2. LITERATURE REVIEW

2.1 Livestock production in the global context

Globalization has brought several positive aspects for enhancing the livestock sector improvement, especially for meat production and consumption. According to the report of the United Nations (UN) the total of meat produced in the global scenario increased dramatically, nearly three times, from 47 mt to 139 mt in two decades. In the developing countries the meat production and consumption has increased rapidly. The populations residing there are consuming 29 kg meat/head. That number has doubled in comparison to 1980 (Moalem, 2006).

In reference to meat types, the world meat consumption consists of 41 % pigs, 29 % birds and 25 % bovines and among that the consumption of pork has increased by 73 % in between 1980-2000 (Ornelas and Gellatley, 2001). Those dynamic developments have increased rapidly in countries with a growing economics, notably in developing countries. It was noticeable that China, India and Brazil have contributed to 96.7 mt out of 148.2 mt (FAO, 2006). Thailand, Malaysia, Vietnam and the Philippines in South East Asia have had a rapid growth in livestock, in particular in the pig and poultry production. In 2003, Thailand was the world's fifth largest broiler exporter (FAO, 2006).

2.2 Pork production and consumption in Vietnam

In the livestock sector, pig farming takes a dominant role. The estimated total of pig population increased from 24.14 to 29.05 millions from 2004 to 2006 (GAIN, 2006). About 40 % of the country's pig population and half of the pork products are produced in the Mekong River Delta and Red River Delta (Dinh *et al.,* 2005). Pork production is mainly based on the household level; about 60 % of the rural population keeps pigs (Costales *et al.,* 2007). There were 548 commercial pig farms which keep

at least 100 pigs (Dinh *et al.,* 2005). That contributes to 20-25 % of the total pork production (GAIN, 2006).

Most of the pork produced in Vietnam is mainly for domestic demands and only 1-2 % for the purpose of export (Nguyen, 2006). Vietnamese's taste prefers fresh meat to frozen (Howard, 2006; Vietnamnews, 2006). It is estimated that 95 % of the households are consuming pork in their daily diets (Dinh *et al.,* 2005). Therefore pigs are slaughtered on a daily basis and most of the pork is consumed as fresh meat within a day (GAIN, 2006). There is also a measurable amount of canned meat, prepared sausages, frozen meat consumed in restaurants and hotels (Dinh *et al.,* 2005). According to a master plan of MARD the aim is to raise animal production to 30 % of the total agricultural value by 2010 (GAIN, 2006). This means that the pork production will have been increased from 1.8 mt in 2003 (FAO-STAT, 2005) to 3.2 mt in 2010 (Vietnamnet, 2006).

2.3 Use of antimicrobials in the food production

An antibiotic is a chemical substance produced by a micro-organism, originally referred to as a natural compound produced by a fungus or another micro-organism that kills bacteria causing diseases in humans or animals. Whereas the term antimicrobial is a broader one than antibiotic, it refers to any substance of natural, semi-synthetic, or synthetic origin that is used to kill or inhibit the growth of micro-organism but causes little or no host damage. The term antimicrobial is used synonymously with the word antibiotic by many people (CDC, 2005a).

2.3.1 Mechanisms of action of antimicrobials

An antimicrobial (either bactericidal or bacteriostatic) has an antimicrobial effect on micro-organisms when it interferes well with their site of activity. Antimicrobial mechanisms of action are (Abedon, 2003):

- Inhibition of cell wall synthesis, such as glycopeptides, vancomycin, β-lactams.
- Disruption of cell membrane function, such as polymixins, surfacants, polyenes, polypeptides.
- Inhibition of protein synthesis, such as aminoglycosides, tetracyclines, phenicols, macrolides.
- Inhibition of nucleic acid synthesis, such as fluroquinolones, metronidazole.
- Action as antimetabolites, such as diaminopyrimidines, flucytosine, sulfanilamide.

2.3.2 Classification of antimicrobials

Antimicrobial drugs can be classified based on three basic features that are based on classes of micro-organisms, effects on target cells and the scope of effectiveness (Fix, 2007). The details of such are described as follows:

Classification based on class of micro-organisms

- Antivirals (amantadine, rimandadine, oseltamivir) have activity against viruses.
- Antifungals (griseofulvin, nystatin) have activity against fungi.
- Antibacterials (sulfonamides, penicillin, streptomycin, tetracycline, chloramphenicol, neomycin) have activity against bacteria.
- Antiprotozoals (pyrimethamine or daraprim) have activity against protozoa.
- Antiparasitics (emetine, quinine, hygromycin, phenothiazine, piperazine) have activity against parasites.

Classification based on effects on target cells

- The bactericidal group includes antimicrobials like streptomycin, aminoglycoside and penicillin that kill micro-organisms.

- The bacteriostatic group includes antimicrobials like sulfonamide, tetracycline that only inhibit the growth of micro-organisms.

Classification based on the scope of effectiveness

- Narrow spectrum group such as macrolides, polypeptides; they are only active against a relatively small subset of organisms, mainly Gram-positive organisms like *Actinomyces*, *Corynebacterium*, *Bacillus*, *Clostridium*.
- Moderate spectrum group such as sulfonamides, aminoglycosides; they are generally effective against the Gram-positive and most systemic, enteric and urinary tract Gram-negative pathogens.
- Narrow and moderate spectrums group such β-lactam antibiotics; they are only effective against Gram-positive organisms while other members can also kill certain Gram-negative bacteria.
- Broad spectrum group such as tetracycline, chloramphenicol; they are effective against all prokaryotes except *Mycobacterium* and *Pseudomonas*.
- Anti-mycobacterial group such as isoniazid; they are effective against *Mycobacterium*.

2.3.3 Development of antimicrobial drugs

Many antimicrobials were described within a few years after the introduction of penicillin. The history of the introduction of the differently known classes of antimicrobials could have shown so far sulphonamide (1938), penicillin (1941), aminoglycoside (1944), cephalosporin (1945), chloramphenicol (1949), tetracycline (1950), macrolides, lincosamide, streptogramin (1952), glycopeptides (1956), rifamycin (1957), nitroimidazole (1959), quinolones (1962) and trimethoprim (1968) (Conly and Johnston, 2005). Since 1980 scientific researches have still invested a lot in generating new kinds of antimicrobials that are active against harmful organisms but there is a lack of development of new antimicrobial drugs (Rapp, 2006; Finch *et al.,* 2005).

2.3.4 The global scenario of the antimicrobial use

The antimicrobial use in the food animal production was introduced in the late 1940s (Mitchell *et al.,* 1998) to treat sick animals and to ensure safe and wholesome meat products (CDC, 2005a). Since 1950 it has been found that many antimicrobials at low concentrations improved the growth rate and efficiency of feed use in food animals and thus antimicrobials has also been used for growth promoting and prophylactic purposes in food producing animals (Barlow, 2003). In the intensive rearing industries, mainly the pig and poultry industry, the use of antimicrobials for non-therapeutic purposes is of a higher tendency (WHO, 2002a; NZSA, 2005).

About half of the total amount of antimicrobials produced, globally, is used in food animals. Among them a large proportion is used not for treating but for preventing disease and for improving efficiency of feed utilization and weight gain (WHO, 2002a). In the European Union (EU) a survey about the usage of antimicrobial in animal health estimated that 2494 tons of active ingredient of purified antimicrobials are used for animals and an equal amount of almost half of the total of the antimicrobials are consumed in the EU (Follet, 2000). The survey also illustrated that the mainly used drugs in food producing animals belonged to the groups of tetracyclines, penicillins and macrolides. Out of that the tetracycline group accounted for two thirds of the total of the sold antimicrobial volumes (Follet, 2000).

In Britain about 434 tons of antimicrobials were administered to animals between 1998 and 2003. This figure rose to 454 tons in 2004. The most widely used types of antimicrobials in food animal production are the older classes (tetracyclines, sulfonamides trimethoprim, β-lactams) and in there tetracyclines contributed around 50 % of the total antimicrobial consumption (VMD, 2006).

According to the International Nonprofit Scientific Society Institute of Food Technologists the total amount of annually produced antimicrobials in the USA

during the 1970-1990 ranged from 31 million to 50 million pounds. About 18.4 million to 30 million pounds were used in agriculture (Klapthor, 2006). Some other reports indicated that about 70 % of the total quantity of antimicrobials used for the purpose of growth promotion and disease prophylaxis (Mellon *et al*, 2001; Valentine, 2001) are equal to 24.6 million pounds; 10.3 million pounds of it for swine (Valentine, 2001). The quantity of antimicrobials used in animal production in the USA has already subsided in recent years (FoodQuality, 2002) but an enormous amount of antimicrobials (penicillins, tetracyclines, and streptogramins) that was prohibited in the EU has been still used, annually (Mellon, 2001).

Data of used antimicrobials in animals in developing countries was more scarce (Istúriz and Carbon, 2000). However, the use of antimicrobials for growth promoters was on a limited scale but many antimicrobial drugs were also used in animals for prophylaxis (Byarugaba, 2004). In Kenya antimicrobial consumption during the period 1995-1999 showed that more than 90 % of the total antimicrobials which were consumed annually, were for therapeutic use and a small percentage of them were used for prophylactic purpose. This study confirmed that antimicrobials were not used for growth promotion (Mitema *et al.*, 2001).

Being aware of the resistance development of micro-organisms the Swann Committee has recommended that antimicrobials used in food animals can be divided into 'feed' and 'therapeutic' classes of drugs. Thereby the feed class does not include drugs that are used for therapeutic purposes in humans or in animals (HR, 2002). However, it is worth to consider that all classes of antimicrobials licensed for disease therapy in humans are also registered for the use in animals in many countries (WHO, 2002a). Some antimicrobials of the tetracycline, penicillin, macrolide classes are still used, therapeutically, in humans and are also used, extensively, for non-therapeutic purposes in the food animal production (Valentine, 2001). It is reported that the USA has used most of the antimicrobials that are used in food producing animals in humans as well (CDC, 2005 c).

2.3.5 The significance of antimicrobial use in food animals

According to the report of the joint FAO/OIE/WHO (2006) the use of antimicrobial compounds in the food animal production has demonstrated benefits like the prevention and treatment of infectious diseases, the controlling and reducing of the spread of zoonotic infections as well as economic benefits through improvements in feed performance and efficiency etc.

However, the use of antimicrobials in those food producing animals has caused mainly adverse effects such as change in intestinal micro-flora, residues in food products, impact on public environment and resistance emergence in micro-organism (VSPA, 2006; Umolu *et al.,* 2006; Miles *et al.,* 2006). This has an impact on the spreading in every country in the world. Besides it is an economic burden, especially, for the patients in developing countries (Okeke *et al.,* 2005). Although not all antimicrobial resistance in a micro-organism is due to the use of antimicrobials, it mainly comes from the use of antimicrobials in animals which can promote the emergence of drug resistance (Olofsson, 2006). Kang *et al*. (2005) showed that the use of antimicrobial drugs in food animals strongly associated with antimicrobial resistance in bacteria. Through the food chain antimicrobial resistant animal bacteria can transfer to the human (WHO, 2002a).

2.4 Antimicrobial resistance in micro-organisms

The mechanisms of antimicrobial resistance are described by using four categories:

- 'Bypass' is based on the structural nature of micro-organism's outer membrane which is considered to be a barrier for drug's entry into the cell. The outer cell wall of *Mycobacterium* makes these bacteria resistant to many antimicrobials (Nikaido, 1994)
- Enzymatic inactivation or modification of the antimicrobial. For example, by the production of the β-lactamase enzymes in *E. coli* (Jacoby, 1994), which destroys the β-lactam-ring's chemical structure of the penicillins; or,

aminoglycoside-modifying enzymes in *Staphylococcus aureus* (Takashi *et al.,* 2001).

- The organism's developed antimicrobial resistance through structural changes of the drug target sites (for example, mutations in the genes*)* lead to a structure of changed drug target sites that inhibit drugs from binding to these target sites (Webber and Paddock, 2001).
- The efflux pump is a self-defense mechanism of a micro-organism whereby a drug that initially enters the cell through the cytoplasmic membrane is subsequently transported, actively, out of the cell before it can contact the action site and exert its effect (Tenover, 2006). The efflux system is considered to be a pump because the ejection process requires energy. This mechanism exists in many situations of natural resistance of specific organisms (Bambeke *et al.,* 2006) and in many kinds of antimicrobials such as tetracyclines (Ramón-García *et al.,* 2006) or fluoroquinolones (Bambeke *et al.,* 2006). Some given bacterial species may lead to an apparently wide range of antimicrobial resistance (Bambeke *et al.,* 2003).

However, more than one of the resistance mechanisms can occur simultaneously in the same microbial strain, such as alterations in the drug target and alterations in the permeation of a drug to reach its target in mechanisms of fluoroquinolone resistance (Nikaido, 1994; Hooper, 2001).

Antimicrobial resistance can be classified as either natural resistance or acquired resistance (Todar, 2002). The natural resistance refers to an organism which has the inherent ability for resisting an antimicrobial. An example for this is the inherent resistance of a Gram-negative bacterium like *E. coli* to penicillin G because there is no reaction site of penicillin G in its structure (Je and Kim, 2005). The acquired resistance refers to a qualitative alteration of the genetic material of the organism as the result of microbes changing in some ways to eliminate the effectiveness of drugs through mutations.

A mutation is a change in the DNA. That means, for example, that a mutation in the *gyrA* gene of *E. coli* leads to the result that ciprofloxacin can not bound an essential bacterial enzyme required for the DNA replication. This allows *E. coli* to continue the DNA replication in the environment with the presence of ciprofloxacin (Rodriguez *et al.,* 2005).

Chromosomal mutations leading to resistance often produce structural changes in the bacterial cell, whereas transferable resistance tends to code enzymes that metabolize antimicrobials. Chromosomal resistance, in general, is a gradual, stepwise process while transferable resistance is often high. The genetic material can be transferred in generations (vertical transfer) or in the same generation or different species of micro-organism by genetic exchange (horizontal transfer). This transfer may occur between species belonging to different families, between bacterial strains of the same species, within species of the same genera or between generations. Although the mutation rate for genes in a bacterial population is low (approx. 10^{-8}) (Todar, 2002) a generation time is very short (from some minutes to some hours) the increase of resistance is enormous (CDC, 2003).

The extra-chromosomal DNA which is responsible for the resistance can reproduce itself within the cell and then spreads to other cells by genetic material exchange in different mechanisms (FDA, 2004).

- Transformation: a naked DNA containing an antimicrobial resistant gene from a lysed cell, a donor micro-organism, is transferred to another cell, a recipient micro-organism which is altering the genotype of the transformed recipient cell.

- Transduction: a DNA containing antimicrobial resistant genes is transferred between cells through bacteriophage or phage. The phage is an intermediate vector of transfer of bacterial genetic material. It therefore does not require cell-to-cell contact. During this process, when a phage infects a bacterium, bacterial DNA containing antimicrobial resistant gene may inadvertently be

incorporated into a new phage DNA. Upon bacterial death and lysis, this new phages go on to infect other bacteria. This brings along genes from the previously infected bacterium.

- Conjugation: a particular kind of circular DNA called plasmid is transferred from one bacterium to another when two cells are in close proximity to each other. The genetic elements responsible for the transfer of antimicrobial resistance are the resistance plasmids or R factors. It is an extra-chromosomal circular DNA which is independent of chromosomal DNA. It poses regions with the resistance genes. By this method a copy of plasmid medicated resistance genes is transferred from one living cell to another in which the recipient cell which is initially susceptible to an antimicrobial agent is now potentially resistant to the same antimicrobial agent. This transfer may occur between bacterial strains of the same species, within species of the same genera, or between species belonging to different families.

Tranposons: The genetic material can transpose from plasmid to chromosome, from plasmid to plasmid or vice versa. The tranposons play an important role in the rapid development of antimicrobial resistance within bacterial populations because of the rapid transfer of tranposons between plasmids in a cell, between plasmids and chromosomes and inter-bacterial transfer of plasmids.

If a bacterium resists to at least 2 antimicrobials then it is called multi-resistant (Refika and Marlyn, 2001) or, informally, a “super bug” (BioMedicine, 2007). The cross-resistance is a phenomenon of one organism acquiring resistance to one drug through direct exposure which turns out to have resistance to one or more drugs to which it has not been exposed (BiologyOnline, 2005).

2.5 An overview of *E. coli*

The total coliform and fecal coliform are referred to as indicator organisms because a quantization of their presence is used to indicate the potential presence of

pathogens in foods. The fecal coliform group includes rod-shaped, Gram-negative, non-spore forming organisms that live in the gastrointestinal tract of humans and other warm blooded animals, ferment lactose at 44.5^{o}C within 48 hour. A member of the fecal coliform group is *Escherichia coli* (Oram, 2005). The name *Escherichia* comes from the name of the paediatrician Escherich, who in 1895 first isolated and characterized this bacterium as "bacterium coli commune" ('enteric bacteria') (Todar, 2005).

E. coli is defined as a Gram-negative, non-spore forming, rod-shaped (1.1-1.5 μm x 2.0-6.0 μm) micro-organism that is often motile by means of flagella or may be non-motile, and which can grow with or without oxygen (Todar, 2005; Fratamico and Smith, 2006). It is catalase positive, oxidase negative, fementative (glucose, lactose, D-mannitol, arabinose and maltose), reduces nitrate and is β-galactosidase positive. Approximately 95 % of *E. coli* strains are indole and methyl red positive, but are Voges-Proskauer and citrate negative (Fratamico and Smith, 2006).

It is characterised by lactose fermentation with gas production and indole production from tryptophan when incubated for 48 ± 2 h at 44°C to separate *E. coli* from other organisms of the fecal coliform group (ISO 7251: 2005). Its involvement in several cases of food poisoning has suggested that *E. coli* should be used as an indicator for sanitary quality (FAO, 1992).

E. coli is an inhabitant of the intestine tract of humans and animals as well as in the environment, water and food. The presence of *E. coli* in food or water is an indication of uncleanliness and careless handing. It also implies that enteric pathogens may be present (Fratamico and Smith, 2006).

The limits of temperature for growth of *E. coli* are 7-46°C and the optimum growth temperature is approximately 37°C but *E. coli* can survive for weeks at -20°C to 4°C. *E. coli* generally grows within the pH range of 4.4–9.0, at an a_w of at least 0.95 (Bell and Kyriakides, 1998).

E. coli grows quickly and its generation time in the intestine is thought to be about 12 hours. Under optimum conditions the generation time is 20 minutes. Some strains of *E. coli* bacteria produce an enzyme called extended-spectrum β-lactamase (ESBL) which helps *E. coli* resist to many types of antimicrobials (HPA, 2005).

Most *E. coli* strains are harmless to hosts, some are useful for producing sources of 'B' and 'K' vitamins for the host but some strains can cause illness such as urinary tract infections, meningitis, peritonitis, mastitis, septicemia and Gram-negative pneumonia. *E. coli* has three kinds of antigens including 'H' or flagellar antigens, 'O' or somatic antigens which are the lipopolysaccharide complexes and 'K' or the capsular antigens which are mainly acidic polysaccharide (Todar, 2005). *E. coli* strains can cause illness such as enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) (Fratamico and Smith, 2006).

2.6 Sources of contamination of *E. coli* in meat

In a living animal the muscles are virtually sterile but other parts of the animal like skins or guts contain an enormous amount of bacteria. Among them there is a large number of *E. coli* which are often excreted or shed in the environment (Brill, 2007). They can survive and replicate outside its host environment i.e. in water, soil, sediments, sand and algae (Byappanhalli *et al.,* 2003; Anderson *et al.,* 2005). Depending on the slaughter hygiene a smaller or higher number of the bacteria can be found in meat during the slaughterhouse operations in particular during skinning, scalding, evisceration, dressing, transport or meat cutting. Contaminated meat is a result of bacterial contamination and this mostly occurs via butchers' hands, tools, contact with the equipment or through water or air (New York Times, 1997; Moldlab, 2003).

Bacterial contamination in meat is quite normal and not totally avoidable. However, the policy is to keep the amount of bacteria as low as possible. Since *E. coli* is as an indicator for sanitary quality (Meng, 2001) the organizations such as

International Commission on Microbiological Specifications of Foods (ICMSF) and Codex Alimentarius have already established microbiological meat standards in there the acceptable level for *E. coli* in fresh meat is not higher than 100 cfu/g (Meng, 2001).

2.7 Resistance in intestinal *E. coli*

Jonathan (2001) has made an experimental attempt to prove that *E. coli* has the ability to become resistant to the antibiotics when having exposure to antimicrobial agents. The development of drug resistance in intestinal bacteria is very different in vitro and in vivo conditions (Yan and Gilbert, 2004). Antimicrobial resistance can be transferred, rapidly, through a susceptible bacterial population in vitro. The possibility of transfer in the normal gut (in vivo), however, can be detected only at a very low rate (Freter *et al.,* 1983; Licht *et al.,* 1999). Evidence obtained from laboratory and epidemiological studies indicated that the persistence of resistant bacteria was related to the persistence of antimicrobial drug use (Andersson, 2003). If an antimicrobial drug is used, continuously, the persistence of resistant organisms will go on. Thus, *E. coli* has often higher degrees of antimicrobials which have a long history of use (Alhaj *et al.,* 2007). Series of studies on the resistance of *E. coli* which were isolated from animals and humans have strongly suggested that those bacteria which are resistant to antimicrobials used in animals would also be resistant to antimicrobials used in humans (VSPA, 2006; Miles *et al.,* 2006; Umolu *et al.,* 2006).

Mayrhofer *et al.* (2006) showed a direct relationship between the degree of antimicrobial use and resistance in *E. coli* isolates. *E. coli* isolated from different animal species was different concerning the degree of resistance (Burch, 2005). *E. coli* isolates from domestic species was resistant to the largest number of antimicrobial agents tested (neomycin, gentamicin, sulphonamides, chloramphenicol, orfloxacin, tetracycline, ampicillin, cephalothin, trimethoprim-sulfamethoxazole, nalidixic acid, nitrofurantoin, and sulfisoxazole) compared with isolates from human excretions, wildlife and surface water (Sayah *et al.,* 2005). Pigs of different ages exhibit different resistance patterns of bacteria because older aged pigs use more

antimicrobials which are affected more by the resistance of fecal *E. coli (*Mathew *et al.,* 1999). *E. coli* from fecal samples of cattle was the frequency of antimicrobial resistant *E. coli* was low, whereas *E. coli* from fecal samples of pigs, which were raised under intensive conditions where antimicrobials were used more often, it was much higher. The prevalence of resistance to one or more of the antimicrobial agents tested in pigs (98.3 %) was much higher than that in cattle (31.1 %) (Lim *et al.,* 2007).

Antimicrobial resistance trend in bacteria has increased (Schröder, 2004; Burch, 2005). Resistant *E. coli* isolated from medical hospitals between 1998 and 2003 in Germany has increased, significantly, over the time (Schröder, 2004). Porcine *E. coli* isolated in the United Kingdom (UK) has had an increase in resistance to tetracycline, trimethoprim, sulphonamide and the fluoroquinolones (Burch, 2005). Further, some intestinal *E. coli* had the ability to resist to some antimicrobials such as sulphonamides, chloramphenicol, ampicillin and cephalothin although these *E. coli* strains had never been in contact with such substances (Bettelheim and Thomas, 1997).

Multi-resistance to many clinically useful antimicrobial drugs has been found in *E. coli* (Lim *et al.,* 2007). It was observed in a variety of sources (humans, wildlife, domestic animals and surface water) (Sayah *et al.,* 2005). In intestinal bacteria plasmids can contribute to exchanging genes encoding antibiotic resistance among them (Nirdnoy, 2005; Petridis *et al.,* 2005; Schjørring *et al.,* 2005). *E. coli* often carries multi-resistant plasmids (Umolu *et al.*, 2006) and it is considered as a reservoir of resistant genes to transfer those plasmids to other species as well as pathogens in humans and animals (Balis *et al.,* 1996; Sunde and Sorum, 2001). Boerlin *et al.* (2005) suggested a possibility of transferability of resistance and virulence genes on plasmids of pathogenic *E. coli* isolated from diarrhea and healthy pigs in Ontario, Canada because of differences between resistance genes in pathogenic isolates and other porcine *E. coli* isolates. Further studies of resistance in *E. coli* have been already found such mechanisms of quinolone resistance by chromosomal mutation and plasmid medicated resistance (Mammeri *et al.,* 2005). *E.*

coli resistant to zidovidine (AZT, an effective drug in HIV treatment) has been found (Kim and Loeb, 1995).

2.8 The management and the use of antimicrobial drugs in food animals in Vietnam

According to a report of a consultant in the field of veterinary drugs (Boisseau, 2002) the use of antimicrobial-containing veterinary medicine products in animal husbandry in Vietnam functions as follows: except of large size farms, the decision to apply antimicrobials to sick animals, the choice of drugs, the dosage, the route of administration, the duration of treatment and the combination of drugs are made by farmers on the basis of their acquired experience and technical and commercial information circulated by veterinary pharmaceutical companies. The farmers use wide-spectrum antimicrobials or combinations of antimicrobials almost all the time. These combinations of antimicrobials might even include wide-spectrum products. The treatment of sick animals was often stopped when symptoms disappeared. In case that the symptoms of the disease would not disappear after 3 days of treatment another antimicrobial was used instead.

Survey conducted by Dinh *et al.* (2003) in 628 farms in the Binh Duong province showed that commonly used antimicrobials were mainly based on farmers' experience without any support of laboratory results and veterinary supervision. That has led to many failures of dosage, of time withdrawal and of the duration of treatment. The survey has also found out that the most frequently used antimicrobials in food producing animals in Binh Duong were tylosin (15 %), colistin (13.2 %), norfloxacin (10.0 %), gentamicin (8.4 %), tetracycline (7.9 %) and ampicillin (7.2 %).

In reference to the management of veterinary drugs, Boisseau (2002) has shown that more than 3000 antimicrobial-containing veterinary medicine products from 4000 veterinary drug shops are sold on markets. The most important sales are those of fluroquinolones (enrofloxacin and norfloxacin), aminoglycosides (gentamicin), tetracyclines, sulfonamides, colistin (combined with ampicillin) and

marcrolides (tylosin and spiramycin). The report has also pointed out that in the most frequently sold antimicrobial-containing veterinary medicine products, even potent wide-spectrum antimicrobials like fluroquinolones, which are always used as single active substances in developed countries, are practically always used in combination with one or two other antimicrobials.

In the aquaculture sector aquatic veterinary drugs are also widely used with 1893 products; in there at least 476 antimicrobial-containing veterinary medicine products (Phan, 2004). A survey on antimicrobial use in aquaculture in the Mekong River Delta found out that 90 % of them are applied for prophylactic and therapeutic treatments. Among 122 antimicrobial-containing veterinary medicine products used, 77, 34, 31, 29 out of them contain compounds of the quinolone, aminoglycoside, polypeptide and sulfonamide groups respectively. Besides, some antimicrobials that have already banned in food animal production such as nitrofuran and chloramphenicol were still found (Nguyen *et al.,* 2004).

Boisseau (2002) has concluded that the whole system, including legislation, implementation of administrative procedures, quality control, distribution and the administration of the management of antimicrobial-containing veterinary medicine products in Vietnam is weak in terms of in-depth assessment. As a result the quantity of antimicrobials used for producing a ton of meat in small pig farms was estimated to be 5 times higher than in large pig farms, where is a possibility of leading to a quick increase of resistant of antimicrobial bacteria.

2.9 Studies on antimicrobial resistance in Vietnam

One of the first studies on antimicrobial resistance in Vietnam which should be mentioned was a study of Sullivan and Nguyen (1971). More than 3,000 isolates of *Shigella, Salmonella, E. coli* have been collected before 1971 from patients with diarrhea for testing of antimicrobial susceptibility with some commonly used antibiotics. Another one testing the antimicrobial susceptibility of *E. coli* isolated from patients' urine in 1977 showed that the rates of sensitivity to kanamycine,

neomycin, chlortetracycline and chloramphenicol were 100 %, 93 %, 64 % and 33 % respectively (Bui, 2003).

Diarrheagenic *E. coli* isolates from children in Hanoi were of low sensitivity against antimicrobials including ampicillin, chloramphenicol, cefuroxime, trimethprim/sulfamethoxazole (Nguyen *et al.,* 2005).

Under the Vietnam National Antimicrobial Resistance Monitoring System (VNARM) commonly pathogenic strains collected from hospitals located in all three regions North, Middle and South such as *E.coli*, *Salmonella* spp., *Shigella* spp., *Enterococcus* spp., *Enterobacter* spp., *Klebsiella* spp., *Morganella* spp., *Proteus* spp., *Haemophilus* spp., *Citrobacter* spp., *Listeria monocytogenes, Pseudomonas aeruginosa, Streptococcus pneumoniae* etc. were tested the antimicrobial susceptibility (Le *et al.*, 2002). Results for resistance in *E. coli* are given in table 1.

Table 1: Antimicrobial resistance in *E. coli* isolated from urine

Drug	No. of isolates	Resistant isolates (%)
ampicillin	224	81.7
cephalothin	103	52.4
cefotaxime	158	12.2
ceftriaxone	124	13.2
ceftazidime	158	5.6
chloramphenicol	219	62.8
gentamicin	234	40.1
tobramycin sulfate	148	25.2
amikacin	216	7.5
trimethoprim-sulfamethoxazole	222	68.6
norfloxacin	148	25.2
ciproxacin	225	34.8
tetracycline	179	69.5
furazolidon	83	8.4

(Source: Le *et al.*, 2002)

Since the 1970s there have also been some studies on antimicrobial resistance of bacteria isolated from animals. Some recent ones are: a study which focuses on the minimum inhibiting concentration (MIC) of antimicrobials (ampicillin, tetracycline, gentamicin, chloramphenicol, trimethoprim/sulfa-methoxazol, streptomycin and kanamycine) relating to the development of *E. coli* which were isolated from diarrheic feces of pigs. The results pointed out that at the permitted concentration 70 to 95 % of the isolates were resistant to streptomycin, tetracycline and chloramphenicol. For the newly used antimicrobials (gentamicin and kanamycine) these rates were 2 to 25 % (Dinh, 1995). Nguyen *et al.* (1996) reported *E. coli* isolated from diarrhea cattle and calves in the Centre of Vietnam were quite sensitive to neomycin, kanamycine and gentamicin but already resistant to ampicillin (91.7 %), erythromycin and chloramphenicol (58.4 %) and others (cefotaxin, trimethoprim/sulfamethoxazol, tetracycline and rifampicin) from 8.4 to 33.3 %.

Results of an examination of factors determining the drug resistance of *E. coli* isolated from piglets suffering of colibacillosis in the Hanoi vicinity indicated that drug resistant *E. coli* isolates were different from farm to farm, depending on how much antimicrobial was used by each farm. Within a farm *E. coli* isolates from piglets with an age of over three days were more resistant to antimicrobial agents than those from the younger ones (Bui and Pham, 1993). Do *et al.* (2003) investigated antimicrobial drug resistance by disk diffusion technique and microplate broth dilution of *E. coli* strains isolated from diarrhea of suckling pigs in Northern provinces. The tendency of high level antimicrobial resistance to commonly used drugs was shown.

While there are some studies reporting the development of *E. coli* isolated from animals, some few studies only focus on *E. coli* isolated from meat. The most recent study on resistance of *E. coli* isolated from pork and chicken meat in the Red River Delta was carried out by To (2004, 2006). The results indicated that isolates from chicken were more resistant to tetracycline, ampicillin, streptomycin, chloramphenicol than that of those from pork. The level of resistance is shown in table 2.

Table 2: Antimicrobial resistance *E. coli* isolated from pork and chicken meat

Antimicrobials	**Antimicrobial resistance of *E. coli* isolates (%)**	
	Chicken meat	**Pork**
tetracycline	56.7	10.0
ampicillin	67.0	10.0
streptomycin	66.7	14.0
chloramphenicol	43.3	11.0
norfloxacin	30.0	0.0
doxycyline	36.7	6.0
ofloxacin	33.3	5.0
gentamicin	6.7	0.0
neomycin	0.0	1.0

(Source: To, 2004)

There was also a more highly considered percentage of multi-resistance in *E. coli* isolates from chicken meat than those from pork (Table 3). Multi-resistance was observed for up to 7 in pork and 8 in chicken meat.

Table 3: Multi-resistance of *E. coli* isolated from pork and chicken meat in the Red River Delta

Multi-resistance against antimicrobials	**Antimicrobial resistance of *E. coli* isolates (%)**	
	Chicken meat	**Pork**
2	23.3	20.0
3	20.0	13.3
4	16.7	3.3
5	10.0	3.3
6	3.3	6.7
7	6.7	3.3
8	3.3	0.0

(Source: To, 2004)

In general, most of all documented reports aimed at objectives on the degrees of resistance in some bacteria with some commonly used antibacterial drugs. In-depth studies on the antimicrobial mechanisms have not been conducted (To, 2006).

2.10 Methods for the detections of *E. coli* in fresh meat

2.10.1 Conventional method

Detection test Test for presumptive *E. coli* in food products (ISO 7251: 2005)

Lauryl Sulfate Tryptose (LST) broth is used as the first selective-enrichment medium. The tubes are incubated at 37°C for up to 48 ± 2 h for gas production. The following step is inoculation in the second selective-enrichment medium (EC medium with 0.15 % (bile salts) at 44°C for up to 48 h for gas production. Finally, the indole test produced from tryptophan in indole-free peptone water at 44°C for 48 h defines the presence of the presumptive *E. coli*.

Interpretation: One sample is considered as presumptive *E. coli* positive when visible gas is produced in the EC broth and indole is produced in the peptone water at 44^{o}C for up to 48 h.

Test for confirming *E. coli* (FAO, 1992)

Inoculate the presumptive *E. coli* in the Levine's Eosin Methylene Blue (L-EMB) agar plate by incubating for 18-24 h at 35°C. The typical *E. coli* colonies on the L-EMB develop a purple color with or without metal sheen. Inoculate in the Plate Count Agar (PCA) slants for 18-24 h at 35°C for Gram stain and biochemical tests of IMViC reactions (IMViC stands for the capital letters of Indole, Methyl red, Voges-Proskauer, and Citrate, respectively).

Interpretation: A presumptive *E. coli* that give the results of Gram-negative non spore-forming rods and the IMViC patterns of "++--" or "-+--" is confirmed to be *E. coli*.

The conventional method is an official method which has already been adopted by the ICMSF recognized by the International Organization for Standardization (ISO). This method is often time consuming and requires much laboratory manual work. Besides, this method might miss some enteropathogenic *E. coli* (pathogenic strains of *E. coli* do not grow at 44°C) but it is the detection of common contamination of *E. coli* in meat that is important in this test rather than the presence of specific types (Todar, 2005).

2.10.2 Rapid methods for the detection of *E. coli*

The petrifilm EC plate method (Fluorogenic assay)

Glucuronidase is present in most strains of *E. coli* but absent in most other enteric micro-organisms. The fluorogenic reagent, 4-methylumbelliferyl β-D-glucuronide (MUG), in the medium is cleaved to release 4-methylumbelliferone (MU) by glucuronidase which is produced by *E. coli* if present in the medium. Under the ultraviolet (UV) light the MU fluorescence is easily visualized around the colonies in the medium (3Mpetrifilm™, 2005).

The advantages of this method are that it is very simple, easy to perform and that it does not require much time. This method has been approved as an official method for enumeration *E. coli* in poultry, meat and seafood by the Association of Official Analytical Chemists (AOAC), coded AOAC 998.08 (McKenzie, 2005). The disadvantage is that the EHEC strains do not possess that particular enzyme, and, thus, go undetected (Todar, 2005). However, this is a great potential method for detecting *E. coli* if fluorogenic reagents can be added to a various selective media.

The LST+MUG method

This method is also based on the principle of the Petrifilm EC Plate method but there is a combination between the selective media (LST) and the fluorogenic reagent MUG together. In the medium coliforms grow and produce visible gas from lactose. *E. coli* produces the gluconidase enzyme which cleaves MUG to release MU having a blue color around colonies under the UV light.

The LST +MUG method has been evaluated by comparative studies with high accuracy (Dogan *et al.,* 2002; Feng, 2001) and is accepted by the AOAC as an official method, coded AOAC 992.30 (McKenzie, 2005).

Other rapid methods for the detection of *E. coli*

The DNA and antibody-based assays are mainly used to detect specific pathogenic *E. coli* in foods (Feng, 2001). Because the number of pathogenic *E. coli* in foods is likely to be very low, a step of enrichment is necessary, especially, in the rapid methods (Todar, 2005). The detailed procedures of rapid methods are available.

Rapid methods have many advantages such as a convenient performance, a low labor input, a low amount of chemicals and a short time for obtaining results. Particularly, those methods can screen a large number of negative samples. Some of them have already been validated as official methods by the AOAC such as AOAC 984.34 for detection of *E. coli* producing enterotoxin and AOAC 986.34 for detection of enteroxigenic *E. coli* (Feng, 2001; McKenzie, 2005). However, some methods require instruments, high technique, cost and high skill. Besides, positive results need to be confirmed by some appropriate official method, which, in many instances, is a cultural method (Feng, 2001).

2.11 Methods for antimicrobial susceptibility testing (Lalitha, 2004)

There are three commonly used methods for antimicrobial susceptibility testing based on the principle applied in each system, including the diffusion susceptibility tests, dilution susceptibility tests and the diffusion and dilution susceptibility tests. All of them are used for in vitro diagnosis in the laboratory. Descriptions of the standard tests are available in the laboratory manuals. Other methods such as automated techniques are variations of these methods.

2.11.1 Dilution susceptibility tests

Dilution susceptibility testing methods yield quantitative data in susceptibility of an organism to a drug. Antimicrobials are prepared in progressive two-fold serial dilutions in broth or agar media to give a range of concentrations. These methods generate the minimum inhibitory concentration (MIC, the lowest concentration of antimicrobial that can inhibit the visible growth of micro-organism after incubation) and the minimum bactericidal concentration (MBC, the lowest concentration of antimicrobial that can prevent the growth of micro-organism after sub-culture on to antimicrobial free media) to an organism concerned. These methods offer the potential to gain information about drug concentrations in the body tissues. Thus, dosages can be established on the basis of a pharmacokinetic description of disposition in the body. They are most often used to determine activity in vitro of new drugs and to determine MIC breakpoints. The MIC value obtained from a standardized procedure of the National Committee on Clinical Laboratory Standards (NCCLS) can be regarded as the reference criteria for defining the susceptibility of a micro-organism. They are also used to confirm resistance and to determine the susceptibility of isolates from blood cultures in prolonged serious infections like endocarditis. The terms ‘susceptible’ and ‘resistant’ have a realistic interpretation. Therefore, MICs are considered to be the ‘gold standard’ for determining the susceptibility of organisms to antimicrobials and to judge the performance of all other methods of susceptibility testing (Andrews, 2006). However, this technique is expensive, and labor intensive (Serrano, 2005).

2.11.2 Diffusion susceptibility tests

This technique was firstly utilized by Beijerinck in 1889 and had developed it further in the 1940s. It is considered as qualitative or semi-quantitative methods. The history and development of antimicrobial susceptibility testing methodology has already recorded some techniques but the agar disk diffusion method (Kirby-Bauer method) has many advantages. The procedure, for instance, is simpler and the obtained results are comparable to the dilution technique. Thus, this method is being widely applied for antimicrobial susceptibility testing in clinical laboratories (Wheat, 2001).

In this method, filter paper disks containing known concentrations of an individual antimicrobial are placed on the surface of appropriate agar medium that has been inoculated with a suspension of an isolate. The culture is incubated for 16-18 h at 35^{o}C. The antimicrobial agent diffuses into the medium creating a halogen zone of inhibition around the antimicrobial disk corresponding to the susceptibility of the isolate to the agent. The diameters of the halogen zones are measured to mm and interpreted for susceptibility testing results into three categories: resistant, intermediate, susceptible on the basis of an appropriately interpretative criterion. This method is now being recommended and becomes the basis of the NCCLS disc diffusion standards. Interpretative criteria of NCCLS have been developed based on international collaborative studies. They are also recommended by the WHO (Lalitha, 2004)

The disk diffusion test is designed to distinguish between sensitive and resistant members of a bacterial population. Antimicrobials that fall into the intermediate category should not be used. This test is useful because antimicrobial concentrations in paper discs are always lower than an underestimation of the antibacterial effects of a drug. This test applies only to fast-growing aerobic bacteria under standard conditions.

2.11.3 Diffusion and dilution susceptibility tests (Etest)

The Etest is a thin, inert and non-plastic strip containing a known gradient range across two-fold dilutions of a conventional MIC method. When an Etest is applied to an inoculated agar surface, the antimicrobial gradient on the strip is transferred into the media. After incubation, a symmetrical inhibition ellipse centered along the strip is seen. The MIC value is read from the scale in terms of μg/ml where the ellipse edge intersects the strip.

The Etest is a quantitative method which is performed using a procedure with a combination of both dilution and diffusion tests. This is a simple, accurate and reliable method to determine the MIC for a wide spectrum of infectious agents. Etest MICs are more precise than conventional MICs based on discontinuous two-fold serial dilutions because their antimicrobial concentration gradient is predefined.

Many factors can influence the results of antimicrobial susceptibility testing such as pH, oxygen, moisture and content of thymidine and thymine of the medium, inoculum preparation, concentration of antimicrobial tested in the carrier and the interpretative criteria. Therefore, the accuracy and reproducibility of these tests are dependent on maintaining a standard set of procedures.