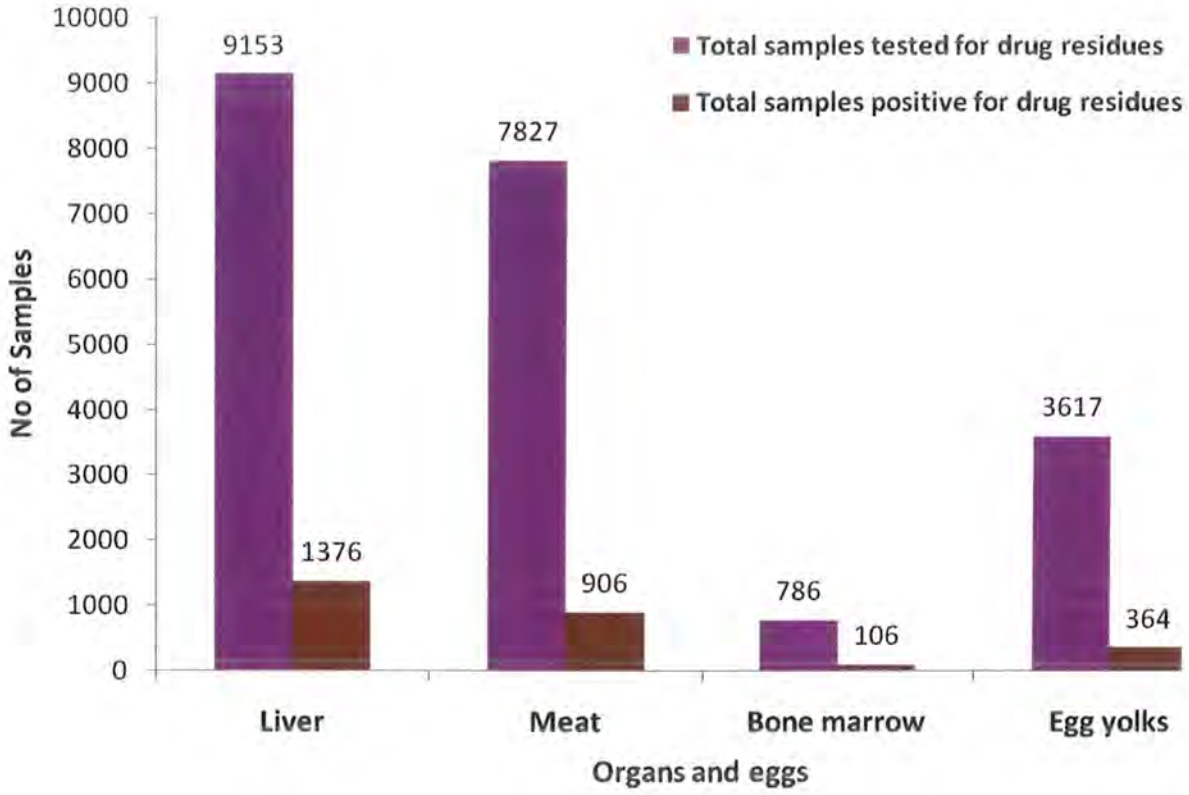

Fig. 13. Distribution of Macrolides, Fluoroquinolones and Sulfonamides residues in poultry liver, meat, bone marrow and egg yolks samples by Microbiological assay during year 2006 through 2008. Numerical values on the violet bars of the graph ■ indicating the total samples assayed in three years. Whereas maroon bars in figure ■ with numerical values showing total positive samples for Macrolides, Fluoroquinolones and Sulfonamides residues among total screened samples of poultry liver, meat, bone marrow and egg yolks.

Commenting on the static results of the drug residues for three years was due to the monitory failure. Significance of the results was always checked between implementation and response. Currently in Pakistan there were no legislative implementation regarding the MRLs. This the dilemma indicated in the present studies. Poultry in Pakistan was increasing 2 to 3 percent annually and total surveyed samples were 12.9 percent positive for drug residues. Considering the static data 3 percent of the total samples were 0.3 percent which is non significant ($P< 0.05$).

TEMPORAL PATTERN OF DRUG RESIDUES

Between three years 2006 and 2008, among total 2752 (100 %) positive poultry samples, 34.4 percent samples were positive for macrolides. During the year 2006 macrolide residues were detected in 4.4 percent samples followed by 4.4 percent in the year 2007 whereas, it was 4.4 percent with slight increased in the drug residues during the year 2008. Fluoroquinolones were detected in 32.9 percent samples during three years, 301 samples were positive for fluoroquinolones residues during the year 2006. During the year 2007 fluoroquinolones residues were detected in 306 samples followed by 298 samples in year 2008. The level of residual contamination was in 32.7 percent samples for sulfonamides residues during the year 2006 through 2008. During three years sulfonamides residues were detected in 4.1 percent in 2006 followed by 4.1 percent in year 2007 whereas, it was 4.4 percent during the year 2008 (Table. 7). As the number of samples collected during the three years were fixed at an average number therefore, these were non significantly different. The reason being was very simple that no legislative measure made to decrease the intensity. Persistency indicates the unchecked behavior of the poultry producers. During 2008 an upward trend has been noted in the drug residues. Currently use of sulfonamides was in increasing trend it is extrapolated that in near future the residues of sulfonamides would be higher side.

Poor population, which is not affordable of poultry meat, commonly used liver, wings and paws of the poultry. Fleshy parts of the poultry meat are coasty as compared with the visceral parts of the poultry. As far as the drug residues are concerned the major portion of the residues metabolized and persists in the liver. Taking in consideration the importance of the liver the major emphasis was given to the liver in the current studies. The presence of higher residues in liver may localize the problem, as most of the people do not consume the soft tissues, like liver

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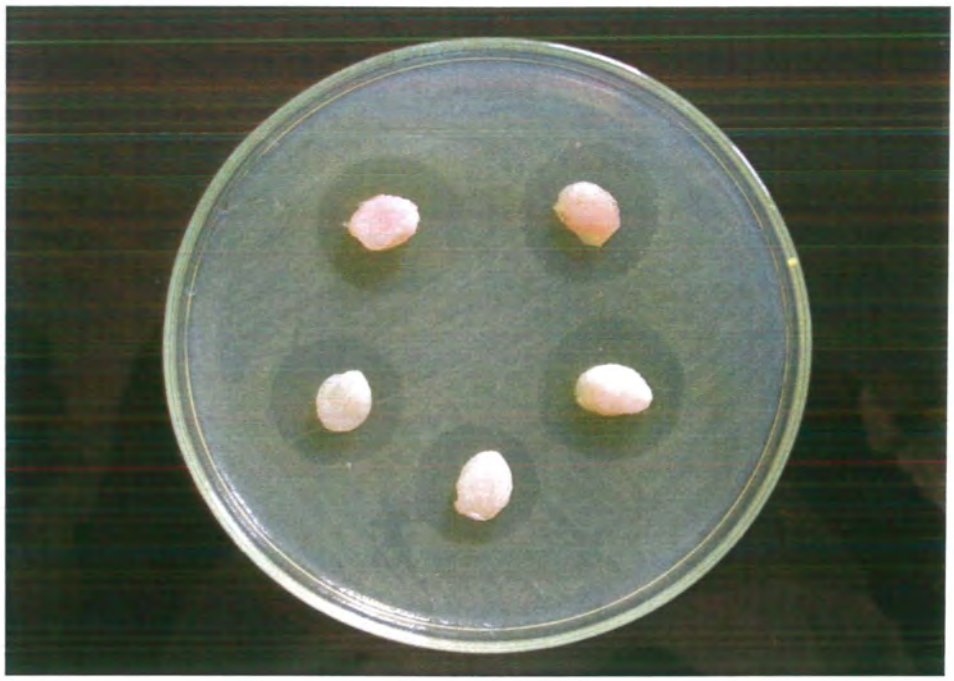

Fig. 14. Showing microbicidal zone around the poultry breast meat samples indicating the presence of antimicrobial drug residues.

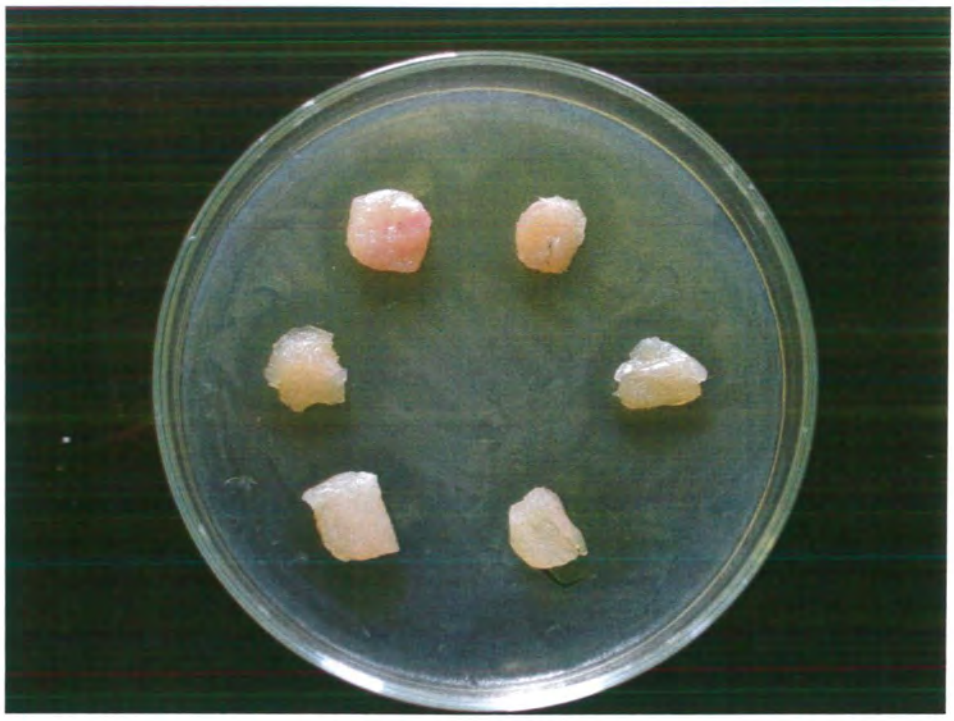

Fig. 15. Showing microbicidal zone around the poultry breast meat samples indicating the presence of antimicrobial drug residues.

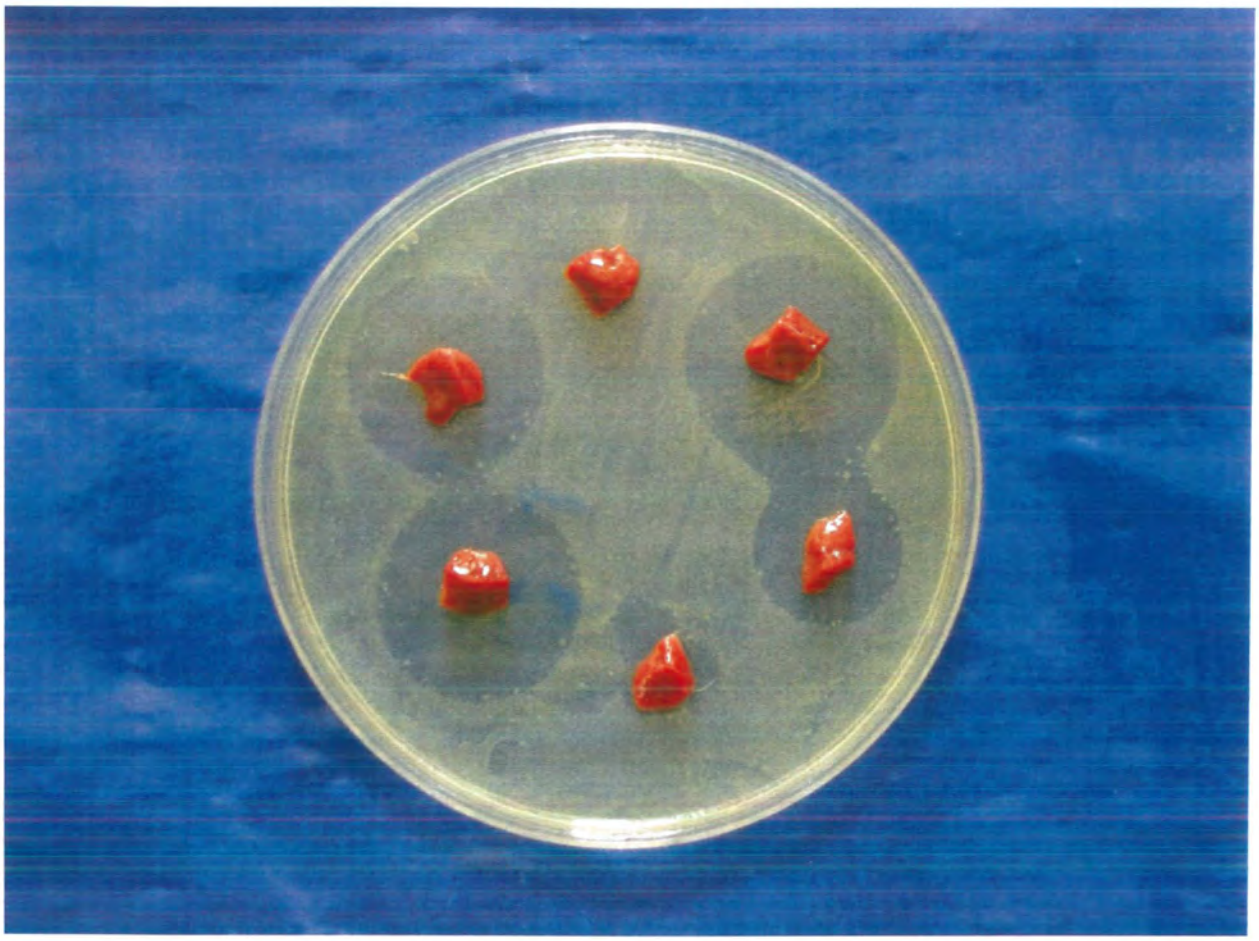

Fig. 16. Showing microbicidal zone around the poultry liver samples indicating the presence of antimicrobial drug residues.

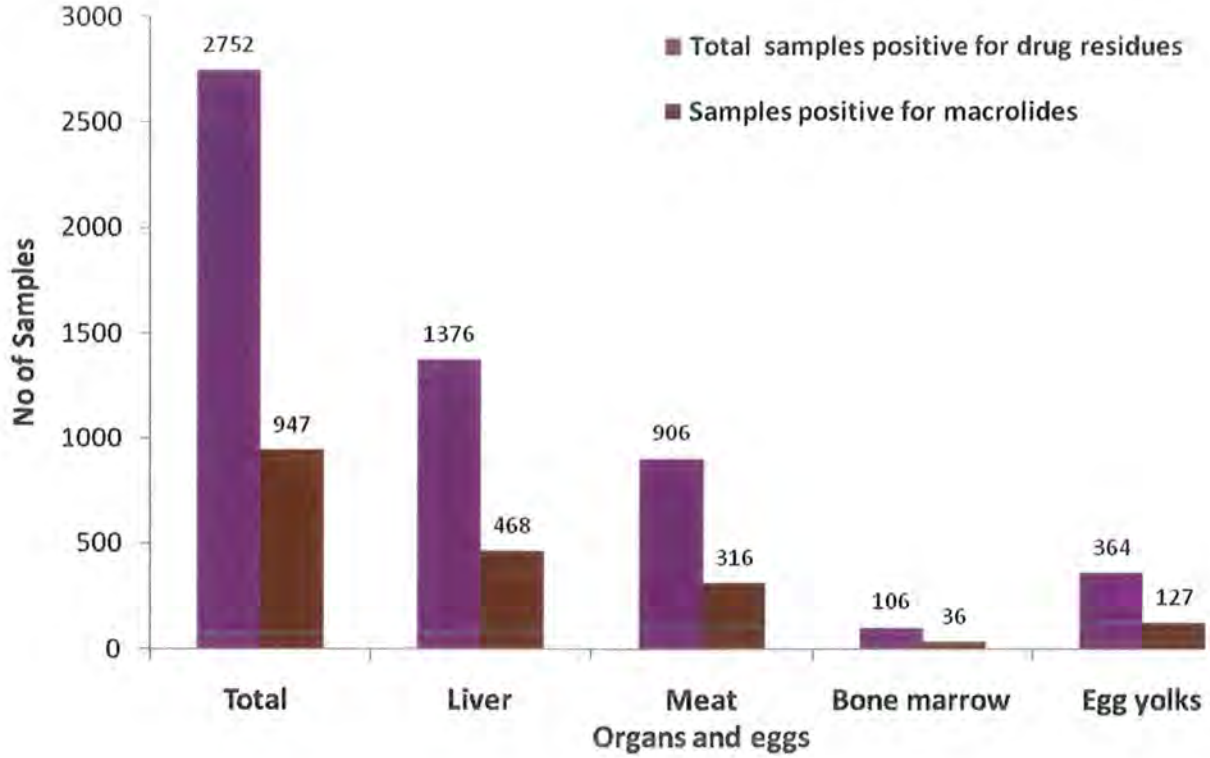

Fig. 17. Distribution of Macrolides residues among total positive poultry liver, meat, bone marrow and egg yolks samples by Microbiological assay during year 2006 through 2008. Numerical values on the violet bars of the graph ■ indicating the positive samples in three years for Macrolides, Fluoroquinolones and Sulfonamides in total screened samples, liver, meat, bone marrow and egg yolks. Whereas maroon bars in figure ■ with numerical values showing positive samples for Macrolides residues among total positive samples, liver, meat, bone marrow and egg yolks.

In Pakistan problem of macrolides, fluoroquinolones and sulfonamides residues are of major concern regarding resistance. Tylosin usage in poultry, in different shape and forms, is leading to the residues pooling in the viscera. Erythromycin is a product, which is commonly used in the human medication therefore; it is of great concern that the drug residues are passing the human being *via* the food chain. Presence of erythromycin in egg yolks indicates the importance of the drug residues. The medication in laying and broiler flocks near to slaughter, needs special attention of the prescribers, farmers and legislation by the food health authorities, to minimize the spread of resistance in term of residues.

Presence of residues in white meat is alarming; therefore the breast meat samples were also collected to rule out the contamination of the edible with macrolides. During the year 2006 among 2571 poultry meat samples were tested for drug residues; 11.7 percent samples were positive for antimicrobial activity and among these 4.1 percent were positive for macrolides residues. During the year 2007, among 2610 meat samples tested; 11.5 percent samples were positive for antimicrobial activity and among these 4.0 percent were positive for macrolide residues. During the year 2008 meat samples tested 11.6 percent samples of bone marrow were positive for antimicrobial activity *in toto* and 4.0 percent were positive for macrolide residues (Table. 7). The pattern of residues was not much changed during the working three years.

Modern *era* consumers demand that industries that make goods/products be open and accountable in their practices. Failure to do so raises concern, and fears about the processes that affect public health. With a vast amount of data and rapid access to it (for example, through the Internet), some health professionals and consumers are asking questions about issues that range from environmental pollution to microwave radiation from recreational electronic devices. Agriculture and its food production practices are not immune from such public scrutiny. Consumers want a wide variety of products at reasonable prices and they also demand safe, wholesome, and nutritious food products. They question agricultural practices meant only to increase productivity and economic return. Consumers are also concerned over the impact of

drug residues in their food. Antimicrobial residues in animal tissues above the legal tolerance do have an impact on human health (Okerman *et al.*, 2004). Tolerances represent the maximal level or concentration of antimicrobial residues permitted in animal tissues at the time of slaughter. The existing evidence exposes the risks associated with drug residues in meat and poultry above the established tolerance. Most residues of veterinary drugs occur in food at such low levels that they rarely pose a chronic or long term health hazard (McCracken *et al.*, 1976). The importance of food safety through the reduction of residues in our food supply cannot be over emphasized. There are five major classes of drugs used in food animals. Everyone who uses drugs on animals has a responsibility to ensure that their use does not result in drug residues in the human food chain. There was need for government and concerned authorities to make certain proper use of antimicrobials in animals and set up a national database to examine infectious diseases and trends in antimicrobial resistance that may result from drug use in food animals could be difficult to treat.

During the year 2006, among 258 samples of bone marrow tested 12.8 percent samples were positive for antimicrobial activity and among these 4.3 percent were positive for macrolides residues. During the year 2007, among 13.4 percent samples were positive for antimicrobial activity and among these 4.6 percent were positive for macrolides residues. During the year 2008 bone marrow were 14.3 percent positive for antimicrobial activity and among these 4.9 percent were positive for macrolides residues (Table. 7).

Taking in consideration the importance of the egg yolks, the eggs were also collected to monitor the presence of macrolides residues in egg yolks. During the year 2006, among 1189 samples of egg yolks tested, 9.9 percent samples results were positive for antimicrobial activity and among these 3.5 percent were found positive for macrolides residues (Table. 7). Whereas in 2007, 3.5 percent were positive for macrolides residues followed by 2008 while the frequency of residues was raised to 3.8 percent (Table. 7). The relatively low frequency of drug residues in eggs as

compared with meat and liver was due to less disease presence in laying flocks and less medication as compared with broiler. However the presence of the residues is alarming. The static frequency and non significant difference indicates that prevalence of drug abuse is consistent in these 3 years.

Incidents of food 'scare' have become almost commonplace in recent years, fuelled by media speculation. Thus expressions such as salmonella, 'mad cow disease' listeriosis and bird flu are becoming familiar to public as well as to specialists in microbiology and agricultural science. Antibiotic use with prescription or veterinary oversight is assumed to be, in general, highly accountable (Schneider *et al.*, 2009). As with many human drugs for which adequate directions can be written for the lay user, some food-animal drugs can be purchased over the counter without a prescription, usually from distributors of animal feed and other animal production supplies. The accountability of use is improved when producers follow industry quality-assurance guidelines and, with the assistance of veterinarians, document the instances of drug use and the practices associated with drug use (Donoghue and Schneider, 2003).

TYLOSIN RESIDUES

Residues of drugs used in the food animal industry threaten human health by being acutely or cumulatively allergenic, toxic, mutagenic, teratogenic or carcinogenic. There was inconclusive evidence that antibiotic residues transferred to humans through food might set up a biological milieu that favors the emergence of microbial strains within the host. Current monitoring systems to detect drugs in milk, meat, and other food products derived from animals are inadequate (Koenen *et al.*, 1995). Screening and monitoring techniques should continue to be deployed to protect consumers against the possible adverse effects of ingesting residues of drugs, some of which could be toxic or may lead to diseases or allergic reactions. The accuracy of testing techniques should be improved to reduce the number of false-positive results that

occur, especially in milk, and more resources are needed to develop testing methods for a wider range of drugs. Improved animal-management practices could substantially reduce the amount of drugs needed for food animals (Pikkemaat *et al.*, 2007).

During the year 2006, among all the screened samples for the presence of macrolides residues in different poultry organs and egg yolks, 311 samples were positive for macrolides residues where 228 were positive for tylosin indicating its higher usage as compared with others macrolides (Table. 8). The spread of tylosin in different poultry organs was higher in the liver 52.2 percent, followed by the meat 32.5 percent, egg yolks 12.3 percent and bone marrow 3.1 percent. During the year 2007, among all the screened samples for the presence of macrolides residues in liver, meat, bone marrow and poultry egg yolks. Macrolides detected in 72.1 percent samples were positive for tylosin residues (Table. 9). The spread of tylosin in different organs was higher in the liver 52.9 percent, followed by the meat 31.3 percent, egg yolks 12.8 percent and bone marrow 3.1 percent. During the year 2008, a total of 321 samples were positive for macrolides residues, whereas 72.3 percent were positive for tylosin (Table. 10). Tylosin residues were higher in the liver 52.2 percent, followed by the meat 31.9 percent, egg yolks 12.1 and bone marrow 3.9 percent.

Although the incidence of tylosin residues during three years was non significant still it was in increasing and/or in stable pattern. Presence of macrolides including tylosin, erythromycin and tilmicosin were detected in the samples. The drug residues of tylosin were higher due to the freely availability of tylosin products to the farmers without prescription and cheaper amongst the other macrolides. The static frequency again indicated unchecked monitory policy to curtail the spread. However the residues levels were at a level position during three years with non significant differences.

Table 8. Tylosin, erythromycin, tilmicosin residues distribution in poultry liver, meat, bone marrow and egg yolks through microbiological (M) assay and spectrophotometric (S) assay during 2006.

ORGAN/ PRODUCT	TYLOSIN (%)	ERYTHROMYCIN (%)	TILMICOSIN (%)	TOTAL (%)
LIVER	119^{A} (52.19)	28^{E} (43.75)	7^{H} (36.84)	154^{M} (49.52)
MEAT	74^{B} (32.46)	25^{E} (39.06)	5^{J} (26.32)	104^{N} (33.44)
BONE MARROW	7^{D} (3.07)	2^{G} (3.13)	2^{K} (10.53)	11^{P} (3.54)
EGG YOLK	28^{C} (12.28)	9^{F} (14.06)	5^{J} (26.32)	42^{O} (13.50)
TOTAL (%)	228 (73.31)	64 (20.58)	19 (6.11)	311

Values with same superscripts (A-P) are non significantly different ($P < 0.05$) whereas, values with different superscripts (A-P) are significantly different within the same column.
Percentages in the table were calculated on the basis of individual column except last row where percentages were made within the row.

Table 9. Tylosin, erythromycin, tilmicosin residues distribution in poultry liver, meat, bone marrow and egg yolks through microbiological (M) assay and spectrophotometric (S) assay during 2007.

ORGAN/ PRODUCT	TYLOSIN (%)	ERYTHROMYCIN (%)	TILMICOSIN (%)	TOTAL (%)
LIVER	120^{A} (52.86)	31^{A} (44.93)	5^{B} (26.32)	156^{A} (49.52)
MEAT	71^{B} (31.28)	27^{A} (39.13)	7^{A} (36.84)	105^{B} (33.33)
BONE MARROW	7^{D} (3.08)	2^{C} (2.90)	3^{C} (15.80)	12^{D} (3.81)
EGG YOLK	29^{C} (12.78)	9^{B} (13.04)	4^{B} (21.05)	42^{C} (13.33)
TOTAL (%)	227^{E} (72.06)	69^{F} (21.90)	19^{G} (6.03)	315

Values with same superscripts (A-G) are non significantly different ($P < 0.05$) whereas, values with different superscripts (A-G) are significantly different within the same column.
Percentages in the table were calculated on the basis of individual column except last row where percentages were made within the row.

Table 10. Tylosin, erythromycin, tilmicosin residues distribution in poultry liver, meat, bone marrow and egg yolks through microbiological (M) assay and spectrophotometric (S) assay during 2008.

ORGAN/ PRODUCT	TYLOSIN (%)	ERYTHROMYCIN (%)	TILMICOSIN (%)	TOTAL (%)
LIVER	121^{A} (52.16)	29^{A} (43.94)	8^{A} (34.78)	158^{E} (49.22)
MEAT	74^{B} (31.90)	25^{A} (37.88)	8^{A} (34.78)	107^{F} (33.33)
BONE MARROW	9^{D} (3.88)	2^{C} (3.03)	2^{C} (8.69)	13^{G} (4.05)
EGG YOLK	28^{C} (12.07)	10^{B} (15.15)	5^{B} (21.74)	43^{F} (13.40)
TOTAL (%)	232^{H} (72.27)	66^{I} (20.56)	23^{J} (7.17)	321

Values with same superscripts (A-J) are non significantly different ($P < 0.05$) whereas, values with different superscripts (A-J) are significantly different within the same column.
Percentages in the table were calculated on the basis of individual column except last row where percentages were made within the row.

All antibacterial preparations and many other drugs have a withdrawal time. This is the amount of time that must elapse after the last dose of the drug was administered to the animal before it can be slaughtered or any of its products used for food. Withdrawal times vary considerably depending upon the individual drug and the formulation of the drug that is used (Myllyniemi *et al.*, 2000). There has been increased concern among consumers about antibiotic and other drug or chemical residues. There has also been increased emphasis from the regulatory agencies to monitor and prevent residues (Dey *et al.*, 2005b). It is imperative that food animal producers utilize in current agricultural practice, raising animals for food depends heavily on the use of drugs. The use of drugs in food animals is fundamental to animal health and for the economics of the industry.

Mean concentration of tylosin among 119 samples of liver was 2.8 ± 1.1 µg/g by microbiological assay compared with the 2.9 ± 1.1 µg/g with spectrophotometric assay in year 2006. In year 2007, mean concentration of tylosin among 120 samples of liver was 2.6 ± 1.2 µg/g by microbiological assay compared with 2.6 ± 1.3 µg/g with spectrophotometric assay. The non significant higher levels detected in spectrophotometric assay were due to the precision of the instrument and sensitivity of the assay. Tylosin residues during the year 2008 in positive 121 liver samples was 3.6 ± 1.2 µg/g by microbiological assay compared with 3.7 ± 1.2 µg/g with spectrophotometric assay. In comparison of three years the maximum tylosin residues were detected during the year 2008 where it was 5.1 µg/g followed by 4.7 µg/g in year 2007 and lowest (4.6 µg/g) in 2006 (Fig.18 & Annex.1.1.1).

There were two sides to the issue of how drug use in food animals affects the health of humans: reported benefits were derived largely from maintenance of good animal health and the reduced chance that disease will spread to humans. Drugs used in food animal production and residues of those drugs could enter human food and increase the risk of ill health in persons who consume products from treated animals. Moreover, the use of antibiotics in food animals could contribute to the emergence of antibiotic resistant microorganisms in animals that could be transmitted to humans and result in infections that these programmes to improve their control on the use of antibiotics and drugs (Le Breton *et al.*, 2007). This will help prevent any erosion of confidence in the milk and meat they produce and market.

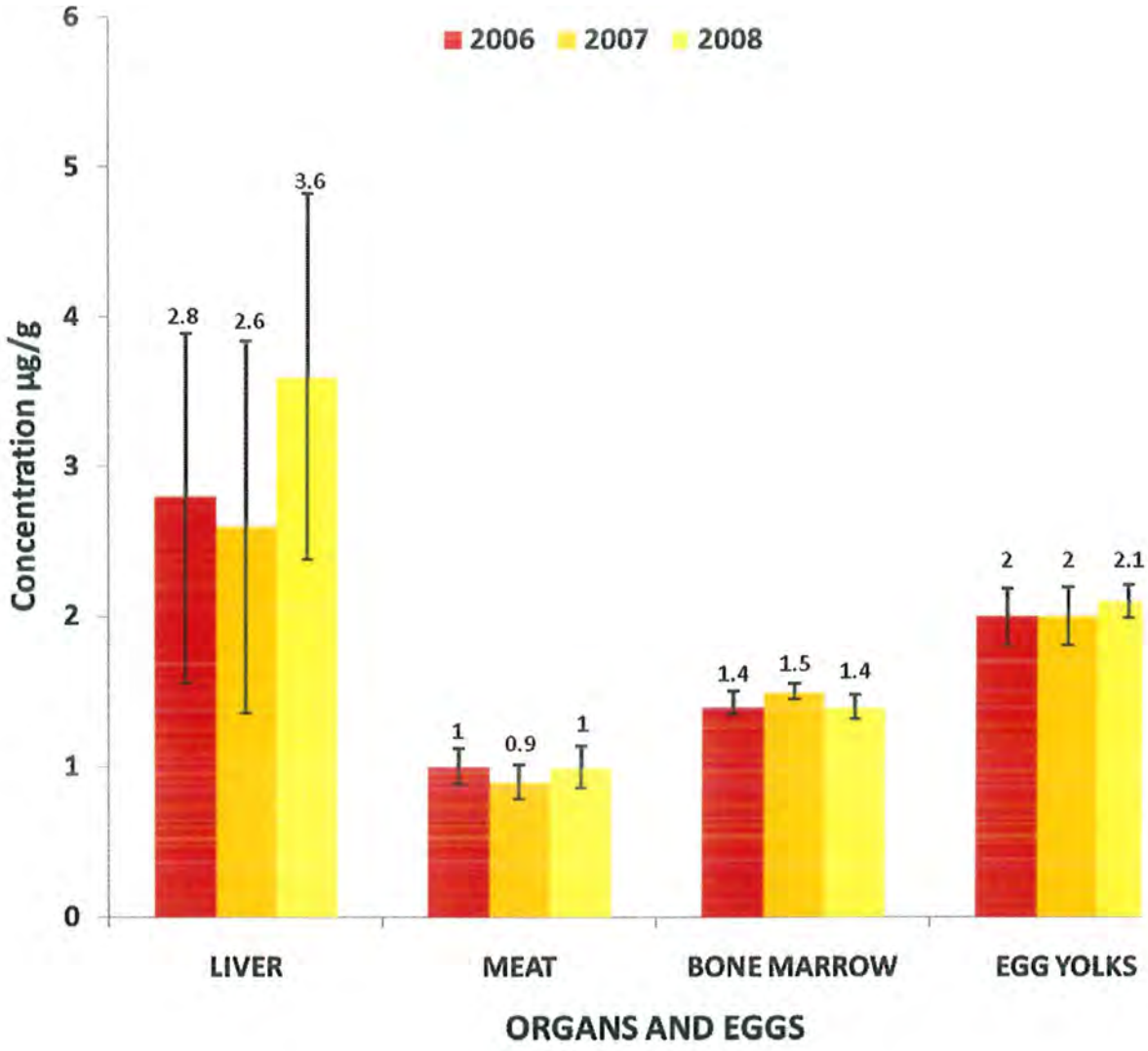

Fig. 18. Tylosin residues mean concentration (µg/g) during year 2006 (■), 2007 (■), 2008 (■) by Microbiological assay in poultry liver, meat, bone marrow and egg yolks samples. Numerical values showing mean concentration of Tylosin residues along with the lines on the bars indicating standard deviation of the mean.

During the year 2006, mean concentration of tylosin among 74 samples of poultry meat was 1.0 ± 0.1 µg/g by microbiological assay compared with the 1.0 ± 0.1 µg/g with spectrophotometric assay. During the year 2007, mean concentration of tylosin among 71 samples of meat was 0.9 ± 0.1 µg/g by microbiological assay compared with the 0.9 ± 0.1 µg/g with spectrophotometric assay. Tylosin residues during the year 2008 in positive 74 samples of meat were 1.0 ± 0.1 µg/g by microbiological assay compared with 1.0 ± 0.2 µg/g with spectrophotometric assay. The minimum tylosin residue through microbiological assay was 0.8 µg/g and it was higher 1.2 µg/g of meat. In spectrophotometric assay the minimum tylosin residues in meat samples was 0.8 µg/g increasing to the level of 1.2 µg/g. In comparison of three years the maximum tylosin residues were detected during the year 2006 and the year 2008 where it was 1.2 µg/g followed by 1.1 µg/g in year 2007 (Fig.18 & Annex.1.1.2).

The therapeutic use of medicinal substances is obviously essential to cure outbreaks of disease whenever they occur. As such treatment is carried out under the direction of a veterinary surgeon. No problems should arise for animals or humans, and particularly as such treatments will be spasmodic rather than continuous (Okerman *et al.*, 2007). However, residues can occur in animal products when large doses are administered immediately prior to slaughter, or in the milk of animals undergoing treatment. Microorganisms can mutate to develop or acquire resistance to antibiotic drugs. Several questions determine whether this resistance will result in an increased hazard for humans.

Tylosin concentration in liver through microbiological assay fluctuated between 2.6 to 3.6 µg/g during 2006 to 2008. The residues were significantly higher in 2008 although the concentration of tylosin in liver samples was non significantly lower in 2007 but over all residues were static or in increasing trend. The lower concentration may be due to the product used having less strength of the tylosin in the product. It was common in Pakistan that the legal limits are 83-105 percent of product purity. Therefore 23 percent variation in the product was also a major reason of difference even with the static data.

According to a number of basic veterinary introductions of antibiotics in feed, was another reason for the irrational use of antibiotics. Prolonged low-dose intake of animal antibiotics that

can weaken the stomach and intestines of harmful microorganisms, inhibit, kill pathogens, enhance disease resistance and can stimulate the secretion of pituitary hormones animals, promote body growth and development, thus accelerating the rate of weight gain. To this end, the pursuit of some farmers to maximize economic benefits, may add feed antibiotics in animal feed (Pikkemaat *et al.*, 2009). There are anti-animal products listed "threshold" low veterinary drug residues of antibiotics was undoubtedly affect the meat, eggs, milk and other livestock and poultry product safety an important factor.

These values were higher in comparison with the MRLs of the tylosin established in USA (0.1 µg/g or 100 µg/kg), UK (0.1 µg/g), and EU (0.1 µg/g). In comparison with MRLs in US and EU the detected residue level in Pakistan were 26 to 36 times higher. The significant higher unlimited values of residues were detected in Pakistan. The picture of residues comparing with other countries were in so high positioned that currently it was difficult to export these material to other countries. Pakistan had targeted the market of Saudi Arabia and Iran, the testing system and MRLs are not yet set in these countries.

Studies have shown that the use of antibiotics can reduce the breeding costs, but for the purpose of prevention and the promotion of growth, long-term use of animals lower than the therapeutic dose of antibiotic resistance in bacteria appears to be accelerating. Appeared resistance in animal breeding and the spread of large-scale breeding animals will be a huge storehouse of resistance genes. This will become increasingly poor animal disease resistance, bacterial resistance growing, leading to treatment of sick livestock; they have to increase the dosage so that more and more antibiotic residues, more concentrated form vicious cycle (Ferrini *et al.*, 2006). There was anti-food was also a serious threat to human health. Interviewed experts pointed out that the accumulation of some antibiotics in food animal tissues, these drug residues can accumulate in the body of livestock and poultry products directly or through the accumulation of environmental release to other plants, and eventually brought together in the human body in various ways, leading to chronic human toxicity and *in vivo* changes in the resistance of normal flora. Human regular intake of low doses of antibiotic residues will gradually accumulate in the body caused by diseases of various organs of the body.

During the year 2006, mean concentration of tylosin among 7 samples of bone marrow was 1.4 ± 0.1 µg/g by microbiological assay compared with 1.5 ± 0.1 µg/g with spectrophotometric assay (Fig.18 & Annex.1.1.3). During the year 2007, mean concentration of tylosin among 7 samples of bone marrow was 1.5 ± 0.1 µg/g by microbiological assay compared with the 1.5 ± 0.1 µg/g with spectrophotometric. Tylosin residues during the year 2008 in 9 positive samples of bone marrow was 1.4 ± 0.1 µg/g by microbiological assay compared with 1.5 ± 0.1 µg/g with spectrophotometric assay.

Since the 90s of last century, the government and relevant departments to address veterinary drug residues in food of animal origin issues a lot of work, such as the development of veterinary drug residue standard detection methods, to develop MRLs of veterinary drugs and off-drug period standards, issued Feed Additive use norms, to carry out the work of veterinary drug residue monitoring and testing, to combat, investigate cases of animal production measures such as use of prohibited drugs. However, in a recently conducted survey it was found that in animal products due to production and marketing chain was still no strict control and restrictions, these measures were still difficult to completely limit has anti-animal products listed. In Shaanxi, detection of veterinary drug residues in each sample batch over more than 1200, but the proportion of animal products in low, not many types of antibiotics involved, the market of poultry products has not been established perfect limit antibiotics veterinary drug residues in the threshold (Ferrini *et al.*, 2006).

During the year 2006, mean concentration of tylosin among 28 samples of egg yolks was 1.9 ± 0.2 µg/g by microbiological assay compared with 2.0 ± 0.2 µg/g with spectrophotometric assay. During the year 2007, mean concentration of tylosin among 29 samples of egg yolks was 2.0 ± 0.2 µg/g, whereas, in year 2008 in 28 positive samples of egg yolks were 2.1 ± 0.1 µg/g by microbiological assay. In comparison of three years the maximum tylosin residues were detected during the year 2007 where it was 2.5 µg/g followed by 2.3 µg/g in year 2006 and 2008 (Fig.18 & Annex.1.1.4).

Comparing the detected concentration of the tylosin residues in egg with MRLs of US, Canada and EU the residues were 0.2 µg/g and in Pakistan the residues detection level was 2.0 to 2.1

µg/g. The residues were 10 times higher in Pakistan than the US and EU MRLs limits. Presence of drug residues in egg were more dangerous in comparison with meat and liver, because daily intake through various products based on the eggs were more in use particularly in contrast with the meat type edibles. The cooking of meat destroyed more residues as compared with the egg where sunny side was up and thermal destruction was lesser.

Reduction of antibiotic residues was an important prerequisite for strict implementation of off drug period, off drugs should not be slaughtering of animals listed, the milk, eggs should not be sold, but because there is no effective detection and monitoring, effective implementation is difficult. Articles reporters found that dairy farmers generally know that milk containing antibiotics should not be listed on the pig, chicken farmers for the animals after treatment of withdrawal, abandoned eggs on the level of awareness is not high (Stead *et al* 2007; Schneider and Lehotay, 2008).

Many countries have developed a "veterinary drugs in foods of animal origin the maximum residue limits," but these veterinary drug residues in the standard method are not fully established. In addition, the current detection method to quantitatively detect liquid-based. the lack of rapid screening and confirmation methods, made a rapid screening kit for veterinary drug residues in products is still small, limiting the overall veterinary drug residue monitoring carried out, should be a method for monitoring residues of veterinary drugs, equipment, technology and so must devote greater efforts for greater improvement in the short term, to meet the testing needs. During the three years tylosin residues in egg yolk were ranging from 1.9 to 2.1 µg/g. The highest tylosin residues were detected during 2008. The trend was in increasing fashion during 2006 to 2008. Although the increase during three years was non significantly higher, still the trend was increasing. During the last 4-5 years egg layer population will be drastically increased due to population pressure the disease condition will be intensified. To check the respiratory diseases farmers were using tylosin in feed during egg production as preventive medicine and as well as the production enhancer. This was the simple reason why tylosin residues were in increasing in eggs.

Several researchers (Stead *et al.*, 2008) also proposed to reduce harmful antibiotic residues of veterinary drugs, the community, particularly the news media should increase the dangers of propaganda and irrational use of antibiotics, to remind consumers vigilance bad mouth, so choose to buy security no resistance food, it has also allowed producers to raise awareness of the rational use of veterinary medicines. The study, conducted by the Bloomberg School's Center for a livable future and Arizona State's Biodesign Institute, looked for drugs and other residues in feather meal, a common additive to chicken, swine, cattle and fish feed. The most important drugs found in the study were fluoroquinolones broad spectrum antibiotics used to treat serious bacterial infections in people, particularly those infections that have become resistant to older antibiotic classes. The banned drugs were found in 8 of 12 samples of feather meal in a multi-state study. The findings were a surprise to scientists because fluoroquinolone use in U.S. poultry production was banned by the U.S. Food and Drug Administration in 2005. This was the first time investigators have examined feather meal, a byproduct of poultry production made from poultry feathers, to determine what drugs poultry may have received prior to their slaughter and sale (Love *et al.*, 2012).

ERYTHROMYCIN RESIDUES

Erythromycin a product commonly used in the human medication therefore; it is of great concern that the drug residues are passing to the human beings *via* the food chain. During the year 2006, among all the screened samples for the presence of drug residues in different poultry organs and egg yolks, 311 samples were positive for macrolides residues and out of these total 20.6 percent were positive for erythromycin indicating its lesser usage as compared with tylosin. The spread of erythromycin in different organs was higher in the liver, followed by the meat, egg yolks and bone marrow (Table. 8). During the year 2007, among all the screened samples for the presence of drug residues in different poultry organs and egg yolks, 315 samples were positive for macrolides residues and out of these 21.9 percent were positive for erythromycin (Table. 9). The spread of erythromycin in different organs was higher in the liver 44.9 percent, followed by the meat 39.1 percent, egg yolks 13.1 percent and bone marrow 2.9 percent. During the year 2008, among 321 positive samples for macrolides residues 20.6 percent were positive for erythromycin indicating its lesser usage as compared with tylosin (Table. 10). The spread of

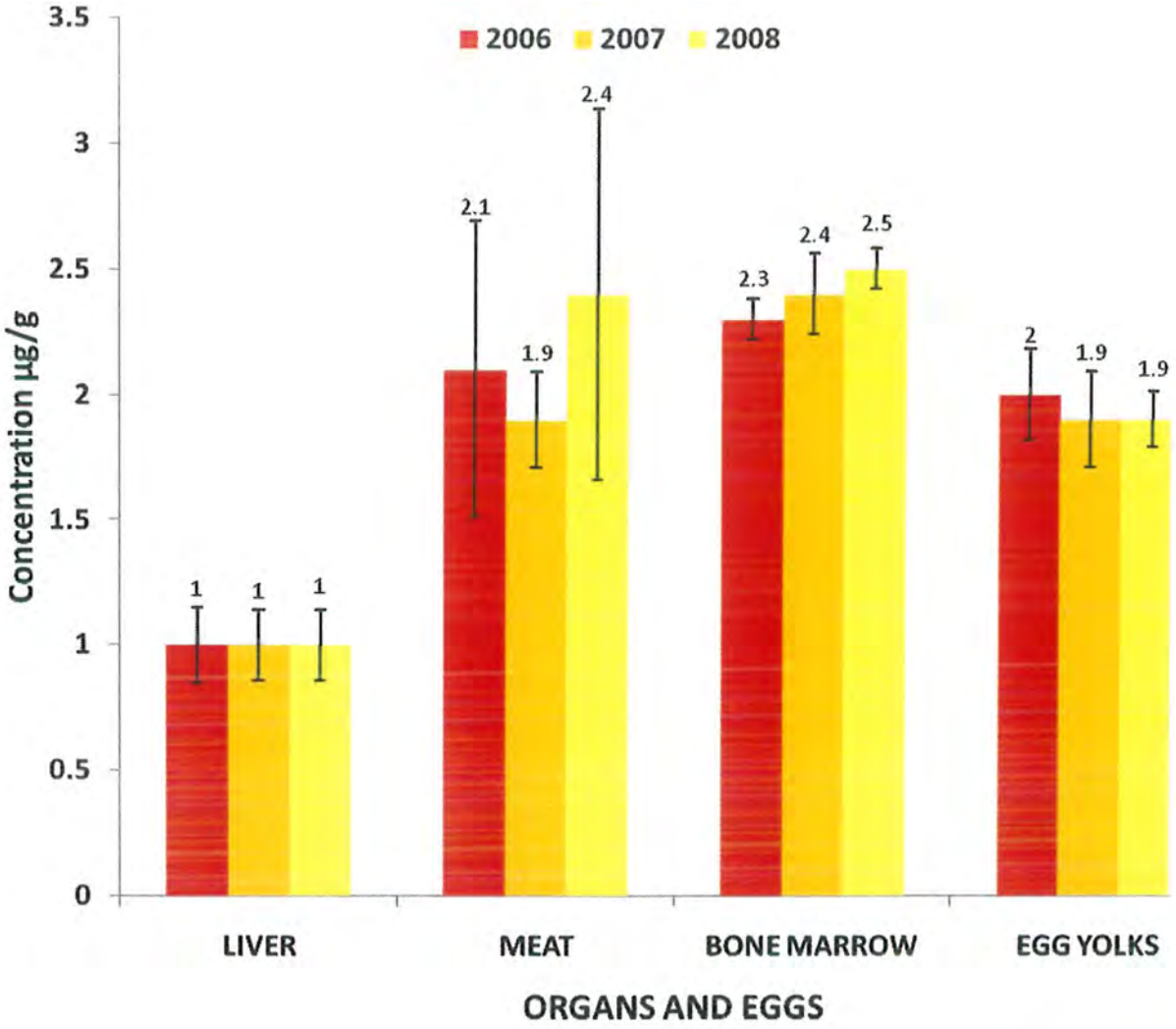

Fig. 19. Erythromycin residues mean concentration (µg/g) during year 2006 ( ), 2007 ( ), 2008 ( ) by Microbiological assay in poultry liver, meat, bone marrow and egg yolks samples. Numerical values showing mean concentration of Erythromycin residues along with the lines on the bars indicating standard deviation of the mean.

Researchers exposed several strains of *E. coli* bacteria to the concentrations of antibiotics found in the feather meal samples, they also discovered the drug residues could select for resistant bacteria. A high enough concentration was found in one of the samples to select for bacteria that were resistant to drugs important to treat infections in humans. FDA should monitor what drugs were going into animal feed was strongly recommended. New FDA guidance documents, which call for voluntary action from industry, will be ineffectual, by looking into feather meal, and uncovering a drug banned nearly 6 years ago, very little confidence that the food animal production industry can be left to regulate itself (Love *et al.*, 2012).

Erythromycin residues during year 2006 in 2 positive samples of bone marrow were 2.3 ± 0.1 µg/g by microbiological assay compared with 2.4 ± 0.1 µg/g with spectrophotometric assay. The erythromycin minimum residue through microbiological assay was 2.3 µg/g in bone marrow and it was higher 2.4 µg/g of bone marrow whereas in spectrophotometric assay the minimum erythromycin residue in bone marrow was 2.3 µg/g increasing to the level of 2.5 µg/g of bone marrow. During the year 2007, the mean concentration of erythromycin between 2 samples of bone marrow was 2.4 ± 0.2 µg/g by microbiological assay compared with 2.5 ± 0.2 µg/g with spectrophotometric assay. Erythromycin residues during the year 2008 in 2 positive samples of bone marrow were 2. 5 ± 0.1 µg/g by microbiological assay compared with 2.5 ± 0.1 µg/g with spectrophotometric assay (Fig.19 & Annex.1.2.3).

A primary reason for the 2005 FDA ban on the use of fluoroquinolones in poultry production was an alarming increase in the rate of the fluoroquinolone resistance among *Campylobacter* bacteria. During this period fluoroquinolone resistance rate was although slow, but not dropped (Choi, *et al.*, 1999). With such a ban, you would expect a decline in resistance to these drugs. The continued use of fluoroquinolones and unintended antibiotic contamination of poultry feed could help explain why high rates of fluoroquinolone-resistant *Campylobacter* continue to be found on commercial poultry meat products over half a decade after the ban. In the U.S.A, antimicrobials are introduced into the feed and water of industrially raised poultry, primarily to make them grow faster, rather than to treat disease. An estimated 13.2 million kg of antibiotics were sold in 2009 to the U.S. poultry and livestock industries, which represented nearly 80

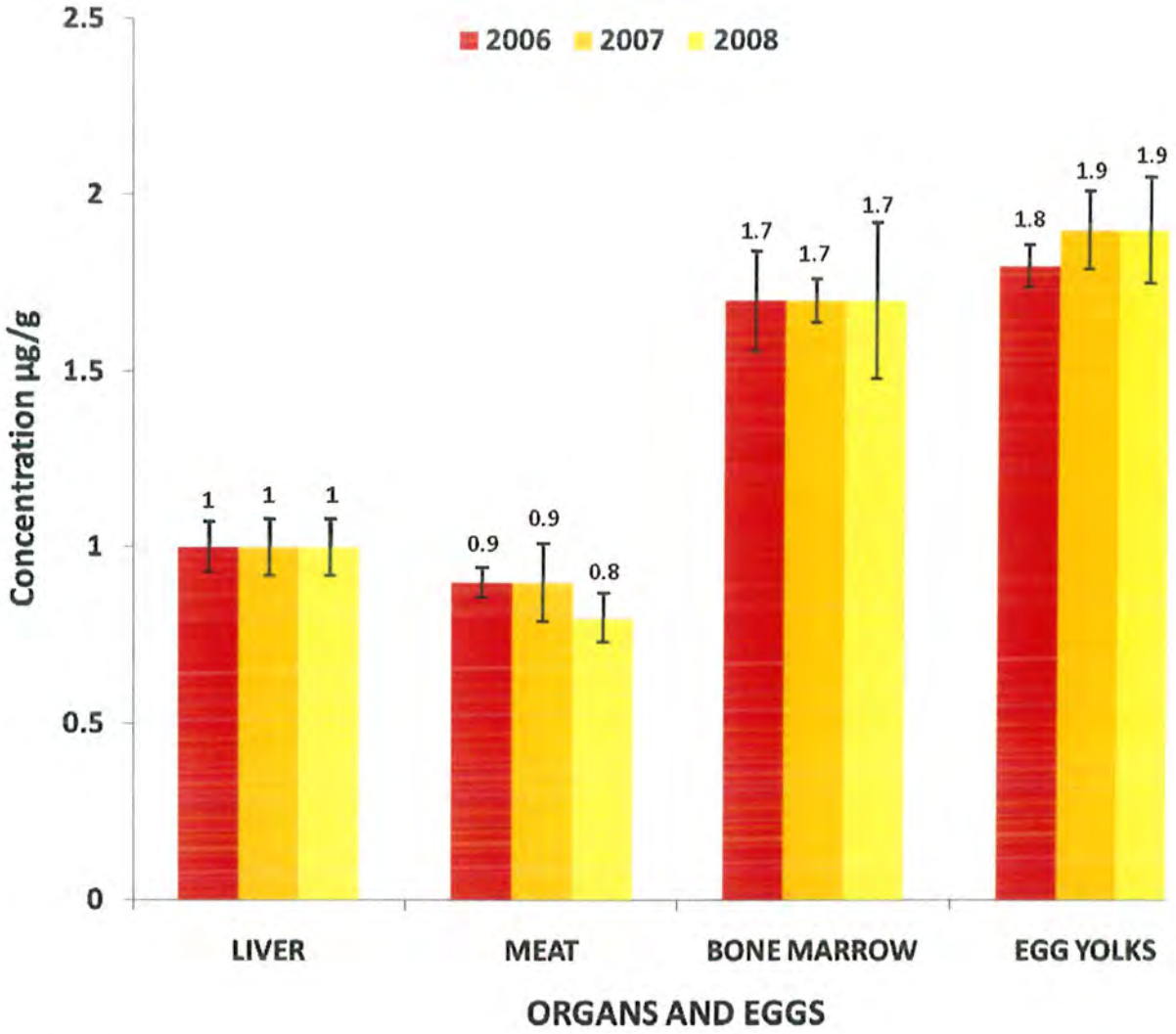

Fig. 20. Tilmicosin residues mean concentration (µg/g) during year 2006 ( ), 2007 ( ), 2008 ( ) by Microbiological assay in poultry liver, meat, bone marrow and egg yolks samples. Numerical values showing mean concentration of Tilmicosin residues along with the lines on the bars indicating standard deviation of the mean.

During 2006, 2007 and 2008 three consecutive years, mean concentration of tilmicosin residues in poultry meat samples was 0.9 ± 0.1 µg/g, 0.9 ± 0.1 µg/g and 0.8 ± 0.1 µg/g by microbiological assay compared with 0.9 ± 0.1 µg/g, 0.9 ± 0.1 µg/g and 0.8 ± 0.1 µg/g with spectrophotometric assay respectively (Fig. 20 & Annex.1.3.2).

Tilmicosin residues were detected in 6.1, 6.0 and 5.9 percent samples, during the year 2006, 2007 and 2008 respectively, among the entire total samples positive for the presence of macrolides residues, in different poultry organs and egg yolks. During the year 2006, the spread of tilmicosin residues in liver were 36.8 percent, followed by the meat 26.3 percent, egg yolks 26.3 percent and bone marrow 10.5 percent. The data was almost in line during the rest of two years. Among the commonly used antimicrobials, the tylosin, erythromycin and tilmicosin were detected in poultry meat, liver, bone marrow and poultry eggs. These residues may persist due to the irrational use of antimicrobials by the farmers, as self medication. Veterinarian, most of the time, followed the withdrawal period of the drug under prescription. Many of the drugs have short or no withdrawal period (Dey, *et al.*, 2005a). Presence of such drugs in poultry eggs and meat lead to the practice where farmer self medicate and in case of failure they sell the flock during medication. The most serious problem needs a due consideration of the authorities. Compensation policy may support to curtail the issue because the poor farmers are not in the capacity to bear the total loss or stay with mortality till clearance of the drug residues.

Tilmicosin residues in bone marrow, during 2006, 2007 and 2008 three consecutive years were 1.7 ± 0.1 µg/g, 1.7 ± 0.1 µg/g and 1.7 ± 0.2 µg/g by microbiological assay compared with 1.7 ± 0.1 µg/g, 1.8 ± 0.1 µg/g and 1.8 ± 0.2 µg/g with spectrophotometric assay respectively. Tilmicosin minimum residues through microbiological assay were 1.6 µg/g in bone marrow and it was higher 1.9 µg/g of bone marrow. In spectrophotometric assay the minimum tilmicosin residues in bone marrow samples was 1.6 µg/g increasing to the level of 1.9 µg/g of bone marrow (Fig. 20 & Annex.1.3.3).

If the farmers do not have proper knowledge regarding residues or they are concealing and not agreed with the soul of the problem that the consumers exposed to drug residue will harm their health through poultry meat, egg, livestock milk and meat. Addition of the sulfamethazine and

sulfadiazine for efficiency for treatment will contaminate the human edibles. Countless harmful effects and bacterial drug resistance will come up as lethal human health threats and risks for national and international consumers (Gaudin *et al.*, 2004). It was time demand a strict legislation for the excessive or unwanted use of antibacterial. Education of the livestock and poultry farmers in the area of good animal production practices for public health safety was needed (Sasanya *et al.*, 2005). Efforts to minimize potential consumer health risk attempts in reduction of the antimicrobials residue level in poultry meat and eggs was the area need due consideration. Fluoroquinolones were used in food animals and has increased in fluoroquinolones resistance, in food borne pathogens and human treatment problems. Fluoroquinolones excessive use in animals was creating resistance in pathogenic bacteria. Fluoroquinolones excessive use in poultry was one of the major causes of development of resistance of drugs in humans. The reason was that residues of these drugs pass on to human, through meat and eggs.

Tilmicosin residues during the year 2006 in 5 positive egg yolks samples were 1.8 ± 0.1 µg/g by microbiological assay compared with 1.9 ± 0.1 µg/g with spectrophotometric assay. During the year 2007, mean concentration of tilmicosin among 4 samples of egg yolks was 1.9 ± 0.1 µg/g by microbiological assay compared with 1.9 ± 0.1 µg/g with spectrophotometric assay. Tilmicosin residues during the year 2008 in 5 positive egg yolks samples were 1.9 ± 0.2 µg/g by microbiological assay compared with 1.9 ± 0.2 µg/g with spectrophotometric assay. The tilmicosin minimum residues through microbiological assay were 1.8 µg/g in egg yolks and it was higher 2.1 µg/g of egg yolks. In spectrophotometric assay the minimum tilmicosin residues in egg yolks samples were 1.8 µg/g increasing to the level of 2.2 µg/g of egg yolks. The non significant higher levels were detected in spectrophotometric assay due to precision of instrument and sensitivity of the assay (Fig. 20 & Annex.1.3.4).

Results of the macrolides residues have been summarized in table 11. Concentration of macrolides residues distributed in different organs of poultry and egg yolks detected through microbiological assay and spectrophotometric assay. The current study throws light on the residual contamination of food chain with macrolides, fluoroquinolones and sulfonamides in poultry meat and eggs. Aims of these investigations were to monitor the flow of residues in

Table 11. Concentration of Tylosin, Erythromycin, Tilmicosin residues distributed in poultry liver, meat, bone marrow and egg yolks, detected through microbiological (M) assay and spectrophotometric (S) assay.

ORGANS	NAME OF DRUG RESIDUE	2006		2007		2008	
		M. assay (µg/g) of sample X ± SD	S. assay (µg/g) of sample X ± SD	M. assay (µg/g) of sample X ± SD	S. assay (µg/g) of sample X ± SD	M. assay (µg/g) of sample X ± SD	S. assay (µg/g) of sample X ± SD
LIVER	Tylosin	2.84 ± 1.09	2.89^{A} ± 1.09	2.58 ± 1.24	2.63^{A} ± 1.25	3.60 ± 1.22	3.65^{A} ± 1.22
	Erythromycin	1.03 ± 0.15	1.09^{B} ± 0.16	1.04 ± 0.14	1.10^{D} ± 0.15	1.04 ± 0.14	1.10^{D} ± 0.15
	Tilmicosin	0.97 ± 0.07	1.00^{C} ± 0.06	0.96 ± 0.08	0.99^{D} ± 0.07	0.95 ± 0.08	0.99^{D} ± 0.07
MEAT	Tylosin	0.99 ± 0.12	1.04^{B} ± 0.13	0.87 ± 0.11	0.91^{D} ± 0.11	0.95 ± 0.14	0.99^{D} ± 0.15
	Erythromycin	2.12 ± 0.59	2.18^{A} ± 0.59	1.88 ± 0.19	1.95^{B} ± 0.20	2.35 ± 0.74	2.42^{B} ± 0.73
	Tilmicosin	0.87 ± 0.04	0.91^{C} ± 0.04	0.89 ± 0.11	0.93^{D} ± 0.11	0.83 ± 0.07	0.86^{D} ± 0.06
BONE MARROW	Tylosin	1.42 ± 0.10	1.48^{B} ± 0.09	1.48 ± 0.05	1.52^{C} ± 0.06	1.44 ± 0.08	1.49^{A} ± 0.07
	Erythromycin	2.34 ± 0.08	2.39^{A} ± 0.08	2.40 ± 0.16	2.45^{A} ± 0.16	2.45 ± 0.08	2.51^{B} ± 0.08
	Tilmicosin	1.66 ± 0.14	1.74^{B} ± 0.16	1.69 ± 0.06	1.77^{B} ± 0.07	1.72 ± 0.22	1.78^{C} ± 0.23
EGG YOLK	Tylosin	1.95 ± 0.18	2.00^{A} ± 0.17	2.03 ± 0.19	2.09^{A} ± 0.18	2.07 ± 0.11	2.12^{B} ± 0.11
	Erythromycin	2.02 ± 0.12	2.07^{A} ± 0.12	1.92 ± 0.16	1.97^{B} ± 0.16	1.93 ± 0.14	1.98^{C} ± 0.14
	Tilmicosin	1.80 ± 0.06	1.87^{B} ± 0.06	1.90 ± 0.11	1.97^{B} ± 0.12	1.90 ± 0.15	1.97^{C} ± 0.15

Values with same superscripts (A-D) are non significantly different ($P < 0.05$) whereas, values with different superscripts (A-D) are significantly different within the same column; X = Mean concentration; SD = Stranded deviation of concentrations; M. assay = microbiological assay; S. assay = spectrophotometric assay.

poultry meat and eggs and consequently the bacterial resistance in human pathogens. Drug resistance is outcome of drug residues, most of the time ignored by the technical personals and farmers needs due consideration. Final goal of the study was to furnish preliminary local data for legislation regarding the withdrawal periods and maximum residual limits. As the poultry farmers and livestock management are not observing the withdrawal period due to the monitory benefits the residues are passing at fast pace in the human food chain through animal origin diets.

FLUOROQUINOLONES RESIDUES

Fluoroquinolones are widely used in poultry as remedy of many bacterial diseases therefore a high percentage of meat and eggs contained with the residues. A total of 21383 samples were collected from slaughter shops and poultry farms and 2752 were positive for antimicrobial activity. Among these 2752 samples, 2338 samples were of poultry meat, liver and bone marrow whereas, 364 samples were of eggs (Table. 6). Three years surveillance results indicated that 905 (32.9 %) samples were positive for fluoroquinolones (norfloxacin, enrofloxacin, ciprofloxacin, sarafloxacin and flumequine) during the years 2006-2008. The spread of fluoroquinolones residues among different organs of poultry was higher in liver (442) followed by meat (306), egg yolks (123) and bone marrow (36). During the year 2006 fluoroquinolones residues were detected in 301 (4.3 %) followed by 306 (4.3 %) in year 2007, whereas, it was 298 (4.1 %) during the year 2008 (Table. 7 & Fig. 21).

Fluoroquinolones are widely used in poultry as remedy of many bacterial diseases therefore a high percentage of meat and eggs do have the residues. A total of 21383 samples were collected from slaughter shops and poultry farms and 2752 were positive. Among these 2752 samples, 2338 samples were of poultry meat, liver and bone marrow whereas, 364 samples were of eggs. During the year 2006, fluoroquinolones included in the studies norfloxacin, enrofloxacin, ciprofloxacin, sarafloxacin and flumequine residues were detected in 301 followed by 306 in year 2007 whereas, it was 298 during the year 2008. Fluoroquinolones residues were highest in liver, followed by meat, bone marrow and egg yolks (Tsai and Kondo, 2001).

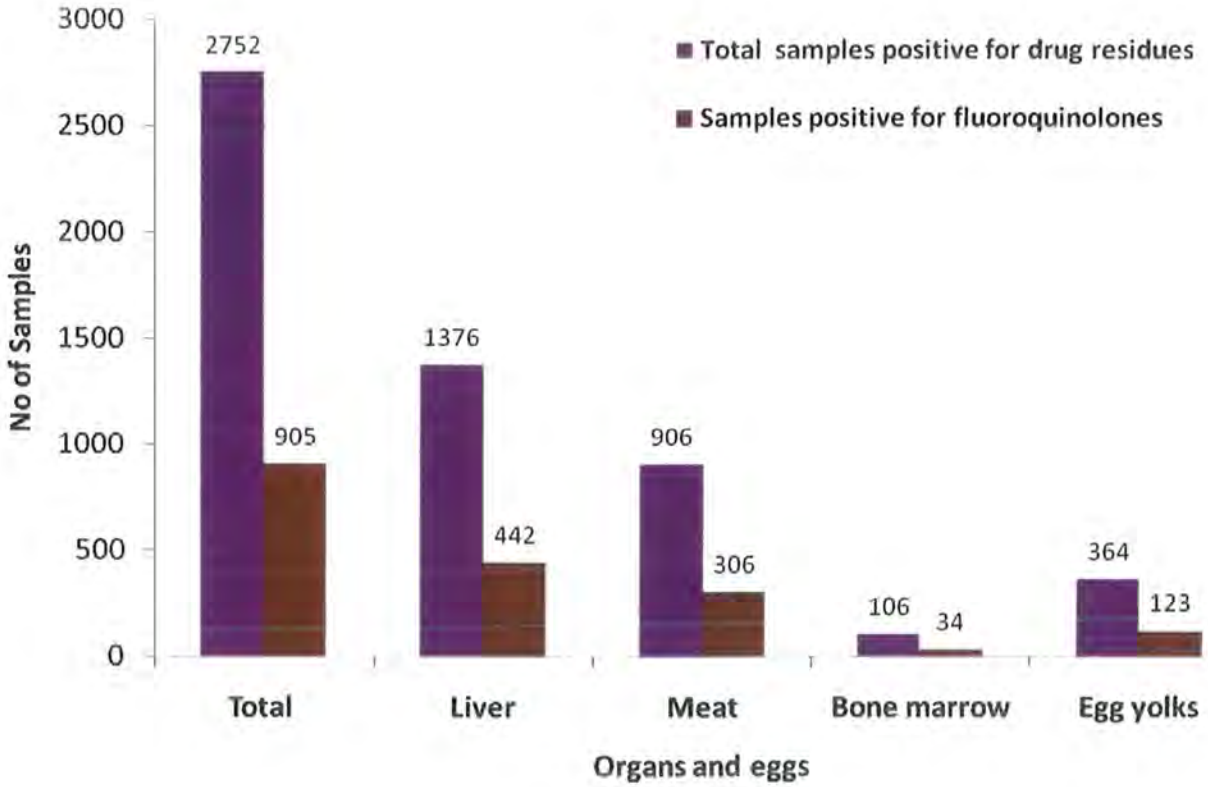

Fig. 21. Distribution of Fluoroquinolones residues among total positive poultry liver, meat, bone marrow and egg yolks samples by Microbiological assay during year 2006 through 2008. Numerical values on the violet bars of the graph ■ indicating the positive samples in three years for Macrolides, Fluoroquinolones and Sulfonamides in total screened samples, liver, meat, bone marrow and egg yolks. Whereas maroon bars in figure ■ with numerical values showing positive samples for Fluoroquinolones residues among total positive samples, liver, meat, bone marrow and egg yolks.

RESIDUES IN POULTRY ORGANS AND EGG YOLKS

During the year 2006, among 3007 samples of liver tested for fluoroquinolones residues, results indicated that 15.0 percent samples were positive for antimicrobial activity and among these positive samples 4.9 percent were positive for fluoroquinolones (norfloxacin, enrofloxacin, ciprofloxacin, sarafloxacin and flumequine) residues. During the year 2007, among 3052 samples of liver, 15.1 percent samples were positive for antimicrobial activity and among these livers 4.9 percent were positive for fluoroquinolones residues. In 3094 liver samples tested, 15.1 percent samples were positive for antimicrobial activity and among these 4.7 percent were positive for fluoroquinolones residues during the year 2008 (Table. 7).

There are some chemotherapeutics commonly used in human being and animals, starting from penicillin to the fluoroquinolones. Synthetic antibacterial fluoroquinolones used in abundance for the prevention and control of human, poultry and large animal diseases. The attraction of usage of fluoroquinolones is due to its pharmacokinetic, bioavailability and withdrawal period. However enrofloxacin the most frequently used in poultry and large animals whereas it's most common known derivatives ciprofloxacin is used in human. Currently ciprofloxacin is also became an attraction in the livestock. Therefore, an assay is needed to distinguish between each fluoroquinolones. Ciprofloxacin and enrofloxacin sensitivity detection limits were 0.01 ppm, formulated for number of drugs (Choma *et al.*, 2005).

During the year 2006, among 2571 meat samples tested for fluoroquinolones residues, 11.7 percent samples were positive for antimicrobial activity and among these 3.9 percent were positive for norfloxacin, enrofloxacin, ciprofloxacin, sarafloxacin and flumequine residues. During the year 2007 totally 11.5 percent samples were positive for antimicrobial activity and among these 3.9 percent were positive for fluoroquinolones residues. The presence was 11.6 percent samples among these 3.8 percent were positive for fluoroquinolones (Table. 7).

Fluoroquinolones are commonly used in the veterinary medication. Screening of residual concentration in the animal origin food will be a step in preventing the transfer of residues to human being and quality control of products. Many countries in the world have fixed the maximum residues limits of fluoroquinolones residues in poultry, sheep, goat and cow. The

Table 13 Norfloxacin, Enrofloxacin, Ciprofloxacin, Sarafloxacin and Flumequine residues distribution in different poultry organs and egg yolks through microbiological (M) assay and spectrophotometric (S) assay during 2007.

ORGAN/ PRODUCT	Norfloxacin (%)	Enrofloxacin (%)	Ciprofloxacin (%)	Sarafloxacin (%)	Flumequine (%)	TOTAL (%)
LIVER	18^{A} (45.00)	75^{E} (48.08)	9^{K} (50.00)	6^{N} (42.86)	41^{R} (52.56)	149 (48.69)
MEAT	15^{B} (37.50)	51^{F} (32.69)	4^{L} (22.22)	3^{P} (21.43)	30^{S} (38.46)	103 (33.66)
BONE MARROW	1^{D} (2.50)	6^{H} (3.85)	1^{M} (5.56)	2^{Q} (14.29)	2^{U} (2.56)	12 (3.92)
EGG YOLK	6^{C} (15.00)	24^{G} (15.38)	4^{L} (22.22)	3P (21.43)	5^{T} (6.41)	42 (12.75)
TOTAL (%)	40 (13.07)	156 (50.98)	18 (5.88)	14 (4.58)	78 (25.49)	306

Values with same superscripts (A-U) are non significantly different ($P < 0.05$) whereas, values with different superscripts (A-U) are significantly different within the same column.
Percentages in the table were calculated on the basis of individual column except last row where percentages were made within the row.

Table 14. Norfloxacin, Enrofloxacin, Ciprofloxacin, Sarafloxacin and Flumequine residues distribution in different poultry organs and egg yolks through microbiological (M) assay and Spectrophotometric (S) assay during 2008.

ORGAN/ PRODUCT	Norfloxacin (%)	Enrofloxacin (%)	Ciprofloxacin (%)	Sarafloxacin (%)	Flumequine (%)	TOTAL (%)
LIVER	15^{A} (40.54)	68^{D} (46.58)	10^{G} (43.48)	7^{M} (50.00)	46^{R} (58.97)	146 (48.99)
MEAT	14^{A} (37.84)	54^{D} (36.99)	7^{H} (30.43)	3^{N} (21.43)	24^{S} (30.77)	102 (34.23)
BONE MARROW	2^{C} (5.41)	4^{F} (2.74)	2^{L} (8.69)	1^{P} (7.14)	1^{U} (1.28)	10 (3.36)
EGG YOLK	6^{B} (16.22)	20^{E} (13.69)	4^{K} (17.39)	3N (21.43)	7^{T} (8.97)	40 (13.42)
TOTAL (%)	37 (12.42)	146 (48.99)	23 (7.72)	14 (4.70)	78 (26.17)	298

Values with same superscripts (A-U) are non significantly different ($P < 0.05$) whereas, values with different superscripts (A-U) are significantly different within the same column.
Percentages in the table were calculated on the basis of individual column except last row where percentages were made within the row.

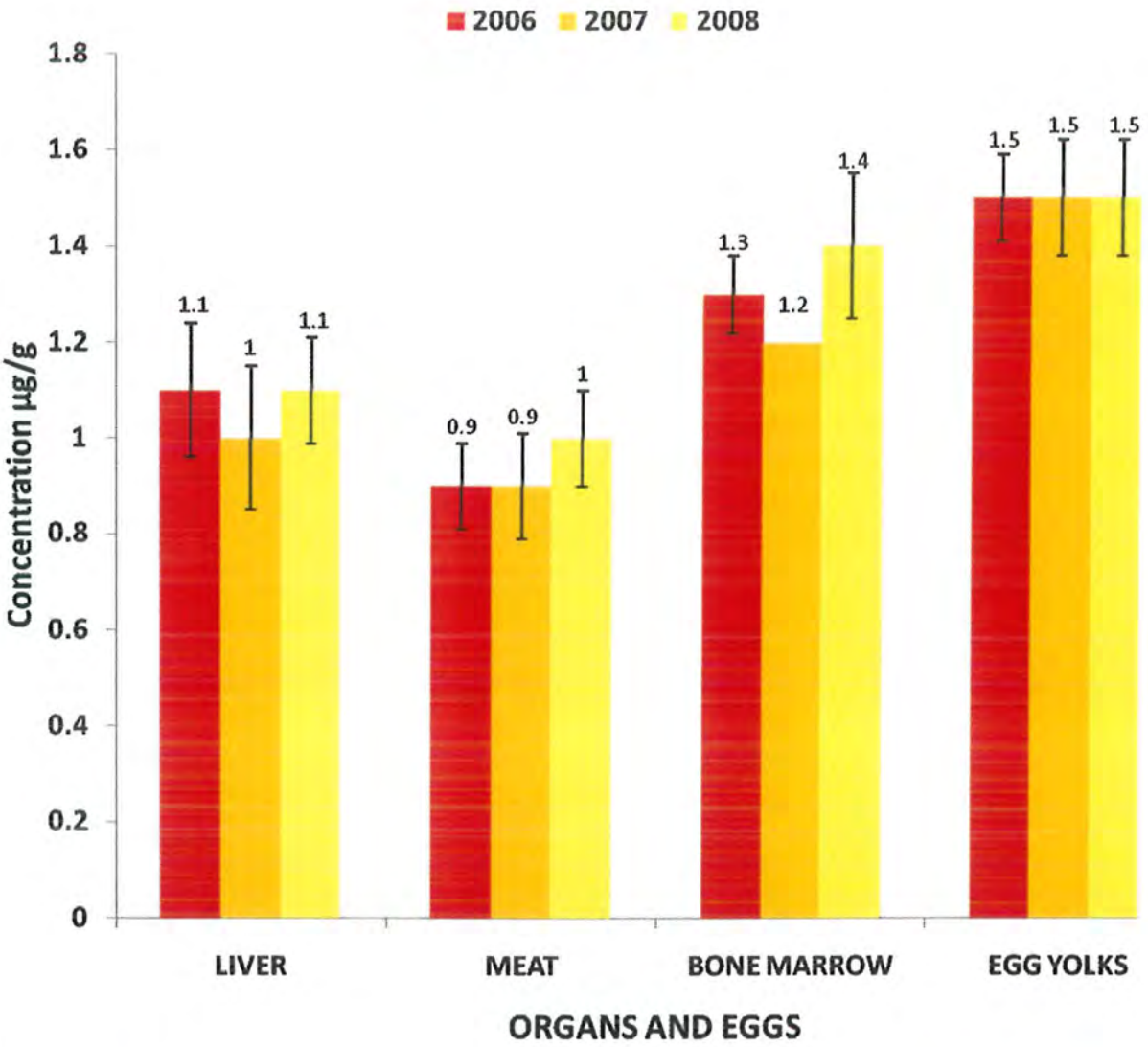

Fig. 22. Norfloxacin residues mean concentration (µg/g) during year 2006 ( ), 2007 ( ), 2008 ( ) by Microbiological assay in poultry liver, meat, bone marrow and egg yolks samples. Numerical values showing mean concentration of Norfloxacin residues along with the lines on the bars indicating standard deviation of the mean.

assay. During the year 2007, mean concentration of norfloxacin among six samples of egg yolks was 1.5 ± 0.1 µg/g by microbiological assay compared with 1.5 ± 0.1 µg/g with spectrophotometric assay. Mean concentration of norfloxacin residues during the year 2008 in 6 positive samples of egg yolks was 1.4 ± 0.1 µg/g by microbiological assay compared with 1.5 ± 0.1 µg/g with spectrophotometric assay (Fig. 22 & Annex. 2.1.4).

ENROFLOXACIN RESIDUES

During the year 2006, among all the screened samples for the presence of fluoroquinolones residues in different poultry organs and egg yolks, out of these total 301 positive samples for fluoroquinolones residues, 51.2 percent were positive for enrofloxacin (Table. 12). The spread of enrofloxacin in different organs was higher in the liver 46.1 percent, followed by the meat 34.4 percent, egg yolks 16.2 percent and bone marrow 3.3 percent. During the year 2007, among all the screened samples for the presence of fluoroquinolones residues in different poultry organs and egg yolks, out of these total 306 positive samples for fluoroquinolones residues 50.9 percent were positive for enrofloxacin (Table. 13). The spread of enrofloxacin in different organs was higher in the liver 48.1 percent, followed by the meat 32.7 percent, egg yolks 15.4 percent and bone marrow 3.9 percent. During the year 2008, among all the screened samples for the presence of fluoroquinolones residues in different poultry organs and egg yolks, out of these total 298 positive samples for fluoroquinolones residues, 49.0 percent were positive for enrofloxacin (Table. 14). The spread of enrofloxacin in different organs was higher in the liver 46.6 percent, followed by the meat 37.0 percent), egg yolks 13.7 percent and bone marrow 2.7 percent. Enrofloxacin is in abundance used without prescription in the poultry. Although withdrawal period of enrofloxacin is 1-2 days therefore, decontaminant of the food is easier by observing the withdrawal period.

During the year first year of study, mean concentration of enrofloxacin among 71 samples of liver was 2.6 ± 1.1 µg/g by microbiological assay compared with 2.7 ± 1.1 µg/g with spectrophotometric assay (Fig. 23 & Annex. 2.2.1). During the second year of study, mean concentration of enrofloxacin among 75 samples of liver was 2.9 ± 1.4 µg/g by microbiological assay compared with 3.0 ± 1.4 µg/g with spectrophotometric assay. Enrofloxacin residues during

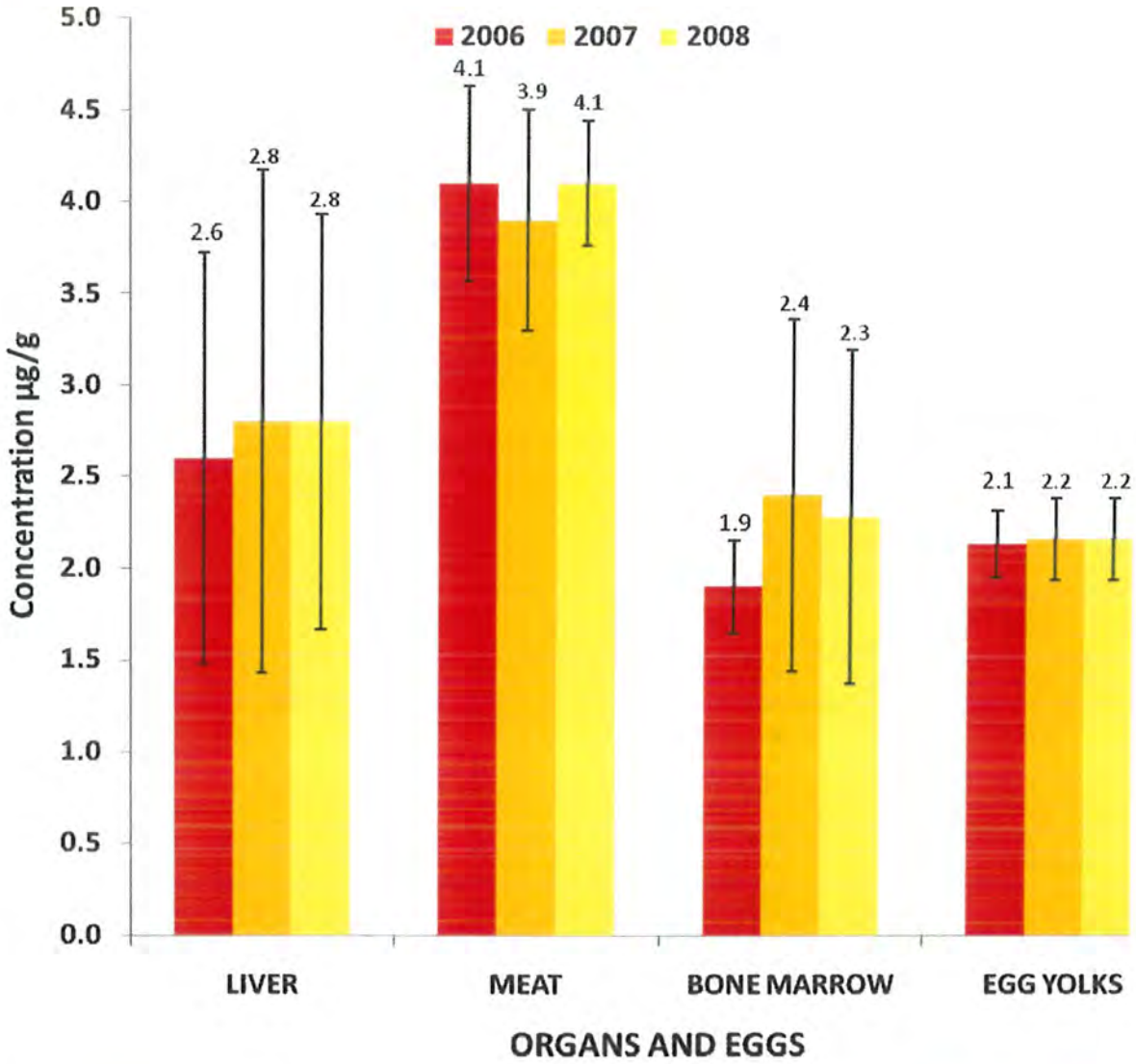

Fig. 23. Enrofloxacin residues mean concentration (µg/g) during year 2006 ( ), 2007 ( ), 2008 ( ) by Microbiological assay in poultry liver, meat, bone marrow and egg yolks samples. Numerical values showing mean concentration of Enrofloxacin residues along with the lines on the bars indicating standard deviation of the mean.

the year third and last year of study in 68 positive samples of liver were 2.8 ± 1.1 µg/g by microbiological assay compared with 2.8 ± 1.1 µg/g with spectrophotometric assay (Fig. 23 & Annex. 2.2.1).

During the year 2006, mean concentration of enrofloxacin among 53 samples of poultry meat was 4.1 ± 0.5 µg/g by microbiological assay compared with 4.3 ± 0.5 µg/g with spectrophotometric assay. The enrofloxacin minimum residue through microbiological assay was 3.1 µg/g in meat and it was higher 4.6 µg/g of meat. In spectrophotometric assay the minimum enrofloxacin residues in meat was 3.2 µg/g increasing to the level of 4.8 µg/g of meat. During the year 2007, mean concentration of enrofloxacin among 51 samples of meat was 3.9 ± 0.6 µg/g by microbiological assay compared with 4.1 ± 0.6 µg/g with spectrophotometric assay. Enrofloxacin residues, during the year 2008 in 54 positive samples of meat were 4.1 ± 0.3 µg/g by microbiological assay compared with 4.3 ± 0.3 µg/g with spectrophotometric assay (Fig. 23 & Annex. 2.2.2).

During the year 2006, mean concentration of enrofloxacin among 5 samples of bone marrow was 1.9 ± 0.2 µg/g by microbiological assay compared with 1.9 ± 0.2 µg/g with spectrophotometric assay. During the year 2007, mean concentration of enrofloxacin among six samples of bone marrow was 2.4 ± 0.9 µg/g by microbiological assay compared with 2.5 ± 0.9 µg/g with spectrophotometric assay. Enrofloxacin residues, during the year 2008 in 4 positive samples of bone marrow were 2.2 ± 0.9 µg/g by microbiological assay compared with 2.3 ± 0.9 µg/g with spectrophotometric assay (Fig. 23 & Annex. 2.2.3).

During the year 2006, mean concentration of enrofloxacin among 25 samples of egg yolks was 2.1 ± 0.1 µg/g by microbiological assay compared with 2.2 ± 0.2 µg/g with spectrophotometric assay. During the year 2007 mean concentration of enrofloxacin among 24 samples of egg yolks was 2.2 ± 0.2 µg/g by microbiological assay compared with 2.2 ± 0.2 µg/g with spectrophotometric assay. Whereas, the mean concentration of enrofloxacin residues, during year 2008 in 20 positive samples of egg yolks was 2.1 ± 0.2 µg/g by microbiological assay compared with 2.2 ± 0.2 µg/g with spectrophotometric assay (Fig. 23 & Annex. 2.2.4).

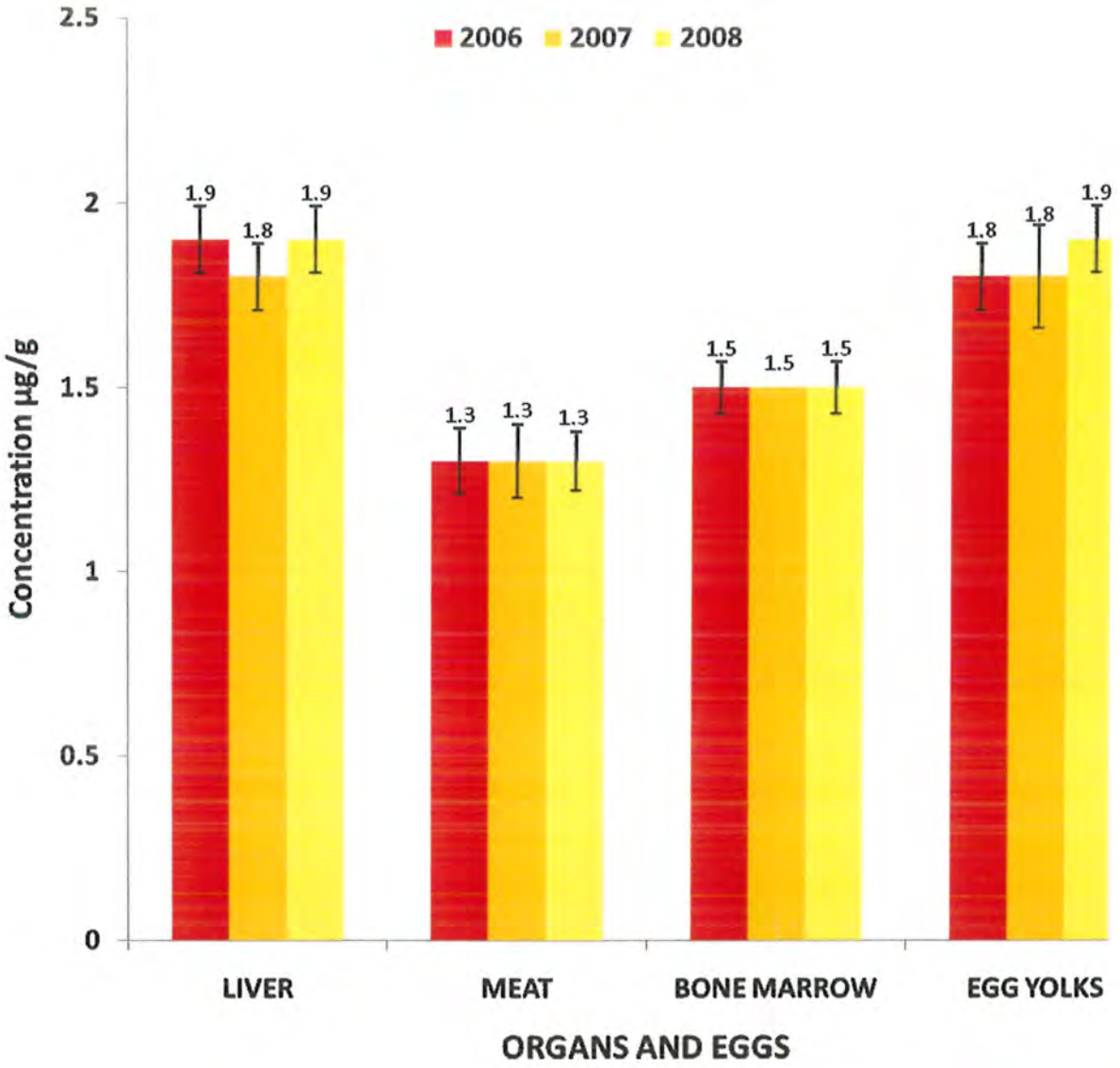

Fig. 24. Ciprofloxacin residues mean concentration (µg/g) during year 2006 ( ), 2007 ( ), 2008 ( ) by Microbiological assay in poultry liver, meat, bone marrow and egg yolks samples. Numerical values showing mean concentration of Ciprofloxacin residues along with the lines on the bars indicating standard deviation of the mean.

During the year 2006, mean concentration of ciprofloxacin between 2 samples of bone marrow was 1.5 ± 0.1 µg/g by microbiological assay compared with 1.5± 0.1 µg/g with spectrophotometric assay (Fig. 24 & Annex. 2.3.3). During the year 2007, concentration of ciprofloxacin in 01 sample of bone marrow was 1.4 µg/g by microbiological assay compared with the 1.5 µg/g with spectrophotometric assay. Mean concentration of ciprofloxacin residues, during the year 2008 in 2 positive samples of bone marrow was 1.5 µg/g by microbiological assay compared with 1.5 µg/g with spectrophotometric assay.

During the year 2006, mean concentration of ciprofloxacin among 5 samples of egg yolks was 1.8 µg/g by microbiological assay compared with 1.8 µg/g with spectrophotometric assay. During year 2007, mean concentration of ciprofloxacin among 4 samples of egg yolks was 1.8 µg/g by microbiological assay compared with 1.8 µg/g with spectrophotometric assay. Ciprofloxacin residues, during the year 2008 in 4 positive samples of egg yolks were 1.8 ± 0.1 µg/g by microbiologically as compared with 1.9 ± 0.1 µg/g with spectrophotometric assay (Fig. 24 & Annex. 2.3.4).

SARAFLOXACIN RESIDUES

During the year 2006, among all the screened samples for the presence of fluoroquinolones residues in different poultry organs and egg yolks, out of these total 301 positive samples for drug residues, 3.3 percent were positive for sarafloxacin (Table. 12). The spread of sarafloxacin in different organs was higher in the liver 50.0 percent, followed by the meat 20.0 percent, egg yolks 20.0 and bone marrow 10.0 percent. During the year 2007, among all the 306 samples positive for fluoroquinolones residues, 4.6 percent were positive for sarafloxacin (Table. 13). The spread of sarafloxacin in different organs was higher in the liver 42.9 percent, followed by the meat 21.4 percent, egg yolks 21.4 percent and bone marrow 14.3 percent. During the year 2008, among all the 298 samples positive for fluoroquinolones residues, 4.7 percent were positive for sarafloxacin (Table. 14). The spread of sarafloxacin in different organs was higher in the liver 50.0 percent, followed by the meat 21.4 percent, egg yolks 21.4 percent and bone marrow 7.1 percent.

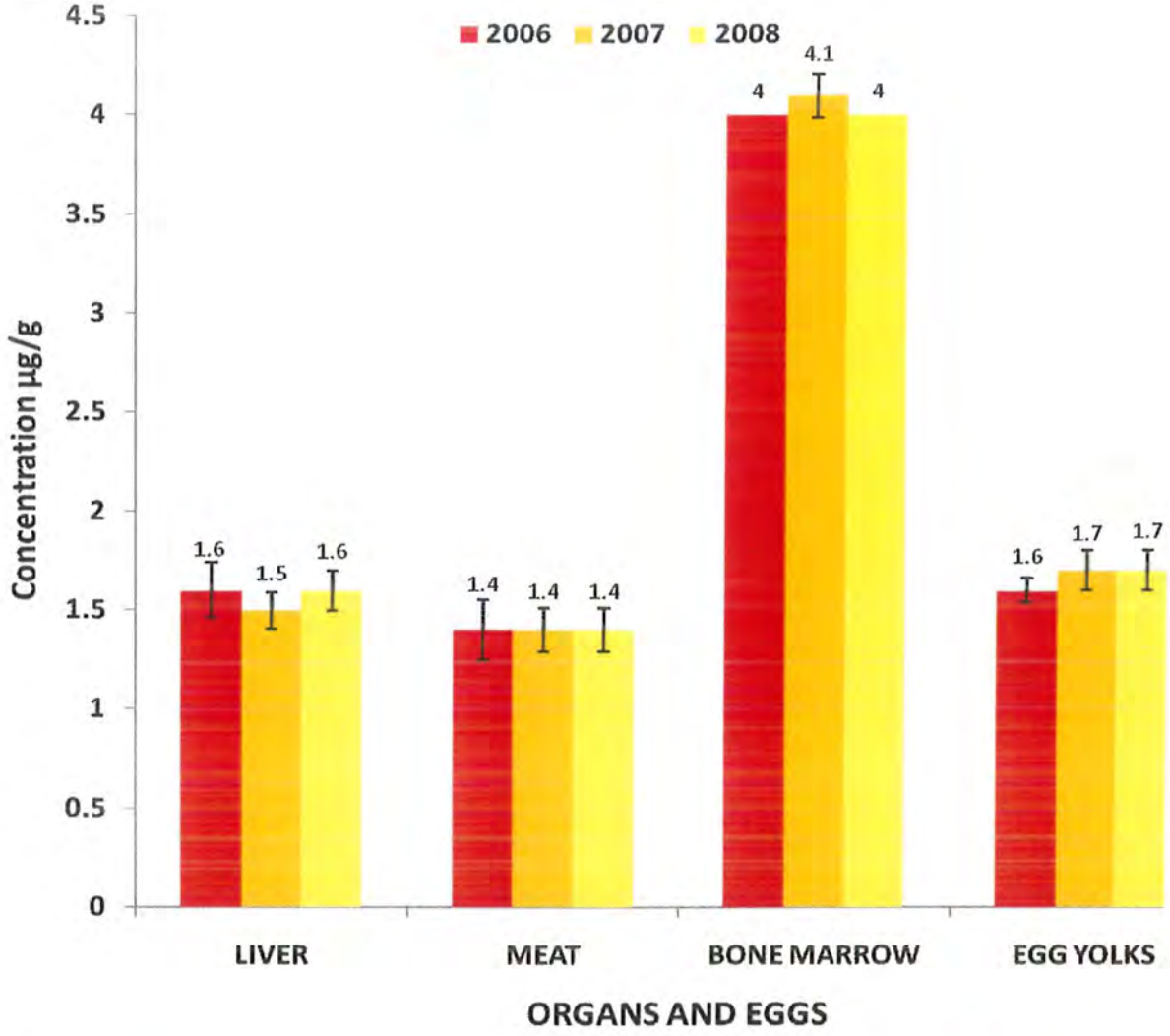

Fig. 25. Sarafloxacin residues mean concentration (µg/g) during year 2006 ( ), 2007 ( ), 2008 ( ) by Microbiological assay in poultry liver, meat, bone marrow and egg yolks samples. Numerical values showing mean concentration of Sarafloxacin residues along with the lines on the bars indicating standard deviation of the mean.

During the year 2007 mean concentration of sarafloxacin among three samples of egg yolks, was 1.7 ± 0.1 µg/g by microbiological assay, compared with 1.7 ± 0.1 µg/g in egg yolks, with spectrophotometric assay. Sarafloxacin residues during the year 2008 in positive 3 samples of egg yolks were 1.7 ± 0.1 µg/g by microbiological assay compared with the 1.7 ± 0.1 µg/g with spectrophotometric assay (Fig. 25 & Annex. 2.4.4).

FLUMEQUINE RESIDUES

During the year 2006, among all the 301 samples positive for fluoroquinolones residues, 26.5 were positive for flumequine (Table. 12). The spread of flumequine in different organs was higher in the liver 60.8 percent, followed by the meat 31.7 percent, egg yolks 5.1 percent and bone marrow 2.5 percent. During the year 2007, among all the 306 positive samples for fluoroquinolones residues, 25.5 percent were positive for flumequine (Table. 13). The spread of flumequine in different organs was higher in the liver 52.6 percent, followed by meat 38.5 percent, egg yolks 6.4 percent and bone marrow 2.6 percent. During the year 2008, among all the 298 samples positive for fluoroquinolones residues, 26.2 percent were positive for flumequine (Table. 14). The spread of flumequine in different organs was higher in the liver 59.0 percent, followed by the meat 30.8 percent, egg yolks 9.0 and bone marrow 1.3 percent.

During the year 2006, the mean concentration of flumequine among 48 samples of liver was 4.0 ± 0.6 µg/g by microbiological assay compared with the 4.3 ± 0.6 µg/g with spectrophotometric assay. During the year 2007, the mean concentration of flumequine among 41 samples of liver was 3.9 ± 0.6 µg/g by microbiological assay compared with the 4.1 ± 0.6 µg/g with spectrophotometric assay. Flumequine residues during the year 2008 in 46 positive samples of liver were 4.0 ± 0.6 µg/g by microbiological assay compared with the 4.2 ± 0.6 µg/g with spectrophotometric assay (Fig. 26 & Annex. 2.5.1).

During the year 2006, mean concentration of flumequine among 25 samples of poultry meat was 2.0 ± 0.2 µg/g by microbiological assay compared with the 2.1 ± 0.2 µg/g with spectrophotometric assay. During the year 2007 mean concentration of flumequine among 30 samples of meat was 2.0 ± 0.2 µg/g by microbiological assay compared with the 2.1 ± 0.2

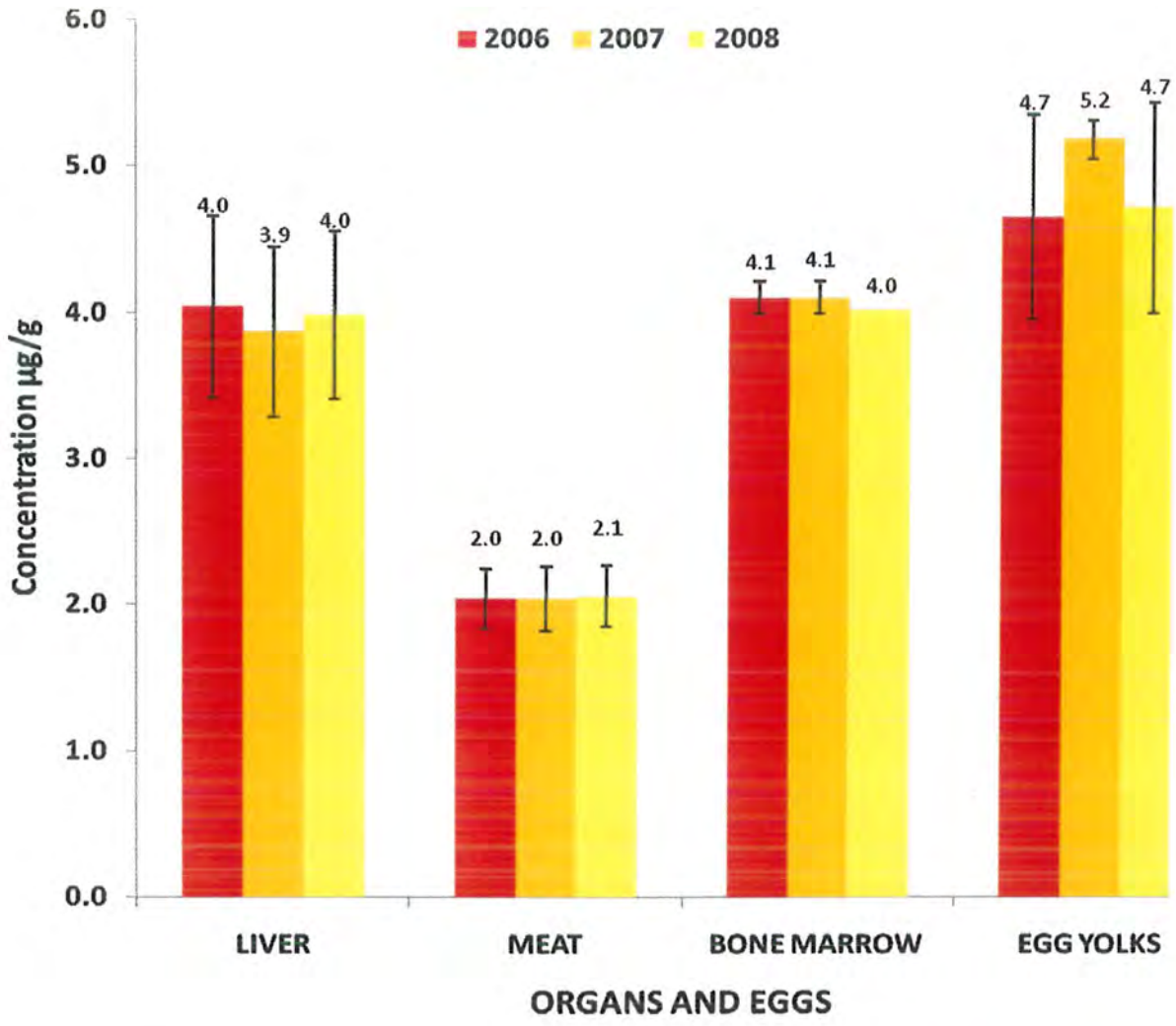

Fig. 26. Flumequine residues mean concentration (µg/g) during year 2006 ( ), 2007 ( ), 2008 ( ) by Microbiological assay in poultry liver, meat, bone marrow and egg yolks samples. Numerical values showing mean concentration of Flumequine residues along with the lines on the bars indicating standard deviation of the mean.

µg/g with spectrophotometric assay. Mean concentration of flumequine residues during the year 2008 in 24 positive samples of meat was 2.1 ± 0.2 µg/g by microbiological assay compared with the 2.1 ± 0.2 µg/g with spectrophotometric assay (Fig. 26 & Annex. 2.5.2).

During the year 2006, mean concentration of flumequine between 2 samples of bone marrow was 4.1 ± 0.1 µg/g by microbiological assay compared with the 4.2 ± 0.1 µg/g with spectrophotometric assay. During the year 2007, mean concentration of flumequine between 2 samples of bone marrow was 4.1 ± 0.1 µg/g by microbiological assay compared with the 4.2 ± 0.1 µg/g with spectrophotometric assay. Concentration of flumequine residues during the year 2008, in positive one sample of bone marrow was 4.0 µg/g by microbiological assay compared with 4.1 µg/g with spectrophotometric assay (Fig. 26 & Annex. 2.5.3).

During the year 2006, mean concentration of flumequine among 4 positive samples of egg yolks was 4.7 ± 0.7 µg/g by microbiological assay compared with the 4.7 ± 0.7 µg/g with spectrophotometric assay. During the year 2007, mean concentration of flumequine among 5 samples of egg yolks was 5.2 ± 0.1 µg/g by microbiological assay compared with the 5.3 ± 0.2 µg/g with spectrophotometric assay. Flumequine residues during the year 2008 in 7 positive samples of egg yolks were 4.7 ± 0.7 µg/g by microbiological assay compared with the 4.7 ± 0.7 µg/g with spectrophotometric assay (Fig. 26 & Annex. 2.5.4).

Results of the fluoroquinolones residues have been summarized in table 15. Concentration of fluoroquinolones residues distributed in different organs of poultry and egg yolks detected through microbiological assay and spectrophotometric assay.

SULFONAMIDES RESIDUES

Sulfonamides are in routine for the treatment and prevention in human and livestock and even as growth promoter is also achieved in animals. The excessive use of sulfadimidine may lead to the serious complication in human being as the chemically induced allergic toxic reaction after consumption of contaminated food having residue of sulfadimidine. Sulfadimidine may be

Table 15. Concentration of Norfloxacin, Enrofloxacin, Ciprofloxacin, Sarafloxacin, Flumequine residues distributed in poultry liver, meat, bone marrow and egg yolks detected through microbiological (M) bioassay and spectrophotometric (S) assay.

ORGANS	NAME OF DRUG RESIDUE	2006		2007		2008	
		M. assay (µg/g) of sample X ± SD	S. assay (µg/g) of sample X ± SD	M. assay (µg/g) of sample X ± SD	S. assay (µg/g) of sample X ± SD	M. assay (µg/g) of sample X ± SD	S. assay (µg/g) of sample X ± SD
LIVER	Norfloxacin	1.07 ± 0.14	1.13^{C} ± 0.15	1.04 ± 0.15	1.10^{B} ± 0.16	1.06 ± 0.11	1.12^{D} ± 0.12
	Enrofloxacin	2.60 ± 1.12	2.66^{B} ± 1.12	2.94 ± 1.37	2.99^{B} ± 1.37	2.82 ± 1.13	2.88^{B} ± 1.13
	Ciprofloxacin	1.86 ± 0.09	1.93^{B} ± 0.09	1.84 ± 0.09	1.90^{C} ± 0.09	1.86 ± 0.09	1.93^{B} ± 0.09
	Sarafloxacin	1.60 ± 0.14	1.65^{C} ± 0.14	1.50 ± 0.09	1.56^{D} ± 0.08	1.60 ± 0.10	1.66^{C} ± 0.10
	Flumequine	4.04 ± 0.62	4.26^{A} ± 0.65	3.87 ± 0.58	4.08^{D} ± 0.62	3.98 ± 0.57	4.21^{A} ± 0.61
MEAT	Norfloxacin	0.91 ± 0.09	0.96^{C} ± 0.12	0.94 ± 0.11	1.00^{D} ± 0.15	0.96 ± 0.10	1.02^{D} ± 0.15
	Enrofloxacin	4.09 ± 0.53	4.31^{A} ± 0.56	3.92 ± 0.60	4.14^{A} ± 0.64	4.14 ± 0.34	4.39^{A} ± 0.36
	Ciprofloxacin	1.32 ± 0.09	1.39^{C} ± 0.09	1.32 ± 0.10	1.38^{D} ± 0.11	1.34 ± 0.08	1.41^{C} ± 0.09
	Sarafloxacin	1.35 ± 0.15	1.41^{C} ± 0.16	1.35 ± 0.11	1.41^{D} ± 0.11	1.35 ± 0.11	1.41^{C} ± 0.16
	Flumequine	2.04 ± 0.20	2.11^{B} ± 0.19	2.04 ± 0.22	2.11^{D} ± 0.21	2.06 ± 0.21	2.13^{B} ± 0.21
BONE MARROW	Norfloxacin	1.30 ± 0.08	1.36^{C} ± 0.08	1.24 ± 0.00	1.30^{A} ± 0.00	1.35 ± 0.15	1.41^{C} ± 0.16
	Enrofloxacin	1.93 ± 0.25	1.99^{A} ± 0.24	2.40 ± 0.96	2.46^{B} ± 0.95	2.28 ± 0.91	2.35^{B} ± 0.90
	Ciprofloxacin	1.51 ± 0.07	1.57^{C} ± 0.07	1.46 ± 0.00	1.51^{D} ± 0.00	1.51 ± 0.07	1.57^{C} ± 0.08
	Sarafloxacin	4.02 ± 0.00	4.10^{A} ± 0.00	4.10 ± 0.11	4.18^{A} ± 0.11	4.02 ± 0.00	4.11^{A} ± 0.00
	Flumequine	4.10 ± 0.11	4.18^{A} ± 0.11	4.10 ± 0.11	4.18^{A} ± 0.10	4.02 ± 0.00	4.10^{A} ± 0.00
EGG YOLK	Norfloxacin	1.47 ± 0.09	1.53^{C} ± 0.09	1.47 ± 0.12	1.53^{D} ± 0.13	1.47 ± 0.12	1.53^{C} ± 0.13
	Enrofloxacin	2.13 ± 0.18	2.19^{B} ± 0.19	2.16 ± 0.22	2.22^{B} ± 0.23	2.16 ± 0.22	2.22^{B} ± 0.22
	Ciprofloxacin	1.78 ± 0.09	1.85^{B} ± 0.09	1.81 ± 0.14	1.88^{C} ± 0.13	1.87 ± 0.09	1.94^{B} ± 0.08
	Sarafloxacin	1.61 ± 0.06	1.66^{C} ± 0.08	1.66 ± 0.10	1.72^{C} ± 0.12	1.66 ± 0.10	1.72^{C} ± 0.11
	Flumequine	4.65 ± 0.70	4.72^{A} ± 0.71	5.18 ± 0.13	5.27^{A} ± 0.15	4.65 ± 0.72	4.71^{A} ± 0.73

Values with same superscripts (A-D) are non significantly different ($P < 0.05$) whereas, values with different superscripts (A-D) are significantly different within the same column; X = Mean concentration; SD = Stranded deviation of concentrations; M. assay = microbiological assay; S. assay = spectrophotometric assay.

detected using variety of chemical method. Liquid chromatography-mass spectrometry, HPLC, thin layer chromatography, paper and gel electrophoresis, ELISA, bio sensing and immunological and microbial bioassay are most commonly employed (Wang *et al.*, 2006).

A total of 21383 samples were collected from slaughter shops and poultry farms and 2752 were positive. Among these 2752 positive samples, 900 (32.7 %) were positive for sulfonamides residues. The spread of sulfonamides among different organs of poultry was higher in liver (466) followed by meat (284), egg yolks (114) and bone marrow (36). During the year 2006, residues of sulfonamides including sulfadiazine, sulfaquinoxaline, sulfachlorpyridazine and sulfadimidine were detected in 291 (4.1 %) followed by 294 (4.1 %) in year 2007 whereas, it was 315 (4.4 %) during the year 2008 (Table. 7 & Fig. 27).

SULFONAMIDES RESIDUES IN POULTRY ORGANS & EGG YOLKS

During the year 2006, among 3007 samples of liver tested for sulfonamides residues, results indicated that 15.0 percent samples were positive for antimicrobial activity and among these positive samples 5.0 percent were positive for sulfonamides residues (Table. 7). During the year 2007, among 3052 samples of liver 15.1 percent samples were positive for antimicrobial activity and among these livers 5.1 percent were positive for sulfonamides residues. In 3094 livers tested samples, 464 samples were positive for antimicrobial activity and among these 160 were positive for sulfonamides; sulfadiazine, sulfaquinoxaline, sulfachlorpyridazine and sulfadimidine residues during the year 2008 (Table. 7).

During the year 2006, among 2571 meat samples tested for sulfonamides residues, 11.7 percent samples were positive for antimicrobial activity and among these 3.7 percent were positive for sulfonamides; sulfadiazine, sulfaquinoxaline, sulfachlorpyridazine and sulfadimidine residues. During the year 2007, among 2610 meat samples tested, 300 samples were positive for antimicrobial activity and among these 92 were positive for sulfonamides residues. During the year 2008, in 2646 meat samples tested, 11.6 percent samples were positive for antimicrobial activity and among these 3.7 percent were positive for sulfonamides residues (Table. 7).

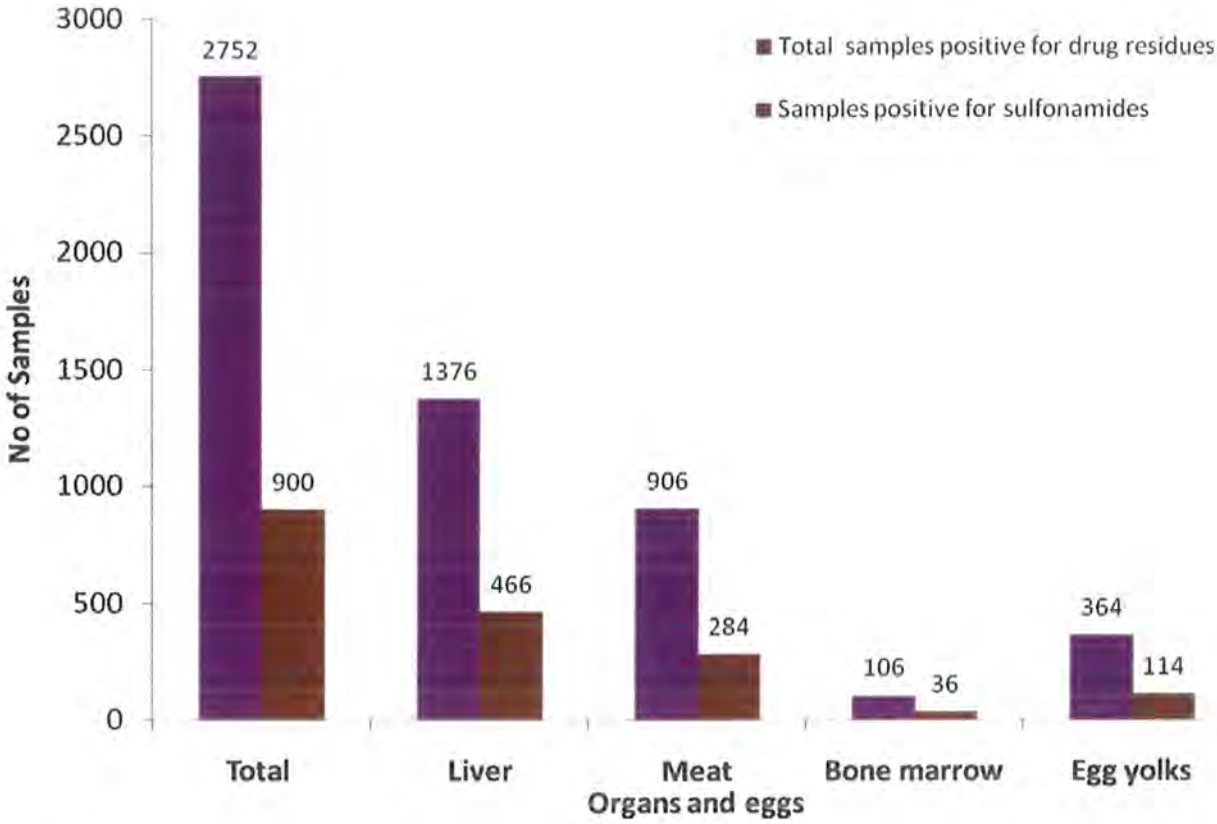

Fig. 27. Distribution of Sulfonamides residues among total positive poultry liver, meat, bone marrow and egg yolks samples by Microbiological assay during year 2006 through 2008. Numerical values on the violet bars of the graph ▮ indicating the positive samples in three years for Macrolides, Fluoroquinolones and Sulfonamides in total screened samples, liver, meat, bone marrow and egg yolks. Whereas maroon bars in figure ▮ with numerical values showing positive samples for Sulfonamides residues among total positive samples, liver, meat, bone marrow and egg yolks.

Animal feed was a watch dog for the human health safety status from form ending at fork. Emergence of rations of pathogen has provided food for thought in the area of contamination transferred from feed to animal and consequently to the human being. Microbes contaminated animal feed was a classical vector of transfer of zoonotic diseases. In United States of America animal feed was most of the time contaminated with the non *typhi Salmonella* called *Salmonella enterica* lead to the infection and carrier stage in human being after colonization in GIT. The infected animals contaminate the meat at slaughter house or during the transportation and spread the infection to the human being after semi cooked or pseudo contamination. Ultimate traceability of source was very tedious work; however several out breaks are blamed to contaminated feed fed to the poultry and livestock herds (Crump *et al.*, 2002).

During the year 2006, among 258 samples of bone marrow tested for sulfonamides residues, 12.8 percent samples were positive for antimicrobial activity and among these 3.9 percent were positive for sulfonamides residues. During the year 2007, among 262 samples of bone marrow tested, 35 samples were positive for antimicrobial activity and among these 11 were positive for sulfonamides residues. During the year 2008, among 266 samples of bone marrow tested, 14.3 percent samples were positive for antimicrobial activity and among these 4.6 percent were positive for sulfonamides residues (Table. 7).

During the year 2006, among 1189 samples of egg yolks tested for sulfonamides residues, 9.9 percent samples results were positive for antimicrobial activity and among these 2.9 percent were found positive for sulfonamides residues. During the year 2007, among 1204 samples of egg yolks tested, 120 samples had antimicrobial activity and among these 36 were positive for sulfonamides residues. During the year 2008, among 1224 samples of egg yolks tested, 10.3 percent samples were positive for antimicrobial activity and among these 3.5 percent were positive for sulfonamides residues (Table. 7).

SULFADIAZINE RESIDUES

During the year 2006, among all the screened samples for the presence of sulfonamides residues in different poultry organs and egg yolks, out of these total 291 positive samples for sulfonamides residues, 33.7 percent were positive for sulfadiazine, (Table. 16). The spread

sulfadiazine of in different organs was higher in the liver 49, followed by the meat 32, egg yolks 13 and bone marrow 4. During the year 2007, among all the screened samples for the presence of sulfonamides residues in different poultry organs and egg yolks out of these total 294 positive samples for drug residues, 34.7 percent were positive for sulfadiazine (Table. 17). The spread of sulfadiazine in different organs was higher in the liver 50.0 percent, followed by the meat 32.4, percent egg yolks 14.7 percent and bone marrow 2.9 percent. During the year 2008, among all the screened samples for the presence of sulfonamides residues in different poultry organs and egg yolks, out of these total 315 positive samples for drug residues, 37.1 percent were positive for sulfadiazine (Table. 18). The spread of sulfadiazine in different organs was higher in the liver 50.4 percent, followed by the meat 26.5 percent, egg yolks 16.2 percent and bone marrow 6.8 percent.

During the year 2006, the mean concentration of sulfadiazine residues among 49 positive samples of liver was 3.9 ± 0.7 µg/g by microbiological assay compared with the 4.1 ± 0.7 µg/g with spectrophotometric assay. Whereas, mean concentration of sulfadiazine during the year 2007 among 51 positive samples of liver was 3.8 ± 0.6 µg/g by microbiological assay compared with the 4.0 ± 0.6 µg/g with spectrophotometric assay. During the year 2008, mean concentration of sulfadiazine in 59 positive samples of liver was 4.0 ± 0.6 µg/g by microbiological assay compared with the 4.2 ± 0.7 µg/g with spectrophotometric assay (Fig. 28 & Annex. 3.1.1).

The spread of sulfadiazine in different organs was higher in liver. During the year 2006, the mean concentration of sulfadiazine among thirteen positive samples of egg yolks was 3.9 ± 0.4 µg/g by microbiological assay compared with the 4.1 ± 0.4 µg/g with spectrophotometric assay. Whereas, the mean concentration of sulfadiazine during the year 2007, among 15 positive samples of egg yolks was 3.9 ± 0.3 µg/g by microbiological assay compared with the 4.1 ± 0.3 µg/g with spectrophotometric assay. Mean concentration of sulfaquinoxaline during the year 2006, among eleven positive samples of egg yolks was 2.2 ± 0.4 µg/g by microbiological assay compared with the 2.3 ± 0.4 µg/g with spectrophotometric assay. The mean concentration of sulfachlorpyridazine among five positive samples of egg yolks was 2.8 ± 0.2 µg/g by

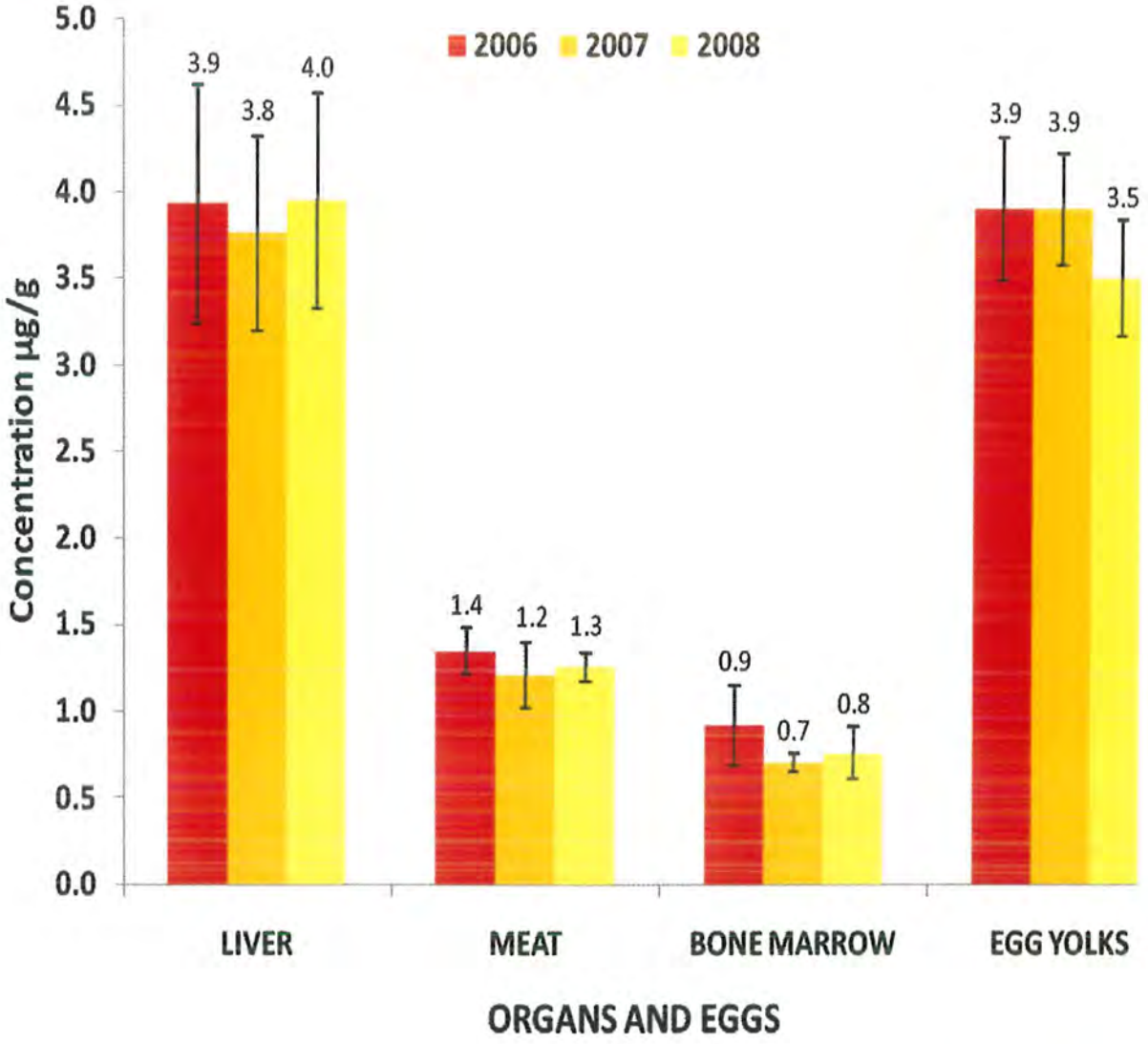

Fig. 28. Sulfadiazine residues mean concentration (µg/g) during year 2006 ( ), 2007 ( ), 2008 ( ) by Microbiological assay in poultry liver, meat, bone marrow and egg yolks samples. Numerical values showing mean concentration of Sulfadiazine residues along with the lines on the bars indicating standard deviation of the mean.

microbiological assay compared with the 3.1 ± 0.2 µg/g with spectrophotometric assay. Whereas, the mean concentration of sulfachlorpyridazine of egg yolks was 2.6 ± 0.2 µg/g by microbiological assay compared with the 2.7 ± 0.3 µg/g with spectrophotometric assay. Mean concentration of sulfadimidine among 6 positive samples of egg yolks was 2.8 ± 0.3 µg/g by microbiological assay compared with the 3.1 ± 0.4 µg/g with spectrophotometric assay.

Sulfadiazine preparations were used in abundance in Pakistan contrary to the other countries. Due to bacteriostatic nature of the drug farmer offered to the flock as preventive medicine against bacterial diseases. The higher incidence of sulfadiazine residues in poultry liver, meat and eggs had also been reported in many countries (Alhindi *et al.*, 2000). The level of sulfadiazine was higher in Pakistan comparing the MRLs limits of US and EU. The range of sulfadiazine residues was 3.8-4.0 µg/g in liver during 2006 to 2008. Although the residues levels were non significantly different still it in increasing trend.

During the year 2006, mean concentration of sulfadiazine residues among 32 positive samples of poultry meat was 1.4 ± 0.1 µg/g by microbiological assay compared with the 1.4 ± 0.1 µg/g with spectrophotometric assay. During the year 2007, mean concentration of sulfadiazine among 33 positive samples of meat was 1.2 ± 0.2 µg/g by microbiological assay compared with the 1.3 ± 0.2 µg/g with spectrophotometric assay. Mean concentration of sulfadiazine residues during the year 2008 in 31 positive samples of meat was 1.3 ± 0.1 µg/g by microbiological assay compared with 1.3 ± 0.1 µg/g with spectrophotometric assay (Fig. 28 & Annex. 3.1.2).

The residues levels of sulfadiazine were almost in static order in meat where it was 1.2-1.4 µg/g. The mean concentration was undisturbed due to the fact that farmers and vet were using frequently sulfadiazine preparation in poultry production. The withdrawal period was not observed leading to the intensification of sulfadiazine in the meat above MRLs of US and EU.

During the year 2006, the mean concentration of sulfadiazine among 4 positive samples of bone marrow was 0.9 ± 0.2 µg/g by microbiological assay compared with the 1.0 ± 0.2 µg/g with spectrophotometric assay. Mean concentration of sulfadiazine during the year 2007, among 3 positive samples of bone marrow was 0.7 ± 0.1 µg/g by microbiological assay compared with

the 0.8 ± 0.0 µg/g with spectrophotometric assay. Whereas, mean concentration of sulfadiazine residues during the year 2008 in 8 positive samples of bone marrow was 0.8 ± 0.2 µg/g by microbiological assay compared with 0.8 ± 0.2 µg/g with spectrophotometric assay (Fig. 28 & Annex. 3.1.3).

Mean concentration of sulfadiazine during the year 2006, among 13 positive samples of egg yolks were 3.9 ± 0.4 µg/g by microbiological assay compared with the 4.1 ± 0.4 µg/g with spectrophotometric assay. Whereas the mean concentration of sulfadiazine, during the year 2007, among 15 positive samples of egg yolks, was 3.9 ± 0.3 µg/g by microbiological assay compared, with 4.1 ± 0.3 µg/g with spectrophotometric assay. Mean concentration of sulfadiazine residues during the year 2008 in 19 positive samples of egg yolks was 3.5 ± 0.3 µg/g by microbiological assay compared with 3.7 ± 0.4 µg/g with spectrophotometric assay (Fig. 28 & Annex. 3.1.4).

Mean concentration of drug residues were 0.7-0.9 µg/g in bone marrow which were the offshoot of distribution of sulfadiazine in all body compartments and parts. The bone marrow was consistently surveyed in these studies which had not frequently studied and a few reports are available. As the poultry paws and bones wee frequently in usage therefore, significance of fixation of MRLs of bone marrow was time demand. The di calcium phosphate was used in tooth paste therefore the direct contact with human was ruled out of the drug residues. Many food companies utilized poultry bone in crushed form to increase the minced meat products to enhance the texture and weight. The rendering material recycled the drug residues in poultry feed had already been reported (Mitchell *et al.*, 2011).

SULFAQUINOXALINE RESIDUES

During the year 2006, among all the screened samples for the presence of sulfonamides residues in different poultry organs and egg yolks out of these total 291 positive samples for sulfonamides residues, 88 (30.2 %) were positive for sulfaquinoxaline (Table. 16). The spread of sulfaquinoxaline residues in different organs was higher in the liver 51.1 percent, followed by the meat 33.0 percent, egg yolks 12.5 percent and bone marrow 3.4 percent. During the year 2007, among all the screened samples for the presence of drug residues in different poultry organs and egg yolks out of these total 294 positive samples for sulfonamides residues, 86 were

positive for sulfaquinoxaline (Table. 17). The spread of sulfaquinoxaline in different organs was higher in the liver 48.8 percent, followed by the meat 32.6 percent, egg yolks 14.0 percent and bone marrow 4.7 percent. During the year 2008, among all the screened samples for the presence of sulfonamides residues in different poultry organs and egg yolks, out of these total 315 positive samples for drug residues, 96 were positive for sulfaquinoxaline (Table. 18). The spread of sulfaquinoxaline in different organs was higher in the liver 46, followed by the meat 31, egg yolks 14 and bone marrow 5.

Mean concentration of sulfaquinoxaline residues during the year 2006, was 2.6 µg/g among 45 samples of liver by microbiological assay compared with the 2.7 µg/g with spectrophotometric assay. During the year 2007, mean concentration of sulfaquinoxaline among 42 samples of liver was 2.5 ± 0.4 µg/g by microbiological assay compared with the 2.6 ± 0.4 µg/g with spectrophotometric assay. Whereas, the mean concentration of sulfaquinoxaline residues during the year 2008 among 46 positive samples of liver was 2.6 ± 0.4 µg/g by microbiological assay compared with the 2.8 ± 0.5 µg/g of liver with spectrophotometric assay (Fig.29 & Annex.3.2.1).

During the year 2006, the mean concentration of sulfaquinoxaline residues among 29 positive samples of meat was 0.8 ± 0.1 µg/g by microbiological assay compared with the 0.8 ± 0.1 µg/g with spectrophotometric assay. Whereas, the mean concentration of sulfaquinoxaline during the year 2007 among 28 positive samples of meat was 0.8 ± 0.1 µg/g by microbiological assay compared with the 0.8 ± 0.1 µg/g with spectrophotometric assay. Mean concentration of sulfaquinoxaline residues during the year 2008 in 31 positive samples of meat was 0.8 ± 0.1 µg/g by microbiological assay compared with the 0.9 ± 0.1 µg/g with spectrophotometric assay (Fig. 29 & Annex. 3.2.2).

Mean concentration of sulfaquinoxaline residues during the year 2006 among 3 samples of bone marrow was 0.4 ± 0.1 µg/g by microbiological assay compared with the 0.5 ± 0.1 µg/g with spectrophotometric assay. Whereas, the mean concentration of sulfaquinoxaline residues during year 2007 among 4 positive samples of bone marrow was 0.4 ± 0.1 µg/g by microbiological assay compared with the 0.5 ± 0.1 µg/g with spectrophotometric assay. During the year 2008,

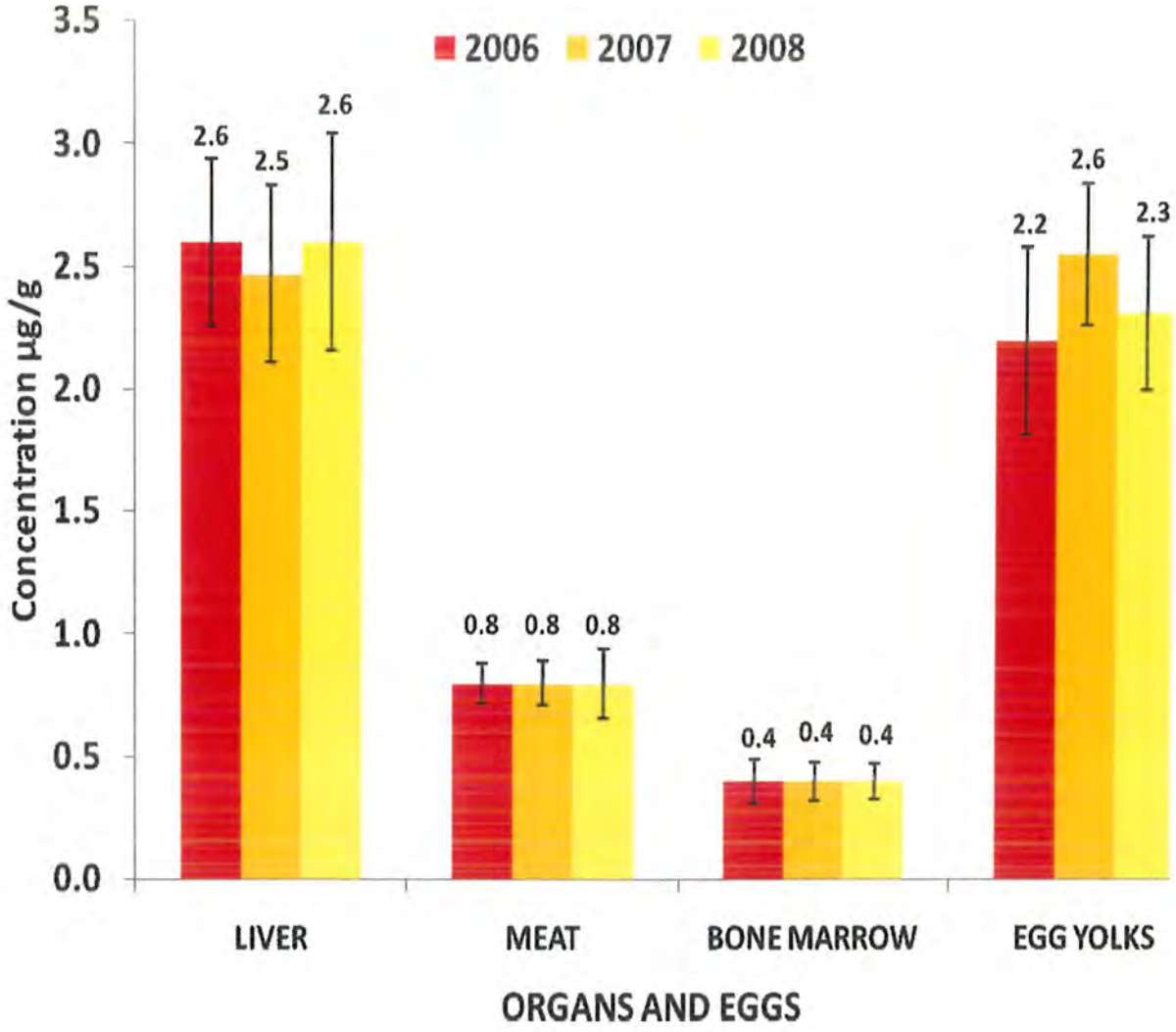

Fig. 29. Sulfaquinoxaline residues mean concentration (µg/g) during year 2006 ( ), 2007 ( ), 2008 ( ) by Microbiological assay in poultry liver, meat, bone marrow and egg yolks samples. Numerical values showing mean concentration of Sulfaquinoxaline residues along with the lines on the bars indicating standard deviation of the mean.

the mean concentration of sulfaquinoxaline residues in positive 5 positive samples of bone marrow was 0.4 ± 0.1 µg/g by microbiological assay compared with the 0.5 ± 0.1 µg/g with spectrophotometric assay (Fig. 29 & Annex. 3.2.3).

Mean concentration of sulfaquinoxaline residues during the year 2006 among 11 positive samples of egg yolks was 2.2 ± 0.4 µg/g by microbiological assay compared with the 2.3 ± 0.4 µg/g with spectrophotometric assay. Whereas, the mean concentration of sulfaquinoxaline residues during the year 2007, among 12 positive samples of egg yolks, was 2.6 ± 0.3 µg/g by microbiological assay compared with the 2.7 ± 0.3 µg/g of egg yolks with spectrophotometric assay. Mean concentration of sulfaquinoxaline residues during the year 2008 in 14 positive samples of egg yolks was 2.3 ± 0.3 µg/g by microbiological assay compared with 2.5 ± 0.4 µg/g with spectrophotometric assay (Fig. 29 & Annex. 3.2.4).

SULFACHLORPYRIDAZINE

During the year 2006, among all the screened samples for the presence of sulfonamides residues in different poultry organs and egg yolks, out of these total 291 positive samples for drug residues, 16.5 percent were positive for sulfachlorpyridazine residues (Table. 16). The spread of sulfachlorpyridazine in different organs was higher in the liver 54.1 percent, followed by the meat 33.3 percent, egg yolks 10.4 percent and bone marrow 2.1 percent. During the year 2007, among all the 294 samples positive for sulfonamides residues, 52 were positive for sulfachlorpyridazine (Table. 17). The spread of sulfachlorpyridazine in different organs was higher in the liver 29, followed by the meat 17, egg yolks 4 and bone marrow 2. During the year 2008, among all the 315 samples positive for sulfonamides residues, 15.9 percent were positive for sulfachlorpyridazine (Table. 18). The spread of sulfachlorpyridazine in different organs was higher in the liver 50.0 percent, followed by the meat 36.0 percent, egg yolks 12.0 percent and bone marrow 2.0 percent.

During the year 2006, the mean concentration of sulfachlorpyridazine residues among 26 positive samples of liver was 2.8 ± 0.4 µg/g by microbiological assay compared with the 2.9 ± 0.5 µg/g with spectrophotometric assay. During the year 2007, the mean concentration of

sulfachlorpyridazine among 29 positive samples of liver was 3.0 ± 0.4 µg/g by microbiological assay compared with the 3.1 ± 0.4 µg/g with spectrophotometric assay. Sulfachlorpyridazine residues during the year 2008 in 25 positive samples of liver were 3.0 ± 0.4 µg/g by microbiological assay compared with the 3.2 ± 0.5 µg/g with spectrophotometric assay. The sulfachlorpyridazine minimum residues through microbiological assay were 2.3 µg/g in liver and it was higher 3.5 µg/g of liver. In spectrophotometric assay the minimum sulfachlorpyridazine residues in liver samples was 2.4 µg/g increasing to the level of 3.6 µg/g of liver (Fig. 30 & Annex. 3.3.1).

Mean concentration of sulfachlorpyridazine residues during the year 2006, among 16 positive samples of meat was 1.0 ± 0.1 µg/g by microbiological assay compared with the 1.1 ± 0.2 µg/g with spectrophotometric assay. However, the mean concentration of sulfachlorpyridazine during the year 2007 among 17 positive samples of meat was 1.0 ± 0.1 µg/g by microbiological assay compared with the 1.1 ± 0.1 µg/g with spectrophotometric assay. Mean concentration of sulfachlorpyridazine residues during the year 2008 in 18 positive samples of meat was 1.0 ± 0.1 µg/g by microbiological assay compared with the 1.1 ± 0.1 µg/g with spectrophotometric assay (Fig. 30 & Annex. 3.3.2).

During the year 2006, concentration of sulfachlorpyridazine in one positive sample of bone marrow was 0.4 µg/g by microbiological assay compared with the 0.5 µg/g with spectrophotometric assay. Mean concentration of sulfachlorpyridazine during the year 2007 between two positive samples of bone marrow was 0.5 µg/g by microbiological assay. Concentration of sulfachlorpyridazine residues during the year 2008 in 01 positive sample of bone marrow was 0.5 µg/g by microbiological assay compared with 0.6 µg/g with spectrophotometric assay (Fig. 30 & Annex. 3.3.3).

During the year 2006, the mean concentration of sulfachlorpyridazine residues among 5 positive samples of egg yolks was 2.8 ± 0.3 µg/g by microbiological assay compared with the 3.0 ± 0.4 µg/g with spectrophotometric assay. Whereas, the mean concentration of sulfachlorpyridazine residues during the year 2007 among 4 positive samples of egg yolks was 2.6 ± 0.2 µg/g by microbiological assay compared with the 2.7 ± 0.3 µg/g with

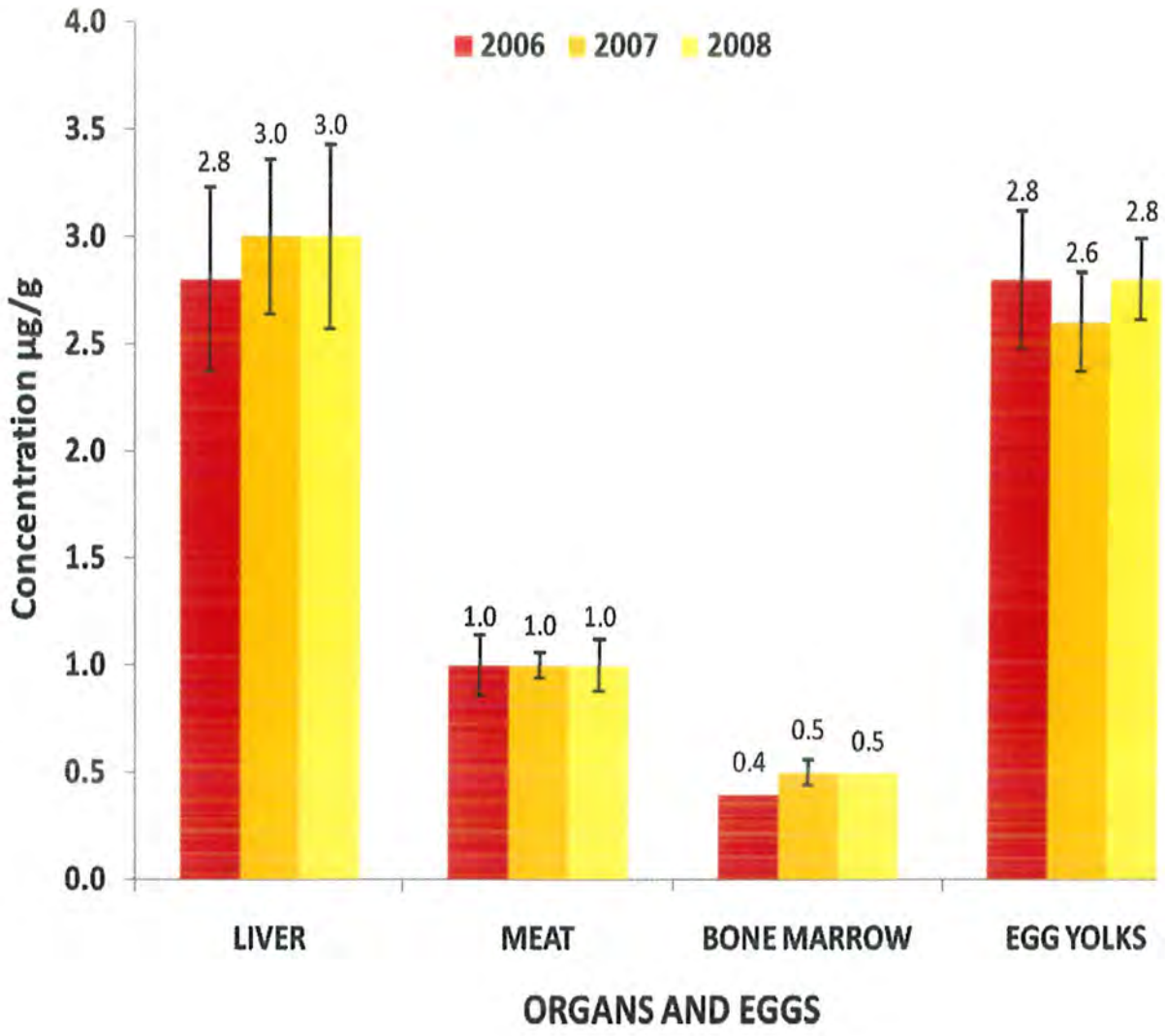

Fig. 30. Sulfachlorpyridazine residues mean concentration (µg/g) during year 2006 ( ), 2007 ( ), 2008 ( ) by Microbiological assay in poultry liver, meat, bone marrow and egg yolks samples. Numerical values showing mean concentration of Sulfachlorpyridazine residues along with the lines on the bars indicating standard deviation of the mean.

spectrophotometric assay. Concentration of sulfachlorpyridazine residues in 6 positive samples of egg yolks was 2.8 ± 0.2 µg/g by microbiological assay during the year 2008 as compared with the 3.1 ± 0.2 µg/g with spectrophotometric assay (Fig. 30 & Annex. 3.3.4).

SULFADIMIDINE RESIDUES

During the year 2006, among all the 291 samples positive for sulfonamides residues 19.6 percent were positive for sulfadimidine (Table. 16). The spread of sulfadimidine in different organs was higher in the liver 54.2 percent, followed by the meat 31.6 percent, egg yolks six 10.5 percent and bone marrow 3.5 percent. During the year 2007, among all the 294 samples positive for sulfonamides residues 54 were positive for sulfadimidine (Table. 17). The spread of sulfadimidine in different organs was higher in the liver 33, followed by the meat 14, egg yolks five and bone marrow 2. During the year 2008, among all the 315 samples positive for sulfonamides residues, 16.5 percent were positive for sulfadimidine (Table. 18). The spread of sulfadimidine residues in different organs was higher in the liver 57.7 percent, followed by the meat 32.7 percent, egg yolks 7.7 and it was 1.9 percent in bone marrow.

During the year 2006, the mean concentration of sulfadimidine among 31 positive samples of liver was 3.1 ± 0.3 µg/g by microbiological assay compared with the 3.2 ± 0.3 µg/g with spectrophotometric assay. During the year 2007, mean concentration of sulfadimidine residues among 33 positive samples of liver was 3.3 ± 0.3 µg/g by microbiological assay compared with the 3.5 ± 0.3 µg/g with spectrophotometric assay. Mean concentration of sulfadimidine residues during the year 2008 in 30 positive samples of liver was 2.9 ± 0.3 µg/g by microbiological assay compared with the 3.1 ± 0.4 µg/g with spectrophotometric assay (Fig. 31 & Annex. 3.4.1).

Mean concentration of sulfadimidine during the year 2006, among 18 positive samples of meat was 1.0 ± 0.1 µg/g by microbiological assay compared with the 1.0 ± 0.1 µg/g with spectrophotometric assay. Whereas, mean concentration of sulfadimidine during the year 2007 among 14 positive samples of meat was 1.3 ± 0.2 µg/g by microbiological assay compared with the 1.3 ± 0.2 µg/g with spectrophotometric assay. Mean concentration of sulfadimidine residues during the year 2008 in 17 positive samples of meat was 1.1 ± 0.2 µg/g by microbiological assay compared with the 1.2 ± 0.2 µg/g with spectrophotometric assay (Fig. 31 & Annex. 3.4.2).

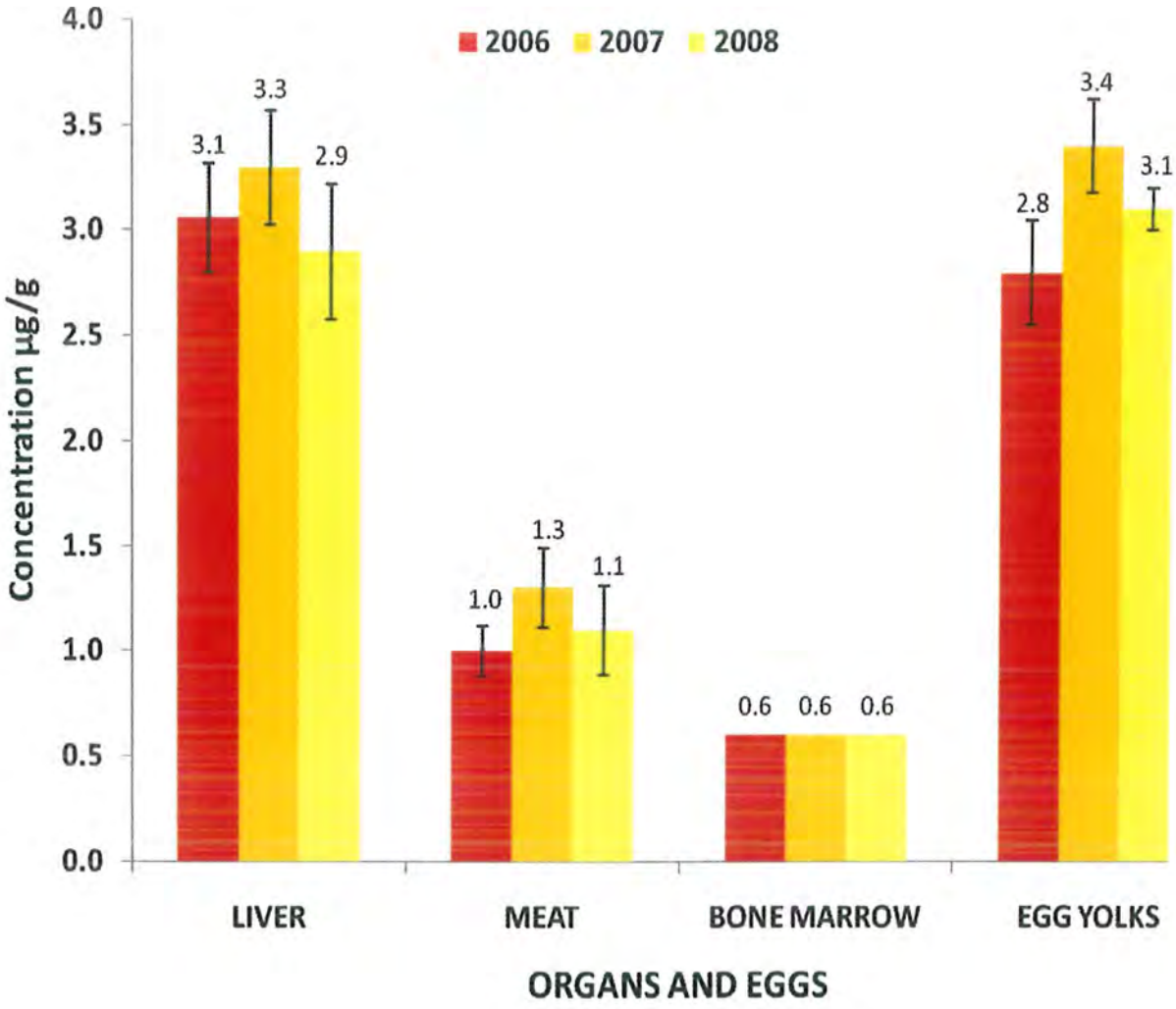

Fig. 31. Sulfadimidine residues mean concentration (µg/g) during year 2006 ( ), 2007 ( ), 2008 ( ) by Microbiological assay in poultry liver, meat, bone marrow and egg yolks samples. Numerical values showing mean concentration of Sulfadimidine residues along with the lines on the bars indicating standard deviation of the mean.

During the year 2006, mean concentration of sulfadimidine between 02 positive samples of bone marrow was 0.6 ± 0.0 µg/g by microbiological assay compared with the 0.6 ± 0.0 µg/g with spectrophotometric assay. Whereas, the mean concentration of sulfadimidine during the year 2007 between 02 positive samples of bone marrow was 0.6 ± 0.0 µg/g by microbiological assay compared with the 0.7 ± 0.0 µg/g with spectrophotometric assay. Concentration of sulfadimidine residues during the year 2008 in 01 positive sample of bone marrow was 0.6 ± 0.0 µg/g by microbiological assay compared with the 0.6 ± 0.0 µg/g with spectrophotometric assay (Fig. 31 & Annex. 3.4.3).

A large proportion of the estimated 23-25 million poultry and their products in Uganda contribute an important source of human food to the country. Therefore, control of animal diseases depends heavily on the use of vaccines as well as antimicrobials and about 10.0 percent of these imported drugs into Uganda were sulfonamides. Sulfonamides such as sulfadiazine, sulfaquinoxaline, sulfamethoxypyridazine and sulfamethazine have been widely used in livestock production (Tarbin *et al.*, 1999). Moreover, irrational antimicrobial usage may leads to emergence of antimicrobial resistance and a very common case of drug resistant pathogen that can infect humans was *Salmonella*, responsible for epidemics of salmonellosis (Threlfall *et al.*, 1994).

During the year 2006, the mean concentration of sulfadimidine among six positive samples of egg yolks was 2.8 ± 0.3 µg/g by microbiological assay compared with the 3.1 ± 0.4 µg/g with spectrophotometric assay. Whereas, mean concentration of sulfadimidine during the year 2007 among five positive samples of egg yolks was 3.4 ± 0.2 µg/g by microbiological assay compared with the 3.5 ± 0.3 µg/g with spectrophotometric assay. Mean concentration of sulfadimidine drug residues during the year 2008 in four positive samples of egg yolks was 3.1 ± 0.1 µg/g by microbiological assay compared with the 3.4 ± 0.0 µg/g with spectrophotometric assay (Fig. 31 & Annex. 3.4.4).

Results of the sulfonamides residues have been summarized in Table 19. Concentration of sulfonamides residues distributed in different organs of poultry and egg yolks, detected through microbiological and spectrophotometric assay.

Table 19. Concentration of Sulfadiazine, Sulfaquinoxaline, Sulfachlorpyridazine, Sulfadimidine residues distributed in poultry liver, meat, bone marrow and egg yolks, detected through microbiological (M) assay and spectrophotometric (S) assay.

ORGANS	NAME OF DRUG RESIDUE	2006		2007		2008	
		M. assay (µg/g) of sample X ± SD	S. assay (µg/g) of sample X ± SD	M. assay (µg/g) of sample X ± SD	S. assay (µg/g) of sample X ± SD	M. assay (µg/g) of sample X ± SD	S. assay (µg/g) of sample X ± SD
LIVER	Sulfadiazine	3.93 ± 0.69	4.12^{A} ± 0.72	3.76 ± 0.56	3.97^{A} ± 0.61	3.95 ± 0.62	4.15^{A} ± 0.66
	Sulfaquinoxaline	2.55 ± 0.34	2.69^{C} ± 0.38	2.47 ± 0.36	2.62^{B} ± 0.41	2.64 ± 0.44	2.78^{C} ± 0.47
	Sulfachlorpyridazine	2.78 ± 0.43	2.93^{C} ± 0.46	2.98 ± 0.36	3.14^{A} ± 0.39	2.99 ± 0.43	3.15^{B} ± 0.46
	Sulfadimidine	3.06 ± 0.26	3.21^{B} ± 0.29	3.32 ± 0.27	3.53^{A} ± 0.31	2.92 ± 0.32	3.07^{B} ± 0.36
MEAT	Sulfadiazine	1.35 ± 0.13	1.43^{D} ± 0.14	1.21 ± 0.19	1.27^{C} ± 0.20	1.26 ± 0.08	1.33^{D} ± 0.10
	Sulfaquinoxaline	0.75 ± 0.08	0.78^{E} ± 0.08	0.80 ± 0.09	0.83^{D} ± 0.09	0.82 ± 0.14	0.85^{E} ± 0.14
	Sulfachlorpyridazine	1.01 ± 0.14	1.07^{D} ± 0.15	1.01 ± 0.06	1.08^{C} ± 0.06	1.01 ± 0.12	1.07^{D} ± 0.12
	Sulfadimidine	0.95 ± 0.12	1.01^{D} ± 0.14	1.25 ± 0.19	1.31^{C} ± 0.20	1.10 ± 0.21	1.16^{D} ± 0.22
BONE MARROW	Sulfadiazine	0.92 ± 0.23	0.98^{D} ± 0.23	0.71 ± 0.05	0.77^{D} ± 0.03	0.76 ± 0.15	0.82^{E} ± 0.15
	Sulfaquinoxaline	0.44 ± 0.09	0.46^{F} ± 0.13	0.43 ± 0.08	0.46^{E} ± 0.13	0.42 ± 0.07	0.45^{F} ± 0.09
	Sulfachlorpyridazine	0.44 ± 0.00	0.46^{E} ± 0.00	0.48 ± 0.06	0.51^{E} ± 0.07	0.52 ± 0.00	0.56^{F} ± 0.00
	Sulfadimidine	0.57 ± 0.00	0.62^{E} ± 0.00	0.64 ± 0.00	0.67^{D} ± 0.00	0.57 ± 0.00	0.62^{F} ± 0.00
EGG YOLK	Sulfadiazine	3.86 ± 0.41	4.11^{A} ± 0.44	3.92 ± 0.32	4.13^{A} ± 0.33	3.46 ± 0.33	3.66^{B} ± 0.40
	Sulfaquinoxaline	2.20 ± 0.38	2.30^{C} ± 0.40	2.55 ± 0.29	2.71^{B} ± 0.32	2.31 ± 0.31	2.47^{B} ± 0.37
	Sulfachlorpyridazine	2.78 ± 0.32	2.99^{C} ± 0.38	2.58 ± 0.23	2.72^{B} ± 0.30	2.84 ± 0.19	3.06^{B} ± 0.24
	Sulfadimidine	2.84 ± 0.25	3.06^{B} ± 0.39	3.38 ± 0.22	3.54^{A} ± 0.25	3.14 ± 0.10	3.36^{B} ± 0.01

Values with same superscripts (A-F) are non significantly different ($P < 0.05$) whereas, values with different superscripts (A-F) are significantly different within the same column; X = Mean concentration; SD = Stranded deviation of concentrations; M. assay = microbiological assay; S. assay = spectrophotometric assay.

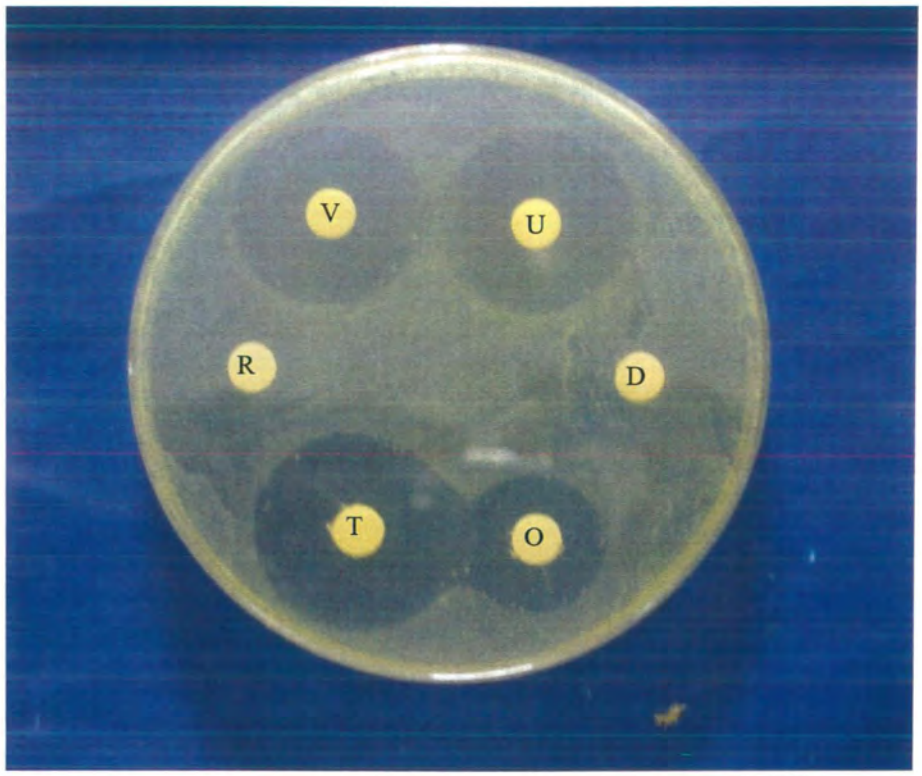

Fig. 32. Microbicidal zone of Enrofloxacin (V), Norfloxacin (U), Sulfadiazine (D), Tilmicosin (O), Tylosin (T), Flumequine (R).

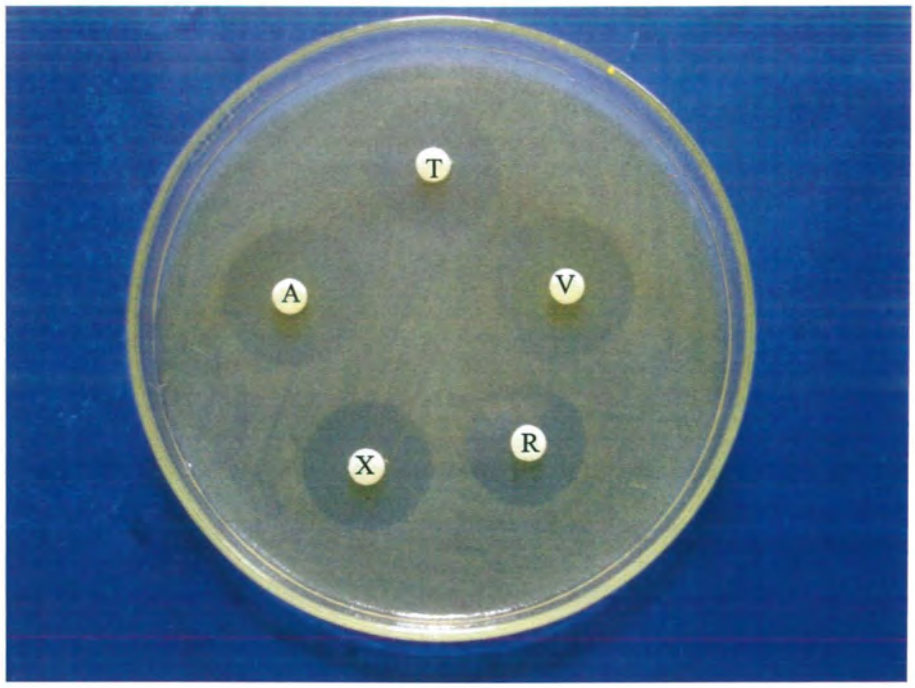

Fig. 33. Microbicidal zone of Tylosin (T), Enrofloxacin (V), Flumequine (R), Ciprofloxacin (X), Sulfadimidine (A).

Retail meat is frequently established polluted with *enterococci* resistant to antimicrobial drugs, but constant intestinal carriage of these ingested contaminants is not apparent. In a randomized, double blind investigation in 18 healthy volunteers was carried out. Three groups of six volunteers were feed with a mixture of 107 cfu of two strains of *Enterococcus faecium* resistant to glycopeptide obtained from poultry purchased at a grocery store, 107 cfu of *Enterococcus faecium* strain resistant to streptogramin obtain from a pig at slaughter and 107 cfu of *Enterococcus faecium* strain vulnerable to both glycopeptide and streptogramin, attained from poultry acquired at a grocery store, respectively. The preparation of 250 ml suspensions in whole milk was well in the prescribed rang of Danish food regulations. Stool samples were examined for one week before and after ingestion as well as at 14 and 35 days. Specific culture techniques were employed to identify resistant *enterococci* in stool samples (White *et al.*, 2001).

Looking into the data, resistance is increasing on the yearly basis about or more than 2 percent. During the year 2006-2008 minimum resistance 37.5 percent observed among *E. coli*, *Salmonella* and *Clostridium* isolates of poultry origin, was against tylosin tartrate of macrolides, whereas, in fluoroquinolones it was minimum against sarafloxacin 21.6 percent and in sulfonamides it was against sulfadimidine 21.1 percent. Resistance parameters once increased against any antimicrobials remain increasing, indicating the persistent use of these antimicrobials during the study period.

EMERGENCE OF RESISTANCE IN *E. coli* STRAINS AGAINST TYLOSIN, ERYTHROMYCIN AND TILMICOSIN

E. coli strains resistant to antimicrobials are frequently present in the gut microflora of poultry. Because numerous of these antimicrobial resistant *E. coli* strains are good colonizers of the gut, these strains persist long subsequent to the withdrawal of antimicrobials. Even if there is no indication that *E. coli* of animal origin cause disease incidents in man, they comprise a very much multitalented pool of transferable drug resistance (R-plasmid), which may be transmitted eventually to some infection causing microbes. Safety is an important requirement for any flock health program. Use of any drug is only acceptable if it causes fewer troubles than it resolve. Protection concepts apply to both human being and chick (Caldwell *et al.*, 2000).

Among the total isolates 971 were screened during the year 2006-2008, tested against representative antimicrobials of macrolides, fluoroquinolones and sulfonamides, 318 isolates of *E. coli* 32.8 percent were tested for these antimicrobials during 2006 followed by 33.0 percent in 2007 and it was 34.3 percent during 2008. The maximum resistance 63.5 percent was observed against norfloxacin, followed by enrofloxacin 51.9 percent, erythromycin 50.3 percent, whereas among sulfonamides highest resistance 40.3 percent was observed against sulfaquinoxaline. The picture of resistance was not good in temporal phase because in comparison with other years. Overall resistance 37.4 percent in 2006 became worst in 2007 where it increases up to 39.4 percent which further intensified the situation in 2008 where it touch the highest level of 42.6 percent. Looking into the data the resistance is increasing on the yearly basis about or more than 2 percent between *E. coli*, in poultry during the years 2006-2008. Minimum resistance 36.2 percent in macrolides was observed against tylosin tartrate, whereas, in fluoroquinolones and sulfonamides it was minimum against sarafloxacin 18.6 percent and sulfadimidine 20.4 percent respectively (Annex. 4.1.1).

During the year 2006 the resistance developed in *E. coli* against tylosin was 36 percent which increased to 39 percent in 2007 and it elevated to its maximum 48 percent in 2008. Straight increasing trend was noted in the *E. coli* against tylosin. Most commonly used therapeutic in poultry was tylosin against avian pathogens therefore, the increasing trend support the emergence of resistance in upward (Fig. 34 & Annex. 4.1.1).

E. coli resistance against erythromycin studied during three years was 50 percent in 2006 and 2008. There was little downfall in the incidence in 2007, although 45 percent was not a less percentage of resistance but comparing the data with 2006 and 2008 where it was 50 percent. The decline was unrealistic but there were many variables which deviate results. The shifting of poultry farms to new areas where the *E. coli* were different in comparison with the older poultry production areas. During the year 2007 most of the control sheds replace the poultry houses with most astringent bio-security and lessen the exposure of birds to pathogens may be the temporary possible cause of decreased incidence of resistance in *E. coli* against erythromycin (Fig. 34 & Annex. 4.1.1).

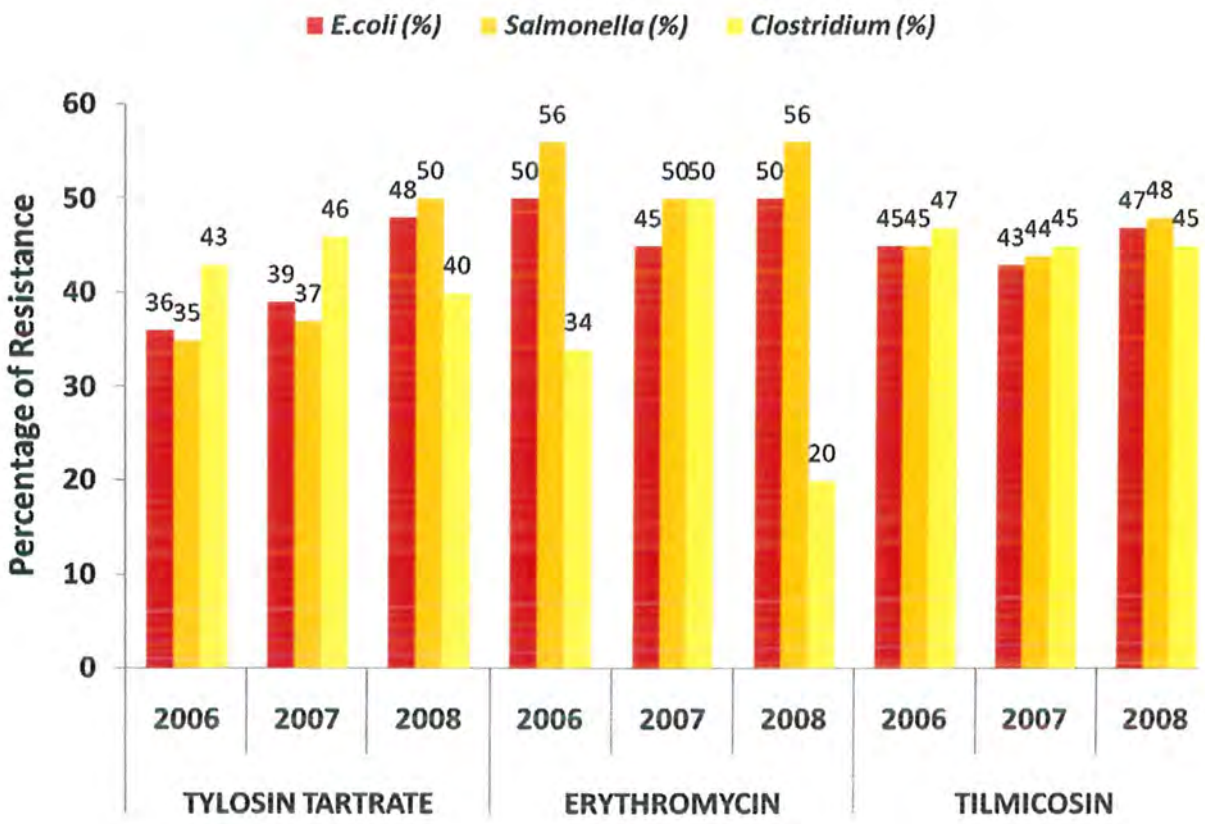

Fig. 34. Prevalence of resistance during the year 2006 through 2008 in *E. coli* ( ), *Salmonella* ( ) *Clostridium* ( ) of poultry isolates against Tylosin, Erythromycin and Tilmicosin by solid media microbiological sensitivity method. Numerical values in the graph showing percentage of resistance.

During the year 2006 resistance in *E. coli* against tilmicosin was 45 percent and in 2007 it was 43 percent where it increased next year in 2008 up to 47 percent. The increasing trend against tilmicosin may be due to the high MIC of tilmicosin against *E. coli* was required. Looking into the data the outcome in the straight increasing trend was noted in incidence of resistance developed against *E. coli*. Tilmicosin was not a drug of choice against *E. coli* still danger of increasing trend will remain there because in poultry *E. coli* was ubiquitous in nature, use of tilmicosin against respiratory diseases will also become the reason of development of resistance in *E. coli* (Fig. 34 & Annex. 4.1.1).

Overall resistance developed in *E. coli* of poultry origin against tylosin and tilmicosin was not of great concern but development of resistance against erythromycin mattered.

EMERGENCE OF RESISTANCE IN *SALMONELLA* STRAINS AGAINST TYLOSIN, ERYTHROMYCIN AND TILMICOSIN

Among the total 689 isolates screened during the years 2006-2008, against representative antimicrobials of macrolides, fluoroquinolones and sulfonamides, 220 isolates of *Salmonella* 31.9 percent were tested for these antimicrobials during 2006 followed by 33.0 percent in 2007 and 34.9 percent during 2008.

During the year 2006 the resistance developed in *Salmonella* against tylosin was 35 percent which increased to 37 percent in 2007 and it elevated to its maximum 50 percent in 2008. Straight increasing trend was noted in the *Salmonella* against tylosin. Most commonly used therapeutic in poultry was tylosin against avian pathogens therefore, the increasing trend support the emergence of resistance in upward.

Salmonella resistance against erythromycin studied during three years was 56 percent in 2006 and 2008. There was little downfall in the incidence in 2007, although 50 percent was not a less percentage of resistance but comparing the data with 2006 and 2008 where it was 56 percent. The decline was unrealistic but there were many variables which deviate results. The shifting of poultry farms to new areas where the *Salmonella* were different in comparison with the older poultry production areas. During the year 2007 most of the control sheds replace the poultry

houses with most astringent bio-security and lessen the exposure of birds to pathogens may be the temporary possible cause of decreased incidence of resistance in *Salmonella* against erythromycin (Fig. 34).

During the year 2006 resistance in *Salmonella* against tilmicosin was 45 percent and in 2007 it was 44 percent where it increased next year in 2008 up to 48 percent. The increasing trend against tilmicosin may be due to the high MIC of tilmicosin against *Salmonella* was required. Looking into the data the outcome in the straight increasing trend was noted in incidence of resistance developed against *Salmonella.* Tilmicosin was not a drug of choice against *Salmonella* still danger of increasing trend will remain there because in poultry *Salmonella* was ubiquitous in nature, use of tilmicosin against respiratory diseases will also become the reason of development of resistance in *Salmonella* (Fig. 34 & Annex. 4.1.2).

Overall resistance developed in *Salmonella* of poultry origin against tylosin and tilmicosin was not of great concern but development of resistance against erythromycin mattered. The maximum resistance 61 percent was observed against norfloxacin, followed by maximum resistance against macrolides was observed in erythromycin 56 percent, whereas among sulfonamides the highest resistance 43 percent was observed against sulfaquinoxaline. The increment in resistance remained increasing in temporal phase in comparison with other years. Overall resistance 39 percent in 2006 became the worst in 2007, where it increases up to 40 percent which further intensified the situation in 2008, where it touch the highest level 44 percent (Fig. 34 & Annex. 4.1.2).

Resistance is increasing on the yearly basis about or more than 2 percent against *Salmonella* in poultry during the years 2006-2008, minimum resistance 35 percent in macrolides was observed against tylosin tartrate, whereas, in fluoroquinolones it was minimum against sarafloxacin 29 percent and in sulfonamides it was against sulfachlorpyridazine 20 percent during 2006. Increasing trend in resistance against any antimicrobials referred to the persistent misuse of the antimicrobials during the year 2006 through 2008 (Fig. 34).

EMERGENCE OF RESISTANCE IN *CLOSTRIDIUM* STRAINS AGAINST TYLOSIN, ERYTHROMYCIN AND TILMICOSIN

During the year 2006-2008 among the total isolates 496 were screened against representative antimicrobials of macrolides, fluoroquinolones and sulfonamides. One hundred sixty one isolates of *Clostridium* 32 percent were tested for these antimicrobials during 2006 followed by 34 percent in 2007 and it was 34 percent during 2008.

During the year 2006 the resistance developed in *Clostridium* against tylosin was 43 percent which increased to 46 percent in 2007 and it elevated to its maximum 40 percent in 2008. Straight increasing trend was noted in the *Clostridium* against tylosin. Most commonly used therapeutic in poultry was tylosin against avian pathogens therefore, the increasing trend support the emergence of resistance in upward (Fig. 34 & Annex. 4.1.3).

Clostridium resistance against erythromycin studied during three years ranged from 20 to 50 percent in 2006 to 2008. There was little downfall in the incidence in 2007, although 50 percent was not a less percentage of resistance but comparing the data with 2006 and 2008 where it was higher than 2007. The decline was unrealistic but there were many variables which the results. The shifting of poultry farms to new areas where the *Clostridium* was different in comparison with the older poultry production areas. During the year 2007 most of the control sheds replace the poultry houses with most astringent bio-security and lessen the exposure of birds to pathogens may be the temporary possible cause of decreased incidence of resistance in *Clostridium* against erythromycin (Fig. 34).

During the year 2006 resistance in *Clostridium* against tilmicosin was 47 percent and in 2007 and 2008 it was 45 percent in next two years. Looking into the data the outcome was static mode in incidence of resistance developed against *Clostridium* was noted. Tilmicosin was a drug of choice against *Clostridium* therefore, the trend remained stable but the percentage of resistance was critical. Overall resistance developed in *Clostridium* of poultry origin against tylosin and tilmicosin was of great concern and same for the erythromycin mattered (Fig. 34).

EMERGENCE OF RESISTANCE IN *E. coli* STRAINS AGAINST NORFLOXACIN, ENROFLOXACIN, CIPROFLOXACIN, SARAFLOXACIN AND FLUMEQUINE

E. coli strains resistant to antimicrobials are frequently present in the gut microflora of poultry. Because numerous of these antimicrobial resistant *E. coli* strains are good colonizers of the gut, these strains persist long subsequent to the withdrawal of antimicrobials. Even if there is no indication that *E. coli* of animal origin cause disease incidents in man, they comprise a very much multitalented pool of transferable drug resistance (R-plasmid), which may be transmitted eventually to some infection causing microbes. Safety is an important requirement for any flock health program. Use of any drug is only acceptable if it causes fewer troubles than it resolve. Protection concepts apply to both human being and chick (Caldwell *et al.*, 2000).

Among the total isolates 971 were screened during the year 2006-2008, tested against representative antimicrobials of fluoroquinolones 318 isolates of *E. coli* 33 percent were tested for these antimicrobials during 2006 followed by 33 percent in 2007 and it was 34 percent during 2008. During the year 2006 maximum resistance 64 percent was observed against norfloxacin, followed by enrofloxacin 52 percent 35 percent against ciprofloxacin, 33 percent against flumequine and 19 percent against sarafloxacin (Fig. 35 & Annex. 4.1.1).

During the year 2006 the resistance developed in *E. coli* against norfloxacin was 64 percent which increased to 70 percent in 2007 and it elevated to its maximum 71 percent in 2008. Straight increasing trend was noted in the *E. coli* against norfloxacin. Most commonly used therapeutic in poultry was norfloxacin against avian pathogens therefore, the increasing trend support the emergence of resistance in upward (Fig. 35).

E. coli resistance against enrofloxacin studied during three years was 52 percent in 2006 which increased to 53 percent in 2007 and it further elevated to its maximum 54 percent during 2008. Straight increasing trend of resistance was noted in the *E. coli* against enrofloxacin during the three years period of study. During the year 2006 resistance in *E. coli* against ciprofloxacin was 35 percent and in 2007 it was 40 percent where it increased next year in 2008 up to 41 percent. The increasing trend was noted in the *E. coli* against ciprofloxacin during the years 2006-2008.

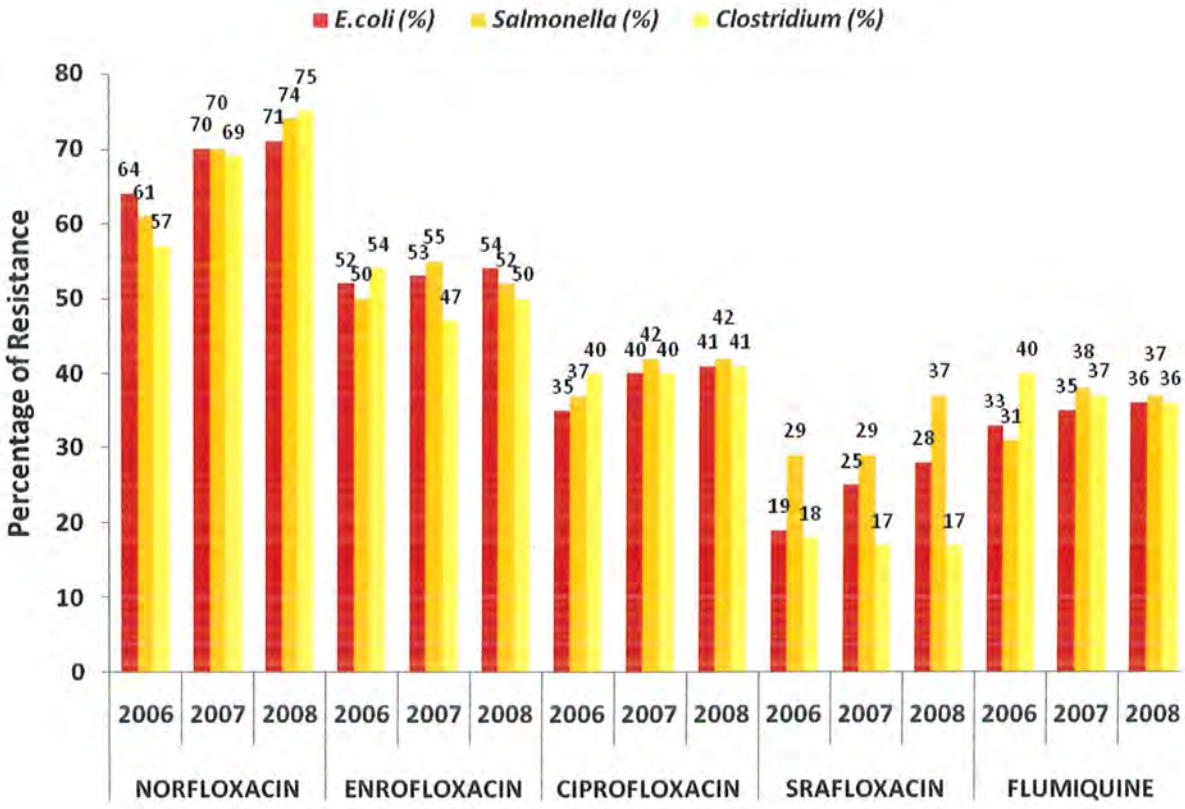

Fig. 35. Prevalence of resistance during the year 2006 through 2008 in *E. coli* ( ), *Salmonella* ( ) *Clostridium* ( ) of poultry isolates against Norfloxacin, Enrofloxacin, Ciprofloxacin, Sarafloxacin and Flumequine by solid media microbiological sensitivity method. Numerical values in the graph showing percentage of resistance.

E. coli resistance against sarafloxacin studied during three years was19 percent in 2006 which increased to 25 percent in 2007 and it further elevated to its maximum 28 percent during 2008. Although resistance in *E. coli* against sarafloxacin was the lowest among the fluoroquinolones group but a rapid and straight increasing trend (19-28 %) was noted in the *E. coli* against sarafloxacin during the three years period of study. The lowest resistance against the sarafloxacin was due to its young usage age as compared with other members of the group. The rapid onset in the resistance was alarming. During the year 2006 resistance in *E. coli* against flumequine was 33 percent and in 2007 it was 35 percent where it increased next year in 2008 up to 36 percent. The increasing trend was noted in the *E. coli* against flumequine during the years 2006 to 2008. Resistance parameters once increased against any antimicrobials remain increasing indicating the persistent use of the antimicrobials during the study period (Fig. 35).

EMERGENCE OF RESISTANCE IN *SALMONELLA* STRAINS AGAINST NORFLOXACIN, ENROFLOXACIN, CIPROFLOXACIN, SARAFLOXACIN AND FLUMEQUINE

Among the total isolates 689 were screened during the year 2006-2008, tested against representative antimicrobials of fluoroquinolones 220 isolates of *Salmonella* were tested for these antimicrobials during 2006 followed by 228 in 2007 and 241during 2008. During the year 2006 maximum resistance 61 percent was observed against norfloxacin, followed by enrofloxacin 50 percent 37 percent against ciprofloxacin, 31 percent against flumequine and 29 percent against sarafloxacin (Fig. 35 & Annex. 4.1.2).

During the year 2006 the resistance developed in *Salmonella* against norfloxacin was 61 percent which increased to 70 percent in 2007 and it elevated to its maximum 74 percent in 2008. Straight increasing trend was noted in the *Salmonella* against norfloxacin. Most commonly used therapeutic in poultry was norfloxacin against avian pathogens therefore, the increasing trend support the emergence of resistance in upward. *Salmonella* resistance against enrofloxacin studied during three years was 50 percent in 2006, showed a little fluctuation in upward direction in 2007 and it was 52 percent during 2008. An overall increasing trend was noted in the *Salmonella* against enrofloxacin during the three years period of study (Fig. 35).

During the year 2006 resistance in *Salmonella* against ciprofloxacin was 37 percent and in 2007 it was 42 percent where it increased next year in 2008 up to 42 percent. The increasing trend was noted in the *Salmonella* against ciprofloxacin during the years 2006 to 2008. *Salmonella* resistance against sarafloxacin studied during three years was 29 percent in 2006 and 2007 and it elevated to its maximum 37 percent during 2008. Although resistance in *Salmonella* against sarafloxacin during was the lowest among the fluoroquinolones group but a rapid and straight increasing trend (29-37 %) was noted in the *Salmonella* against sarafloxacin during the year 2007 to 2008 (Fig. 35 & Annex. 4.1.2).

During the year 2006 resistance in *Salmonella* against flumequine was 31 percent and in 2007 it was 38 percent where it increased next year in 2008 up to 37 percent. An overall increasing trend was noted in the *Salmonella* against flumequine during the years 2006 to 2008.

EMERGENCE OF RESISTANCE IN *CLOSTRIDIUM* STRAINS AGAINST NORFLOXACIN, ENROFLOXACIN, CIPROFLOXACIN, SARAFLOXACIN AND FLUMEQUINE

Among the total isolates 496 were screened during the year 2006-2008, tested against representative antimicrobials of fluoroquinolones 161 isolates of *Clostridium* were tested for these antimicrobials resistance during 2006 followed by 169 in 2007 and 166 during 2008. During the year 2006 maximum resistance 57 percent was observed against norfloxacin, followed by enrofloxacin 54 percent 40 percent against ciprofloxacin, 40 percent against flumequine and 18 percent against sarafloxacin and it was almost similar in fashion to *E. coli* and *Clostridium* isolates (Fig. 35 & Annex. 4.1.3).

During the year 2006 the resistance developed in *Clostridium* against norfloxacin was 57 percent which increased to 69 percent in 2007 and it elevated to its maximum 75 percent in 2008. Straight increasing trend was noted in the *Clostridium* against norfloxacin. Norfloxacin was the drug of choice in 1990 in Pakistan which replaced with enrofloxacin 1995 and in 2000 with ciprofloxacin. Due to commonly used therapeutic in poultry norfloxacin against avian pathogens the increasing trend supports the emergence of resistance in upward (Fig. 35).

Clostridium resistance against enrofloxacin studied during three years was 54 percent in 2006, decreased to 47 percent in 2007 but it became 50 percent during 2008. An overall decreasing trend (54- 50 %) was observed in the *Clostridium* against enrofloxacin during the three years period of study. During the year 2006 and 2007 resistance in *Clostridium* against ciprofloxacin remained static at the level of 40 percent and it increased during 2008 up to 41 percent. The increasing trend was noted in the *Clostridium* against ciprofloxacin during the years 2006 to 2008. A slight decreasing trend (18-17 %) was observed in *Clostridium* resistance against sarafloxacin during three years 2006 - 2008. *Clostridium* resistance against flumequine adopted almost a similar fashion (40-36 %) during three years 2006 to 2008 (Fig. 35).

EMERGENCE OF RESISTANCE IN *E. coli* STRAINS AGAINST SULFADIAZINE, SULFAQUINOXALINE, SULFACHLORPYRIDAZINE AND SULFADIMIDINE

Among the total isolates 971 were screened during the year 2006-2008, tested against representative antimicrobials of sulfonamides 318 isolates of *E. coli* were tested for these antimicrobials during 2006 followed by 320 in 2007 and it was 333 during 2008. During the year 2006 maximum resistance 34 percent was observed against sulfadiazine, followed by sulfaquinoxaline 40 percent, against sulfachlorpyridazine 21 percent and against sulfadimidine 20 percent. During the year 2006 the resistance observed in *E. coli* against sulfadiazine was 34 percent which increased to 37 percent in 2007 and it elevated to its maximum 40 percent in 2008. Straight increasing trend was noted in the *E. coli* against sulfadiazine. Sulfadiazine against avian pathogens was most commonly used therapeutic agent therefore, the increasing trend support the emergence of resistance in upward due to its massive usage (Fig. 36 & Annex. 4.1.1).

E. coli resistance against sulfaquinoxaline studied during three years was 40 percent in 2006 which decreased to 36 percent in 2007 and it further elevated to its maximum 42 percent during 2008. An overall increasing trend was noted in the *E. coli* against sulfaquinoxaline during the three years period of study. During the year 2006 resistance in *E. coli* against sulfachlorpyridazine was 21 percent and in 2007 it was 25 percent where it increased next year

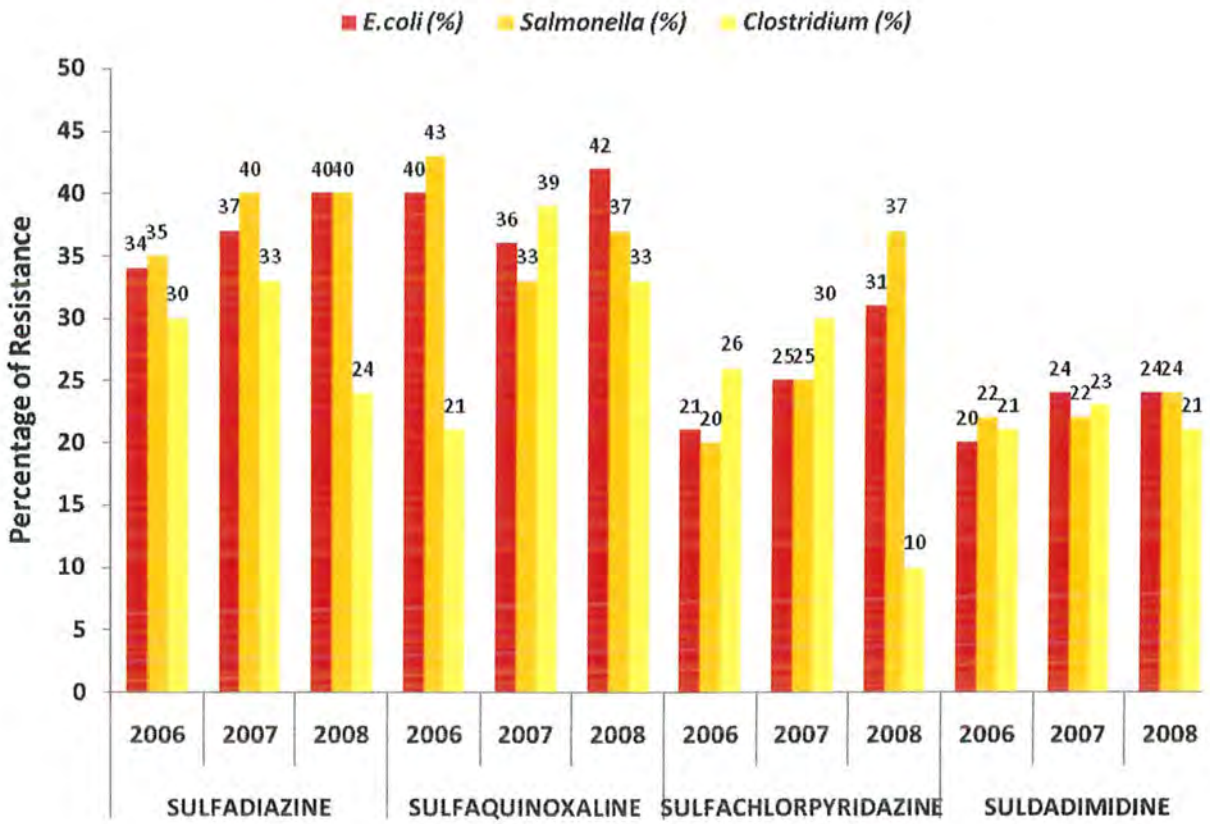

Fig. 36. Prevalence of resistance during the year 2006 through 2008 in *E. coli* ( ), *Salmonella* ( ) *Clostridium* ( ) of poultry isolates against Sulfadiazine, Sulfaquinoxaline, Sulfachlorpyridazine and Sulfadimidine by solid media microbiological sensitivity method. Numerical values in the graph showing percentage of resistance.

in 2008 up to 31 percent. Although resistance in *E. coli* against sulfachlorpyridazine during was the lower level among the sulfonamides group but a very rapid and straight increasing trend (21-31 %) was noted in the *E. coli* against sulfachlorpyridazine during the period of three years.

E. coli resistance against sulfadimidine studied during three years was 20 percent in 2006 which increased to 24 percent in 2007 and it remain static during 2008. The increasing trend was noted in the *E. coli* against sulfadimidine during the years 2006 to 2008 (Fig. 36).

EMERGENCE OF RESISTANCE IN *SALMONELLA* STRAINS AGAINST SULFADIAZINE, SULFAQUINOXALINE, SULFACHLORPYRIDAZINE AND SULFADIMIDINE

Among the total isolates 689 were screened during the year 2006-2008, tested against representative antimicrobials of sulfonamides 220 isolates of *Salmonella* were tested for these antimicrobials during 2006 followed by 228 in 2007 and 241 during 2008. During the year 2006 maximum resistance 43 percent was observed against sulfaquinoxaline, followed by sulfadiazine 35 percent, against sulfadimidine 22 percent and against sulfachlorpyridazine 20 percent. During the year 2006 *Salmonella* resistance against sulfadiazine was 35 percent which increased to 40 percent in 2007 and 2008. An increasing trend was noted in the *Salmonella* against sulfadiazine. *Salmonella* resistance against sulfaquinoxaline studied during three years was 43 percent in 2006, showed a little fluctuation in downward direction in 2007 (33 %) and it was 37 percent during 2008 (Fig. 36 & Annex. 4.1.2).

Salmonella resistance against sulfachlorpyridazine studied during three years was 20 percent in 2006, it increased up to 25 percent in 2007 and it elevated to its maximum 37 percent during 2008. Resistance in *Salmonella* against sulfachlorpyridazine among the sulfonamides group was in very a rapid and straight increasing fashion (20-37 %) during the year 2007 to 2008. During the year 2006 and 2007 resistance in *Salmonella* against sulfadimidine was 22 percent and it increased next year in 2008 up to 24 percent. An overall increasing trend was noted in the *Salmonella* against sulfadimidine during the years 2006 to 2008 (Fig. 36).

EMERGENCE OF RESISTANCE IN *CLOSTRIDIUM* STRAINS AGAINST SULFADIAZINE, SULFAQUINOXALINE, SULFACHLORPYRIDAZINE AND SULFADIMIDINE

Among the total isolates 496 were screened during the year 2006-2008, tested against representative antimicrobials of fluoroquinolones 161 isolates of *Clostridium* were tested for these antimicrobials during 2006 followed by 169 in 2007 and 166 during 2008. During the year 2006 maximum resistance 30 percent was observed against sulfadiazine, followed by sulfachlorpyridazine 26 percent and against sulfaquinoxaline and sulfadimidine was 21 percent. During the year 2006 the resistance developed in *Clostridium* against sulfadiazine was 30 percent which increased to 33 percent in 2007 and it declined to 24 percent in 2008. *Clostridium* resistance against sulfaquinoxaline studied during three years was 21 percent in 2006, very rapid increased to 39 percent in 2007 but it became 33 percent during 2008. In spite of fluctuation during 2007, an overall increasing trend (21-33 %) was observed in the *Clostridium* against sulfaquinoxaline during the three years period of study (Fig. 36 & Annex. 4.1.3).

During the year 2006 resistance in *Clostridium* against sulfachlorpyridazine was 26 percent, it raised up to 30 percent in 2007 but it declined during 2008 to 10 percent. A straight decreasing trend (20-10 %) was observed in *Clostridium* resistance against sulfachlorpyridazine during three years 2006 to 2008. During the year 2006 the resistance developed in *Clostridium* against sulfadimidine was 21 percent which increased to 23 percent in 2007 and it declined to 21 percent in 2008 (Fig. 36).

MICROBIAL RESISTANCE IN HUMAN

During the three years (2006-2008), 2496 human isolates were screened out of which 828 isolates were antibiographed during the years 2006 whereas, 780 during 2007 and 888 were screened during 2008. These samples were shared with local hospital and pathology laboratories to get inter laboratory optimization of the results. The aim on one side was to confirm the resistance and sensitivity for the patients and on other side the results helped the laboratories to minimize the error. These results were interpreted to draw the information regarding the loosing potential of the drugs.

Antibiography of 828 isolates was conducted during the year 2006 against representative antimicrobials of macrolides and fluoroquinolones. Among tested isolates, 190 (22.9 %) were resistant to these antimicrobials. The maximum resistance (24.3 %) was observed against ciprofloxacin, whereas, it was 21.6 percent for erythromycin. The picture of resistance was not good in temporal phase because in comparison with other years. Overall resistance (23.0 %) in 2006 became worst in 2007 where it increases up to 26.2 percent which turned the situation more gloomy in 2008 where it touch the level of 28.9 percent (Fig. 37).

Among the total isolates 838 screened during the years 2006 through 2008 against representative antimicrobials of macrolides and fluoroquinolones, 278 isolates of *Salmonella* (33.2 %) were tested for these antimicrobials during 2006, followed by 262 (31.3 %) in 2007 and 298 (35.6 %) during 2008. The resistance 24.8 percent was observed against ciprofloxacin, followed by erythromycin 21.2 percent. Among the total 1204 isolates screened during the year 2006-2008, against representative antimicrobials of macrolides and fluoroquinolones, 380 isolates of *Staphylococcus* 31.5 percent were tested for these antimicrobials during 2006, followed by 30.4 percent in 2007 and 38.0 percent during 2008. An overall 20.0 percent resistance was observed against erythromycin followed by 16.8 percent against ciprofloxacin (Fig. 37 & Annex. 4.2).

EMERGENCE OF RESISTANCE IN *E. coli* STRAINS AGAINST ERYTHROMYCIN AND CIPROFLOXACIN

Among the total isolates 454 screened during the year 2006-2008, were tested against erythromycin and ciprofloxacin representative antimicrobials of macrolides and fluoroquinolones groups, 37 percent isolates of *E. coli* were tested for these antimicrobials during 2006 followed by 34 percent in 2007 and it was 29 percent during 2008. During the year 2006 maximum resistance 40 percent was observed against ciprofloxacin and erythromycin was 26 percent. During 2006 resistance in *E. coli* isolates against ciprofloxacin was 40 percent, it increased to 41 percent in 2007 which turned the situation more gloomy in 2008 where it touch the level of 45 percent. Whereas during 2006 resistance in *E. coli* isolates against erythromycin was 26 percent, it increased to 29 percent in 2007 which turned the situation gloomier in 2008 where it touches the level of 33 percent (Fig. 37 & Annex. 4.2.1).

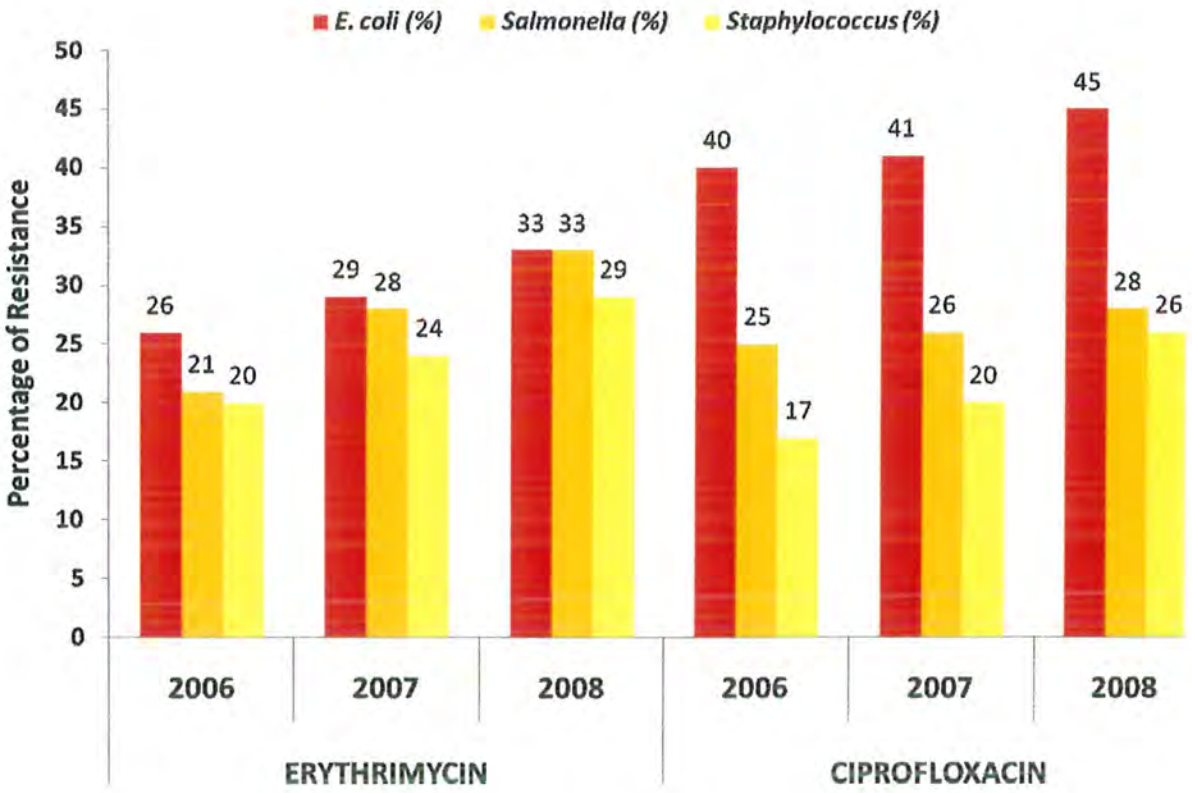

Fig. 37. Prevalence of resistance during the year 2006 through 2008 in *E. coli* ( ), *Salmonella* ( ) *Staphylococcus* ( ) of poultry isolates against Ciprofloxacin and Erythromycin by solid media microbiological sensitivity method. Numerical values in the graph showing percentage of resistance.

EMERGENCE OF RESISTANCE IN *SALMONELLA* STRAINS AGAINST ERYTHROMYCIN AND CIPROFLOXACIN

Among the total isolates 838 screened during the year 2006-2008, were tested against erythromycin and ciprofloxacin representative antimicrobials of macrolides and fluoroquinolones groups, 278 isolates of *Salmonella* were tested for these antimicrobials during 2006 followed by 262 in 2007 and it was 298 during 2008. During the year 2006 maximum resistance 25 percent was observed against ciprofloxacin and erythromycin was 21 percent (Fig. 37 & Annex. 4.2.2).

During 2006 resistance in *Salmonella* isolates against ciprofloxacin was 25 percent, it increased to 26 percent in 2007 which turned the situation more gloomy in 2008 where it touch the level of 28 percent. Straight increasing trend in resistance among *Salmonella* isolates against ciprofloxacin was prevailing during the three years study period. Resistance among *Salmonella* isolates against erythromycin during 2006 was 21 percent; it increased to 28 percent in 2007 which turned the situation gloomier in 2008 where it touched the level of 33 percent (Fig. 37).

EMERGENCE OF RESISTANCE IN *STAPHYLOCOCCUS* STRAINS AGAINST ERYTHROMYCIN AND CIPROFLOXACIN

Among the total isolates 1204 screened during the year 2006-2008, were tested against erythromycin and ciprofloxacin representative antimicrobials of macrolides and fluoroquinolones groups, 380 isolates of *Staphylococcus* were tested for these antimicrobials during 2006 followed by 366 in 2007 and it was 458 during 2008. During the year 2006 maximum resistance 20 percent was observed against erythromycin and against ciprofloxacin 17 percent. During 2006 resistance in *Staphylococcus* isolates against ciprofloxacin was 17 percent, it increased to 20 percent in 2007 which turned the situation more gloomy in 2008 where it touch the level of 26 percent. A very sharp increase in resistance among *Staphylococcus* isolates against ciprofloxacin (17.3 %) during three years period was alarming. Resistance among *Staphylococcus* isolates against erythromycin during 2006 was 20 percent; it increased to 24 percent in 2007 which turned the situation gloomier in 2008 where it touched the level of 29 percent (Fig. 37 & Annex. 4.2.3).

CONCLUSIONS

Most of the countries rely upon a monitoring infrastructure including dozens of routine field laboratories, but still a strong need for the development and implementation of adequate microbial screening methods and more regular proficiency testing to reveal the shortcomings in first line of defense in antimicrobial residue monitoring. Currently Pakistan is exporting chicken products and whole processed meat to Middle East, Central Asia, Afghanistan and Iran. Without establishment of control measures it is difficult to check the transmission of residues in these countries. Therefore an effort was made to draw the attention of the authorities to the health hazard disguised in the shape of food.

MCSTAR developed to screen residues of tylosin, erythromycin, tilmicosin (macrolides), norfloxacin, enrofloxacin, ciprofloxacin, sarafloxacin, flumequine (fluoroquinolones), sulfadiazine, sulfaquinoxaline, sulfachlorpyridazine and sulfadimidine (sulfonamides) antimicrobials in poultry meat, liver, bone marrow and egg yolks, was same or higher with the sensitivity of MRL and also had augmented advantage of screening test in time and cost in comparison with high cost of HPLC coupled with tandem mass spectrometry.

Larger based residue monitoring plans ensure better protection levels for consumers, than control systems designed simply as a proportion of production units. The occurrences of drug residues in poultry products in and around Rawalpindi-Islamabad Pakistan were determined. Drug residues surveillance studies gave an insight into the aggravated residues status, as 12.87 percent poultry meat, liver, bone marrow and egg yolks samples were found positive. Higher incidence of resistance among *E. coli*, *Salmonella*, *Clostridum* and *Staphylococcus* was part of the studies.

Blunders in the normal safety procedures for processing of animal foods may lead to exposure of human to more virulent animal pathogens than in its less-antibiotic-resistant form. Medics are handicapped due to the lesser new antimicrobials to substitute for remedies to which resistance has been developed. Prolong low dose usage of antimicrobials; accelerate the mutation of drug-resistant bacteria. In these instances an increased dose for curative purposes is used,

leading to more residues in meat, eggs or milk consequently a threat in term of pathological changes in the body.

Final goal of the study was to furnish preliminary local data to drag the attention of the concerned authorities regarding the withdrawal periods and egg discarding period and maximum residual limits. In addition, a cheaper and practicable testing method/technique MCSTAR will be helpful for future studies of other antimicrobials.

SUGGESTIONS AND RECOMMENDATIONS

- Antimicrobials as growth promoters and therapeutics must be stopped at Farmers end.
- Sale of drugs must be available on prescription of qualified registered Veterinarian.
- Residues monitoring laboratories may be established by the Government.
- Antimicrobials may be discouraged as growth promoters and prophylactics at feed mills.
- Antimicrobials which are common in human and poultry their usage must be restricted.
- Withdrawal periods should be observed.
- MRLs and ADIs must be notified for each residue by the Government.
- Strict control over the free availability of raw materials of antimicrobials in market.

FUTURE PROSPECTS

MCSTAR test developed in these studies was an economical and practical approach to monitor the residues of drugs in preliminary stages. Countries like Pakistan currently are not in the position to afford the cost of HPLC and other costly techniques. In the current studies residues of three groups were investigated. Still a number of antibacterial drugs used in poultry needed to be investigated. Further studies are required to complete the list of residues in future from A-Z. Transferability of gene encoding antimicrobial resistance needs further investigations as it is the land mark of final consequences of residues.

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APPENDICES

Annex.1.1.1. Tylosin residues distribution in poultry liver through, microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
4	14	1.45	1.47	11	13	1.35	1.40	6	12	1.24	1.27
21	17	1.76	1.83	20	14	1.45	1.47	7	17	1.76	1.83
17	18	1.87	1.92	9	17	1.76	1.83	8	19	1.97	2.02
13	20	2.08	2.13	10	18	1.87	1.92	9	22	2.28	2.33
18	24	2.51	2.56	15	20	2.08	2.13	14	23	2.39	2.44
10	25	3.62	3.67	11	22	2.28	2.33	17	25	3.62	3.67
14	26	4.18	4.23	13	24	2.51	2.56	18	27	4.34	4.39
16	27	4.34	4.39	14	28	4.50	4.56	20	30	4.82	4.87
6	28	4.50	4.56	17	29	4.66	4.72	22	31	5.00	5.05
119	X ± SD	2.84±1.09	2.89±1.09	120		2.58±1.24	2.63±1.25	121		3.60±1.22	3.65±1.22

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.1.1.2. Tylosin residues distribution in poultry meat through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
18	8	0.84	0.89	26	7	0.76	0.79	14	7	0.76	0.79
21	9	0.92	0.96	13	8	0.84	0.89	14	8	0.84	0.89
16	10	1.04	1.09	17	9	0.92	0.96	18	9	0.92	0.96
19	11	1.16	1.22	15	10	1.04	1.09	12	10	1.04	1.09
								16	11	1.16	1.22
74	X ± SD	0.99±0.12	1.04±0.13	71		0.87±0.11	0.91±0.11	74		0.95±0.14	0.99±0.15

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.1.1.3. Tylosin residues distribution in bone marrow through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
4	13	1.35	1.42	5	14	1.45	1.49	3	13	1.35	1.42
1	14	1.45	1.49	2	15	1.56	1.61	4	14	1.45	1.49
2	15	1.56	1.61					1	15	1.56	1.61
7	X ± SD	1.42±0.10	1.48±0.09	7		1.48±0.05	1.52±0.06	9		1.44±0.08	1.49±0.07

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.1.1.4. Tylosin residues distribution in poultry egg yolks through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
4	16	1.66	1.71	5	17	1.76	1.85	4	18	1.87	1.92
3	17	1.76	1.85	4	18	1.87	1.92	6	19	2.00	2.05
5	18	1.87	1.92	6	19	2.00	2.05	5	20	2.10	2.13
5	19	2.00	2.05	4	20	2.10	2.13	9	21	2.12	2.16
7	20	2.10	2.13	6	21	2.12	2.16	4	22	2.25	2.31
2	21	2.12	2.16	3	23	2.35	2.42				
2	22	2.25	2.31	1	24	2.45	2.51				
28	X ± SD	1.95±0.18	2.00±0.17	29		2.03±0.19	2.09±0.18	28		2.07±0.11	2.12±0.11

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter;
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.1.2.1. Erythromycin residues distribution in poultry liver through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
6	8	0.83	0.87	6	8	0.83	0.87	6	8	0.83	0.87
5	9	0.93	0.97	5	9	0.93	0.97	4	9	0.93	0.97
5	10	1.04	1.09	7	10	1.04	1.09	6	10	1.04	1.09
6	11	1.10	1.19	7	11	1.10	1.19	7	11	1.10	1.19
6	12	1.24	1.30	6	12	1.24	1.30	6	12	1.24	1.30
28	X ± SD	1.03±0.15	1.09±0.16	31		1.04±0.14	1.10±0.15	29		1.04±0.14	1.10±0.15

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.1.2.2. Erythromycin residues distribution in poultry meat through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
3	16	1.66	1.70	7	16	1.66	1.70	5	17	1.76	1.83
5	17	1.76	1.83	5	17	1.76	1.83	5	18	1.87	1.94
5	18	1.87	1.94	5	18	1.87	1.94	2	19	1.97	2.02
2	19	1.97	2.02	2	19	1.97	2.02	5	20	2.08	2.16
5	20	2.08	2.16	6	20	2.08	2.16	2	22	2.28	2.33
2	22	2.28	2.33	2	22	2.28	2.33	6	25	3.62	3.67
3	25	3.62	3.67								
25	X ± SD	2.12±0.59	2.18±0.59	27		1.88±0.19	1.95±0.20	25		2.35±0.74	2.42±0.73

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.1.2.3. Erythromycin residues distribution in bone marrow through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
1	22	2.28	2.33	1	22	2.28	2.33	1	23	2.39	2.45
1	23	2.39	2.45	1	24	2.51	2.56	1	24	2.51	2.56
2	X ± SD	2.34±0.08	2.39±0.08	2		2.40±0.16	2.45±0.16	2		2.45±0.08	2.51±0.08

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.1. 2.4. Erythromycin residues distribution in poultry egg yolks through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
2	18	1.82	1.87	3	17	1.76	1.82	2	17	1.76	1.82
4	20	2.04	2.09	2	18	1.82	1.87	3	18	1.82	1.87
3	21	2.12	2.17	2	20	2.04	2.09	4	20	2.04	2.09
				2	21	2.12	2.17	1	21	2.12	2.17
9	X ± SD	2.02±0.12	2.07±0.12	9		1.92±0.16	1.97±0.16	10		1.93±0.14	1.98±0.14

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.1.3.1. Tilmicosin residues distribution in poultry liver through, microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
1	8	0.83	0.87	1	8	0.83	0.87	2	8	0.83	0.87
3	9	0.96	1.01	2	9	0.96	1.01	3	9	0.96	1.01
3	10	1.02	1.04	2	10	1.02	1.04	3	10	1.02	1.04
7	X ± SD	0.97±0.07	1.00±0.06	5		0.96±0.08	0.99±0.07	8		0.95±0.08	0.99±0.07

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.1.3.2. Tilmicosin residues distribution in poultry meat through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
3	8	0.84	0.88	1	7	0.75	0.79	3	7	0.75	0.79
2	9	0.92	0.95	3	8	0.84	0.88	3	8	0.84	0.88
				2	9	0.92	0.95	2	9	0.92	0.94
				1	10	1.09	1.15				
5	X ± SD	0.87±0.04	0.91±0.04	7		0.89±0.11	0.93±0.11	8		0.83±0.07	0.86±0.06

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.1.3.3. Tilmicosin residues distribution in bone marrow through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
1	15	1.56	1.62	2	16	1.66	1.73	1	15	1.56	1.62
1	17	1.76	1.85	1	17	1.76	1.85	1	18	1.87	1.94
2	X ±	1.66±0.14	1.74±0.16	3		1.69±0.06	1.77±0.07	2		1.72±0.22	1.78±0.23

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.1.3.4. Tilmicosin residues distribution in egg yolks through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
3	17	1.76	1.83	2	17	1.76	1.83	2	17	1.76	1.83
2	18	1.87	1.94	1	18	1.87	1.94	1	18	1.87	1.94
				1	19	2.00	2.09	1	19	2.00	2.09
								1	20	2.10	2.16
5	X ±	1.80±0.06	1.87±0.06	4		1.90±0.11	1.97±0.12	5		1.90±0.15	1.97±0.15

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.2.1.1. Norfloxacin residues in poultry liver through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
2	8	0.83	0.87	3	8	0.83	0.87	4	9	0.93	0.97
3	9	0.93	0.97	4	9	0.93	0.97	5	10	1.04	1.09
1	10	1.04	1.09	2	10	1.04	1.09	3	11	1.10	1.19
6	11	1.10	1.19	5	11	1.10	1.19	3	12	1.24	1.30
4	12	1.24	1.30	4	12	1.24	1.30				
16	X ± SD	1.07±0.14	1.13±0.15	18		1.04±0.15	1.10±0.16	15		1.06±0.11	1.12±0.12

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.2.1.2. Norfloxacin residues in poultry meat through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
7	8	0.83	0.87	5	8	0.83	0.87	3	8	0.83	0.87
6	9	0.93	0.97	6	9	0.93	0.97	6	9	0.93	0.97
2	10	1.04	1.09	2	10	1.04	1.09	3	10	1.04	1.09
1	11	1.14	1.32	2	11	1.14	1.32	2	11	1.14	1.32
16	X ± SD	0.91±0.09	0.96±0.12	15		0.94±0.11	1.00±0.15	14		0.96±0.10	1.02±0.15

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.2.1.3. Norfloxacin residues in poultry bone marrow through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
1	12	1.24	1.30	1	12	1.24	1.30	1	12	1.24	1.30
1	13	1.35	1.41					1	14	1.45	1.53
2	X ± SD	1.30±0.08	1.36±0.08	1		1.24±0.00	1.30±0.00	2		1.35±0.15	1.41±0.16

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.2.1.4. Norfloxacin residues in poultry egg yolks through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
1	13	1.35	1.39	2	13	1.35	1.41	2	13	1.35	1.4
2	14	1.45	1.51	2	14	1.45	1.51	2	14	1.45	1.51
2	15	1.56	1.61	1	15	1.56	1.61	1	15	1.56	1.61
				1	16	1.66	1.74	1	16	1.66	1.74
5	X ± SD	1.47±0.09	1.53±0.09	6		1.47±0.12	1.53±0.13	6		1.47±0.12	1.53±0.13

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.2.2.1. Enrofloxacin residues in poultry liver through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
4	13	1.35	1.40	8	12	1.24	1.27	6	14	1.45	1.48
9	14	1.45	1.48	8	13	1.35	1.40	7	17	1.76	1.83
12	17	1.76	1.83	11	18	1.87	1.94	13	20	2.08	2.15
5	19	1.97	2.02	5	20	2.08	2.15	8	22	2.28	2.36
9	22	2.28	2.36	7	23	2.39	2.44	12	24	2.51	2.56
11	23	2.39	2.44	5	24	2.51	2.56	4	25	3.62	3.67
6	25	3.62	3.67	14	27	4.34	4.39	9	26	4.18	4.25
8	27	4.34	4.39	8	28	4.50	4.56	5	29	4.66	4.72
5	28	4.50	4.56	4	29	4.66	4.72	4	31	5.00	5.05
2	30	4.82	4.87	5	30	4.82	4.87				
71	X ± SD	2.60±1.12	2.66±1.12	75		2.94±1.37	2.99±1.37	68		2.82±1.13	2.88±1.13

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.2.2.2. Enrofloxacin residues in poultry meat through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
6	22	3.07	3.22	11	22	3.07	3.22	19	24	3.85	4.04
5	23	3.44	3.59	9	23	3.44	3.59	23	25	4.07	4.40
9	24	3.85	4.04	14	25	4.07	4.40	8	28	4.59	4.81
10	25	4.07	4.40	17	28	4.59	4.81	4	30	4.96	5.18
23	28	4.59	4.81								
53	X ± SD	4.09±0.53	4.31±0.56	51		3.92±0.60	4.14±0.64	54		4.14±0.34	4.39±0.36

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.2.2.3. Enrofloxacin residues in poultry bone marrow through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
1	16	1.66	1.72	2	16	1.66	1.72	1	16	1.66	1.72
1	17	1.76	1.83	1	17	1.76	1.83	1	17	1.76	1.83
1	18	1.87	1.94	1	20	2.08	2.16	1	20	2.08	2.16
1	20	2.08	2.16	2	25	3.62	3.67	1	25	3.62	3.67
1	22	2.28	2.32								
5	X ± SD	1.93±0.25	1.99±0.24	6		2.40±0.96	2.46±0.95	4		2.28±0.91	2.35±0.90

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.2.2.4. Enrofloxacin residues in poultry egg yolks through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
3	18	1.87	1.92	4	18	1.87	1.92	3	18	1.87	1.92
4	19	1.97	2.02	6	19	1.97	2.02	5	19	1.97	2.02
8	20	2.08	2.16	1	20	2.08	2.16	1	20	2.08	2.16
4	21	2.18	2.24	2	21	2.18	2.24	3	21	2.18	2.24
2	22	2.28	2.35	5	22	2.28	2.35	3	22	2.28	2.35
2	23	2.39	2.45	3	23	2.39	2.45	3	23	2.39	2.45
2	24	2.51	2.59	3	24	2.51	2.59	2	24	2.51	2.59
25	X ± SD	2.13±0.18	2.19±0.19	24		2.16±0.22	2.22±0.23	20		2.16±0.22	2.22±0.22

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.2.3.1. Ciprofloxacin residues in poultry liver through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
2	17	1.76	1.81	4	17	1.76	1.81	3	17	1.76	1.81
2	18	1.86	1.94	2	18	1.86	1.94	3	18	1.86	1.94
3	19	1.97	2.02	3	19	1.97	2.02	4	19	1.97	2.02
7	X ± SD	1.86±0.09	1.93±0.09	9		1.84±0.09	1.9±0.09	10		1.86±0.09	1.93±0.09

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.2.3.2. Ciprofloxacin residues in poultry meat through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
2	12	1.24	1.30	2	12	1.24	1.30	2	12	1.24	1.30
2	13	1.35	1.41	1	13	1.35	1.41	3	13	1.35	1.41
1	14	1.44	1.52	1	14	1.44	1.52	2	14	1.44	1.52
5	X ± SD	1.32±0.09	1.39±0.09	4		1.32±0.10	1.38±0.11	7		1.34±0.08	1.41±0.09

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.2.3.3. Ciprofloxacin residues in poultry bone marrow through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
1	14	1.46	1.52	1	14	1.46	1.51	1	15	1.56	1.63
1	15	1.56	1.62					1	14	1.46	1.51
2	X ± SD	1.51±0.07	1.57±0.07	1		1.46±0.00	1.51±0.00	2		1.51±0.07	1.57±0.08

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.2.3.4. Ciprofloxacin residues in poultry egg yolks through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
1	16	1.65	1.72	1	16	1.65	1.72	1	17	1.76	1.83
2	17	1.76	1.83	1	17	1.76	1.83	2	18	1.87	1.94
2	18	1.87	1.94	1	18	1.87	1.94	1	19	1.97	2.03
				1	19	1.97	2.02				
5	X ± SD	1.78±0.09	1.85±0.09	4		1.81±0.14	1.88±0.13	4		1.87±0.09	1.94±0.08

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.2.4.1. Sarafloxacin residues in poultry liver through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
2	14	1.45	1.51	4	14	1.45	1.51	1	14	1.45	1.51
2	16	1.66	1.71	1	15	1.56	1.62	3	15	1.56	1.62
1	17	1.76	1.82	1	16	1.66	1.71	2	16	1.66	1.71
								1	17	1.76	1.82
5	X ± SD	1.60±0.14	1.65±0.14	6		1.50±0.09	1.56±0.08	7		1.60±0.10	1.66±0.10

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.2.4.2. Sarafloxacin residues in poultry meat through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
1	12	1.24	1.30	1	13	1.35	1.41	1	12	1.24	1.30
1	14	1.45	1.52	1	12	1.24	1.30	1	13	1.35	1.41
				1	14	1.45	1.52	1	14	1.45	1.52
2	X ± SD	1.35±0.15	1.41±0.16	3		1.35±0.11	1.41±0.11	3		1.35±0.11	1.41±0.11

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.2.4.3. Sarafloxacin residues in poultry bone marrow through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
1	25	4.02	4.10	1	25	4.02	4.10	1	25	4.02	4.11
				1	26	4.18	4.26				
1	X ± SD	4.02±0.00	4.10±0.00	2		4.10±0.11	4.18±0.11	1		4.02±0.00	4.11±0.00

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.2.4.4. Sarafloxacin residues in poultry egg yolks through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
1	15	1.56	1.60	1	15	1.56	1.60	1	15	1.56	1.61
1	16	1.65	1.72	1	16	1.65	1.72	1	16	1.65	1.72
				1	17	1.76	1.83	1	17	1.76	1.83
2	X ± SD	1.61±0.06	1.66±0.08	3		1.66±0.10	1.72±0.12	3		1.66±0.10	1.72±0.11

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.2.5.1. Flumequine residues in poultry liver through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
8	22	3.07	3.22	9	22	3.07	3.22	6	22	3.07	3.22
6	23	3.44	3.59	5	23	3.44	3.59	8	23	3.44	3.59
5	24	3.85	4.04	9	24	3.85	4.04	6	24	3.85	4.04
11	25	4.07	4.40	8	25	4.07	4.40	13	25	4.07	4.40
13	28	4.59	4.81	8	28	4.59	4.81	9	28	4.59	4.81
5	30	4.96	5.18	2	30	4.96	5.18	4	30	4.96	5.18
48	X ± SD	4.04±0.62	4.26±0.65	41		3.87±0.58	4.08±0.62	46		3.98±0.57	4.21±0.61

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.2.5.2. Flumequine residues in poultry meat through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
5	17	1.76	1.85	7	17	1.76	1.85	5	17	1.76	1.85
3	18	1.87	1.92	4	18	1.87	1.92	2	18	1.87	1.92
2	19	1.97	2.02	2	19	1.97	2.02	2	19	1.97	2.02
8	20	2.08	2.16	7	20	2.08	2.16	7	20	2.08	2.16
4	21	2.18	2.24	5	21	2.18	2.24	4	21	2.18	2.24
3	23	2.39	2.45	5	23	2.39	2.45	4	23	2.39	2.45
25	X ± SD	2.04±0.20	2.11±0.19	30		2.04±0.22	2.11±0.21	24		2.06±0.21	2.13±0.21

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.2.5.3. Flumequine residues in poultry bone marrow through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
1	25	4.02	4.10	1	25	4.02	4.10	1	25	4.02	4.10
1	26	4.18	4.26	1	26	4.18	4.26				
2	X ± SD	4.10±0.11	4.18±0.11	2		4.10±0.11	4.18±0.10	1		4.02±0.00	4.10±0.00

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.2.5.4. Flumequine residues in poultry egg yolks through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
1	29	3.62	3.67	1	31	5.00	5.05	2	29	3.62	3.67
1	30	4.82	4.87	2	32	5.15	5.24	1	30	4.82	4.87
1	31	5.00	5.05	2	33	5.31	5.41	2	31	5.00	5.05
1	32	5.15	5.24					1	32	5.15	5.24
								1	33	5.31	5.41
4	X ± SD	4.65±0.70	4.72±0.71	5		5.18±0.13	5.27±0.15	7		4.65±0.72	4.71±0.73

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter;
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.3.1.1. Sulfadiazine residues in poultry liver through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
9	22	3.07	3.22	15	22	3.07	3.22	11	22	3.07	3.22
11	23	3.44	3.59	7	23	3.44	3.59	9	23	3.44	3.59
13	24	3.85	4.04	8	24	3.85	4.04	12	24	3.85	4.04
6	28	4.59	4.81	11	25	4.07	4.4	8	25	4.07	4.4
10	29	4.96	5.18	10	28	4.59	4.81	13	28	4.59	4.81
								6	29	4.96	5.18
49	X ± SD	3.93±0.69	4.12±0.72	51		3.76±0.56	3.97±0.61	59		3.95±0.62	4.15±0.66

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.3.1.2. Sulfadiazine residues in poultry meat through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
5	13	1.14	1.19	15	12	1.02	1.07	9	13	1.14	1.19
7	14	1.28	1.34	9	14	1.28	1.34	14	14	1.28	1.34
12	15	1.35	1.46	4	15	1.35	1.46	8	15	1.35	1.46
8	16	1.53	1.60	5	16	1.53	1.60				
32	X ± SD	1.35±0.13	1.43±0.14	33		1.21±0.19	1.27±0.20	31		1.26±0.08	1.33±0.10

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.3.1.3. Sulfadiazine residues in poultry bone marrow through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
1	12	0.68	0.75	2	12	0.68	0.75	1	11	0.64	0.67
1	13	0.77	0.81	1	13	0.77	0.81	3	12	0.68	0.75
2	14	1.11	1.17					3	13	0.77	0.81
								1	14	1.11	1.17
4	X ± SD	0.92±0.23	0.98±0.23	3		0.71±0.05	0.77±0.03	8		0.76±0.15	0.82±0.15

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.3.1.4. Sulfadiazine residues in poultry egg yolks through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
3	23	3.26	3.40	7	24	3.64	3.82	2	22	2.91	3.05
6	25	3.85	4.17	3	25	3.85	4.17	8	23	3.26	3.40
4	27	4.34	4.55	5	27	4.34	4.55	3	24	3.64	3.82
								6	25	3.85	4.17
13	X ± SD	3.86±0.41	4.11±0.44	15		3.92±0.32	4.13±0.33	19		3.46±0.33	3.66±0.40

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.3.2.1. Sulfaquinoxaline residues in poultry liver through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
8	19	2.08	2.18	15	19	2.08	2.18	11	19	2.08	2.18
13	20	2.33	2.43	7	20	2.33	2.43	5	20	2.33	2.43
7	21	2.60	2.73	3	21	2.60	2.73	10	21	2.60	2.73
9	22	2.75	2.98	12	22	2.75	2.98	7	22	2.75	2.98
8	23	3.10	3.25	5	23	3.10	3.25	6	23	3.10	3.25
								7	24	3.35	3.52
45	X ± SD	2.55±0.34	2.69±0.38	42		2.47±0.36	2.62±0.41	46		2.64±0.44	2.78±0.47

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.3.2.2. Sulfaquinoxaline residues in poultry meat through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
16	11	0.69	0.72	7	11	0.69	0.72	11	11	0.69	0.72
9	12	0.77	0.80	12	12	0.77	0.80	7	12	0.77	0.80
4	13	0.91	0.94	9	13	0.91	0.94	10	13	0.91	0.94
								3	14	1.12	1.17
29	X ± SD	0.75±0.08	0.78±0.08	28		0.80±0.09	0.83±0.09	31		0.82±0.14	0.85±0.14

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.3.2.3. Sulfaquinoxaline residues in poultry bone marrow through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
1	8	0.35	0.37	1	8	0.35	0.37	2	8	0.35	0.37
1	10	0.46	0.50	1	9	0.39	0.42	1	9	0.39	0.42
1	11	0.52	0.55	1	10	0.46	0.50	1	10	0.46	0.50
				1	11	0.52	0.55	1	11	0.52	0.55
3	X ± SD	0.44±0.09	0.46±0.13	4		0.43±0.08	0.46±0.13	5		0.42±0.07	0.45±0.09

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.3.2.4. Sulfaquinoxaline residues in poultry egg yolks through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
6	19	1.96	2.05	4	20	2.2	2.3	5	19	1.96	2.05
3	20	2.20	2.30	5	21	2.60	2.81	2	20	2.20	2.30
2	22	2.93	3.07	3	22	2.93	3.07	7	21	2.60	2.81
11	X ± SD	2.20±0.38	2.30±0.40	12		2.55±0.29	2.71±0.32	14		2.31±0.31	2.47±0.37

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.3.3.1 Sulfachlorpyridazine residues in poultry liver through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
8	20	2.32	2.43	3	20	2.32	2.43	5	20	2.32	2.43
6	21	2.60	2.71	4	21	2.60	2.71	2	21	2.60	2.71
4	22	2.91	3.05	10	22	2.91	3.05	6	22	2.91	3.05
3	23	3.08	3.33	5	23	3.08	3.33	4	23	3.08	3.33
5	24	3.47	3.64	7	24	3.47	3.64	8	24	3.47	3.64
26	X ± SD	2.78±0.43	2.93±0.46	29		2.98±0.36	3.14±0.39	25		2.99±0.43	3.15±0.46

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.3.3.2. Sulfachlorpyridazine residues in poultry meat through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
3	11	0.77	0.81	7	13	0.97	1.02	5	12	0.86	0.92
4	13	0.97	1.02	8	14	1.02	1.10	3	13	0.97	1.02
3	14	1.02	1.10	2	15	1.15	1.21	4	14	1.02	1.10
6	15	1.15	1.21					6	15	1.15	1.21
16	X ± SD	1.01±0.14	1.07±0.15	17		1.01±0.06	1.08±0.06	18		1.01±0.12	1.07±0.12

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.3.3.3. Sulfachlorpyridazine residues in poultry bone marrow through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
1	10	0.44	0.46	1	10	0.44	0.46	1	11	0.52	0.56
				1	11	0.52	0.56				
1	X ± SD	0.44±0.00	0.46±0.00	2		0.48±0.06	0.51±0.07	1		0.52±0.00	0.56±0.00

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.3.3.4. Sulfachlorpyridazine residues in poultry egg yolks through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
1	20	2.20	2.31	3	21	2.46	2.57	1	21	2.46	2.57
4	22	2.92	3.16	1	22	2.92	3.16	5	22	2.92	3.16
5	X ± SD	2.78±0.32	2.99±0.38	4		2.58±0.23	2.72±0.30	6		2.84±0.19	3.06±0.24

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.3.4.1. Sulfadimidine residues in poultry liver through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
4	21	2.57	2.69	5	22	2.88	3.00	11	21	2.57	2.69
9	22	2.88	3.00	10	23	3.22	3.38	8	22	2.88	3.00
15	23	3.22	3.38	14	24	3.41	3.69	7	23	3.22	3.38
3	24	3.41	3.69	4	25	3.84	4.03	4	24	3.41	3.69
31	X ± SD	3.06±0.26	3.21±0.29	33		3.32±0.27	3.53±0.31	30		2.92±0.32	3.07±0.36

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.3.4.2. Sulfadimidine residues in poultry meat through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
9	12	0.85	0.89	4	13	0.96	1.00	3	12	0.85	0.89
4	13	0.96	1.00	2	15	1.28	1.35	5	13	0.96	1.00
5	14	1.13	1.22	8	16	1.38	1.45	4	14	1.13	1.22
18	X ± SD	0.95±0.12	1.01±0.14	14		1.25±0.19	1.31±0.20	17		1.10±0.21	1.16±0.22

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.3.4.3. Sulfadimidine residues in poultry bone marrow through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
2	11	0.57	0.62	2	12	0.64	0.67	1	11	0.57	0.62
2	X ± SD	0.57±0.00	0.62±0.00	2		0.64±0.00	0.67±0.00	1		0.57±0.00	0.62±0.00

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.3.4.4. Sulfadimidine residues in poultry egg yolks through microbiological (M) assay and spectrophotometric (S) assay.

2006				2007				2008			
No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)	No of sample	M. assay of sample		S. assay of sample (µg/g)
	Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)			Inhibition zone (mm)	Conc. (µg/g)	
1	16	2.43	2.35	3	23	3.22	3.36	2	22	3.05	3.35
2	21	2.74	2.98	2	24	3.63	3.81	2	23	3.22	3.36
3	22	3.05	3.35								
6	X ± SD	2.84±0.25	3.06±0.39	5		3.38±0.22	3.54±0.25	4		3.14±0.10	3.36±0.01

X = Mean of concentration; SD = Stranded deviation; Conc. = Concentration; µg/g = microgram per gram; mm = millimeter.
M. assay = Microbiological assay; S. assay = Spectrophotometric assay.

Annex.4.1. Susceptibility pattern of 2156 isolates of *E. coli, Salmonella* and *Clostridium* of poultry origin during years 2006-2008.

ANTIMICROBIALS TESTED	SUSCEPTIBILITY								
	2006			*2007*			*2008*		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
TYLOSIN TARTRATE	372 (53.22)	65 (9.30)	262 (37.48)	366 (51.05)	64 (8.93)	287 (40.03)	320 (43.24)	73 (9.86)	347 (46.89)
ERYTHROMYCIN	271 (38.77)	89 (12.73)	339 (48.50)	258 (35.98)	115 (16.04)	344 (47.98)	378 (51.08)	28 (3.78)	334 (45.14)
TILMICOSIN	324 (46.35)	59 (8.44)	316 (45.21)	316 (44.07)	85 (11.85)	316 (44.07)	183 (24.73)	208 (28.11)	349 (47.16)
NORFLOXACIN	245 (35.05)	25 (3.58)	429 (61.37)	162 (22.59)	57 (7.95)	498 (69.46)	126 (17.03)	74 (10.00)	540 (72.97)
ENROFLOXACIN	284 (40.63)	54 (7.73)	361 (51.65)	267 (37.24)	77 (10.74)	373 (52.02)	274 (37.03)	79 (10.68)	387 (52.30)
CIPROFLOXACIN	366 (52.36)	77 (11.02)	256 (36.62)	351 (48.95)	74 (10.32)	292 (40.73)	227 (30.68)	209 (28.24)	304 (41.08)
SARAFLOXACIN	488 (69.81)	60 (8.58)	151 (21.60)	424 (59.14)	119 (16.60)	174 (24.27)	441 (59.59)	88 (11.89)	211 (28.51)
FLUMEQUINE	380 (54.36)	79 (11.30)	240 (34.33)	340 (47.42)	115 (16.04)	262 (36.54)	420 (56.76)	51 (6.89)	269 (36.35)
SULFADIAZINE	342 (48.93)	123 (17.60)	234 (33.48)	314 (43.79)	138 (19.25)	265 (36.96)	319 (43.11)	152 (20.54)	269 (36.35)
SULFAQUINOXALINE	304 (43.49)	140 (20.03)	255 (36.48)	300 (41.84)	160 (22.32)	257 (35.84)	314 (42.43)	143 (19.32)	283 (38.24)
SULFACHLORPYRIDAZINE	436 (62.37)	110 (15.74)	153 (21.89)	386 (53.84)	144 (20.08)	187 (26.08)	405 (54.73)	127 (17.16)	208 (28.11)
SULFADIMIDINE	460 (65.81)	91 (13.02)	148 (21.17)	440 (61.37)	112 (15.62)	165 (23.01)	481 (65.00)	88 (11.89)	171 (23.11)
TOTAL (%)	356 (50.93)	81 (11.59)	262 (37.48)	327 (45.61)	105 (14.64)	285 (39.75)	324 (43.78)	110 (14.86)	306 (41.35)

S = Sensitive (> 20 mm); I = Intermediate (< 15 mm); R = Resistant (≤ 8 mm)

Annex.4.1.1. Susceptibility pattern of 971 *E. coli* isolates of poultry origin during years 2006-2008.

ANTIMICROBIALS TESTED	SUSCEPTIBILITY								
	2006			*2007*			*2008*		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
TYLOSIN TARTRATE	164 (51.57)	39 (12.26)	115 (36.16)	166 (51.88)	29 (9.06)	125 (39.06)	136 (40.84)	37 (11.11)	160 (48.05)
ERYTHROMYCIN	126 (39.62)	32 (10.06)	160 (50.31)	145 (45.31)	30 (9.38)	145 (45.31)	138 (41.44)	28 (8.41)	167 (50.15)
TILMICOSIN	151 (47.48)	25 (7.86)	142 (44.65)	138 (43.13)	43 (13.44)	139 (43.44)	96 (28.83)	79 (23.72)	158 (47.45)
NORFLOXACIN	109 (34.28)	7 (2.20)	202 (63.52)	82 (25.63)	15 (4.69)	223 (69.69)	64 (19.22)	32 (9.61)	237 (71.17)
ENROFLOXACIN	121 (38.05)	32 (10.06)	165 (51.89)	116 (36.25)	35 (10.94)	169 (52.81)	111 (33.33)	44 (13.21)	178 (53.45)
CIPROFLOXACIN	166 (52.20)	41 (12.89)	111 (34.91)	152 (47.50)	39 (12.19)	129 (40.31)	106 (31.83)	91 (27.33)	136 (40.84)
SARAFLOXACIN	230 (72.33)	29 (9.12)	59 (18.55)	185 (57.81)	54 (16.88)	81 (25.31)	181 (54.35)	59 (17.72)	93 (27.93)
FLUMEQUINE	173 (54.40)	39 (12.26)	106 (33.33)	159 (49.69)	49 (15.31)	112 (35.00)	171 (51.35)	41 (12.31)	121 (36.34)
SULFADIAZINE	154 (48.43)	57 (17.92)	107 (33.65)	144 (45.00)	57 (17.81)	119 (37.19)	150 (45.05)	50 (15.02)	133 (39.94)
SULFAQUINOXALINE	158 (49.69)	32 (10.06)	128 (40.25)	146 (45.63)	59 (18.44)	115 (35.94)	138 (41.44)	56 (16.82)	139 (41.74)
SULFACHLORPYRIDAZINE	204 (64.15)	46 (14.47)	68 (21.38)	187 (58.44)	54 (16.88)	79 (24.69)	179 (53.75)	51 (15.32)	103 (30.93)
SULFADIMIDINE	212 (66.67)	41 (12.89)	65 (20.44)	192 (60.00)	52 (16.25)	76 (23.75)	198 (59.46)	56 (16.82)	79 (23.72)
TOTAL (%)	164 (51.57)	35 (11.01)	119 (37.42)	151 (47.19)	43 (13.44)	126 (39.38)	139 (41.74)	52 (15.62)	142 (42.64)

S = Sensitive (> 20 mm); I = Intermediate (< 15 mm); R = Resistant (≤ 8 mm)

Annex.4.1.2. Susceptibility pattern of 689 *Salmonella* isolates of poultry origin during the years 2006-2008.

ANTIMICROBIALS TESTED	SUSCEPTIBILITY								
	2006			*2007*			*2008*		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
TYLOSIN TARTRATE	117 (53.18)	26 (11.82)	77 (35.00)	122 (53.51)	22 (9.65)	84 (36.84)	96 (39.83)	24 (9.96)	121 (50.21)
ERYTHROMYCIN	64 (29.09)	32 (14.55)	124 (56.36)	57 (25.00)	57 (25.00)	114 (50.00)	107 (44.40)	0 (0.00)	134 (55.60)
TILMICOSIN	106 (48.18)	16 (7.27)	98 (44.55)	110 (48.25)	17 (7.46)	101 (44.30)	34 (14.11)	91 (37.76)	116 (48.13)
NORFLOXACIN	73 (33.18)	12 (5.46)	135 (61.36)	59 (25.88)	10 (4.39)	159 (69.74)	31 (12.86)	32 (13.28)	178 (73.86)
ENROFLOXACIN	89 (40.45)	22 (10.00)	109 (49.55)	72 (31.58)	31 (13.60)	125 (54.82)	80 (33.20)	35 (14.52)	126 (52.28)
CIPROFLOXACIN	112 (50.91)	27 (12.27)	81 (36.82)	98 (42.98)	35 (15.35)	95 (41.67)	92 (38.17)	49 (20.33)	100 (41.49)
SARAFLOXACIN	126 (57.27)	31 (14.09)	63 (28.64)	98 (42.98)	65 (28.51)	65 (28.51)	122 (50.62)	29 (12.03)	90 (37.34)
FLUMEQUINE	121 (55.00)	30 (13.64)	69 (31.36)	107 (46.93)	34 (14.91)	87 (38.16)	142 (58.92)	10 (4.15)	89 (36.93)
SULFADIAZINE	107 (48.64)	35 (15.91)	78 (35.45)	106 (46.49)	32 (14.04)	90 (39.47)	113 (46.89)	32 (13.28)	96 (39.83)
SULFAQUINOXALINE	126 (57.27)	0 (0.00)	94 (42.73)	114 (50.00)	38 (16.67)	76 (33.33)	121 (50.21)	31 (12.86)	89 (36.93)
SULFACHLORPYRIDAZINE	133 (60.45)	44 (20.00)	43 (19.55)	114 (50.00)	57 (25.00)	57 (25.00)	110 (45.64)	43 (17.84)	88 (36.51)
SULFADIMIDINE	146 (66.36)	25 (11.36)	49 (22.27)	143 (62.72)	34 (14.91)	51 (22.37)	152 (63.07)	32 (13.28)	57 (23.65)
TOTAL (%)	110 (50.00)	25 (11.36)	85 (38.64)	100 (43.86)	36 (15.79)	92 (40.35)	100 (41.50)	34 (14.11)	107 (44.40)

S = Sensitive (> 20 mm); I = Intermediate (< 15 mm); R = Resistant (≤ 8 mm).

Annex.4.2.2. Susceptibility pattern of 838 isolates of *Salmonella* from human origin during the years 2006-2008.

ANTIMICROBIALS TESTED	SUSCEPTIBILITY								
	2006			*2007*			*2008*		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
ERYTHROMYCIN	184 (9.19)	35 (12.59)	59 (21.22)	127 (48.47)	63 (24.05)	72 (27.48)	124 (41.61)	75 (25.17)	99 (33.22)
CIPROFLOXACIN	186 (66.91)	23 (8.27)	69 (24.82)	147 (56.11)	47 (17.94)	68 (25.95)	146 (48.99)	69 (23.15)	83 (27.85)
TOTAL (%)	185 (66.55)	29 (10.43)	64 (23.02)	137 (52.29)	55 (20.99)	70 (26.72)	135 (45.30)	72 (24.16)	91 (30.54)

S = Sensitive (> 20 mm); I = Intermediate (< 15 mm); R = Resistant (≤ 8 mm)

Annex.4.2.3. Susceptibility pattern of 1204 isolates of *Staphylococcus* from human origin during the years 2006-2008.

ANTIMICROBIALS TESTED	SUSCEPTIBILITY								
	2006			*2007*			*2008*		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
ERYTHROMYCIN	252 (66.32)	52 (13.68)	76 (20.00)	177 (48.36)	100 (27.32)	89 (24.32)	172 (37.55)	152 (33.19)	134 (29.26)
CIPROFLOXACIN	278 (73.16)	38 (10.00)	64 (16.84)	245 (66.94)	48 (13.11)	73 (19.95)	292 (81.56)	72 (20.11)	94 (26.26)
TOTAL (%)	265 (69.74)	45 (11.84)	70 (18.42)	211 (57.65)	74 (20.22)	81 (22.13)	232 (64.80)	112 (31.28)	114 (31.84)

S = Sensitive (> 20 mm); I = Intermediate (< 15 mm); R = Resistant (≤ 8 mm).