II. REVIEW OF LITERATURE

2.1. Overview on antibiotics

2.1.1. Definition of antibiotics (Sande and Mandell, 1985; Bywater, 1991)

2.1.1.1. Definition

Antibiotics are chemical substances produced by various species of microorganisms and other living systems that are capable in small concentrations of inhibiting the growth of or killing bacteria and other microorganisms. These organisms can be bacteria, viruses, fungi, or protozoa. A particular group of these agents is made up of drugs called antibiotics, from the Greek word anti ("against") and bios ("life"). Some antibiotics are produced from living organisms such as bacteria, fungi, and molds. Others are wholly or in part synthetic – that is, produced artificially.

2.1.1.2. Natural antibiotics

Natural antibiotics are chemical substances produced by various species of microorganisms (bacteria and fungi) that are able to suppress or kill the growth of bacteria. Hundreds of natural antibiotics have been identified, and nearly 100 have been developed to the stage where they are of value in the therapy of infectious diseases. The first identified natural antibiotic was benzylpenicillin. Other examples are streptomycin, chloramphenicol, tetracyclines and macrolides.

2.1.1.3. Semi-synthetic antibiotics

Semi-synthetic antibiotics are derivatives of natural antibiotics. They are obtained by small alterations in structural formulas of natural antibiotics. For example, soon after the introduction of benzylpenicillin, a small variation in the growth medium for the *Penicillium* altered the side chain of the benzylpenicillin structure by a single oxygen atom, resulting in phenoxymethylpenicilin. This derivative is acid-stable and is suitable for oral administration. After chemical identification of natural antibiotics many derivatives have been, or are still produced and tested for their antibacterial activity. Other examples of semi-synthetic antibiotics are the penicillinase resistant semi-synthetic penicillins such as nafcillin, cloxacillin and flucloxacillin.

2.1.1.4. Synthetic antibiotics

Synthetic antibiotics formerly called chemotherapeutics are chemically synthesized. The first compound with chemotherapeutic activity that was used therapeutically was prontosil rubrum, an azo dye structurally related to sulfanilamide (Forth *et al*., 1983). Soon afterwards the sulfonamides were developed, and they still play an important role in therapy of infectious diseases. More recent examples of synthetic antibiotics are the nitrofurans and the quinolones.

2.1.1.5. Mechanisms of action

Antibiotics can be bacteriostatic (bacteria stopped from multiplying) or bactericidal (bacteria killed). To perform either of these functions, antibiotics must be brought into contact with the bacteria.

Mechanisms of action of antibiotics are divided into four categories:

- inhibition of cell wall synthesis (β-lactam antibiotics, vancomycin, bacitracin);
- damage to cell membrane function (polymyxins, polyenes);
- inhibition of nucleic acid function (nitroimidazoles, nitrofurans, quinolones, rifampicin) or intermediate metabolism (sulfonamides, trimethoprim);

- inhibition of protein synthesis (aminoglycosides, fenicols, lincosamides, macrolides, streptogramins, pleuromutilins, tetracyclines).

Groups of antibiotics can be classified based on their scope of effectiveness. Narrow-spectrum antibiotics have an antibacterial effect on a relatively small number of species whereas broad-spectrum antibiotics are active against a variety of organisms (Carlson and Fangman, 2000).

2.1.1.6. Resistance and side effects

The term antibiotic resistance can be used in two ways: microbiological and clinical resistance. Microbiological resistance refers to resistant organisms that possess any kind of resistance mechanism or resistance gene. This term may be qualified in a quantitative way as “moderately or highly resistant” or as “low-level or high-level resistance”. Clinical resistance refers to the classification of bacteria as susceptible or resistant depending on whether an infection with the bacterium responds to therapy or not (EMEA, 1999).

When one is exposed continually to an antibiotic for an illness of long duration (such as rheumatic fever), the targeted bacteria may develop their own defense against the drug. An enzyme that can destroy the drug may be produced by the bacteria, or the cell wall can become resistant to being broken by the action of the antibiotic. When this happens, and it does most frequently in response to long or frequent treatment with penicillin or streptomycin, the patient is said to be "fast" against the drug. For example, one may be penicillin-fast, meaning penicillin is no longer able to fight against the infection, and consequently another type of antibiotic must be given.

Side effects range from slight headache to a major allergic reaction. One of the more common side effects is diarrhea, which results from the antibiotic disrupting the balance of intestinal flora, the "good bacteria" that dwell inside the human digestive system. Other side effects can result from interaction between the antibiotic and other

drugs, such as elevated risk of tendon damage from administration of a quinolone antibiotic with a systemic corticosteroid.

Allergic reactions to antibiotics are usually seen as rashes on the skin, but severe anemia, stomach disorders and deafness can occasionally result. It was once thought that allergic reactions to antibiotics - penicillin in particular - were frequent and permanent. Recent studies suggest, however, that many people outgrow their sensitivity or never were allergic. The large number of antibiotics that are now available offers a choice of treatment that can, in most instances, avoid allergy-causing drugs.

It is important to remember that all drugs can cause both wanted and unwanted effects on the body. The unwanted ones are called side effects. These must be balanced against the desired effects in determining if a particular drug will do more harm than good. It is a fact that all drugs have the potential to be both beneficial and harmful.

2.1.1.7. History and Future

Antibiotics have a short history which began in the early 20th century when the antibiotic penicillin was discovered. Since then antibiotics have played very important role in human as well as animal health. In 1928 Sir Alexander Fleming, a British bacteriologist, noticed that a mold growing in one of his laboratory cultures was able to destroy that bacterial cultures. Since the mold that produced the substance that killed the bacteria was a species of *Penicillium*, he named the germ-killing substance penicillin. The first use of an antibiotic, however, is not known, as folk medicine has used various molds to fight infections throughout history. In 1935 the German chemist Gerhard Domagk discovered the first sulfa drug called prontosil. In 1941 penicillin was used to treat serious infections. The results were dramatic because patients who received the drug made rapid and complete recoveries. Bacitracin, chlortetracycline, and streptomycin, which are naturally occurring antibiotics, were discovered by 1948. The penicillin ring was finally isolated in 1959

by British and United States scientists, and the way was open for the development of semi-synthetic penicillin. This was the beginning of an era that has been called the golden age of chemotherapy. Since 1948, a large number of substances that inhibit or kill bacteria have been discovered.

The future of antibiotics can be identified with the development of antiviral drugs to treat emerging serious viral diseases and with the progressive improvement of current antibiotics to overcome the antibiotic drug resistance of pathogens.

2.1.2. Antibiotic production and use in animal production

Antibiotics are used for animals as well as humans to prevent and treat infections. In animal husbandry, they are also mixed in feeds as growth promoters. In addition antibiotics are used on a large scale in horticulture and agriculture (EMEA, 1999). Antibiotics used for growth-promotant purposes constitute a large proportion of the total antibiotic usage, but the scale of the problem is difficult to estimate since there is few information published on the overall quantities of antibiotics used in animals or human subjects (Barton, 2000). Approximately 42 % of all veterinary pharmaceuticals used worldwide are used as feed additives, 19 % are used as anti-infectives (e.g., antibacterials, antifungals and antivirals), 13 % as parasiticides, 11 % are used as biologicals and 15 % represent other pharmaceuticals. In volume and money value antimicrobials represent the largest proportion of pharmaceutical sales of any drugs used in animal production (Miller, 1993). It has been estimated that as much as 50 % of total antibiotic production (by weight) is used in animals and plants, with 50-80 % used in some countries for growth promotion or disease prophylaxis and the rest used for therapeutic purposes (WHO, 2001).

Alone in the United States around 15 million pounds of antibiotics are administered to farm animals annually (Walter and Veith, 2005). By 1954, U.S. farmers were using roughly 490,000 pounds of antibiotics a year for livestock feed. Six years later that figure was over one million pounds. In 1984, it was between 12 and 15 million pounds. Today, U.S. livestock is fed more than 24 million pounds of

antibiotics for other purposes than treating disease. Antibiotic use is present in all aspects of livestock production: poultry, dairy, beef and pork. In the swine industry alone, antibiotics are currently used in almost 90 % of starter feeds, in 75 % of grower feeds and in more than 50 % of finishing feeds (DeVore, 2002).

In Australia import statistics for the years 1992-1993 to 1996-1997 show that 55.8 % of antibiotics imported were for use in stock feed, 36.4 % for human use and 7.8 % for veterinary use (JETACAR, 1999).

The worldwide use of antibiotics for animal health purposes in 1996 was estimated at 27,000 tons with about 25 % of global usage in the EU. Within the EU 50 % of this usage is estimated to arise from prescriptions issued for therapeutic purposes while 25 % arose from feed additive usage for growth promotion and another 25 % for ionophore feed additives primarily used to prevent coccidiosis in poultry. Sales of animal health antibiotics (excluding coccidiostatics) in 1997 within the EU plus Switzerland were estimated at a total of 5,093 tons, therapeutics accounting for 3,494 tons (69 % of the total) and growth promoters for 1599 tons (31 %). Out of the estimated total usage of antibiotics within the EU plus Switzerland in 1997 (10,493 tons), human health antibiotics (estimated at 5,400 tons) accounted for 52% whereas therapeutic animal antibiotics accounted for 33 % and growth promoters for 15 % (Boatman/FEDESA, 1998).

The most commonly used antimicrobials for food-producing animals are the β-lactams, tetracyclines, aminoglycosides, lincosamides, macrolides, pleuromutilins and sulfonamides. In addition, quinolones have been available for more than 25 years (Myllyniemi, 2004). Antimicrobials are administered to animals by injections (intravenously, intramuscularly, or subcutaneously), orally in feed or water, topically on the skin and by intramammary and intrauterine infusions (Michell *et al.*, 1998). Knowledge of the antimicrobial spectrum of different antimicrobial substances as well as on pharmacokinetics and pharmacodynamics of the species requiring treatment is of importance for the outcome of the treatment (MAF, 2003).

2.1.3. Antibiotic residues in meat

Residues of veterinary drugs include the parent compounds and/or their metabolites in any edible portion of the animal product, and include residues of associated impurities of the veterinary drug concerned (CAC, 2003). Theoretically, all of administration routes of antibiotics may lead to residues appearing in foods of animal origin such as milk, meat and eggs (Johnston, 1998). In a survey conducted in 1969 of 5,000 samples of tissue, urine and/or feces samples collected from swine, beef cattle, veal calves, lambs and poultry at the time of slaughter in Illinois found antibiotic residue in 27 %, 9 %, 17 %, 21 % and 20 %, respectively (Huber, 1971). Current data estimate that 1% of all animal products in the United States and Europe contain antibiotic residues, though at very low levels (Prescott and Baggot, 1988).

Results of a statutory survey in the UK in 1996 indicate that antimicrobials were detected in six (4 penicillin G, 2 streptomycin) out of 17,000 sheep kidney samples tested. In one out of 2,300 cattle samples, oxytetracycline was found with completely unacceptable concentration of 7,620 μg/kg above the maximum residue limit (MRL) of 600 μg/kg. Out of over 12,300 samples collected from pigs, 64 contained antimicrobials (chlortetracycline being most common, and found in 44 of the 64 samples) (Gracey *et al.*, 1999).

In Ireland, among 140 pork samples tested for antibiotic residues during the period of 1996-1997, chlortetracycline at levels less than MRL was found in 35 (25 %) samples and greater than MRL in 7 (5 %) samples, whereas those results for the period 1997-1998 are 5 (12 %) and 0 (0 %), respectively (TEAGASC, 2001).

In Sweden, 10,688 samples were tested for veterinary drug residues in the year 2000, among them four samples (0.037 %) contained residues above MRL. Two bovine and one pig kidney samples contained residues of penicillins above MRL. One bovine kidney sample contained residues of tetracyclines above MRL (Tillbaka, 2001).

In Korea, violative residues of tetracyclines, sulfonamides and aminoglycosides were detected in beef and pork samples taken from slaughtering establishments and import shipments (Lee *et al.*, 2001).

In the United States, a survey (table 1) of all violative carcasses in 1993 revealed that the drugs most frequently causing residues were penicillin (20 %), streptomycin (10 %), oxytetracycline (10 %), sulfamethazine (9 %), tetracycline (4 %), gentamicin (4 %) and neomycin (3 %). The slaughter classes most often associated with residues were culled dairy cows, veal calves and market hogs (Paige, 1994). Injectables were responsible for 46 % of the violative residues in meat followed by oral administration at 20 % (feed, water and bolus) and intramammary infusions at 7 % (Mitchell *et al.*, 1998).

Table 1. Violative antibiotic residues in animals in the United States 1993 (Paige, 1994)

Types of antibiotics	**% of violation**	**Classes of animals**	**% of violation**
Penicillin	20	Culled dairy cow	30
Streptomycin	10	Bob veal	40
Oxytetracycline	10	Market hog	6
Sulfamethazine	9	Sow	2
Tetracycline	4		
Gentamicin	4		
Neomycin	3		

A study from Nairobi, Kenya on cattle meat reports that out of 250 samples analyzed in 2001, 114 (45.6 %) contain tetracycline residues of which 60 (24 %) are liver, 35 (14 %), are kidney, and 19 (7.6 %) are muscle samples. The mean residue levels of tetracycline ranging from 524 to 1,046 µg/kg exceed the MRL for beef edible tissues. Oxytetracycline and chlortetracycline are detected in 110 (44 %) and 4 (1.6 %) samples, respectively (Muriuki *et al*., 2001).

There are several factors contributing to the residue problem such as poor treatment records or failure to identify treated animals. Most violations result from the use of a drug in some manner that is inconsistent with the labeling. This occurs primarily through not observing label withdrawal time as well as “extra-label” use of the drug. Treatment involving any other method than what is stated on the product label (e.g., different species, increased dosage, different route of administration, different frequency of treatment) are classified as extra-label usage, and withdrawal times are difficult or impossible to determine in these situations (Paige, 1994; Apley, 2003).

Overuse of antibiotics in animal production and their residues in foods of animal origin may cause some problems such as the potential for allergic reactions in sensitized individuals (penicillin), toxicity such as aplasia of the bone marrow (chloramphenicol), effects on the human gut microbial populations, the emergence of resistant bacteria within animals and the transfer of the antibiotic resistance genes to the human pathogens (Mitchell *et al.*, 1998). In animal products, antibiotic residues may interfere with further processing if this depends on a fermentation reaction (Gracey *et al.*, 1999). Besides residue problem and its adverse consequences above, overuse of antibiotics may cause environmental pollution (Tuan and Munekage, 2004; Hirsch *et al.*, 1999; Sczesny *et al.*, 2003).

Control of antibiotic residues has been emerged as one of the most concerned questions in animal production and food safety. Control mechanisms include control of the distribution, use, determination of safe residue levels and residue detection technologies to be employed. Many international, national and local organizations as well as scientific institutions have been involved in this domain. On the international level, the Codex Alimentarius Commission, whose guidelines are set by the Codex Committee on Residues of Veterinary Drugs in Food (CCRVDF) based on the scientific advice of the Joint WHO/FAO Expert Committee on Food Additives (JECFA); further, the European Agency for the Evaluation of Medicinal Products (EMEA), the Office International des Epizooties (OIE) and the Consultation Mondiale de l’Industrie de la Santé Animale (COMISA).

The safety evaluation of antibiotic residues is performed and based on several criteria established specifically for each substance in question and each target product. The first criterion is the no-observable-effect level (NOEL) or the dosage level (mg/kg or ppm) at which no any adverse effects are observed as established by animal bioassay toxicological studies. These studies use the most sensitive testing methods available in the most sensitive animal species (e.g., teratogenicity, carcinogenicity, mutagenicity or immunopathological effects).

The second criterion is the Acceptable Daily Intake (ADI), an estimate of the amount of a veterinary drug, expressed on a body weight basis, that can be ingested daily over a lifetime without appreciable health risk (standard human, bodyweight = 60 kg) (CAC, 2003). The estimate was carried out by the JECFA. ADI is determined by the NOEL and the safety factor (SF) which varies from 100 to 1000 (Mitchell *et al.*, 1998).

The last criterion, Maximum Residue Limit for Veterinary Drugs (MRLVD), is the maximum concentration of residue resulting from the use of a veterinary drug (expressed in mg/kg or μg/kg on a fresh weight basis) that is recommended by the Codex Alimentarius Commission to be legally permitted or recognized as acceptable in or on a food (CAC, 2003).

2.1.4. Vietnam: antibiotic use and residues in animals

According to Boisseau (2002) there are more than 3,000 antimicrobial-containing veterinary medical products (VMPs) registered to be imported, manufactured, and marketed by 51 local veterinary pharmaceutical companies among which 66.3 % VMPs contain more than one antimicrobial (table 2&3). Significant deficiencies in the registration of VMPs open the door widely to bad management of VMPs. Antibiotics are usually applied to sick animals by farmers without any veterinary prescription and supervision and laboratory diagnosis.

Table 2. The combination of antimicrobials in VMPs in Vietnam (Boisseau, 2002)

Number of VMPs	Number of antimicrobials per VMP	% of the total number of VMPs
882	1	33.7
1244	2	47.5
404	3	15.5
70	4	2.7
15	5	0.7

Table 3. The ten antimicrobials most frequently present among the registered VMPs in Vietnam (Boisseau, 2002)

Antimicrobials	Number of VMPs
Colistin	549
Sulfonamides	394
Norfloxacin	309
Tylosin	281
Oxytetracycline	157
Enrofloxacin	232
Ampicillin	205
Gentamicin	195
Tiamulin	184
Flumequin	154

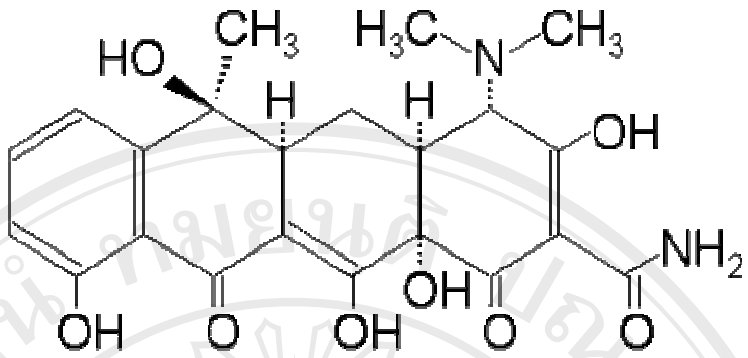
A study (An *et al.*, 2002) on antibiotic use and residues in chicken in Ho Chi Minh City reports that among 36 currently used antibiotics the eight most commonly used antibiotics include colistin, enrofloxacin, daveridin, sulfadimidin, trimethoprim, norfloxacin, oxytetracycline, gentamicin, and oxolynic acid. Imprudent use of antibiotics was found in 32.61 % of chicken farms particularly 23.3 % for violation of dosage regimens. 44.54 % of the farms did not observe the prescribed withdrawal time.

Another study (Thuận *et al.*, 2003) on antibiotic use and residues in the southern province of Binh Duong indicates that the six most commonly used antibiotics are tylosin, colistin, norfloxacin, gentamicin, tetracycline, and ampicillin. Injudicious use of antibiotics was found in 17.1 %, mainly dosage regimen violation. Appropriate withdrawal time was not observed in 40.13 % of the farms.

In Vietnam, there have been few studies on antibiotic residues. A study in Ho Chi Minh City (An *et al.*, 2002) reports that antibiotic residues were found in 42 (60 %) out of 70 suspect chicken samples. These antibiotics are enrofloxacin, norfloxacin, tylosin, tetracycline, sulfadimidin, sulfaquinoxalin, and sulfadiazine. Results of a similar study in the southern province of Binh Duong (Thuận *et al.*, 2003) show that 47 % of suspect chicken samples and 62.50 % of suspect pork samples containing antibiotic residues such as chloramphenicol, oxytetracycline, chlortetracycline, norfloxacin, and tylosin.

2.1.5. Tetracycline

Tetracycline is any kind of antibiotics which is produced by the bacteria of the genus *Streptomyces.* They are effective against a wide range of gram positive and gram-negative bacteria, interfering with protein synthesis in these microorganisms. Tetracycline is used to treat many bacterial infections, such as Rocky Mountain spotted fever, some eye, respiratory, intestinal, and urinary infections, some kinds of acne, and some diseases especially the infecting microorganism is resistant to where the infecting microorganism is resistant to penicillin. The first drug of the tetracycline family, chlortetracycline, was introduced in 1948. The term tetracycline obviously implies its chemical structure with four (tetra-) cycles as follows:



Tetracycline

(Source: Wikipedia Encyclopedia, 2004)

Tetracycline may cause a permanent discoloration of developing teeth. Furthermore, this antibiotic is one of drugs that are capable of acting as teratogens (Medicinenet, 2004). Therefore, it should not be administered to pregnant and lactating women and growing children under six years old. Because of the development of strains of microorganisms resistant to the tetracyclines, these antibiotics have lost some of their usefulness. Aureomycin is a trade name for the derivative chlortetracycline, and Terramycin is a trade name for oxytetracycline (Wikipedia Encyclopedia, 2004).

As known as a broad-spectrum antibiotic, tetracycline can be effectively used against a wide variety of bacteria including Haemophilus influenzae, Streptococcus pneumoniae, Mycoplasma pneumoniae, Chlamydia psittaci, Chlamydia trachomatis, Neisseria gonorrhoeae, and many others (Medicinenet, 1998).

The tetracycline group is one of the most commonly used antibiotics in animal production. According to Boatman/FEDESA (1998), approximately two-thirds of the animal health therapeutic antibiotics sold in 1997 in the EU and Switzerland were tetracyclines (66 %). In the United States, tetracyclines are the most commonly used antibiotics, approved for use in animals (disease treatment and prevention; growth promotion), plants, and humans. Species on which tetracyclines can be used include beef cattle, dairy cows, fowl, honeybees, poultry, sheep, swine, catfish, trout, salmon,

lobster, and certain plants (GAO, 1999). They are the commonly used antibiotics in feeding operations, and they are prevalent in animal products, for example milk and meat, purchased from supermarkets and other stores (Walter and Veith, 2005). In Vietnam, 257 out of more than 3,000 VMPs contain oxytetracycline.

The 36th Joint FAO/WHO Expert Committee on Food Additives (JECFA) meeting in 1990 established MRLs for oxytetracycline of 600 μg/kg in kidney; 300 μg/kg in liver; 100 μg/kg in muscle; 100 μg/kg in milk; 200 μg/kg in eggs; and 10 μg/kg in fat for all species for which residue depletion data were provided (cattle, swine, sheep, chickens, turkeys and fish). These MRLs were approved through the Codex Alimentarius Commission in 1994. The 45th JECFA meeting in 1995 concluded that both tetracycline and chlortetracycline are of low toxicity: LD_{50} values in mice and rats vary between 2,150 and > 5,000 mg/kg bodyweight (bw), there is no evidence of reproductive or developmental toxicity and there is no evidence of carcinogenic effects or of a genotoxic potential. The lowest overall NOELs are 100 and 250 mg/kg bw/day for chlortetracycline and tetracycline, respectively. The antimicrobial potency of chlortetracycline and tetracycline is comparable to the antimicrobial potency of oxytetracycline. The spectrum of antimicrobial activity is comparable for tetracycline, chlortetracycline and oxytetracycline. The residue distribution for oxytetracycline, tetracycline and chlortetracycline in food-producing animals is comparable (EMEA, 1995). The same ADIs and MRLs, except milk, were allocated to chlortetracycline and tetracycline as those previously allocated to oxytetracycline at the 36th meeting of JECFA. The MRLs allocated to the tetracyclines were defined as applying to both individual tetracyclines or the sum of the combined tetracycline residues. The ADI of 0-3 μg/kg of body weight previously assigned to oxytetracycline was converted to a group ADI with chlortetracycline and tetracycline at that meeting. It was recommended that the MRL of 10 μg/kg for oxytetracycline in fat be withdrawn and that MRLs in fat for chlortetracycline and tetracycline are not required. This recommendation was raised based on the evidence that tetracyclines have the affinity to liver, spleen, bone marrow, teeth; but diffusion in liquor and in fatty tissue is poor. That is why an MRL for tetracyclines in fat is not really necessary (Forth *et al.*, 1983). Allocated MRLs for tetracyclines can be

satisfactorily monitored by a combination of the microbiological (screening for antibiotic residues) and chemical (identification and quantification) analyses that are presently available. Target tissues for the analysis of all three tetracyclines were kidney and muscle in cattle, pigs and poultry and, based on limited data, kidney was the target tissue in sheep (FAO, 1997).

In addition, tetracyclines are poorly metabolized in animals (Nielsen and Hansen, 1996). Therefore, they can also occur in animal slurry with significant amounts that may pollute the environment (Sczesny *et al.*, 2003). However, a potential risk for the environment cannot be assessed yet as very little is known about the not excludible causal connection between the occurrence of resistant bacteria and the low environmental concentrations of antibiotics (Hirsch *et al*., 1999).

2.2. Methods for detection of antibiotic residues

There are six types of detection methods commonly used for the detection of antimicrobial residues in food, including microbial growth inhibition assays, microbial receptor assays, enzymatic colorimetric assays, receptor binding assays, chromatographic methods and immunoassays.

The assays are either qualitative, quantitative or semi-quantitative. Qualitative assays employ a predetermined cutoff value to classify samples as positive or negative relative to a specific drug concentration. Quantitative assays require that positive controls covering a wide range of drug concentration be tested with each sample set, thus permitting residue quantification by interpolation from a standard curve. Such assays require a precise instrumentation to measure the test response and to determine the standard curve. Semi-quantitative assays are similar to quantitative assays except that the test results are interpreted relative to a range of drug concentrations (e.g., negative, low positive, high positive) reflected by the range of positive controls run with test samples (Mitchell *et al.*, 1998).

The qualitative and semi-quantitative are mostly classified as screening assays. A screening assay may be defined as an assay that gives a reliable and accurate indication that the analyte of interest is not present in the sample at unsafe or violative levels (O'Rangers, 1993). Quantitative assays require more technical expertise; therefore their primary use has been found in laboratory confirmation applications (O'Rangers, 1993).

2.2.1. Microbial growth inhibition assays

These are the earliest methods used for the detection of antimicrobial residues in food based on the detection of growth inhibition of various sensitive bacterial strains. Such methods, originally developed for use in clinical medicine, were based on microbial agar diffusion tests of the inhibition of acid production of coagulation by starter organism (Mitchell *et al.*, 1998). The basic microbial inhibition assay format involves a standard culture of a test organism, usually *Bacillus stearothermophilus, Bacillus subtilis, Bacillus cereus, Micrococcus luteus, Escherichia coli, Bacillus megaterium* or *Streptococcus thermophilus* seeded in an agar or liquid growth medium which is then inoculated with a milk, urine, tissue or tissue liquid sample and incubated for periods of up to several hours. The sample can be applied directly to the medium, in stainless steel cylinders (peni-cylinder) or on a filter paper disk impregnated with liquid sample. The presence of an inhibitory substance is indicated by zones of growth inhibition or a change in the color of the medium (with pH and redox indicators) (Mitchell *et al.*, 1998). The major disadvantages of microbial inhibition assays are that they are not very specific for antibiotic identification purposes, are qualitative, have limited detection levels to many antibiotics and require several hours before results are available (2.5 to 18 hours). Growth inhibition tests are subject to the effects of many natural inhibitory substances found in foods of animal origin such as lysozyme, lactoferrin, lactoperoxidase, somatic cells, complement, defensins, long-chain fatty acids, bile and lactic acid. These compounds may give false positive test results. Advantages of these tests are that they are inexpensive, are easy to perform, are adaptable to the screening of large numbers of samples and have reasonably broad antimicrobial detection spectrum (Mitchell *et al*., 1998; Gracey *et*

al., 1999; Nouws *et al.*, 1998; Chang *et al.*, 2000). Some examples of these assays include the four-plate-test method (FPT) (Gracey *et al.*, 1999), modified four-plate-test method (MFPT) (Chang *et al.*, 2000), five-plate test or STAR protocol (Gaudin *et al.*, 2004), and six-plate method (Myllyniemi *et al.*, 2001).

2.2.2. Microbial receptor assays

The CHARM I and II tests are qualitative microbial receptor assays for the rapid detection of β-lactams, macrolides, aminoglycosides, tetracyclines, chloramphenicol and sulfonamides in milk and tissue. Although there are analogous in test principle to the radioimmunoassay (RIA), by strict definition the CHARM I and II tests cannot be classified as RIAs. The CHARM I test for β-lactams in milk was the first AOAC-recognized rapid test for the detection of β-lactams in milk with a test time of 15 min (Charm and Chi, 1988).

2.2.3. Enzymatic colorimetric assays

The Penzyme test is a qualitative enzymatic method for the rapid detection of β-lactams antibiotics in milk. Test results are available in 20 min. The test principle is based on the detection of the inactivation of an enzyme by β-lactam antibiotics (Mitchell *et al.*, 1998).

2.2.4. Receptor binding assays

The SNAP and Delvo-X-Press tests for β-lactam antibiotics in milk are qualitative enzyme linked receptor binding assays in which β-lactams are captured by a penicillin binding protein conjugated to an enzyme (horseradish peroxidase) (Mitchell *et al.*, 1995).

2.2.5. Chromatographic analysis

Chromatography is commonly used for separating the components of a solution. The process of liquid chromatography (LC) was first discovered in 1906 by Tsvett when he separated the chlorophyll pigments in green leaves by passing an ether solution of these pigments through a tube of solid powdered calcium carbonate. Chromatography was not used extensively until 25 years later when many new applications were developed. But these early methods tended to be very slow and inefficient, had poor resolution and quantitative ability and were difficult to automate. The development of paper chromatography in the 1940s and thin layer chromatography in the 1950s improved speed and resolution of LC was greatly improved and it was used more extensively. Further improvements in the chromatographic process (e.g., instrumentation) led to the development of High Performance Liquid Chromatography (HPLC) in the late 1960s and allowed the potential of chromatography to be realized (Lindsay, 1992).

The initial application of chromatographic methods for the detection of drug residues in foods was very limited due to the sensitivity required and poor recovery from the more complex food matrices (Shaikh, 1993). In the early 1980s the methods for detection of residue were developed, primarily for the detection of β-lactams in milk and meat (Mitchell *et al.*, 1998). The ability of chromatographic methods to specifically identify and quantitate very low levels of chemical residues has led to their use primarily as confirmation tests for screening test positive samples (Mitchell *et al.*, 1998). There are several types of chromatographic methods currently and of use for residue analysis. These include GC (gas chromatography), TLC (thin layer chromatography), TLC/BA (thin-layer chromatography/bioautography) and HPLC (high performance liquid chromatography). Due to the polar, non-volatile and heat sensitive nature of most antibiotics, HPLC is the most commonly used detection method for residue analysis (Shaikh, 1993). Combination of several methods, for example, HPLC combined with positive-ion electrospray ionization mass spectrometry, can be used successfully in the quantitative determination of residues in

tissues and feed as well (Cherlet *et al.*, 2003; Sczesny *et al.*, 2003; Zurhelle *et al.*, 2000; Kühne *et al.*, 2000).

2.2.6. Immunoassays

These tests are based on the antigen-antibody reaction which possess a high specificity. Typical examples of immunoassays include the radioimmunoassay (RIA) developed in 1959 and the enzyme-linked immunosorbent assay (ELISA or EIA) developed in 1971. The semi-quantitative ELISA is easily adopted in predicting tissue residues for tetracycline antibiotics in live pigs (Lee *et al.*, 2001). Some examples of commercial test kits commonly used for drug residue testing in milk and tissue include the Lactek tests for milk and Cite Sulfa Trio and EZ-screen Quick Card for various types of matrices (Mitchell *et al.*, 1998).