ANALYSIS OF TETRACYCLINE RESIDUES IN MARKETED PORK IN HANOI, VIETNAM

DUONG VAN NHIEM

MASTER OF SCIENCE
IN VETERINARY PUBLIC HEALTH

CHIANG MAI UNIVERSITY AND FREIE UNIVERSITÄT BERLIN
SEPTEMBER 2005
ISBN 974-9887-73-5

ANALYSIS OF TETRACYCLINE RESIDUES IN MARKETED PORK IN HANOI, VIETNAM

DUONG VAN NHIEM

A THESIS SUBMITTED TO CHIANG MAI UNIVERSITY AND FREIE UNIVERSITÄT BERLIN IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN VETERINARY PUBLIC HEALTH

CHIANG MAI UNIVERSITY AND FREIE UNIVERSITÄT BERLIN
SEPTEMBER 2005
ISBN 974-9887-73-5

ANALYSIS OF TETRACYCLINE RESIDUES IN MARKETED PORK IN HANOI, VIETNAM

DUONG VAN NHIEM

THE THESIS HAS BEEN APPROVED
TO BE A PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE
IN VETERINARY PUBLIC HEALTH

EXAMINING COMMITTEE

..... CHAIRPERSON
Associate Professor Dr. Peter Paulsen

..... CHAIRPERSON
Dr. Witaya Suriyasathaporn

23 September 2005

Thesis Title Analysis of Tetracycline Residues in Marketed Pork in Hanoi Vietnam

Author Mr. Duong Van Nhiem

Degree Master of Science (Veterinary Public Health)

Thesis Advisory Committee Assoc. Prof. Dr. Peter Paulsen Chairperson (FU-Berlin)

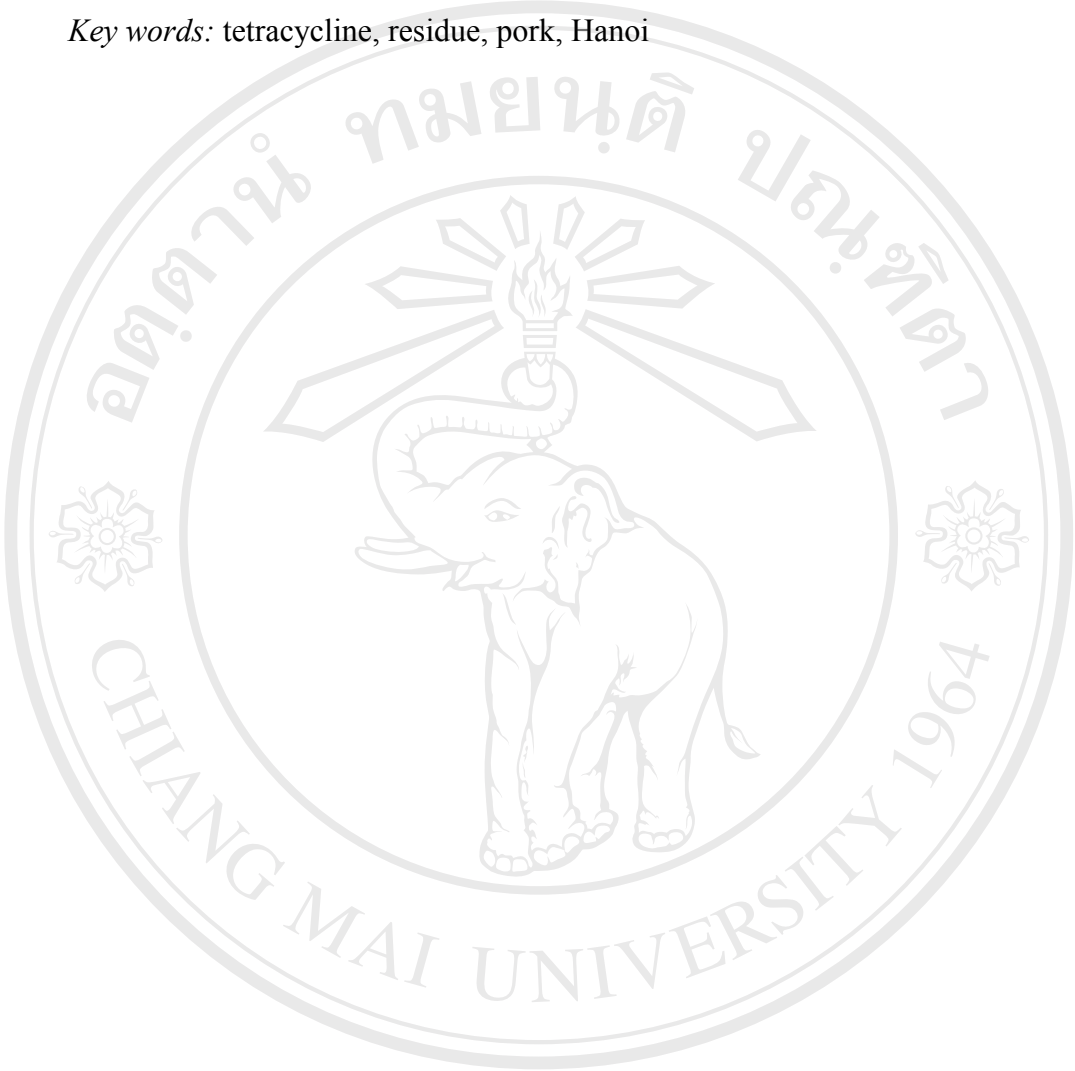
Dr. Witaya Suriyasathaporn Chairperson (CMU)

ABSTRACT

A cross-sectional survey was designed to investigate the proportion of tetracycline residues in marketed pork in suburb and urban districts in Hanoi. A total of 290 raw muscle samples were randomly collected from open markets in these districts. The samples were qualitatively screened for tetracycline residues using the agar inhibition test, and the *Bacillus cereus* (ATCC 11778) as the reference bacterial strain. The positive and inconclusive samples were then analyzed using High Performance Liquid Chromatography (HPLC). To calculate the proportion of the antibiotic residues, samples positive with either of the above tests were defined as positive results. According to this definition, 16 out of 290 samples (5.5 %) were positive. The proportion of positive samples from shops in suburb districts was significantly ($P<0.05$) different from those collected from shops in urban districts. So, the factor of region was identified as a risk factor of tetracycline residue proportion in raw pork with an odds ratio (OR) of 4.03 (95%CI=1.12, 14.45). Among samples analyzed by HPLC six samples were confirmed containing at least one compound of the tetracycline group with concentrations ranging from 51.57 to 167.40 µg/kg. A total amount of tetracycline residues that exceeds the MRL (100 µg/kg or ppb) for pig muscle was only found in two samples of which one contained a total 192.26 µg/kg

(oxytetracycline 97.11 µg/kg and chlortetracycline 95.15 µg/kg) and the other contained 167.4 µg/kg oxytetracycline only.

Key words: tetracycline, residue, pork, Hanoi



ชื่อเรื่องวิทยานิพนธ์	การวิเคราะห์การตกค้างของยาเตตราไซคลินในเนื้อสุกรที่ขายในตลาดสดเมืองฮานอย ประเทศเวียดนาม
ผู้เขียน	นาย Duong Van Nhiem
ปริญญา	วิทยาศาสตร์มหาบัณฑิต (สัตวแพทย์สาธารณสุข)
คณะกรรมการที่ปรึกษาวิทยานิพนธ์	รศ.ดร. Peter Paulsen ประธานกรรมการ(FU-Berlin) อ.น.สพ.ดร.วิทยา สุริยาสถาพร ประธานกรรมการ(CMU)

บทคัดย่อ

ทำการสร้างการสำรวจแบบตัดขวางเพื่อหาสัดส่วนการตกค้างของยาเตตราไซคลินในเนื้อสุกรที่ขายในตลาด เขตเมือง และเขตชนบท ในเมืองฮานอย ประเทศเวียดนาม โดยการสุ่มเก็บตัวอย่างเนื้อสุกรดิบ จากตลาดสดในพื้นที่ที่ทำการศึกษาจำนวนทั้งหมด 290 ตัวอย่าง ตัวอย่างที่เก็บได้ถูกตรวจคัดกรองเบื้องต้นสำหรับการตกค้างของยาเตตราไซคลินด้วยวิธี Agar inhibition test ที่ใช้ *Bacillus cereus* (ATCC 11778) เป็นเชื้ออ้างอิงสำหรับทดสอบ ตัวอย่างที่ให้ผลบวกและตัวอย่างที่ให้ผลชัดเจนหรือไม่ให้ผลลบ ถูกนำวิเคราะห์โดยวิธี High Performance Liquid Chromatography (HPLC) การคำนวณหาสัดส่วนของสารตกค้างทำโดยกำหนดว่าตัวอย่างที่ให้ผลบวกเป็นตัวอย่างที่ได้ผลบวกอย่างน้อย 1 วิธี ตามคำจำกัดความดังกล่าวพบว่า 16 จาก 290 ตัวอย่างให้ผลบวก (5.5%) สัดส่วนของตัวอย่างที่ให้ผลบวกในเขตชนบทมีความแตกต่างกับสัดส่วนของตัวอย่างที่เก็บในร้านขายเนื้อในเขตเมืองอย่างมีนัยสำคัญ ($P<0.05$) ซึ่งแสดงว่าปัจจัยพื้นที่ในการขายเนื้อสุกรเป็นปัจจัยเสี่ยงของการตกค้างของยาเตตราไซคลินในเนื้อสุกร โดย Odd Ratio เท่ากับ 4.03 (95% CI= 1.12,14.45) จากตัวอย่างที่นำมาตรวจซ้ำด้วย HPLC พบว่ามี 6 ตัวอย่างที่มียาเตตราไซคลินอย่างน้อย 1 กลุ่มตกค้างอยู่ในเนื้อสุกร ปริมาณที่ตรวจพบอยู่ในช่วง 51.57-167.40 ไมโครกรัมต่อกิโลกรัม โดยพบว่ามี 2 ตัวอย่างที่มีปริมาณยาเตตราไซคลินตกค้างในเนื้อ สูงกว่าค่า MRL สำหรับกล้ามเนื้อสุกร (100ไมโครกรัมต่อกิโลกรัม) ตัวอย่างแรกพบปริมาณ 192.26 ไมโครกรัมต่อ

กิโลกรัม (ออกซิเตตราไซคลิน 97.11 ไมโครกรัมต่อกิโลกรัม และ คลอเตตราไซคลิน 95.15 ไมโครกรัมต่อกิโลกรัม) และอีกตัวอย่างที่เหลือมีการตกค้างเฉพาะ ออกซิเตตราไซคลิน ชนิดเดียว ในปริมาณ 167.40 ไมโครกรัมต่อกิโลกรัม

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

ABBREVIATIONS AND SYMBOLS

%	Percent
µg	Microgram
ADI	Acceptable Daily Intake
AFSSA	Agence Française de Sécurité Sanitaire des Aliments
AOAC	The Association of Official Analytical Chemists
bw	Bodyweight
CAC	Codex Alimentarius Commission
CCRVDF	Codex Committee on Residues of Veterinary Drugs in Food
CI	Confidence Interval
COMISA	Consultation Mondiale de l'Industrie de la Santé Animale
CTC	Chlortetracycline
ELISA or EIA	Enzyme-Linked Immunosorbent Assay
EMEA	European Agency for Evaluation of Medicinal Products
EU	European Union
FAO	Food and Agriculture Organization
FEDESA	European federation of Animal Health
FPT	Four Plate Test
g	Gram
GAO	United States General Accounting Office
GC	Gas Chromatography
GDP	Gross Domestic Product
HPLC	High Performance Liquid Chromatography
JECFA	Joint Expert Committee on Food Additives
JETACAR	Joint Expert Technical Advisory Committee on Antibiotic Resistance
kg	Kilogram
LC	Liquid Chromatography
LD_{50}	Lethal Dose for 50% of a given population
LOD	Limit of detection

LOQ	Limit of quantification
MAF	Ministry of Agriculture and Fisheries
MARD	Ministry of Agriculture and Rural Development
MFPT	Modified Four Plate Test
MRL	Maximum Residue Limit
MRLVD	Maximum Residue Limit for Veterinary Drugs
NCSS	Number Cruncher Statistical System
NOEL	No Observable Effect Level
^{o}C	Degree Celsius
OIE	Office International des Epizooties
OR	Odds Ratio
OTC	Oxytetracycline
PE	Polyethylene
ppb	Part per billion
RIA	Radioimmunoassay
SF	Safety Factor
Spr	Spring
Sub	Suburban
TC	Tetracycline
TEAGASC	Irish Agriculture and Food Development Authority
TLC	Thin Layer Chromatography
TLC/BA	Thin-Layer Chromatography/Bioautography
UK	United Kingdom
Urb	Urban
UV	Ultraviolet
VMP	Veterinary Medical Products
WHO	World Health Organization
Win	Winter

I. INTRODUCTION

Vietnam is an agricultural country. About 75 % of the population resides in rural areas and is employed in the agricultural production. The agricultural production provides around 24.3 % of the gross domestic products (GDP) in the country. Livestock production is one of the major sectors of agricultural production besides paddy rice farming, accounting for 22.3 % of the production value or 5.4 % of the GDP in Vietnam (FAO, 2004).

Pork is the staple product of livestock production in Vietnam. According to statistic data collected by the Ministry of Agriculture and Rural Development (MARD), annual pork production reached 1.8 million tons in 2003, accounting for 77 % of the total meat production in the country (Mạnh and Toàn, 2003). The majority of the pork production is domestically consumed, and the rest is exported. Hanoi, the capital city of the country with a population of 3.029 million people (VietnamNet, 2004), excluding a quite big number of tourists and in-official immigrants, daily consumes between 180 and 200 tons of pork (Kim Liên, 2004). Nevertheless, meat quality and safety is remaining a public health issue. Studying and monitoring residues in meat, including antibiotics, is a quite new field in the country.

Tetracyclines, antibiotics produced by the *Streptomyces* bacterium, is a broad-spectrum antibiotic which show activity against gram-positive and gram-negative bacteria, including anaerobes (Goodman *et al.,* 1985) and have been widely used for the treatment of infectious diseases and as an additive in animal foodstuffs (Pilar *et al.*, 2004). This is one of the five most commonly used groups of antimicrobials in food animals (Mitchell *et al.*, 1998). Among more than 3,000 antimicrobial-containing veterinary medical products (VMPs) registered to be imported, manufactured, and marketed by 51 veterinary pharmaceutical companies in Vietnam,

257 VMPs contain oxytetracycline – a compound of the tetracycline group (Boisseau, 2002).

The widespread use of antibiotics, especially along with a poor management and imprudent use of antibiotics, in livestock industry may pose a very high risk of antibiotic residue in meat and other animal products. With regard to public health, the consumers incur adverse consequences of antibiotic residues in food. No one is alert to the risk of antibiotic residue because of the lack of comprehensive studies on this issue. In addition, it do not exist any information about antibiotic residue so that people could choose or buy meat products without antibiotic residue. Furthermore, the farmer household small-sized or backyard animal raising model and the uncontrolled animal slaughtering is resulting in the fact that a lot of poor quality meat, including meat with high antibiotic residue or contamination, is marketed and is adversely affecting human health.

Therefore, it should also be one of the most emerging issues regarding food hygiene and safety.

The objectives of this study were therefore (i) to investigate the proportion of tetracycline residues in raw pork marketed on Hanoi's markets and to preliminarily identify potential risk factors; and, (ii) to quantitatively analyze tetracycline residues in the pork samples that had showed positive or inconclusive results in the previous qualitative analysis.

Results of the study would primarily help food professionals, veterinary authorities, and the consumers to be aware of possible antibiotic residual risks confronting them in their product or food. They possibly know that this problem prompts to take urgent measures of dealing, prevention and control. In addition, this study would provide the information about the factors associated with the antibiotic residues. The information would be useful for consumers to choose products with the lowest risk.

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

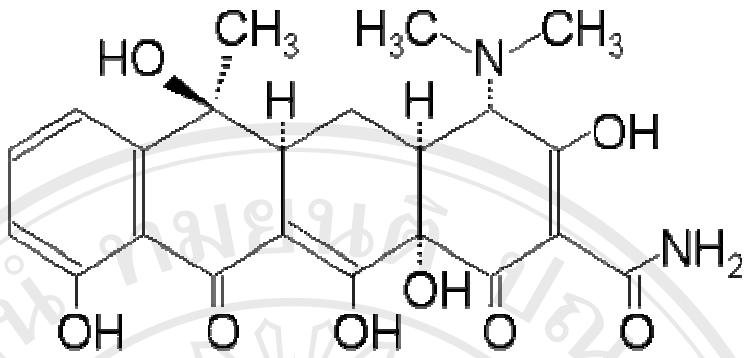
A study (An *et al.*, 2002) on antibiotic use and residues in chicken in Ho Chi Minh City reports that among 36 currently used antibiotics the eight most commonly used antibiotics include colistin, enrofloxacin, daveridin, sulfadimidin, trimethoprim, norfloxacin, oxytetracycline, gentamicin, and oxolynic 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).

III. MATERIALS AND METHODS

3.1. Study design

3.1.1. Study site

Hanoi, the capital city, is located in the Red River Delta in North Vietnam, is comprised of fourteen districts, five suburb and nine urban districts.

Table 4. Study site and distribution of samples

No.	Districts	Numbers of samples by season		Total number of samples
		Winter	Spring	
1	Gia Lam	25	20	45
2	Soc Son	13	16	29
3	Dong Anh	16	17	33
4	Tu Liem	16	12	28
5	Thanh Tri	7	13	20
6	Cau Giay	8	10	18
7	Long Bien	11	16	27
8	Hoang Mai	9	11	20
9	Tay Ho	5	6	11
10	Thanh Xuan	12	10	22
11	Hai Ba Trung	4	5	9
12	Ba Dinh	1	4	5
13	Hoan Kiem	5	8	13
14	Dong Da	5	5	10
	Total	137	153	290

Climatically, Hanoi falls in the subtropical zone, influenced by tropical humid monsoon. There are four distinct seasons, namely spring (February-April), summer (May-July), autumn (August-October) and winter (November-January). It can also be divided into two: the rainy (May-September) and the dry (October-April) season. The average temperature in summer is 29.2^0C, in winter 17.2^0C and for the whole year 23.2^0C. There are about 114 rainy days a year with a mean rainfall of 1,800mm/year (Vietnamtourism, 2005).

Table 4 shows the study site and the distribution of samples. The study was conducted in the five suburb districts namely Gia Lam, Soc Son, Dong Anh, Tu Liem, and Thanh Tri and in the nine urban districts Cau Giay, Long Bien, Hoang Mai, Tay Ho,Thanh Xuan, Hai Ba Trung, Ba Dinh, Hoan Kiem, and Dong Da of Hanoi.

3.1.2. Study population and sample size determination

Permanent regular markets in all above districts were selected as sampling sites; meat shops in the markets were defined as study units. However, only a certain percentage of these shops, which were sufficed to a statistically minimum required number of samples, were selected. The number of samples was estimated by using the computer program Win-Episcope 2.0. An expected prevalence of antibiotic residues at detectable levels in pork was 10% (An *et al.*, 2002; Thuận *et al.*, 2003). Based on the expected prevalence of 10%, the study population (N) in 14 districts of 1200, 95% confidence level, and 5% of accepted error, the required sample size (n) was 125 (10.33% of the population). To satisfy the requirement on sample size (one-tailed) for estimating difference between two percentages (expected proportion in group 1 as 5%, group 2 as 15%; 95% confidence level, and power 85%), the sample size was estimated as 127 samples for one time and 254 for two times of sampling. In actual, 290 samples altogether were collected by two times of sampling that satisfied both requirements.

3.2. Methods

3.2.1. Sampling and data collection

The sampling was conducted in two seasons, namely early winter and early spring. Meat shops were randomly selected from each market. The sampling was not repeated for previously selected samples. In other words, a shop selected by the first sampling round was then not selected in the second round. This means that 290 different shops were selected. From each shop, one muscle sample of approximately 300 to 400 grams was collected, wrapped in P.E. bag and put in a cool box with ice. Samples were then transported to the laboratories and stored in freezers at a temperature of no higher than -18^0C until analysis. Number and distribution of samples are shown in table 4. Relevant information such as location of the market, origin of meat, type of abattoir, product(s) offered on the same shop, and shop-owner's profile was also obtained simultaneously at the time of sampling by using a questionnaire (Appendix 1). This collected information would be subsequently used in the analysis of risk factor.

3.2.2. Methods for analysis

3.2.2.1. Microbiological inhibition test

This method was used as a screening test. All samples were analyzed by using the microbiological inhibition test with *Bacillus cereus* ATCC 11778 as a reference bacterium, oxytetracycline discs (Mast Diagnostics 0.5 µg/disc) as control, on agar test pH 6 (Merck) (SOP RES 31 V.8, 2002; Myllyniemi *et al*., 2001; Nouws *et al*., 1998). The sterile bottles of medium were melted and sterilized in an autoclave at 121^0C for 15 minutes; subsequently placed in a waterbath at 55^0C and left for at least 30 minutes until they reached the temperature of the waterbath. Appropriate volumes of the *Bacillus cereus* spore suspension were added to the medium, gently mixed and poured into 90mm-diameter sterile Petri dishes on a leveling platform with 5ml/plate.

Muscle samples were removed from the freezer and placed at room temperature for up to 20 minutes. An 8mm-diameter cylindrical core from each sample was cut using a stainless cork borer. The core was subsequently cut into slices of 2mm thickness using a sterile scalpel blade. Two slices from each sample were placed opposite each other on a plate using forceps; a positive control disc was placed in the center of the plate. The plates were incubated at 30^0C for approximately 18 hours. Plates were read against a black background with a light from underneath. The zones of inhibition given by the tissue slices and control discs were measured to the nearest mm using a ruler. Positive results were indicated by the complete inhibition of growth around both meat slices in a zone of 12 mm diameter or greater (the annular zone not less than 2 mm wide). Negative results were indicated by no inhibition of growth around the meat slices. The inconclusive samples, which showed the annular inhibition zone less than 2 mm wide or incomplete, were then, together with the positive ones, analyzed by the high performance liquid chromatography (HPLC) mentioned below.

3.2.2.2. Analysis by HPLC

Samples that were previously considered as inconclusive results, as well as samples which were indicated as positive by microbiological inhibition test were subsequently analyzed by HPLC. These samples underwent three principal stages in the sample preparation (i) homogenization and extraction of the sample residues by EDTA/Mc Ilvain buffer; (ii) precipitation of proteins using trichloroacetic acid and filtration; and, (iii) cleanup on solid-phase extraction cartridges C_{18}. Tetracyclines are separated on a C_{18} stationary phase and detected by UV absorption at 355 nm. The amount of tetracycline is calculated by interpolation from a calibration curve determined for each of the three compounds: oxytetracycline, tetracycline and chlortetracycline, taking into account the calculated recovery. The detailed procedures are mentioned in the standard of Agence Française de Sécurité Sanitaire des Aliments (AFSSA) for “Determination of Tetracycline residues in kidney and muscle by high performance liquid chromatography” (Appendix 2).

3.2.3. Further data processing

A sample that was indicated as a positive result by either above-mentioned methods was defined as a positive result in this study. Data were described using percentage and range. The inferential statistics were Fisher's exact test for identification of risk factors. A statistical software package used for analyzing data was NCSS version 2000. A confidence level of 95% (α=0.05) was defined. Odds ratio was calculated as given in table 5.

Table 5. 2-by-2 table of results of each factor to calculate odds ratio

		(+)	(-)	Total
Exposure	(+)	a	b	a+b
	(-)	c	d	c+d
		a+c	b+d	a+b+c+d

Odds Ratio (OR) = (a/b)/(c/d) = ad/bc

Quantitative data obtained from HPLC analysis, and the data collected from the questionnaire survey were analyzed using descriptive statistics such as percentage, mean and range.

IV. RESULTS

4.1. Descriptive data

4.1.1. Results of the questionnaire survey

Results of the questionnaire survey are shown in table 6. These results refer to the distribution of selected factors related to meat shops including the shop per se and its owner as well.

It was found that 131 (45.9 %) out of 290 shops were selling other meat product(s) apart from pork. In all shops, meat was not wrapped. A great majority (245 out of 290 or 84.5 %) of the shops obtained meat from small household abattoirs, and the rest 15.5 % were supplied by municipal slaughterhouses.

Relating to the origin of meat, meat in most shops (238 out of 290 or 82.1 %) originated from Hanoi itself including urban and sub-urban areas, meat transported from the surrounding provinces such as Bac Ninh, Hai Duong, Ha Tay, etc. accounting for as low as 17.9 % of the shops.

Size of a shop was defined based on its daily sold amount from up-to 50 kg/day to greater than 250 kg/day. The results show that a majority (174 out of 290 or 60 %) of shops sold an average amount ranging from greater than 50 to 100 kg/day each followed by the number of shops that sold up-to 50 kg/day with 78 shops or 26.9 %. There were few shops having greater sold amount of than 150 kg daily, particularly 1 %, 0.7 % and 0.7 % for average amount per day of >150 – 200, >200 – 250 , and >250, respectively.

Table 6. Distribution of selected factors related to meat shops

Factors		No. of shops selected (n)	%
Product(s) offered	Pork only	159	54.10
	Pork and other(s)	131	45.90
Wrapping	Wrapped	0	0
	Non-wrapped	290	100
Type of abattoir	Municipal	45	15.50
	Household	245	84.50
Origin of meat	Hanoi	238	82.10
	Other provinces	52	17.90
Daily sold amount (kg)	Up to 50	78	26.90
	>50 – 100	174	60
	>100 – 150	31	10.70
	>150 – 200	3	1
	>200 – 250	2	0.70
	>250	2	0.70
Residence of owner	Urban Hanoi	111	38.30
	Suburban Hanoi	170	58.60
	Other provinces	9	3.10
Education attainment of owner	Primary	125	43.10
	Junior secondary	132	45.50
	Senior secondary	33	11.40
	Professional	0	0
Sex	Male	8	2.76
	Female	282	97.24
Age (year)	Up to 30	41	14.14
	>30 – 40	139	47.93
	>40 – 50	95	32.76
	>50	15	5.17
Experience (year)	Up to 3	26	8.97
	>3 – 10	158	54.48
	>10	106	36.55

With regard to the owners, the individual profiles such as residence, education attainment, sex, age, and experience years were taken into account. Results in table 6 show that most (170 out of 290 or 58.6 %) shop owners resided in suburban areas of the city, only 9 or 3.1 % came from other provinces, and 111 or 38.3 % had residence in urban areas of Hanoi. Results on the education attainment of the owners indicate that none of the shop owners had experienced a professional training course on meat

and food hygiene or any other professional courses or higher education. All of them attained a highest education level at primary school (43.1 %), junior secondary school (45.5 %), or senior secondary school (11.4 %). Most of the shop owners were female accounting for 282 out of 290 or 97.24 %. Number of owners aging from >30 – 40 years old was highest occupying 139 out of 290 or 47.93 %, followed by groups of >40 – 50 and up-to 30 with 32.76 % and 14 %, respectively. There were few (15 out of290 or 5.17 %) persons older than 50. In terms of number of experience years or seniority, most (54.48 %) of the owners had from more than three to ten years experience in their job. A minority (8.97 %) of them had little experience with not more than three years in their job.

4.1.2. Results of the qualitative analysis

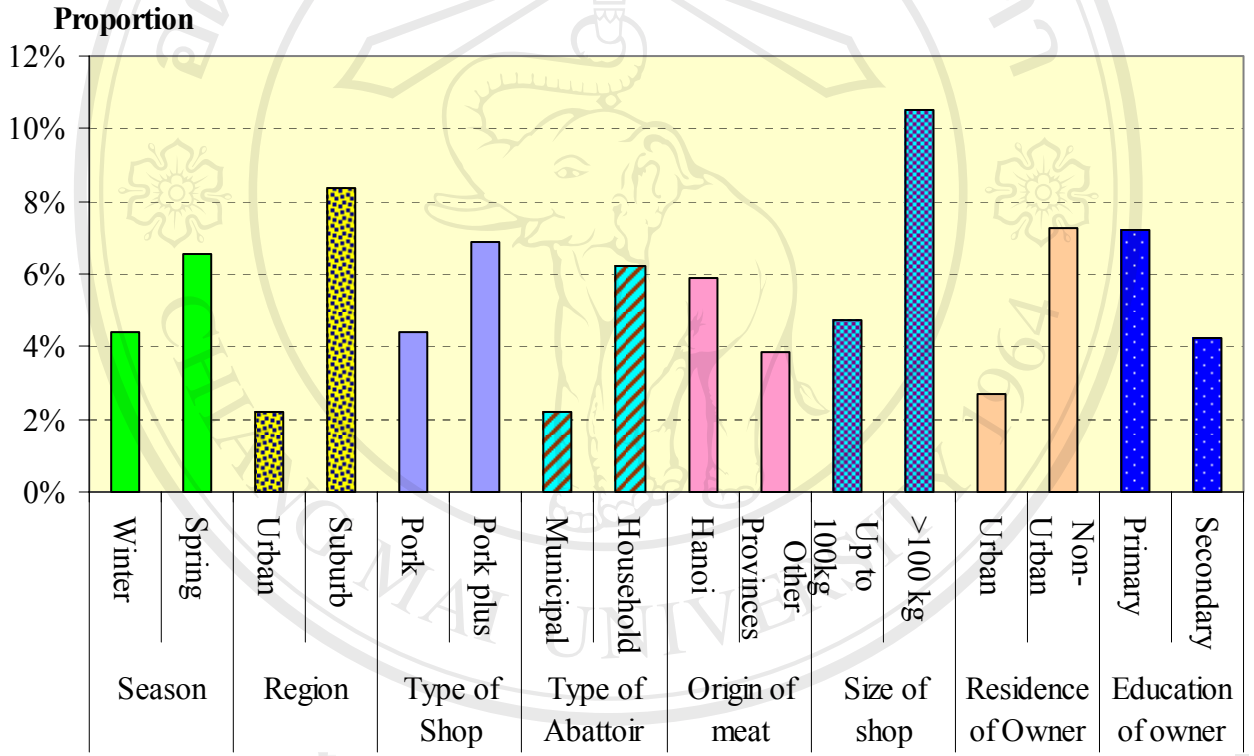
Fifteen samples were positive, and 16 samples were inconclusive for tetracycline residues in the qualitative assay. Of the 16 inconclusive samples, one was confirmed by HPLC. These fifteen positive samples in the qualitative assay and one sample confirmed by HPLC were all defined as positive samples in qualitative analysis to calculate the proportion of tetracycline residues in pork.

The results in table 7 show that 16 samples positive to tetracycline antibiotic residues, or (5.52%) out of total 290 analyzed samples, were detected. The proportion of positive results separated into season, geographical region, type of shop identified by kind of product(s) offered, type of abattoir, origin of meat, daily sold amount or size of shop, owner's residence and education attainment are shown in figure 1.

Table 7. Proportions of Tetracycline-positive muscle samples and analysis of potential risk factors

Variables	No. of samples (n)	No. of Positive samples	% Positive samples	95% CI	Fisher's Exact Test P-value (α = 0.05)	Odds Ratio (95% CI)
Season						
Early Winter	137	6	4.38	[1.79, 9.70]	0.294514	-
Early Spring	153	10	6.54	[3.36, 12.01]		
Region						
Urban	135	3	2.22	[0.58, 6.86]	**0.018414**	**4.03**
Sub-urban	155	13	8.39	[4.72, 14.21]		[1.12, 14.45]
Type of shop						
Pork only	159	7	4.40	[1.94, 9.21]	0.254633	-
Pork and other(s)	131	9	6.87	[3.39, 13.01]		
Type of abattoir						
Municipal	45	1	2.22	[0.13, 14.41]	0.257001	-
Household	245	15	6.22	[3.59, 10.10]		
Origin of meat						
Hanoi	238	14	5.88	[3.38, 9.88]	0.427928	-
Other provinces	52	2	3.85	[0.67, 14.33]		
Daily sold amount						
Up to 100 kg	252	12	4.76	[2.60, 8.38]	0.142307	-
>100 kg	38	4	10.53	[3.43, 25.74]		
Residence of owner						
Urban	111	3	2.70	[0.70, 8.28]	0.078638	-
Non-urban	179	13	7.26	[4.08, 12.36]		
Education attainment of owner						
Primary	125	9	7.20	[3.55, 13.61]	0.201916	-
Secondary	165	7	4.24	[1.87, 8.89]		
Total	290	16	5.52	[3.29, 8.98]	-	-

Figure 1. Proportions of Tetracycline-positive muscle samples by selected factors



4.1.3. Content of tetracycline compounds residues in pork

Fifteen positive and sixteen inconclusive samples from the screening test were analyzed by using HPLC. Tetracycline compounds were found and quantified in only six samples. Results of HPLC confirmation and quantification of tetracycline compounds in pork are shown in table 8 and illustrated in figure 2.

Table 8. Concentration of tetracycline compounds in pork quantified by HPLC

ID No.	Season		Region		Concentration (ppb)			Total	>MRL (100 ppb)
	Win	Spr	Urb	Sub	OTC	TC	CTC		
42	X			X	167.40	-	-	167.40	X
46	X			X	-	63.41	-	63.41	
107	X			X	97.11	-	95.15	192.26	X
15		X		X	-	59.51	-	59.51	
40		X		X	-	51.77	-	51.77	
78		X		X	-	68.14	-	68.14	
		Mean			132.60	60.70	95.15	100.42	

Note:

Win: Winter

Spr: Spring

Urb: Urban

Sub: Suburban

OTC: oxytetracycline;

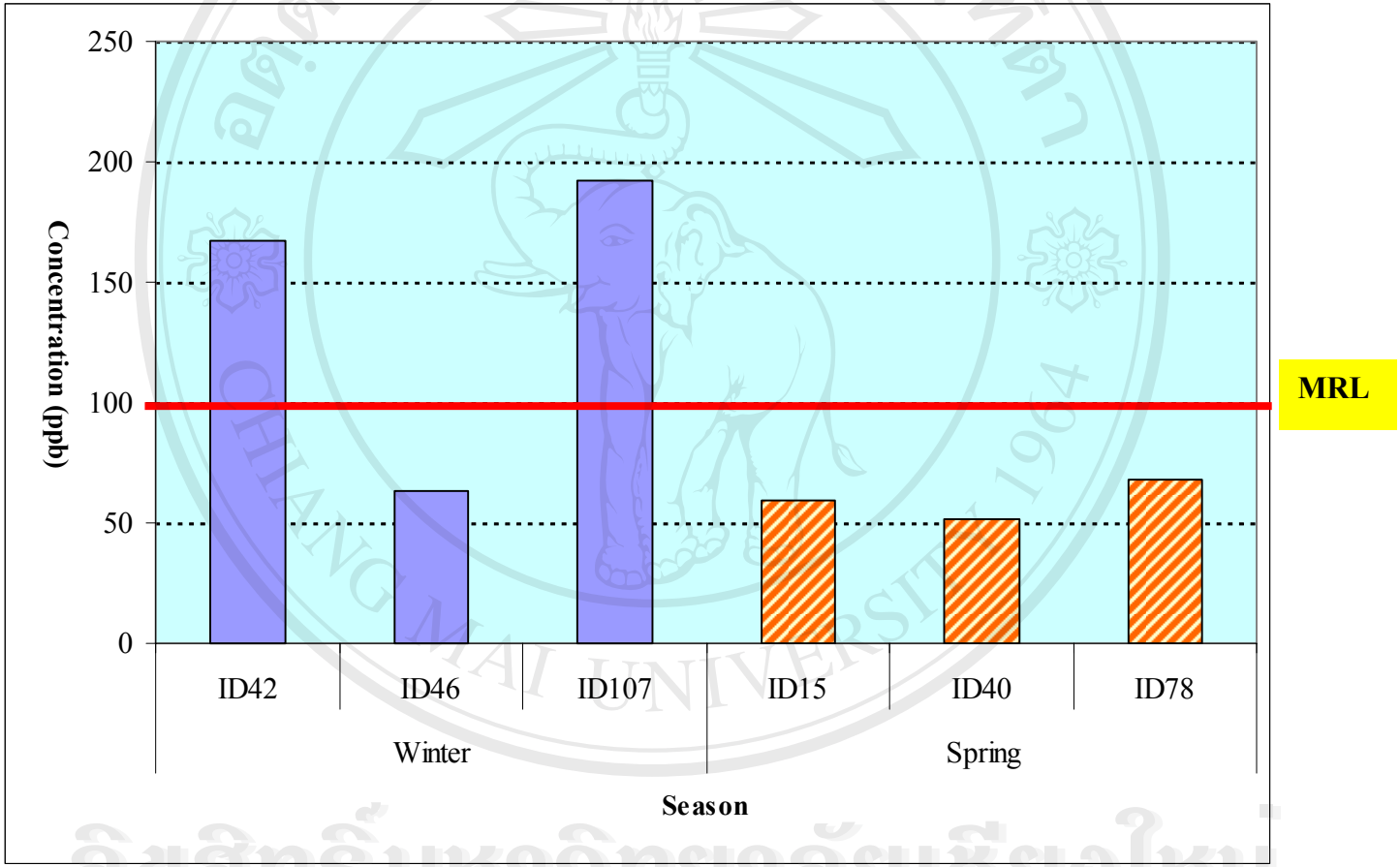
TC: tetracycline;

CTC: chlortetracycline;

X: yes;

- : not detected.

Figure 2. Total concentration of tetracycline compounds corresponding to the MRL



The results indicate that six samples (three in the winter time and the other three in the spring time) were detected containing at least one compound of the tetracycline group with concentrations ranging from 51.77 to 167.40 µg/kg. Particularly, oxytetracycline was identified in two samples with concentrations of 167.40 and 97.11 µg/kg; tetracycline ranging from 51.77 to 68.14 µg/kg in four samples; and chlortetracycline found in only one sample with a concentration of 95.15 µg/kg . Among the six samples, one contained two compounds of tetracycline group namely oxytetracycline and chlortetracycline with 97.11 and 95.15 µg/kg, respectively. Total of these two compounds as 192.26 µg/kg exceeds the MRL for pig muscle. Another one alone contained oxytetracycline alone at concentration of 167.40 µg/kg exceeding the MRL. Thus two out of six samples, in which tetracyclines were found, contained an amount of these substances in combination exceeding the MRL.

4.2. Inferential analysis

Table 7 demonstrates that most differences, except the one between urban and suburban markets, are not significantly associated with antibiotic residue. The significant difference in the antibiotic residue proportions between the two areas – urban and suburban is shown. An odds ratio of 4.03 in this study indicated that raw pork offered on the suburban markets was about four times more likely in being contaminated by antibiotics at detectable levels than those sold on the urban markets.

V. DISCUSSION AND CONCLUSION

5.1. Discussion

5.1.1. Proportion of tetracycline antibiotic residues

A proportion of 5.52% samples indicated positive to only tetracyclines antibiotic residue implies that there might be a much higher proportion of residues of antibiotics. In general, the frequency of utilization of tetracycline group accounts for 7.95% total utilization of antibiotics in pig and chicken production (Thuận *et al.*, 2003). The injudicious use of antibiotics (dosage regimen violation, no observance of appropriate withdrawal time, etc.) is commonly seen at animal farms (Boisseau, 2002; Thuận *et al.*, 2003). In addition, according to Boisseau (2002), a combination of antibiotics is quite popular in the country with 66.3% VMPs containing more than one antimicrobial.

This proportion is much higher one. It was reported that in Western countries such as the UK (Gracey *et al.*, 1999) 44 out of over 12,300 samples collected from pigs contain chlortetracycline. However, this proportion is much lower than one found in Ireland with 42 % and 12 % pork samples detected positive to chlortetracycline for the period 1996/1997 and 1997/1998, respectively (TEAGASC, 2001).

To date, no similar study on the prevalence of tetracycline residues in pork in the country has been reported. However, a study in the southern province of Binh Duong reports proportion of tetracycline residues in incurred (suspicious) pork samples with oxytetracycline detected in 7 (14.6%) and chlortetracycline 6 (12.5%) out of 48 samples (Thuận *et al.*, 2003).

A significant difference between the two selected areas in antibiotic residue proportion was found in this study. An odd ratio (OR) calculated as 4.03 showed a four-time higher likelihood in getting product contaminated with the antibiotic. A

possible explanation for this might be the socio-economic conditions of the two areas. The living standard is usually lower in the suburban area than the urban area. In the suburban, consumers' awareness of food hygiene and safety is generally poorer and a less strict inspection of slaughter and trade of animals are assumed. It might be possible that the shop owners tend to buy cheaper products of live animals. The cheap products might be generally originated from sick or even dead animals that had very likely undergone an antibiotic treatment just before slaughter.

Although there is no statistically significant difference (α =0.05) in antibiotic residue proportion in relation to residence of owner, the P-value of 0.08 that is very close to the significant level ($P \leq 0.05$) may be explained by a fact that most meat shops located in the urban area are owned by people from this area, and similarly for those in suburban area.

5.1.2. Concentration of tetracycline compounds in pork

All the six samples, in which quantities of tetracycline compounds were confirmed and quantified, were collected from the suburban markets. This may support the conclusion above that there is a higher risk of getting antibiotic residues in pork in the suburban areas than getting them in the urban area.

Three tetracycline compounds were identified in samples. This finding may possibly indicate that these three substances were being still used widely in animal production and that the withdrawal time for these antibiotics was not properly observed.

There were two (0.69 %) and four (1.38 %) of 290 samples containing a total amount of tetracyclines above and below the MRL, respectively.

The finding in the present study show that two compounds of the tetracycline group were found in the same sample and perhaps goes in line with other study's finding that the combination of antimicrobials in VMPs is very common and

imprudently used in the country, even two or more substances with the same pharmacologic characteristics are combined in a VMP. Once animals got sick, farmers buy VMPs and administer to the animals without any veterinary prescription and diagnosis (Boisseau, 2002). So, several VMPs may be used at the same time. This potentially results in a likelihood of applying compounds of the same drug family, for example tetracyclines, to an animal.

Ten screening-positive samples and 15 screening-inconclusive samples were not found to contain tetracycline compounds by HPLC analysis. The reason may be the well-known low specificity of the microbial growth inhibition test (Mitchell *et al*., 1998; Gracey *et al.*, 1999; Nouws *et al.*, 1998; Chang *et al.*, 2000). This means that either other antibiotics than tetracyclines or/and that other non-specific antimicrobial factors may be present in the samples.

5.1.3. Experimental design and outcomes of the study

This study followed a regular procedure in analysis of antibiotic residue: a screening followed by a confirmation test. The microbiological inhibition screening test using *Bacillus cereus* as a microbial test strain on agar test pH 6 showed it's effectiveness in separating out a majority of the tetracycline negative samples. As in other studies, in a number of samples generating inhibition zones in the microbiological growth inhibition test, no tetracyclines were demonstrated by the confirmatory method (De Wasch *et al*., 1998).

However, it would be more cost effective if several kinds of antibiotic residues were screened at the same time by using several test organisms and agar media with different pH levels such as 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). The confirmation test by HPLC is a sophisticated method with a very high sensitivity and specificity, and a low detection limit, but expensive in identifying and quantifying tetracycline residues in pork.

The results of the present study somewhat reflect the problem of antibiotic residue in Hanoi. These were limited to only residues of tetracycline compounds in marketed pork in Hanoi area. They do not represent residues of antibiotics in animal products of the whole country. Further studies would provide a more comprehensive view on this matter.

Despite limitations of methods and results in this study, it is recommended that the microbiological inhibition screening test using *Bacillus cereus* on agar test pH 6 coupled with HPLC should be used widely to test tetracycline residues in pork in the country. A similar protocol may be used for testing these substances in other kinds of meat such as beef and chicken. There should be further studies on residues of other commonly used antibiotics such as penicillin, streptomycin, gentamicin, tylosin and sulfonamides in animal products in the city and the whole country.

5.2. Conclusion

1. Out of 290 marketed pork samples, fifteen (5.17 %) were positive and 16 (5.52 %) were inconclusive with the microbial growth inhibition test (a screening test with *Bacillus cereus* as a test strain on test agar pH 6). Out of the 15 screening-positive samples, five were found to contain tetracycline compounds with HPLC method, as well as one of the 16 screening-inconclusive samples. In the suburban regions there is a higher risk to obtain meat with tetracycline residues than in shops in the urban areas.
2. Residues of three compounds of the tetracycline group are found with HPLC method in six (2.07 %) out of 290 marketed pork samples with individual concentrations ranging from 51.77 to 167.40 µg/kg, and sum concentration in each sample ranging from 51.77 to 192.26 µg/kg. Two (0.69 %) of 290 samples contain a sum concentration exceeding the MRL.

REFERENCES

Agence Française de Sécurité Sanitaire des Aliments (AFSSA): "Determination of Tetracycline residues in kidney and muscle by high performance liquid chromatography". MV/ITC//P06/25AN – Version 2.

An, V. T. T, Tuân, N. N., Pho, N. N. (2002): Antibiotics use and residues in chicken in Ho Chi Minh City. *Khoa Học Kỹ Thuật Thú Y (Veterinary Science and Technique)* **9** (2), 53-57.

Apley, M. (2003): How do violative residues happen in swine?. National Pork Board fact sheet, http://www.porkscience.org/documents/Other/PORK%20SAFETY%20FCTSHT-drug%20re.pdf, accessed 2004 Sep 15th.

Barton, M. D. (2000): Antibiotic use in animal feed and its impact on human health. Nutr. Res. Rev. **13** (2), 279-299.

Boatman M. (1998): Survey of Antimicrobial Usage in Animal Health in the European Union. Boatman Consulting, Sept. 1998, by order of FEDESA.

Boisseau, J. (2002): Registration of veterinary drugs containing antimicrobials. Report of the project Strengthening of Veterinary Services in Vietnam (SVSV) ALA/96/20.

Bywater J. (1991): The control of infectious diseases: Chemotherapy. In: Veterinary Applied Pharmacology & Therapeutics, 5th Edition, Balliere Tindall, London.

CAC (2003): Glossary of Terms and Definitions (Residues of Veterinary Drugs in Foods). CAC/MISC 5-1993, Amended 2003.

Carlson, M.S., Fangman, T.J. (2000): Swine Antibiotics and Feed Additives: Food Safety Considerations, http://muextension.missouri.edu/xplor/agguides/ansci/g02353.htm, accessed 2004 Sep 10th.

Chang, C. S., Tai, T.F., Li, H. P. (2000): Evaluating the Applicability of the Modified Four-Plate Test on the Detection of Antimicrobial Agent Residues in Pork. *J. Food and Drug Analysis.* **8**(1), 25-34.

Charm, S. E. and Chi, R. (1988): Microbial receptor assay for rapid detection and identification of seven families of antimicrobial drugs in milk: collaborative study. *J. Assoc. Off. Anal. Chem*. **71**, 304-316.

Cherlet, M., Schelkens, M., Croubels, S., Backer, P. D. (2003): Quantitative multi-residue analysis of tetracyclines and their 4-epimers in pig tissues by high performance liquid chromatography combined with positive-ion electrospray ionization mass spectrometry. *Analitica Chimica Acta* **492**, 199-213.

DeWasch, K., Okerman, L., DeBrabender, H., VanHoof, J., DeBacker, P. (1998): Detection of residues of tetracycline antibiotics in pork and chicken meat: correlation between results of screening and confirmatory tests. *Analyst* **123**, 2737-2741.

DeVore, B. (2002): Antibiotics, Agriculture & Resistance. *The Land Stewardship Letter*, 1-4, http://www.landstewardshipproject.org/pdf/antibio_reprint.pdf, accessed 2005 Jun 15th.

EMEA (1999): Antibiotic resistance in the European Union associated with therapeutic use of veterinary medicines. Report and qualitative risk assessment by the Committee for Veterinary Medicinal Products (CVMP). London: EMEA/CVMP/342/99-corr-Final.

EMEA (1995): Oxytetracycline, Tetracycline, Chlortetracycline. Summary Report (3) by the Committee for Veterinary Medicinal Products (CVMP). London: EMEA/MRL/023/95.

FAO (1997): Residues of some veterinary drugs in animals and foods: The chlortetracycline and tetracycline monographs prepared by the 47th Joint FAO/WHO Expert Committee on Food Additives). FAO Food and Nutrition Paper 41/9.

FAO (2004): Review of the livestock sector in the Mekong countries. Livestock sector report, http://www.fao.org/ag/againfo/resources/en/publications/sector_reports/lsr_mekong.pdf, accessed 2004 Sep 16th.

Forth, W., Henschler, D., Rummel, W. (1983): Allgemeine und spezielle Pharmakologie. Bibl. Institut, Mannheim, p. 566-570.

GAO (1999): The Agricultural Use of Antibiotics and Its Implications for Human Health. Food Safety. GAO/RCED-99-74.

Gaudin, V., Maris, P., Fuselier, R., Ribouchon, J. L., Cadieu, N., Rault, A. (2004): Validation of a microbiologicacl method: the STAR protocol, a five-plate test, for the screening of antibiotic residues in milk. *J. Food Additives and Contaminants*. **21**(5), 422-433.

Goodman, G.A., Goodman, L.S., Rall, T.W., Murad, F. (Eds.) (1985): The Pharmacological Basis of Therapeutics. Seventh ed., Mac-Millan, New York.

Gracey, J. F., Collins, D. S., Huey, R. J. (1999): Meat hygiene. 10th ed. London: Harcourt Brace and Company.

Hirsch, R., Ternes, T., Haberer, K., Kratz, K. L. (1999): Occurrence of antibiotics in the aquatic environment. *The Science of the Total Environment* **225**, 109-118.

Huber, W.G. (1971): The impact of antibiotic drugs and their residues. *Adv. Vet. Sci. Comp. Med.* **15**, 101-132.

JETACAR – Joint Expert Technical Advisory Committee on Antibiotic Resistance (1999): The use of antibiotic in food-producing animals: antibiotic-resistant bacteria in animals and humans. Canberra: Commonwealth of Australia, http://www.health.gov.au/internet/wcms/Publishing.nsf/Content/health-pubs-jetacar.htm, accessed 2005 Jul 20th.

Johnston, A. (1998): Use of antimicrobial drugs in veterinary practice. *Br. Med. J.* **317**, 665-667.

Kim Liên (2004): Hà Nội vẫn khan hiếm thịt sạch, http://www.vneconomy.com.vn/vie/index.php?param=article&catid=0999&id=040706154906, accessed 2004 Jul 24th. Báo điện tử - Thời báo Kinh tế Việt Nam (Vietnam Economy) updated 06/07/2004.

Kühne, M., Wegmann, S., Kobe, A., Fries, R., (2000): Tetracycline residues in bones of slaughtered animals. *Food Control*, **11**, 175-180.

Lee, H. J., Lee, M. H., Ryu, P. D., Lee, H., Cho, M. H. (2001): Enzyme-linked immunosorbent assay for screening the plasma residues of tetracycline antibiotics in pigs. *J. Vet. Med. Sci.* **63**(5), 553-556.

Lindsay, S. (1992): History and basic principles. In J. Barnes (ed.): High Performance Liquid Chromatography. John Wiley and Sons, Toronto, Ontario. pp. 1-7.

MAF (2003): Recommendations for the use of antimicrobial agents in the treatment of the most significant infectious diseases in animals. The Ministry of Agriculture and Forestry. Memorandum 2003:9a. Helsinki, Finland.

Mạnh, N. T, Toàn, N. M. (2003): Vì sao lợn Việt Nam có sức cạnh tranh thấp, http://www.vista.gov.vn/Anphamdientu/tapchitrongnuoc/hdkh/2003/so10/17.htm, accessed 2004 Aug 28th.

Medicinenet (1998): Tetracycline, http://www.medicinenet.com/tetracycline/article.htm, accessed 2005 Jul 15th.

Medicinenet (2004): Dangerous Drug for Baby during Pregnancy, http://www.medicinenet.com/script/main/art.asp?articlekey=9337, accessed 2005 Jul 15th.

Miller, D.J.S. (1993): Present state and trends in the use of veterinary drugs. Proceedings of the EuroResidue II conference, Veldhoven, The Netherlands, 3-5 May.

Mitchell, J., Griffiths, M.W., McEwen, S.A., McNab, W.B., Yee, A.J. (1998): Antimicrobial drug residues in milk and meat: causes, concerns, prevalence, regulations, tests, and test performance. *J. Food Protection* **61**(6), 742-56.

Mitchell, M., Bodkin, B., Martin, J. (1995): Detection of beta-lactam antibiotics in bulk tank milk. *J. Food Prot*. **58**, 577-578.

Muriuki, F.K., Ogara, W.O., Njeruh, F.M., Mitema, E.S. (2001): Tetracycline residue levels in cattle meat from Nairobi slaughter house in Kenya. *J. Vet. Sci.* **2**(2), 97-101.

Myllyniemi, A. L. (2004): Development of microbiological methods for the detection and identification of antimicrobial residues in meat. Helsinki: University of Helsinki, Faculty of Veterinary Medicine, Dissertation.

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

Nielsen, P., Hansen, G. P. (1996): Bioavailability of oxytetracycline, tetracycline and chlortetracycline after oral administration to fed and fasted pigs. *J. Vet. Pharmacol. Ther.* **19**, 305-311.

Nouws, J. F. M., Loeffen, G., Schouten, J., Egmond, H. V., Keukens, H., Stegeman, H. (1998): Testing of raw milk for tetracycline residues. *J Dairy Sci.* **81**(9), 2341-5.

O'Rangers, J. J. (1993): Utilization of new screening tests for animal drug residues detection. Proceedings of the EuroResidue II Conference, Veldhoven, The Netherlands, 3-5 May.

Paige, J. C. (1994): Analysis of tissue residues. *FDA Vet*. **9**(6), 4-6.

Pilar Vinas, Nuria Balsalobre, Carmen López-Erroz, Manuel Hernández-Córdoba (2004): Liquid chromatography with ultraviolet absorbance detection for the analysis of tetracycline residues in honey. *Chromatogr. A* **1022**, 125-129.

Prescott, J.F., Baggot, J.D. (1988): Antibiotic residues. In: Antimicrobial therapy in veterinary medicine. Boston: Blackwell Scientific Publications, Inc. pp. 341-346.

Sande M.A., Mandell, G.L. (1985): Chemotherapy of Microbial Diseases. In: The Pharmacological Basis of Therapeutics, 7th Edition, Macmillan Publishing Co.

Sczesny, S., Nau, H., Hamscher, G. (2003): Residue analysis of tetracyclines in the environment by HPLC coupled with a microbiological assay and tandem mass spectrometry. *J. Agric. Food Chem*. **51**, 697-703.

Shaikh, B. (1993): Liquid chromatographic analysis of antibacterial drug residues in food products and animal origin. *J. Chromatogr*. **643**, 369-378.

SOP RES 31 V.8 (2002): The detection of residues of anti-bacterial substances in animal tissues (six plate method). Veterinary Sciences Division, Stoney Road, Stormont, BELFAST BT4 3SD.

TEAGASC (2001): Food Residue Database. Irish Agriculture and Food Development Authority, http://www.teagasc.ie/research/reports/foodprocessing/4548/eopr-4548.htm, accessed 2004 Jul 17th.

Thuận, D. T., Tuân, N. N., An, V. T. T, Hiền, L. T., Lâm, V. B., Ninh, K. T. (2003): Initial survey on antibiotics utilization in farms and its residue in pork and chickens raised in Binh Duong province. *Khoa Học Kỹ Thuật Thú Y (Veterinary Science and Technique),* **10** (1), 50-58.

Tillbaka (2001): Examination of residues in live animals and animal products: Results of the control 2000. National Food Administration, http://www.slv.se/upload/dokument/Rapporter/Lakemedel/slvrapp6_2001_residues.pdf, accessed 2004 Aug 29th.

Tuan, X. L., Munekage, Y. (2004): Residues of selected antibiotics in water and mud from shrimp ponds in mangrove areas in Viet Nam. *Marine Pollution Bulletin.* **49**, 922-929.

VietnamNet (2004): Hanoi: population exceeds 3mil, http://english.vietnamnet.vn/social/2004/12/352674/, accessed 2005 Jul 27th.

Vietnamtourism (2005): Vietnam country and people: Climate, http://www.vietnamtourism.com/e_pages/country/overview.asp, accessed 2005 Jun 17th.

Walter, J. Veith (2005): Dietary research: Why No Animal Products, http://www.amazingdiscoveries.org/amazingdiet/modern_animal_husbandry.htm , accessed 2005 Jun 13th.

WHO (2001): Antibiotic Resistance: synthesis of recommendations by expert policy groups. Alliance for the Prudent Use of Antibiotics. WHO/CDS/DRS/2001.10.

Wikipedia Encyclopedia (2004): Tetracycline, http://encyclopedia.thefreedictionary.com/tetracycline, accessed 2004 Aug 25th.

Zurhelle, G., Seitz, E. M., Petz, M. (2000): Automated residue analysis of tetracyclines and their metabolites in whole egg, egg white, egg yolk and hen's plasma utilizing a modified ASTED system. *J. Chromatogr. B* **739**, 191-203.

APPENDICES

Appendix 1

QUESTIONNAIRE

(for butchers/meat shops)

NOTE:

1. *This questionnaire is designed for a survey on butchers (meat shops) in Hanoi at which meat (muscle) samples are collected for* ***study purpose only****.*
2. *Data and information gathered via this survey are maintained* ***confidential****.*
3. *There is* ***only one appropriate answer*** *to each question unless otherwise specified.*

1- Date of sampling: ……

2- Location (district): ……

3- Wrapping of products:

….. wrapped

…… non-wrapped

4- Type(s) of meat offered at the same shop (more than one type may be selected)

……pork

……beef

……chicken

……processed meat product(s)

5- Origin of meat:

…….slaughterhouse of the city (specify)……

…….private small abattoir(s) in Hanoi

…….province (specify)……..

6- Estimated amount of meat (pork) sold a day……kg

7- Owner's name: ……

8- Residence:

…..inner-city

…..suburb area

…..other province (specify)……

9- Sex:

……..Male

……..Female

10- Age ……..

11- Educational/professional attainment:

…… primary school

…….junior secondary school

…….senior secondary school

…….high school/professional training course

12- Number of experienced years in business: ……years

……..

Thank you!

Appendix 2

METHOD FOR DETERMINATION OF TETRACYCLINE RESIDUES IN MEAT

(According to the standard of Agence Française de Sécurité Sanitaire des Aliments (AFSSA) for "Determination of Tetracycline residues in kidney and muscle by high performance liquid chromatography")

1- SCOPE AND FIELD OF APPLICATION

The present method allows the determination of the residues of four compounds and their epimers, oxytetracycline (OTC) and epi-oxytetracycline (epi-OTC), tetracycline (TC) and epi-tetracycline (epi-TC), chlortetracycline (CTC) and epi-chlortetracycline (epi-CTC) and doxycycline (DC). It is suitable for pork and bovine kidney and muscle. The detection limits in kidney are 80 µg/kg, 60 µg/kg, 170 µg/kg and 160 µg/kg for OTC, TC, CTC and DC respectively and 8 µg/kg, 9 µg/kg, 15 µg/kg and 12 µg/kg for OTC, TC, CTC and DC in muscle according to the criteria of the decision 93/256/EEC. Fortified samples from 300 µg/kg to 2400 µg/kg in kidney and from 50 µg/kg to 400 µg/kg in muscle have been analyzed in accordance with the criteria of this decision. Maximum residue limits for the sum of each tetracycline and its epimer (excluding epi-doxycycline) have been set up at 600 µg/kg in kidney and 100 µg/kg in muscle.

2- PRINCIPLE

There are three principal stages in the samples preparation:

- Homogenization and extraction of the sample residues by EDTA/Mc Ilvain buffer,
- Precipitation of proteins using trichloroacetic acid and filtration,
- Cleanup on solid-phase extraction cartridges C_{18} and injection.

Tetracyclines are separated on a C_{18} stationary phase and detected by UV absorption at 355 nm. The amount of tetracycline is calculated by interpolation from a calibration curve determined for each of the 4 compounds, taking into account the calculated recovery.

3- CHEMICALS AND REAGENTS

Unless otherwise specified, all reagents are of analytical grade. Demineralized water is obtained from an ultra pure water system (Millipore).

3.1- Acetonitrile (Merck, Art. 1.14291).

3.2- Methanol (Merck, Art. 1.06009).

3.3- Trichloroacetic acid (Prolabo, Art. 20742-293): dissolve 50 g in 50 ml ultra pure water to obtain a 1g/ml solution.

3.4- Oxalic acid dehydrate (Prolabo, Art. 28582.291): dissolve 1.20 g in 1 l ultra pure water to obtain a 0.01 M solution. Filter through a 0.45 µm unit under vacuum (4.1.20).

3.5- 0.01 M oxalic acid solution in methanol: dissolve 1.26 g in 1 l methanol.

3.6- Citric acid monohydrate (Merck, Art. 244): dissolve 21 g in 1 l ultra pure water.

3.7- Disodium phosphate anhydrous (Merck, Art. 1.06586): dissolve 28.4 g in 1 l ultra pure water.

3.8- Disodium ethylenediaminetetraacetate dehydrate (EDTA), (Prolabo 20 302 293).

3.9- Mc Ilvain buffer: mix 1 l citric acid solution (3.6) with 625 ml disodium phosphate solution (3.7) and adjust pH to 4.0 ± 0.05 if necessary.

3.10- Mc Ilvain buffer/ETDA solution: prepare a 0.1 M ETDA solution in Mc Ilvain buffer (60.5 g ETDA in 1.625 l).

3.11- Mobile phase: acetonitrile (3.1) and 0.01 M oxalic acid (3.4) in a gradient mode.

3.12- Standards:

3.12.1- Oxytetracycline hydrochloride, potency (Pfizer).

3.12.2- Tetracycline hydrochloride (Virbac).

3.12.3- Chlortetracycline hydrochloride (Vetoquinol).

3.12.4- Doxycycline hyclate (Veprol).

3.12.5- Epi-oxytetracycline (Acros).

3.12.6- Epi-tetracycline (Acros).

3.12.7- Epi-chlortetracycline (Acros).

3.13- Stock solutions:

N.B.: because interferences may occur between some of the standards, they will not be injected simultaneously for the quantification. For example, chlortetracycline standard contains tetracycline. Each stock solution will be prepared with two tetracyclines as here under.

3.13.1- OTC and CTC stock standard solution: prepare a methanolic solution containing 1 mg/ml of OTC and CTC (+ their respective epimer if these epimers are contained in the standard).

3.13.2- TC and DC stock standard solution: prepare a methanolic solution containing 1 mg/ml of TC and DC (+ their respective epimer if these epimers are contained in the standard).

3.13.3- Epi-OTC, epi-TC and epi-CTC stock standard solution: prepare 3 methanolic solutions containing each 1 mg/ml of epimer.

3.14- Working solutions for analysis of kidney samples:

3.14.1- Two 100 µg/ml intermediate solutions are obtained by diluting the two stock solutions (3.13.1- and 3.13.2-) with methanol. These solutions can be stored two weeks at $+4^0$C

3.14.2- Working solutions are obtained by diluting each of the two intermediate solutions (3.14.1) with 0.01 M oxalic acid in methanol/water solution (30/70) to obtain concentrations of 0.75; 1.5; 3 and 6 µg/ml. These solutions are prepared freshly every day in amber flasks.

3.14.3- Working solutions containing 1 µg/ml of each epimer are obtained by diluting each of the stock standard solutions (3.13.3) with 0.01 M oxalic acid in methanol/water solution (30/70). These solutions will be used only for epimers identification and not for quantification. The amount of tetracycline + the corresponding epimer contained in a kidney sample is calculated by comparison with the standard of tetracycline only.

3.15- Working solutions for the analysis of muscle samples

3.15.1- Two 50 µg/ml intermediate solutions are obtained by diluting the two stock solutions 3.13.1- and 3.13.2- with methanol. These solutions can be stored two weeks at $+4^0$C

3.15.2- Working solutions are obtained by diluting each of the two intermediate solutions (3.15.1) with 0.01 M oxalic acid in methanol/water solution (30/70) to obtain concentrations of 0.125; 0.25; 0.5 and 1 µg/ml. These solutions are prepared freshly every day in amber flasks.

3.15.3- Working solutions containing 1 µg/ml of each epimer are obtained by diluting each of the stock standard solutions (3.13.3) with 0.01 M oxalic acid in

methanol/water solution (30/70). These solutions will be used only for epimers identification and not for quantification. The amount of tetracycline + the corresponding epimer contained in a kidney sample is calculated by comparison with the standard of tetracycline only.

3.16- Control kidney

3.17- Control muscle

3.18- Spiking solutions

3.18.1- Spiking solutions for kidney samples

Spiking solutions of 6 µg/ml are obtained by diluting the two stock solutions with ultra pure water. These solutions can be stored at $+4^0$C for 24 hours.

3.18.2- Spiking solutions for muscle samples

Two 100 µg/ml intermediate solutions are obtained by diluting the two stock solutions (3.13.1 and 3.13.2) with methanol. Spiking solutions of 1 µg/ml are obtained by diluting with the hundredth these two intermediate solutions. These solutions can be stored at $+4^0$C for 24 hours.

3.19- Spiked control samples

The control samples allow to calculate the recovery and ensure the quality of the analysis.

3.19.1- Kidney spiked samples

Prepared fortified kidney samples by adding 500 µl of spiking solutions (3.18.1) to 5g of control kidney (3.16) to obtain a spiking level of 600 µg/kg. Stir 30 seconds. The kidney sample is frozen until analysis.

3.19.2- Muscle spiked samples:

Prepare fortified muscle samples by adding 500 µl of spiking solutions (3.18.2) to 5g of control muscle (3.17) to obtain a spiking level of 100 µg/kg. Stir 30 seconds. The muscle sample is frozen until analysis.

4. APPARATUS

4.1- Laboratory equipment

4.1.1- Polypropylene centrifuge tubes, 50 ml capacity, with caps.

4.1.2- Glass tubes, 30 ml capacity.

4.1.3- Polypropylene tubes, 5 ml capacity.

4.1.4- Amber volumetric flasks, 25 ml, 50 ml, 100 ml, 200 ml and 1000 ml.

4.1.5- Graduated glass pipettes, 2 ml, 5 ml, 20 ml and 25 ml.

4.1.6- Automatic pipettes type Gilson P1000.

4.1.7- Blender type moulinette (Moulinex).

4.1.8- Analytic and precision balance model PB302 (Mettler – Toledo).

4.1.9- High precision analytic balance type A120S (Sartorius).

4.1.10- Solvent dispensers (Brandt).

4.1.11- pH-meter (Tacussel).

4.1.12- Electric stirrer type vortex (Bioblock).

4.1.13- Rotary stirrer type Rheax 2 (Heidolph).

4.1.14- Magnetic stirrer type Nuova II (Bioblock).

4.1.15- Cooled centrifuge model GR 4.22 (Jouan).

4.1.16- Solid phase extraction cartridges Bond-Elut C18, 3 cc, 200 mg (Varian).

4.1.17- Solid phase extraction manifold (Supelco), adaptors, needles (Analytichem).

4.1.18- Vacuum pump, 0.4 bar, 12 w (Bioblock).

4.1.19- Whatman disposable filter funnels, 25 mm diameter (Whatman, Art. 1922-1800) or 50 ml reservoirs containing these same filters.

4.1.20- Membrance filter holder with filter paper model HVLP 0.45 μm (Millipore).

4.1.21- Refrigerated ultra-speed centrifuge model MR 1822 (Jouan)

4.2- High Performance Liquid Chromatography equipment

4.2.1- Series 1050 quaternary gradient pump (Hewlett Packard).

4.2.2- Series 1050 UV-VIS detector (Hewlett Packard).

4.2.3- Vectra 486/66VL computer (Hewlett Packard) and HPLC 2D Chemstation software.

4.2.4- Series 1100 autosampler (Hewlett Packard).

4.2.5- Analytical column: Purospher RP 18-e, 5 μm, 4 x 4 mm I.D. guard column (Merck).

5. STORAGE OF SAMLES AND SAMPLING

Sample must be stored at about -20^{0}C. They must be thawed just before the analysis and then ground (4.1.7).

6. PROCEDURE

NB: Tetracyclines are sensitive to light. Care must be taken to protect solutions from light during the manipulations.

6.1- Extraction

6.1.1- Weigh out 5 ± 0.1 g of ground kidney or muscle into a centrifuge tube (4.1.1).

6.1.2- Add 25 ml Mc Ilvain buffer/ETDA solution (3.10) and stir for about 30 s (4.1.12).

6.1.3- Stir for 15 min at 100 rpm with the rotary stirrer (4.1.13).

6.1.4- Centrifuge 10 min at 4000 g about 4^0C. Do not leave the samples for a long time in this state because of problems of stability.

6.2- Proteins precipitation

6.2.1- Transfer the supernatant in a glass tube (4.1.2), place this tube in a beaker on the magnetic stirrer (4.1.14).

6.2.2- Add slowly 2.5 ml of 1 g/ml trichloroacetic acid solution (3.3) with constant stirring. Then stir more rapidly for a further 1 min. Remove the magnetic stirrer.

6.2.3- Centrifuge 5 min at about 3000 g.

6.3- Cleanup

6.3.1- Activate the cartridge Bond Elut with 1 ml methanol, 1 ml ultra pure water and 1 ml Mc Ilvain buffer. (3.9).

6.3.2- Connect a filter funnel or a reservoir containing a filter (4.1.19) to the cartridge.

6.3.3- Transfer the sample solution into the funnel and pull it through the filter with the vacuum pump (4.1.18) at a flow rate of no more than 2 drops/s. Do not allow the cartridge to dry at this step.

6.3.4- Flush the cartridge with 1 ml ultra pure water.

6.3.5- Dry the cartridge for 5 min using the vacuum pump.

6.3.6- Remove the filter and elute slowly with 1 ml 0.01 M oxalic acid in methanol (3.5) and next with 1 ml ultra pure water into a polypropylene tube (4.1.3).

6.3.7- The samples are centrifuged 3 min at 20,000 g at about 4^0C before injection of a 100 µl volume into the chromatographic system.

6.4- Chromatographic conditions

6.4.1- Gradient mobile phase

Time	Acetonitrile, %	0.01 M oxalic acid, %
0 min	13	87
15 min	36	64
Post-time: 5 min.		

6.4.2- Flow rate: 0.8 ml/min.

6.4.3- UV detector wavelength: 355 nm.

N.B.: in case of chlortetracycline analysis, the wavelength can be set at 375 nm, which is the more adjusted wavelength for chlortetracycline detection.

6.4.4- Retention times:

Epi-oxytetracycline: 5.9 min
Oxytetracycline: 6.0 min
Epi-tetracycline: 6.1 min
Tetracycline: 7.1 min
Epi-chlotetracycline: 9.3 min
Chlortetracycline: 10.8 min
Doxycycline: 12.1 min

7- CALCULATION OF RESULTS

The following calculations can be executed directly by the HPLC 2D Chemstation software.

7.1- Derive the calibration curve from the results obtained with the working standards solutions. Peaks corresponding to the tetracyclines and to their respective epimer have to be taken into account if possible. Then, determine the curve equation:

$y = ax + b$

y = peak area (TC + epimer)

x= concentration (ng/ml)

a = slope

b = intercept

7.2- Calculation of the recovery:

This result is obtained from the spiked control sample.

Determine the control sample final concentration (Cf) using the curve equation (7.1) as:

$$Cf = \frac{Yf - b}{a}$$

Cf = final concentration of the injected extract

Yf = tetracycline peak area + epi-tetracycline peak area

a = slope

b = intercept

Calculate the recovery as:

$$R = \frac{Cf}{F.Ct}$$

R = recovery

Cf = final concentration of the injected extract determined above

Ct = true concentration or spiking concentration (600 µg/kg = MRL)

F = concentration factor (2.5 in this case).

Check the quality of the analysis: this last is validated if the calculated recovery is in accordance with the limits establishing during the method validation:

$Rm - 3.SD \leq R \leq Rm + 3.SD$

R = recovery

Rm = mean recovery determined during validation

SD = standards deviation of the mean recovery

Calculate the concentration of tetracycline + epi-tetracycline present in the sample to be analyzed (Ca) using the calibration curve and taking account the calculated recovery:

$$Ca = \frac{Cf}{F} \times \frac{1}{R}$$

Ca = concentration of tetracycline + epi-tetracycline present in the sample to be analyzed.

Cf = final concentration of the injected extract.

R = recovery

F = concentration factor (2.5 in this case).

CURRICULUM VITAE

1. Personal data

- Name: Duong Van Nhiem
- Date of birth: 28th October 1970
- Nationality: Vietnamese
- Marital status: Married
- Home address: Tu The (village) – Tri Qua (commune)
THUAN THANH – BAC NINH - VIETNAM
Tel.: (+84) 0241.866 524
Mobile: (+84) 0915.086 521
Email: dvnhiem@yahoo.com

2. Present working place: Hanoi Agricultural University (HAU)
Faculty of Animal Science and Veterinary Medicine (FASVM)
GIALAM – HANOI - VIETNAM
Tel.: (+84) 04.8768 270
Fax: (+84) 04.8276 653; 04.8276 554
E-mail: dvnhiem@yahoo.com

- Work position: Lecturer
- Work experience: May 1995 – present: Lecturer in the subject "Veterinary Inspection", FASVM, HAU

3. Education background:

1976 – 1981	Primary school in Bac Ninh province
1981 – 1984	Junior Secondary school in Bac Ninh
1984 – 1987	Senior Secondary school in Bac Ninh
1989 – 1994	Bachelor of science in Veterinary Medicine, Faculty of Animal Science and Veterinary Medicine, Hanoi Agricultural University, VIETNAM
2000 – 2002	Master's degree in Development Management, University of the

Philippines at Los Baños, Philippines

4. Foreign language: English

5. Professional training: 1- FAO-sponsored Training course on Processing of low cost meat products (University of Agriculture and Forestry, Ho Chi Minh City, Vietnam, July – August 1997.

2- The fourth OIE/FAO-APHCA Workshop on WTO's Sanitary and Phyto-sanitary (SPS) Agreement (Chiang Mai University, July 2004).

DECLARATION

I, the undersigned, declare that the thesis is my original work and has not been presented for a degree in any university.

Name: **Duong Van Nhiem**

Signature.....

Date of submission.....