EVALUATION OF THE PERFORMANCE OF *Bacillus cereus* AND *Bacillus subtilis* AS TEST ORGANISMS FOR ASSAY OF TETRACYCLINES AND BETA-LACTAMS IN CHICKEN MEAT

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DECLARATION AND RECOMMENDATION

Declaration

This thesis is my original work and has not been presented, wholly or in part, for an award of degree in any other university.

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SM14/1878/07

Signed:……. Date:…….

Recommendation

We wish to confirm that this thesis was carried out under our supervision and has our approval to be presented for examination as per the Egerton University regulations.

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DEDICATION

This work is dedicated to my father Stanley Mwangi and my mother Mary Mwangi for being a great source of inspiration and encouragement. Mum and dad, you made this happen.

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ABSTRACT

The uncontrolled and unrestricted use of antimicrobials may lead to the accumulation of undesirable drug residues in the treated animals and their products. To avoid adverse effects from antibiotic residue consumption such as drug resistance, regulations such as maximum residue limits (MRLs), acceptable daily intakes (ADIs) for humans and withholding times for pharmacologically active substances have been set. However, in the Kenyan poultry industry there lacks affordable, easy to perform antibiotic residues screening methods. The aim of this study was to evaluate the performance of *Bacillus cereus* and *Bacillus subtilis* as test organisms for assay of tetracyclines and beta-lactam antibiotics in chicken meat. Microbiological detection was achieved by agar well diffusion using 24 combinations of the two test bacteria, three medium pH, two poultry organs and two antibiotics. The test bacteria grew optimally at a pH range of 6-7.3. Inhibition zones increased significantly ($p<0.001$) when both test organisms were used to detect oxytetracycline (OTC) at a decreasing pH in both liver and kidney tissues. Zone diameters decreased significantly ($p<0.001$) when *B. subtilis* was used to detect penicillin G (PEN G) at decreasing pH from 7.3, 7.0 to 6. The zones increased significantly ($p<0.001$) when *B. cereus* was used to detect the same antibiotic in both kidney and liver samples at decreasing pH. The inhibition zone means were significantly ($p<0.001$) different when the effect of the organism was tested. Moreover *B. cereus* was more sensitive to OTC while *B. subtilis* for PEN G. There was significant ($p<0.001$) difference in the detection of PEN G in both kidney and liver samples at the different pH values although high antibiotic concentrations produced zones that were not significantly different. The inhibition zones differed significantly ($p<0.001$) at all OTC concentrations tested in kidney and liver. It was concluded that the pH of the growth media influences the growth of test organisms. Although OTC and PEN G were reliably detected below the MRLs, of 50ng/g in both liver and kidney for PEN G and 600ng/g in liver and 1200ng/g in kidney for OTC, by *B. cereus* and *B. subtilis* the pH of the growth media, type of organ and the test organism affected both the detection and the LODs of these antibiotics. *Bacillus subtilis* plate at pH 7.3 and a *B. cereus* plate at pH 7 can be used effectively for routine screening for residues of PEN G and OTC respectively in chicken kidney and liver tissues. The *B. subtilis* plate had LOD of 0.1ng/ml in both liver and kidney tissues. The LODs were 131.3ng/ml and 33.4ng/ml, in liver and kidney respectively, on the *B. cereus* plate. This screening test is technically simple and can be carried out in any laboratory.

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LIST OF ABBREVIATIONS

ADI	Acceptable daily intake
BCOK	*Bacillus cereus* oxytetracycline kidney
BCOL	*Bacillus cereus* oxytetracycline liver
BCPK	*Bacillus cereus* penicillin G kidney
BCPL	*Bacillus cereus* penicillin G liver
BSOK	*Bacillus subtilis* oxytetracycline kidney
BSOL	*Bacillus subtilis* oxytetracycline liver
BSPK	*Bacillus subtilis* penicillin G kidney
BSPL	*Bacillus subtilis* penicillin G liver
CAC	Codex Alimentarius Commission
CTC	Chlorotetracycline
GC-MS	Gas chromatography-mass spectrometry
HPLC	High performance liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LOD	Limits of detection
MHA	Mueller Hinton agar
MIC	Minimum inhibitory concentration
MRL	Maximum residue limit
OTC	Oxytetracycline
PEN G	Penicillin G
TC	Tetracycline

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CHAPTER ONE
INTRODUCTION

1.1 Background

The availability of a ready market for broiler meat, low capital requirement, minimal space requirement, proximity to hatcheries and the availability of a wide selection of animal feeds in urban areas are some factors that have contributed to the increased popularity of broiler meat production. This represents an increased opportunity to generate income (MOA, 2001).

Various antimicrobials are usually administered via feed or drinking water for the prevention and treatment of infectious diseases in poultry such as infectious coryza, mycoplasmosis and fowl cholera among others. They have also been used to enhance feed efficiency, promote growth and improve productivity (Mitema *et al*., 2001; Hermes, 2003; Gaudin *et al*., 2004; Bergwett, 2005). In particular, broiler chicken are grown actively often with antibiotics to attain maximum weight within a short period of time (Nonga *et al*., 2009). The uncontrolled and unlimited use of these antibiotics may however lead to the accumulation of undesirable residues in the treated animals and their products. A residue is any substance that is left behind in the tissue of an animal which has been treated with the substance. Drug residues are as a result of the failure of the elimination of the drug from the edible portions of the animal because withdrawal period was not observed. These residues may have adverse heath effects on both the animals and human beings (Lee *et al.*, 2001; McCracken *et al.*, 2005).

The occurrence of antibiotic residues in foods causes adverse public health effects such as drug resistance and hypersensitivity that could be life threatening (Waltner-Toews and McEwen, 1994; Khachatourians, 1998). To protect the public against possible health risks caused by such hazards, regulations regarding veterinary use of drugs including withholding periods after antibiotics therapy and tolerance levels have been formulated (WHO/FAO, 1999) and followed in developed countries (Lee *et al.*, 2001; Donoghue, 2003). However, such regulations are not usually adhered to, especially in developing countries where routine monitoring of drug residues in food is not done (Shitandi and Sternesjo, 2001).

Detection of antibiotic residues is done by microbiological, immunochemical, biosensor, spectrophotometric or chromatographic methods (Garcia *et al*., 1998; Muriuki *et al*., 2001; Ahmad *et al*., 2004; Pikkemaat *et al*., 2008). However, examination of routine samples for the presence of residues of all possible antibiotics with chemical methods is too expensive in

practice. Therefore, samples should first be screened with easy, quick and inexpensive methods, in order to select samples that contain or may contain unsafe levels of antibiotic residues from the great majority of samples that do not contain such residues (Okerman *et al*., 2001). Microbiological screening tests are widely used because they are cheap and easy to perform.

This study validated a screening method for detecting tetracycline and beta-lactam antibiotics in poultry kidney and liver by *Bacillus subtilis* and *Bacillus cereus*.

1.2 Statement of the Problem

Extensive and uncontrolled use of antibiotics in broiler chicken production leads to presence of antibiotic residues in poultry meat. In the Kenyan poultry industry there lacks affordable, easy to perform antibiotic residues screening methods with the capability for a high sample throughput and which can be used to rapidly sift large numbers of samples for suspect or potential non-compliant results. There is therefore need to develop a screening method that would detect the presence of an antibiotic or class of antibiotics at the maximum residue limits (MRL) for permitted substances.

1.3 Objectives

1.3.1 General objective

To evaluate the performance of *B. cereus* and *B. subtilis* as test organisms for assay of tetracycline and beta-lactam antibiotics in chicken meat.

1.3.2 Specific objectives

1. To determine the effect of pH on the growth of *B. cereus* and *B. subtilis* test organisms on Mueller Hinton agar.
2. To determine the effect of pH on the detection of oxytetracycline (OTC) and penicillin G (PEN G) by *B. cereus* and *B. subtilis* in chicken liver and kidney fortified with known concentrations of these antibiotics.
3. To establish the limits of detection (LODs) of PEN G and OTC and evaluate whether they are reliably detectable at maximum residue limits (MRLs) in chicken liver and kidney tissues.
4. To determine which organ should be used as the best test material for screening PEN G and OTC with the test bacterium and medium combinations of this study.

1.4 Hypotheses

1. The media pH has no effect on the growth of *B. cereus* and *B. subtilis* test organisms on Mueller Hinton agar.
2. The media pH has no effect on the detection of PEN G and OTC residues in chicken liver and kidney by *B. cereus* and *B. subtilis*.
3. Penicillin G and OTC residues are not reliably detectable at or below MRLs by the two test organisms.
4. There is no difference in the use of chicken liver and kidney in detecting PEN G and OTC residues by the two test organisms.

1.5 Justification

Approximately 14.6 tonnes of active antibiotics are annually used in food animal production in Kenya. Tetracyclines and penicillin derivatives are the mostly used antibiotics in poultry production because they are broad spectrum antibiotics and are cheap and easily obtained over the counter in veterinary shops. Unfortunately, this has led to the presence of their residues in meat especially when withdrawal periods are not observed. Cooking and/or cold storage act as minimal safeguards in destroying antibiotic residues and thus concerns about food safety are increasing in developing countries where urbanization and changing lifestyles are associated with greater dependence on marketed foods.

Methods for screening of veterinary drugs have been developed. However, most of these are expensive and laboratory and local conditions also tend to differ and hence the necessity of determining the operational characteristics of a method in a different test region such as Kenya. There is need for cheaper method that can screen for residues in chicken meat quickly, easily and inexpensively. This would form a basis for wider adaptability so that samples containing residues can be selected from a majority of samples that do not contain drug residues, for further quantitative testing.

CHAPTER TWO
LITERATURE REVIEW

2.1 Antibiotics in Food Producing Animals

A wide range of antibiotics are used in food producing animals not only to treat disease but also to maintain health, promote growth and enhance feed efficiency (Gustafson and Bowen, 1997). Antibiotic usage has facilitated the efficient production of poultry, allowing the consumer to purchase, at a reasonable cost, high quality meat and eggs. Antibiotic usage has also enhanced the health and well-being of poultry by reducing the incidence of disease (Donoghue, 2003).

Important classes of antimicrobials used in poultry production include sulphonamides, β-lactams, tetracyclines, aminoglycosides, macrolides, peptide antibiotics and ionophores (O'Keeffe and Kennedy, 1998). The latter two classes of drugs are used primarily as feed additive growth promoters rather than as therapeutic antibiotics (O'Keeffe and Kennedy, 1998; Mitema *et al*., 2001). Certain antibiotics such as chloramphenicol, previously used in animal husbandry are now prohibited for use in food-producing animals. Prohibition of such drugs is based on their potential toxicity for susceptible humans (O'Keeffe and Kennedy, 1998). At the same time, others like the nitrofurans have mutagenic, carcinogenic and bound-residue characteristics (O'Keeffe and Kennedy, 1998). It is also important to note that about 10% of all people given beta-lactam antibiotics develop allergic reactions to them (Rossi, 2004).

2.1.1 Beta-lactam antibiotics

The β-lactam group is one of the most important families of antibiotics used in veterinary medicine and has been widely used for decades in animal husbandry (Kowalski and Konieczna, 2007). These drugs are widely used in cattle, swine and poultry to treat infections and as feed or drinking water additives to prevent diseases. These antibiotics include a series of drugs composed of one consistent structure, the beta-lactam ring with side groups that can be modified to alter the biochemistry of the molecule (Fig. 1). They include penicillins, cephalosporins, carbapenems and monobactams (James and Gurk-Turner, 2001). Penicillins include penicillin G, ampicillin, amoxillin and methicillin (Mitema *et al*., 2001).

Beta-lactam antibiotics act on the penicillin binding proteins on the inner surface of the bacterial cell membrane. When bound, these antibiotics inactivate cell wall synthesis in a pathogen, through interference with production of cell wall peptidoglycans, with a bacteriocidal effect on the bacteria due to lysis of the cell in hypo- or iso-osmotic environment (Jacoby and

Munoz-Price, 2005).

The concentrations of β-lactam antibiotics in most tissues are equal to those in the serum (Hollenstein *et al*., 2000). They undergo minimal or no metabolism with excretion via kidneys (Tune, 1997; Katsumi, 1999). These broad-spectrum drugs are used to treat gram-positive and gram-negative bacterial infections and their residues have been found in chicken tissues (Teh and Rigg 1992; Okerman *et al*., 2001; Popelka *et al*., 2005; Loinda *et al*., 2008).

Benzylpenicillin (penicillin G) is widely used in Kenya to treat specific infections and also as a prophylactic. It is administered as one or more of a variety of salts which are used to prolong the activity of the drug. These can be the soluble sodium or potassium salts or the longer acting procaine and benzathine salts. Concern has been shown over the possible presence of residues of this drug in foods of animal origin due to the occurrence of penicillin hypersensitivity in humans and development and transfer of antibiotic resistance between animals and man (Rose *et al*., 1997; Mitema *et al*., 2001; Shitandi and Kihumbu, 2005).

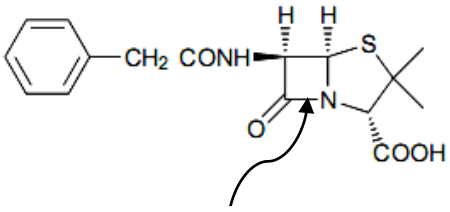

Figure 1: Structure of benzylpenicillin (Rose *et al*., 1997).

2.1.2 Tetracyclines

Tetracyclines are of great clinical importance because they posses a wide range of antimicrobial activity against aerobic and anaerobic Gram-positive and Gram negative bacteria and thus broad spectrum antibiotics (Schneider and Lehotay, 2004). They are also effective against some microorganisms that are resistant to cell-wall-inhibitor antimicrobial agents such as *Rickettsia, Mycoplasm pneumoniae, Chalamydia spp*., *Ureplasma* and some atypical *Mycobacteria* and *Plasmodium spp*. (Kapusnik-Uner *et al.,* 1996).

Tetracyclines include the naturally-occurring such as tetracycline, chlortetracycline, oxytetracycline, demeclocycline and the semi-synthetic such as doxycycline, lymecycline, meclocycline, methacycline, minocycline and rolitetracycline. Tetracycline appears to have a greater effect on the selection of resistant bacterial strains than the other naturally-occurring

tetracyclines, but in most cases microorganisms that are resistant to one of the tetracyclines are frequently resistant to the other compounds in this class (WHO/FAO, 1998).

Tetracyclines have a common ring structure (Fig 2) and their bacteriostatic activity is by inhibition of protein synthesis in the pathogenic cell (Roberts, 1996). They bind to 30S subunits of bacterial ribosomes and prevent attachment of aminoacyl-tRNA to the ribosomal receptor site, thus altering protein synthesis. Transcription is important for cell multiplication and therefore bacterial survival. The inhibitory effect of tetracyclines is usually reversible when the drug is removed (Chopra *et al*., 1981; Chopra *et al*., 1992; Roberts, 1996; WHO/FAO, 1998).

These antibiotics are incompletely absorbed from the human gastrointestinal tract with 30% chlortetracycline and 60-80% oxytetracycline and tetracycline are absorbed from an empty stomach. The absorption is impaired by cations such as calcium, magnesium, iron and aluminium which form complexes with the drug (Neuvonen, 1976; Cook *et al*., 1993). These drugs undergo minimal metabolism and they are mainly excreted in urine and faeces in their microbiologically inactive forms. These drugs are widely distributed in the body with the highest concentrations of residue being found in kidney and liver. This tissue distribution pattern is comparable in all food producing animals (Neuvonen, 1976; WHO/FAO, 1998; Agwuh and MacGowan, 2006).

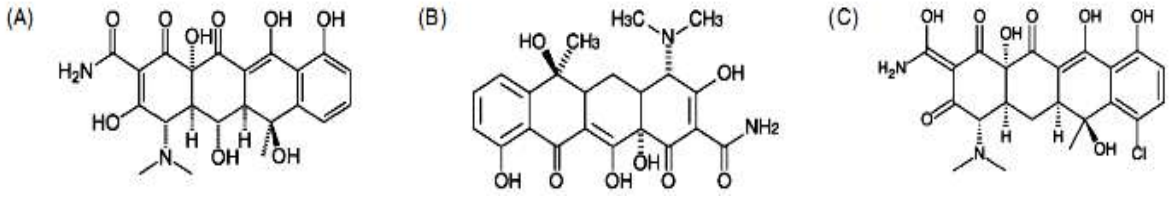

Figure 2. Chemical structures of (A) oxytetracycline (OTC), (B) tetracycline (TC) and (C) chlortetracycline (CTC) (Biswas *et al*., 2007).

In Kenya, tetracyclines are the most widely used group of antibiotics and they have a wide range of trade names (Mitema *et al*., 2001). Indeed, oxytetracycline residues in poultry products give rise to concern because the drug is used indiscriminately by farmers and feed millers due to their low cost (Omija *et al*., 1994). Residues of these veterinary drugs have been reported in slaughtered chicken (Omija *et al*., 1994; Al-Ghamdi *et al*., 2000; Okerman *et al*., 2001; Salehzadeh *et al*., 2006; Shahid *et al*., 2007; Loinda *et al*., 2008; Nonga *et al*., 2009).

2.2 Antibiotic Residues and their Effects

Drugs given to birds orally or parentally may be found in tissues, particularly when the

birds are slaughtered without the observance of withdrawal period, or when eggs are harvested within the withdrawal period of the drug (Anadon *et al*., 1994; Donoghue and Hairston, 2000; Kan and Petz, 2000). Inappropriate use could be due to unintentional misuse such as not following label directions or intentional, illegal usage or recirculation via litter. To guard against these possibilities, post-approval monitoring programs are carried out in developed countries (Pikkemaat, 2009). This extensive effort requires collection and analysis of field samples. Tissue samples are analyzed for numerous analytes including antibiotics to determine if they exceed established MRLs. If a violation is determined, corrective action is taken to prevent recurrence (Donoghue, 2003). The presence of antibiotic residues in food animals raises public health, environmental and technological concerns.

2.2.1 Health concerns

The concern that antibiotic residues may contribute in an accumulative way to human ill-health is quite prevalent. The use of tetracyclines in pregnancy is dangerous as they are teratogenic to the developing fetus (O'Keeffe and Kennedy, 1998). The overuse of tetracyclines in animal production and therefore their residues in food system poses a potential allergic reaction in sensitized individuals. Sub-therapeutic and therapeutic levels of tetracyclines may also perturb human gut microflora by introducing drug resistant strains and altering the metabolic activity of the microflora, its resistant microorganism's barrier effects, and its ecological balance. Therapeutic doses are occasionally associated with discolored teeth, allergic reactions, or peripheral blood changes (Paige *et al*., 1997; Mitchell *et al*., 1998; van-den-Bogaard and Stoobberingh, 2000; Saenz *et al*., 2001).

Hypersensitivity to β-lactam compounds is especially prevalent (de Weck, 1983). Allergic reactions such as dermatitis, pruritis and urticaria in pre-sensitized individuals caused by β lactam residues in milk have been documented (Dewdney and Edwards, 1984). As little as 0.01 IU/ml of milk could cause an allergic reaction in a sensitive individual (Waltner-Toews and McEwen, 1994).

2.2.2 Drug resistance

Addressing the issue of antimicrobial resistance has become one of the most urgent public health priorities today (Karraouan *et al*., 2009). The use of antibiotics and antimicrobials in food animals' production has contributed significantly to the pool of antibiotic resistant

bacterial populations that do not respond to treatments commonly used for human illnesses (Khachatourians, 1998). This is because many of the antimicrobials administered to food animals are either identical to or related to drugs used in human medicine, including penicillins, tetracyclines, cephalosporins and fluoroquinolones (McDermott *et al*., 2003). Development and spread of antibiotic resistance represents a serious threat with potential public health implications (WHO, 2000; Lee *et al*., 2001). Dissemination of resistance traits could narrow the line of defense against bacterial infections to only a few antibiotic agents and could increase health care costs (Lee *et al*., 2001). Indeed, multi-drug resistance among food borne pathogens and other bacteria is becoming more common and is threatening the ability to successfully treat certain infections (Levy, 1998).

The enterococci which form part of the normal resident flora of both humans and some animals have become resistant to cephalosporins, penicillins, and aminoglycosides. This has made them important opportunistic pathogens and one of the most common causes of nosocomial infections (van den Bogaard *et al*., 2002; Petersen and Dalsgaard, 2003). More than 90% of *Staphylococcus aureus* and between 50% and 70% of coagulase-negative *Staphylococci* are resistant to antibiotics (O'Brien, 1987). *Streptococcus* spp. such as *S. pneumoniae*, *S. pyogenes*, and *S. agalactiae* have also become resistant to antibiotics (Magee and Quinn, 1991; Borzani *et al*., 1997; Boost *et al*., 2003; Apgar *et al*., 2005). Some major food and waterborne bacterial pathogens such as *Campylobacter* spp., *Salmonella* spp., *Shigella* spp., *Vibrio* spp., and *Escherichia coli* have also developed tolerance to antibiotics (Scuderi *et al*., 2000; Threlfall *et al*., 2000; Petersen and Dalsgaard 2003).

Antibiotic resistance development in bacteria is mediated by both selective pressure due to antibiotic use and the presence of resistance genes (Tenover and Hughes, 1996; McCormick, 2003; Popelka *et al*., 2005). However, drug concentration, long-term exposure, organism type, antibiotic type and host immune status are factors which are thought to influence the development of drug resistance (Sjogren *et al*., 1992). Low-level, long-term exposure to antibiotics may in particular have a greater selective potential than short-term, full-dose therapeutic use (Lopez-Lozano *et al*., 2000; Kummerer, 2004).

Four major mechanisms by which microorganisms develop resistance to antibiotics have been identified. These include alterations in the target site of the antibiotic, such as changes in penicillin binding proteins, drug degradation and enzymatic inactivation of the antibiotic as is the

case in penicillinases (Normark and Normark, 2002; Gaynor and Mankin, 2003; Belcher, 2005). Other mechanisms include changes in cell wall permeability that prevents access to antibiotics and increases in the activity of efflux pumps in the cell wall which prevent accumulation of antibiotic within the cell (Roberts, 1996; Mazel and Davies, 1999; Bonomo and Rossolini, 2008).

2.2.3 Environmental concerns

After treatment, most antibiotics are excreted by animals through urine and faeces either unaltered or as metabolites (Sarmah *et al*., 2006). This makes them potentially hazardous to bacteria and other organisms in the environment (Baguer *et al*., 2000). Different types of drugs have different anticipated exposure routes to the environment (Jørgensen and Halling-Sørensen, 2000). Since most antibiotics are water-soluble, up to 90% of a dose can be excreted in urine and up to 75% in animal faeces (Winckler and Grafe 2001; De Liguoro *et al*., 2003). The most prevalent antibiotics found in the environment such as surface waters belong to the macrolide and the sulfonamide groups (Heberer, 2002). Low concentrations of tetracyclines, penicillins or fluoroquinolones have only been found in some cases (Hirsch *et al*., 1999; Heberer, 2002; Kolpin *et al*., 2002).

Some commonly used antibiotics that persist in the soil and remain in surface waters and soils for over a year have been identified. These include erythromycin, cyclophosphamide, sulfadimidin and tetracycline (Zuccato *et al*., 2000; Quack *et al*., 2005). Antibiotic metabolites have also been found to be able to be transformed back to their original active substances once in the environment (Hirsch *et al*., 1999). However, it has been difficult to ascertain whether these residues are due to poor waste water management or if they are due to inputs from agriculture (Halling-Sørensen, 1998).

Although little is known about the extent of environmental occurrence, transport and ultimate fate and effects of pharmaceuticals in general (Kummerer, 2003), it is known that antibiotics are not bio-accumulative, but their continual release into the environment gives them persistent quality (Erickson, 2002), added to the effect of low biodegradation rates in soil (Al-Ahmad *et al*., 1999). Wide spectrum resistance to antibiotics is the chronic effect associated with soil microflora which receives antibiotic residues (Lee *et al*., 2001).

2.2.4 Technological concerns

The presence of antimicrobial substances in foods may have serious toxicological and

technological consequences. Successful and safe manufacture of dry and semi-dry fermented sausage for example, depends upon the use of high quality raw materials and the acidifying action of lactic acid bacteria (LAB) commonly added as starter cultures at the meat formulation stage (Halley and Blaszyk, 1998). There, LAB including pediococci or lactobacilli dependably reduce meat pH which facilitates drying and at the same time inhibit growth of pathogenic bacteria (Geisen *et al*., 1992; Incze, 1992). A variety of factors such as low culture viability, incorrect concentrations of carbohydrate, nitrate, nitrite, salt or inappropriate fermentation temperature are associated with delays in achieving the target pH during fermentation (Wallentin and Borch, 1991) whereas antibiotic residues in raw meats may sometimes be involved in cases where culture failures are observed (Halley and Blaszyk, 1998). The presence of antibiotics in milk has also been observed to delay if not totally prevent the bacteriological processes used in the manufacture of certain dairy products (Katla *et al*., 2001).

2.3 Food Safety, Antibiotic Residues Regulation and Detection Methods

2.3.1 Food safety

The presence of antibiotic residues in foodstuffs of animal origin is one of the most important indices for their safety (Donoghue and Hairston, 2000; Pavlov *et al*., 2008). Several different types of toxicological and pharmacological studies to assess the safety of antibiotics in edible tissues have to be done in order for a drug to be approved for use in food producing animals (Donoghue, 2003; WHO/FAO, 2009). For toxicological testing, acute and chronic dosing studies have to be carried out. The acute studies evaluate animals for problems such as allergic reactions, whereas long-term chronic studies identify problems such as cancer associated with a particular drug. These studies determine a dose that does not create any health problems (WHO/FAO, 2009). This is called no observable effect level (NOEL) and is the antibiotic dose at which no adverse effect on an animal's health can be observed (O'Keeffe and Kennedy, 1998; Donoghue, 2003). Once established, this information is used to calculate a tolerance concentration for the antibiotic in edible tissue (Donoghue, 2003).

Pharmacokinetic studies are then performed to ensure the tolerance is not exceeded for the proposed dosing, according to label directions (Donoghue, 2003; WHO/FAO, 2009). Usually, a radiolabeled carbon isotope (C^{14}) antibiotic depletion study is conducted. Dosing is done and thereafter edible tissues are evaluated at different time points to determine when total residue concentrations decrease below the tolerance (Donoghue, 2003). Approval or disapproval

of the drug in consideration of the label directions of withdrawal period then follows.

2.3.2 Regulations concerning antibiotics residues

Regulations concerning drug residues have been formulated. The World Health Organization (WHO) and the Food and Agriculture Organization (FAO) have set maximum residue limits (MRLs), acceptable daily intakes (ADIs) for humans and withholding times for pharmacologically active substances including antimicrobial agents prior to marketing (WHO/FAO, 2009).

The maximum residue limits (MRL) is the maximum concentration of a residue, expressed as µg per kg food, legally permitted in or on food commodities and animal feeds. MRLs have been established for all antibiotics allowed for use in food producing animals (WHO/FAO, 2009). MRLs are usually given in µg/kg, but in order to compare them to the limits of detection (LODs) they can be expressed in ng/100 mg (Okerman *et al*., 2001) or ng/g. The MRLs for CTC, OTC, TC in poultry muscle, liver, kidney and eggs are 200, 600, 1200 and 400 µg/kg, respectively while the MRL for benzylpenicillin in poultry muscle, liver and kidney is 50 µg/kg (WHO/FAO, 1999).

Acceptable daily intake (ADI) value is an estimate of the amount of residue, expressed as µg per kg bodyweight that can be ingested daily over a lifetime period without appreciable health risk. The group ADI of CTC, OTC and TC is 0-30 µg/kg body (WHO/FAO, 1999). The ADI for benzylpenicillin is 30 µg-penicillin/person/day. It is recommended that residues of benzylpenicillin and procaine benzylpenicillin should be kept below this level (WHO/FAO, 1998). Presence of drug residues in edible tissues at levels below the MRLs are not considered a causative agent for induction of bacterial resistance because ADIs, which the MRLs respect, are increasingly set on the basis of establishing minimum inhibitory concentration (MIC) values (WHO/FAO, 2009). MIC values are the concentration of antibiotic which has no inhibitory effect on the bacteria in the human gut (O'Keeffe and Kennedy, 1998).

2.3.3 Thermal stability of antibiotics residues

Testing of antibiotic residues is ordinarily done on raw products. However, products are normally cooked or processed in some manner before they are consumed. Research on the effects of cooking temperatures on the stability of various antibiotics indicates that there is variability in the stability exhibited depending on the antibiotic tested (Ibrahim and Moats, 1994;

Furusawa and Hanabusa, 2002). While most antimicrobials ultimately demonstrate reduction in potency when subjected to cooking conditions, the temperature conditions under which these stability studies are performed are generally more severe than heat conditions used in cooking. Normal cooking procedures or methods involving low temperatures or short holding periods fail to eliminate antibiotic residues (CAC, 2001; Loksuwan and Tomongkol 2005).

Studies done on various methods of cooking such as microwaving, boiling, braising, grilling, frying and roasting gave varying degrees of OTC and PEN G residue reduction (Rose *et al*., 1996; Rose *et al*., 1997). Results in these studies showed that OTC was unstable in water at 100°C with a half-life of about 2 min, but more stable in oil at 180°C where the half-life was about 8 min. Substantial net reductions in OTC of 35-94% were observed, with temperature during cooking having the largest impact on the loss. Migration from the tissue into the surrounding liquid or meat juices was observed during the cooking processes (Rose *et al*., 1996).

Benzylpenicillin was stable at 65°C but not stable at temperatures above 100°C with half-life times varying between 15 and 60 min in water at 100°C and 65°C, 5% ethanol, 5% sodium bicarbonate, pH 5.5 buffer at 100°C and in hot cooking oil at 140°C and 180°C. The drug loss due to cooking was proportional to the harshness of the cooking regime. Boiling had the least effect on residue reduction while frying had the highest effect. Net reductions of 4-90% were recorded (Rose *et al*., 1997). It was also reported that much of the residue migrated with juices which exuded from the tissue as it was cooked. Therefore, exposure to residues of PEN G may be reduced by discarding any juices which come from the meat as it is cooked which is usually not the case (Rose *et al*., 1997). Antibiotics residues are also found to be stable in cold storage (Mercer, 1975; Boison *et al*., 1992; Rose *et al*., 1996). Considering these facts therefore, it is only in order to remove products that contain antibiotics residues from the consumer line and screening of raw products is therefore of the essence.

The fact that the occurrence or avoidance of antibiotics residues in food is to a greater or lesser extent under the control of the food producer, processor and consumer should be underscored. The extent to which residues in food do or can constitute a food safety hazard for the consumer is the fundamental question (O'Keeffe and Kennedy, 1998). The most effective approach to minimize potential health hazard associated with consumption of OTC and PEN G residues in cooked animal products would be the adherence to the guidelines regarding to MRLs and ADIs.

2.3.4 Antibiotic residues detection methods

Much of the analytical methodology for veterinary drugs has been developed and validated using spiked tissues (Cooper *et al*., 1998) or standard antimicrobial solutions (Myllyniemi *et al*., 2000). Detection of antibiotic residues is done by microbiological, immunochemical, biosensor, spectrophotometric or chromatographic methods (Garcia *et al*., 1998; Muriuki *et al*., 2001; Ahmad *et al*., 2004; Pikkemaat *et al*., 2008). Microbiological agar diffusion tests are widely used to screen for antimicrobial residues in animal tissues. These methods rely on their ability to inhibit growth of sensitive bacteria (Koenen-Dierick and De Beer, 1998; Myllyniemi *et al*., 1999; Myllyniemi *et al*., 2000; Popelka *et al*., 2005). Usually these assays utilize the genus *Bacillus* because of its high sensitivity to the majority of antibiotics (Popelka *et al*., 2003; Popelka *et al*., 2005). Test plates contain a variety of test bacteria, media pH values and substances that either block or are synergistic to the action of certain antimicrobials (Pikkemaat *et al*., 2009). A screening method should identify samples that contain a residue concentration at the level of interest or legal requirement, usually the maximum residue limit (MRL). It should be a method of high sample-throughput optimized to prevent false-negative results that is high sensitivity, and to give an acceptable number of false-positive results (Mitchell and Yee, 1999). Microbiological tests have the advantage of being multi-residue tests because inhibition is caused by a wide range of antibiotics and they are used as screening tests for antibiotics in milk, meat and other animal tissues (Koenen-Dierick and De Beer, 1998; Popelka *et al*., 2005).

In developed countries tests like *Bacillus stearothermophilus var. calidolactis* disc assay (BsDA) and the four plate test (FPT) have been used to analyse meat samples, which are laid directly on the agar medium, which is inoculated with an antibiotic sensitive bacterial strain (Pikkemaat, 2009). BsDA consists of a plate utilizing a spore suspension of *B. stearothermophilus var. calidolactis*. The FPT four plate test is intended to detect residues of antibiotics in muscle tissue of slaughtered animals. The FPT consists of plates with *B. subtilis* (BGA) spore suspension and *Kocuria rhizophila* bacterial suspension. The FPT is qualitative and recommended for screening only (Pikkemaat, 2009). The FPT is based on the inhibition of growth of the micro-organism, which is included in the test. This inhibition becomes visible as a clear zone around the sample in cases where antibiotic substances are present at or above the LOD of the plate. The size of this zone is dependent on the antibiotic concentration where

concentrations above the LOD will lead to larger zones (Popelka *et al*., 2005).

The Premi® Test has also been developed and is used for the detection of antibiotics in meat and is based on the inhibition of growth of *B. stearothermophilus*, a thermophilic bacterium sensitive to many antibiotics (Reybroeck, 2000). Premi® Test is an ampoule, containing an agar medium, imbedded spores of *B. stearothermophilus var. calidolactis* and a colour indicator. Premi® Test combines the principle of an agar diffusion test with colour change of the indicator. In case of an active metabolism of the included microorganism, the test will change colour from purple to yellow. When growth of microorganism is inhibited due to presence of an antibiotic at or above the LOD, the test will remain purple (Stead *et al*., 2004; Popelka *et al*., 2005).

Confirmation, identification and quantification of residues in an inhibitor positive test result should be done to check if a legally set MRL has been exceeded. A post-screening method using microbiological identification may be used as a preliminary identification step before chemical analyses (Calderon *et al*., 1996; Myllyniemi *et al*., 1999). However violation of the MRL regulation can only be proven with fully validated chromatographic methods such as high-performance liquid chromatography (HPLC) (Moats, 1997) , or by hyphenated techniques such as gas chromatography-mass spectrometry (GC-MS) (Pfennig *et al*., 1998) and liquid chromatography-mass spectrometry (LC-MS) (De Wasch *et al*., 1998; Chico *et al*., 2008). These methods however, require highly sophisticated and expensive equipment.

Examination of routine samples for the presence of residues of all possible antibiotics with chromatographic methods is too expensive in practice as each antibiotic family requires a different extraction and detection procedure, and many chromatographic methods are not even able to detect all members of the same family up to the MRL level. Therefore, samples should first be screened with easy, quick and inexpensive methods, in order to select samples that contain or may contain unsafe levels of antibiotic residues from the great majority of samples that do not contain such residues (Okerman *et al*., 2001). Microbiological screening tests are widely used for the screening of residues of antimicrobial substances because they are cheap and easy to perform in comparison with specific chemical methods.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Bacterial Suspensions

Bacillus subtilis BGA (E. Merck, Darmstadt, Federal Republic of Germany) and *Bacillus cereus* (Difco Laboratories, Detroit, Michigan, USA) were used as spore suspensions. Five batches of Mueller Hinton agar at pH 5, 6, 7, 7.3 and 8 were prepared for use. Mueller Hinton agar was prepared as per the manufacturer's instruction and the pH adjusted appropriately using 0.1M HCl or 0.1M NaOH. After autoclaving at a pressure of 15 psi at a temperature of 121°C for 15 minutes, it was cooled to 45-55°C. The spore suspension of *B. cereus* and *B. subtilis* was inoculated into the molten agar and mixed thoroughly to ensure uniform distribution. Approximately 10^5 spores per ml were determined by spectrophotometry and inoculated. Sterile Petri plates (diameter 90mm) were filled with 15ml of the inoculated media and incubated at 30°C for 18-24 hours to determine the effect of pH of bacterial growth.

3.2 Test Plates

Six different plates were used for antibiotic detection: plate I, Mueller Hinton agar (MHA) pH 6, plate II, MHA pH 7, plate III MHA pH 7.3, plate IV MHA pH 6, plate V MHA pH 7, and plate VI MHA pH 7.3. Plate I, II and III were seeded with *B. subtilis* while plate IV, V and VI were seeded with *B. cereus*. The test plates were coded such that the first two letters represented the test organism, the third letter the antibiotic, the fourth letter poultry organ to be used while the fifth number represented the pH e.g. BSOL7 representing plate II with OTC spiked chicken liver fluid. Plate composition and incubation conditions are shown in appendix 1. The sterile Petri dishes (diameter 90 mm) were filled with 15ml of the prepared and seeded media. After solidification, the media was used immediately or stored at 2-5°C for a maximum of 5 days.

Seven holes with diameters of 10mm were punched into the agar layer and filled with 100µl of the artificially spiked kidney or liver fluid at different concentration of the antibiotics, an antibiotic-free negative control and a positive reference standard fortified with the established MRL for PEN G and OTC. The holes were distanced at least 30mm from each other. After a pre-diffusion period of about 1 hour, at room temperature, the plates were incubated at 30°C for 13-18 hours. The samples and the standards were run in triplicates. The diameters of the zones of inhibition, from the edges of the wells, were measured and an inhibition zone of $\geq$ 2mm was

considered as positive. These diameters were measured minus the diameter of the punch hole which was 10mm (Omija *et al*., 1994; Aila *et al*., 2009; Nonga *et al*., 2009).

3.3 Stock Antibiotic Solutions

Stock solutions of 0.05g/l and 1.2g/l for PEN G and OTC respectively were prepared in distilled water. *In vitro* sensitivity was carried out by serial dilutions of the stock solutions for both OTC and PEN G. Dilutions were made in distilled water and/or kidney/liver supernatants. PEN G and OTC were obtained from Sigma Chemical Company (St. Louis, MO, USA).

3.4 Control Samples

Fifty µg/l of PEN G and 600µg/l and 1200µg/l of OTC, which are MRL concentrations for liver and kidney respectively, of each drug were used as positive control in the search for the limits of detection of each drug in the two tissues. Liver or kidney tissues that were free of any antimicrobial drugs were used as the negative control.

3.5 Preparation of Fortified Liver and Kidney

One hundred frozen samples consisting of 50 livers and 50 kidneys from chicken that had not been treated with antibiotics were homogenized with distilled water at a ratio of 1:2 (tissue: distilled water) and the homogenates centrifuged for 5 min at 3000g to eliminate tissue debris. Supernatants from these extracts were used to dilute stock solutions to produce working solutions standards of 0.15µg/ml - 2.4µg/ml and 0.0125 - 0.083µg/ml of OTC and PEN G, respectively. These are dilutions of the established MRLs for each antibiotic for the two tissues (Shitandi and Kihumbu, 2004; Aila *et al*., 2009).

3.6 Determination of Limits of Detection

To verify the detection limits, the spiked supernatants were added to the holes on the agar such that each hole containing a replicate of each drug concentration received 100µl of test solution. Each concentration was analyzed in three replicates. The plates were allowed to stand for 1 h to allow the supernatant to diffuse into the media. They were then incubated at 30°C for 13-18h. The zones of inhibition were then measured using a caliper. A regression line of log concentration (µg/ml/well) vs inhibition zone diameters was used to calculate the LOD (Koenen-Dierick *et al*., 1995). This was done using GraphPad Prism 5 statistical software (GraphPad Software, Inc. 2009).

3.7 Data Analysis

The data was analyzed using GraphPad Prism 5 statistical software (GraphPad Software, Inc. 2009). Two way analysis of variance (ANOVA) was applied to test for the significant differences in mean inhibition zones among the test organisms, pH and concentrations combinations. Bonferroni posttests were carried out to compare replicate means. Comparisons were considered significantly different at p values <0.001. Regression analysis of concentration and the inhibition zone diameters was used to determine the LODs.

CHAPTER FOUR

RESULTS

4.1 Cultural and Incubation Conditions

The five batches of Mueller Hinton agar prepared showed different growth of both *B. subtilis* and *B. cereus* when 10^5 cfu/ml was used. No growth was observed at pH 5 and 8 while growth was observed at pH values of 6, 7 and 7.3. There was maximal growth after 14-16 hours at 30°C.

4.2 Examination of Samples

Poultry liver and kidney samples with known concentrations of OTC and PEN G were analysed and the influence of the type of organ and pH on the sensitivity of the two organisms was also examined. Tests were carried out for the presence of OTC and PEN G residues that ranged from 0.25 to 1.4 times the MRL for poultry tissues. Concentrations of OTC of 0.15-0.86µg/ml in liver; 0.3-1.7µg/ml in kidney and concentrations of PEN G of 0.0125-0.083µg/ml in both liver and kidney were tested.

Table 1: Average inhibition zones (mean±SE) obtained from the analysis of chicken kidney spiked with different levels of OTC onto Mueller Hinton agar at different pH.

OTC concentration (µg/ml)	Mean zones of inhibition (mm)					
	B. subtilis			*B. cereus*		
	pH 6.0	pH 7.0	pH 7.3	pH 6.0	pH 7.0	pH 7.3
0	0	0	0	0	0	0
0.3	4.6±0.1	4.3±0.1	2.2±0.2	8.7±0.1	8.5±0.1	2.5±0.1
0.4	5.7±0.1	5.9±0.1	3.2±0.2	10.1±0.1	9.2±0.1	4.1±0.2
0.6	6.9±0.1	8.1±0.2	4.5±0.2	12.1±0.1	10.9±0.1	5.4±0.2
1.2*	8.7±0.1	9.9±0.2	5.7±0.2	14.5±0.1	12.1±0.1	7.4±0.2
1.5	10.3±0.1	12.1±0.1	6.4±0.2	15.7±0.1	12.9±0.1	8.9±0.2
1.7	12.0±0.1	14.2±0.1	7.0±0.2	17.6±0.0	13.9±0.1	9.7±0.2

*MRL concentration for OTC in chicken kidney; SE-standard error

Positive results showed inhibition activities around the well while negative results showed no inhibition. Negative controls showed no inhibition of test organism. *Bacillus cereus*

was found to be more sensitive to OTC as larger zones of inhibition were observed.

Plate 1: Inhibition zones on plate IV (*B. cereus*; pH6) caused by OTC spiked chicken liver

Plate 2 : Inhibition zones on plate V (*B. cereus*; pH 7) caused by OTC spiked chicken liver

Inhibition zones were smaller when PEN G was used with the same bacteria/media/pH combination. On the other hand, *B. subtilis* was more sensitive to PEN G than OTC based on larger inhibition zone diameters measured. The zones of inhibition are presented as mean±SE in tables 1, 2, 3 and 4.

Table 2: Average inhibition zones (mean±SE) obtained from the analysis of chicken liver spiked with different levels of OTC onto Mueller Hinton agar at different pH.

OTC concentration (µg/ml)	Mean zones of inhibition (mm)					
	B. subtilis			*B. cereus*		
	pH 6.0	pH 7.0	pH 7.3	pH 6.0	pH 7.0	pH 7.3
0	0	0	0	0	0	0
0.15	0.1±0.0	1.7±0.1	1.3±0.1	1.5±0.1	2.7±0.1	2.0±0.1
0.2	0.2±0.0	2.5±0.1	2.2±0.1	3.1±0.1	4.3±0.1	3.3±0.2
0.3	0.8±0.1	3.5±0.1	3.0±0.1	4.4±0.1	5.3±0.1	4.9±0.2
0.6*	2.6±0.1	4.3±0.1	4.1±0.2	6.2±0.2	7.6±0.1	7.5±0.2
0.75	3.6±0.1	4.8±0.1	4.7±0.2	7.7±0.2	9.3±0.1	8.5±0.2
0.86	4.6±0.1	5.4±0.1	5.7±0.2	9.0±0.1	10.6±0.1	9.5±0.2

*MRL concentration for OTC in chicken liver; SE-standard error

4.2.1 The effect of pH on detection of PEN G and OTC

Change in pH had an effect on drug detection based on the size of the inhibition zone. Zones of inhibition increased in size with OTC and PEN G concentration for all pH. Inhibition zones increased significantly ($p<0.001$) when both test organisms were used to detect OTC at a decreasing pH in both liver and kidney (Table 1 and 2). When OTC was tested in liver using the two test organisms at varying pH, there was high significant ($p<0.001$) difference in mean inhibition zones between BSOL6 and BSOL7, BSOL6 and BSOL7.3 and BCOL6 and BCOL7. There was also a significant ($p<0.001$) difference between BCOL7 and BCOL7.3 except in the 0.6µg/ml concentration ($p>0.05$). The 0.6µg/ml and 0.75µg/ml concentrations in BCOL6 and BCOL7.3 were highly significant ($p<0.001$), 0.15µg/ml, 0.3µg/ml and 0.86µg/ml slightly significant ($p<0.05$) while the difference in the 0.2µg/ml concentration was not significant ($p>0.05$). There was no significant ($p>0.05$) difference between BSOL7 and BSOL7.3 except in 0.3µg/ml concentration which was slightly significant ($p<0.05$). There was no significant ($p>0.05$) difference in mean inhibition zones in all the plates compared at 0µg/ml concentration.

Testing of OTC in kidney showed significant ($p<0.001$) differences in mean inhibition zones of BSOK6 and BSOK7.3, BSOK7 and BSOK7.3, BCOK6 and BCOK7.3 and BCOK7 and

BCOK7.3. In BSOK6 and BSOK7, there was no significant ($p>0.05$) difference in mean inhibition zones at 0.3µg/ml and 0.4µg/ml while the difference at other concentrations was strongly significant ($p<0.001$). There was significant ($p<0.001$) difference in mean inhibition zones observed in BCOK6 and BCOK7 except at 0.3µg/ml concentration ($p>0.05$). There was no significant ($p>0.05$) difference in mean inhibition zones in all the plates compared at 0µg/ml concentration.

Table 3: Average inhibition zones (mean±SE) obtained from the analysis of chicken kidney spiked with different levels of PEN G onto Mueller Hinton agar at different pH.

PEN G concentration (µg/ml)	Mean zones of inhibition (mm)					
	B. subtilis			*B. cereus*		
	pH 6.0	pH 7.0	pH 7.3	pH 6.0	pH 7.0	pH 7.3
0	0	0	0	0	0	0
0.0125	0	12.9±0.1	14.2±0.1	0	4.6±0.1	2.5±0.1
0.0167	0	13.8±0.1	15.3±0.1	0	5.8±0.1	3.7±0.1
0.025	0.6±0.1	14.8±0.1	16.8±0.1	0	6.8±0.1	5.4±0.1
0.05*	3.1±0.1	15.5±0.1	17.6±0.1	0	7.7±0.1	6.1±0.2
0.0625	4.7±0.1	16.7±0.1	18.6±0.1	0.7±0.0	8.2±0.1	7.3±0.1
0.083	6.3±0.1	17.6±0.1	19.8±0.1	0.7±0.0	8.9±0.1	9.0±0.1

*MRL concentration for PEN G in chicken kidney; SE-standard error

Zone diameters decreased significantly ($p<0.001$) when *B. subtilis* was used to detect PEN G at decreasing pH from 7.3, 7.0 to 6. The zones increased significantly ($p<0.001$) when *B. cereus* was used to detect the same antibiotic in both kidney and liver samples at decreasing pH (Table 3 and 4). There was significant ($p<0.001$) difference in the inhibition zones measured in BSPL6 and BSPL7, BSPL6 and BSPL7.3, BSPL7 and BSPL7.3, BCPL6 and BCPL7, BCPL6 and BCPL7.3, BCPL7 and BCPL7.3, BSPK6 and BSPK7, BSPK6 and BSPK7.3, BSPK7 and BSPK7.3, BCPK6 and BCPK7, BCPK6 and BCPK7.3 and BCPK7 and BCPK7.3. There was no significant ($p>0.05$) difference in mean inhibition zones in the plates compared at 0µg/ml concentration.

Table 4: Average inhibition zones (mean±SE) obtained from the analysis of chicken liver spiked with different levels of PEN G onto Mueller Hinton agar at different pH.

PEN G concentration (µg/ml)	Mean zones of inhibition (mm)					
	B. subtilis			*B. cereus*		
	pH 6.0	pH 7.0	pH 7.3	pH 6.0	pH 7.0	pH 7.3
0	0	0	0	0	0	0
0.0125	0	12.9±0.1	14.1±0.1	0	5.2±0.1	2.4±0.1
0.0167	0	13.6±0.1	15.4±0.1	0	6.1±0.1	3.5±0.1
0.025	1.6±0.1	14.7±0.1	16.8±0.1	0	7.1±0.1	5.1±0.2
0.05*	4.6±0.1	15.6±0.1	17.6±0.1	0	8.0±0.1	5.9±0.2
0.0625	6.6±0.2	16.5±0.1	18.5±0.1	1.2±0.0	9.1±0.1	7.3±0.2
0.083	8.7±0.1	17.5±0.1	19.7±0.1	1.5±0.0	10.4±0.1	8.8±0.2

*MRL concentration for PEN G in chicken liver; SE-standard error

4.2.2 The effect of the test organism on detection of PEN G and OTC

When all other factors were held constant and the two test organisms studied, the inhibition zone means were significantly ($p<0.001$) different between BSOK6 and BCOK6, BSOK7 and BCOK7, BSOL6 and BCOL6, BSOL7 and BCOL7 and BSOL7.3 and BCOL7.3 except in BSOK7.3 and BCOK7.3 where 0.3µg/ml concentration produced zones that were not significantly ($p>0.05$) different (Table 1 and 2).

Mean inhibition zones differed significantly ($p<0.001$) between BSPK7 and BCPK7, BSPK7.3 and BCPK7.3, BSPL7 and BCPL7 and BSPL7.3 and BCPL7.3. In BSPK6 and BCPK6 and BSPL6 and BCPL6, there were no significant ($p>0.05$) differences in measured zones at 0.0125µg/ml and 0.0167µg/ml although zones differed significantly ($p<0.001$) at other concentrations. There was no significant ($p>0.05$) difference in mean inhibition zones in the plates compared at 0µg/ml concentration (Table 3 and 4).

4.2.3 The effect of the type of organ on detection of PEN G and OTC

When *B. subtilis* was used, there was no significant ($p>0.05$) difference in the detection of PEN G in both kidney and liver samples at the different pH values. However, 0.025µg/ml, 0.05µg/ml, 0.0625µg/ml and 0.083µg/ml produced inhibition zones that were significantly

($p<0.001$) different when BSPL6 and BSPK6 were compared. When *B. cereus* was used, there was no significant ($p>0.05$) difference in mean zone diameters although at 0.0625µg/ml and 0.083µg/ml zones were strongly different between BCPL6 and BCPK6 and between BCPL7 and BCPK7 ($p<0.001$). At 0.025µg/ml and 0.05µg/ml, the zones were slightly different ($p<0.05$) between BCPL7.3 and BCPK7.3 and between BCPL7 and BCPK7 respectively. The 0.0125µg/ml concentration produced inhibition zones that differed significantly ($p<0.001$) when BCPL7 and BCPK7 were compared. The inhibition zones differed significantly ($p<0.001$) at all OTC concentrations tested in kidney and liver.

4.3 Limits of Detection

The two test organisms were able to detect both OTC and PEN G at the legally acceptable levels. However PEN G could not be detected at these levels by *B. cereus* when pH was reduced to 6. PEN G spiked liver and kidney produced large zones at pH 7 and 7.3 on the *B. subtilis* plate. The zones were however much reduced at pH 6 on the same plate. At 1.2µg/ml and 0.6µg/ml, the zones of inhibition caused by OTC increased when the pH was reduced on both the *B. subtilis* and *B. cereus* plates. Table 5 shows the mean inhibition zones obtained from liver and kidney spiked with the appropriate MRL concentrations.

Table 5: Mean inhibition zones (mm) caused by control samples at MRL at pH 6, 7 and 7.3

Plate	Antibiotic	MRL (µg/kg)	Mean inhibition zone (mm)			n
			pH 6	pH 7	pH 7.3	
B. subtilis	PEN G	50[1]	3.1 (0.9)[3]	15.5 (0.5)	16.6 (1.4)	50
		50[2]	4.7 (1.0)	15.6 (0.5)	16.5 (1.6)	50
	OTC	1200[1]	8.7 (0.8)	9.9 (1.1)	5.7 (1.4)	50
		600[2]	2.6 (0.7)	4.3 (0.4)	4.0 (1.3)	50
B. cereus	PEN G	50[1]	0.0 (0.0)	7.7 (0.6)	6.1 (1.1)	50
		50[2]	0.0 (0.0)	8.4 (0.4)	5.9 (1.1)	50
	OTC	1200[1]	14.5 (0.7)	12.1 (0.5)	7.4 (1.2)	50
		600[2]	6.2 (1.2)	7.6 (1.0)	7.5 (1.6)	50

[1]Kidney MRL; [2]liver MRL for chicken; [3]standard deviation in brackets

The detection levels were very low which is indicative of high sensitivity of the test organisms. The LODs were 1.5-6 times below the MRLs except for PEN G which had a detection limit above the MRL in liver and kidney on plate VI (*B. cereus*, pH 6) (Table 6). The regression curves for OTC and PEN G in plate I-VI that were used to calculate respective LODs are shown in Figures 3-6.

Table 6: Limits of detection for OTC and PEN G in chicken kidney and liver at pH 6, 7 and 7.3

			Limits of detection (ng/ml)					
			B. cereus			*B. subtilis*		
Tissue	Antibiotic	[1]MRL (ng/g)	pH 6	pH 7	pH 7.3	pH 6	pH 7	pH 7.3
Liver	OTC	600	167.8	131.3	149.7	391.4	164.4	201.8
	PEN G	50	126.90[2]	3.7	11.2	24.0	0.1	0.1
Kidney	OTC	1200	74.1	33.4	256.6	169.7	201.2	262.5
	PEN G	50	662.2[2]	3.2	10.6	29.3	0.1	0.1

[1]Chicken tissues MRLs; [2]LOD above the MRLs

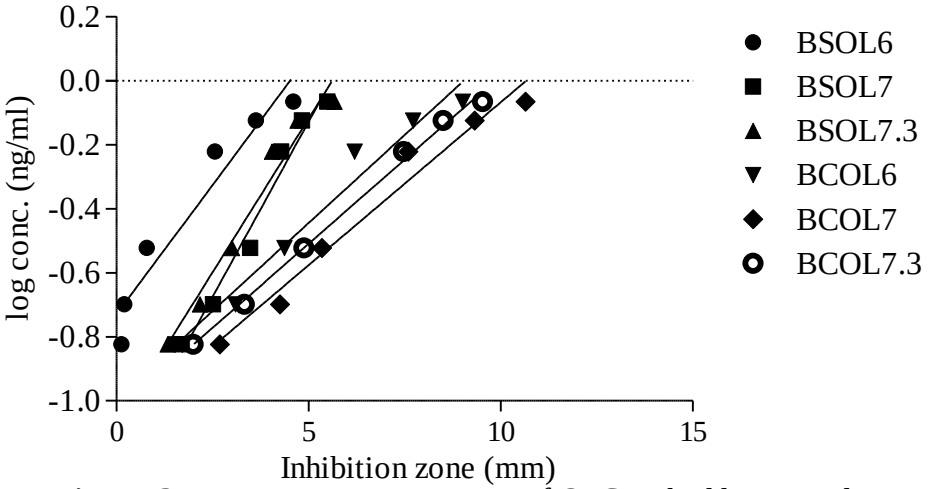

Figure 3: Linear regression curves of OTC spiked liver on plates I-VI

R^2: BSOL6=0.9301, p<0.0019; BSOL7=0.9748, p<0.0002; BSOL7.3=0.9679, p<0.0004; BCOL6=0.9708, p<0.0003; BCOL7=0.9701, p<0.0003; BCOL7.3=0.9955, p<0.0001. The curves were used to calculate LOD of OTC in chicken liver on plates I-VI.

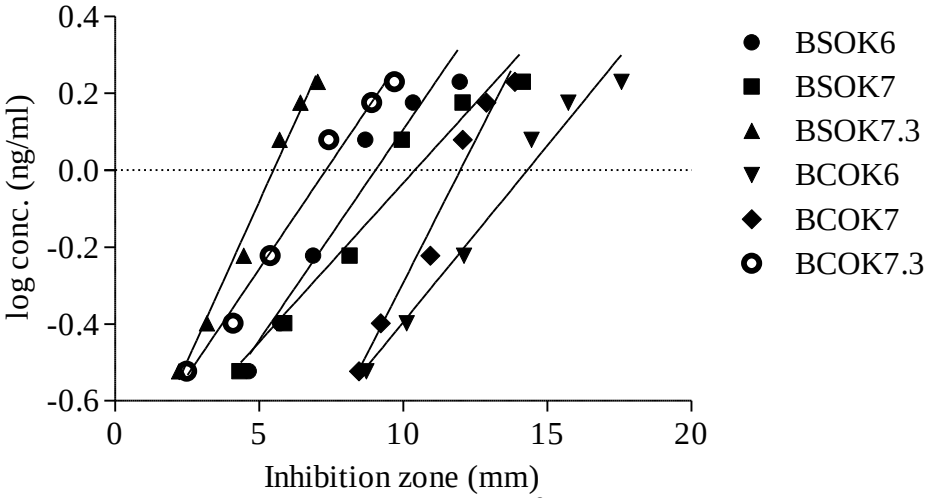

Figure 4: Linear regression curves of OTC spiked kidney on plates I-

R^2: BSOK6=0.9475, p<0.0011; BSOK7=0.9527, p<0.0009; BSOK7.3=0.9874, p<0.0001; BCOK6=0.9762, p<0.0002; BCOK7=0.9732, p<0.0003, BCOK7.3=0.9845, p<0.0001. The curves were used to calculate LOD of OTC in chicken kidney on plates I-VI.

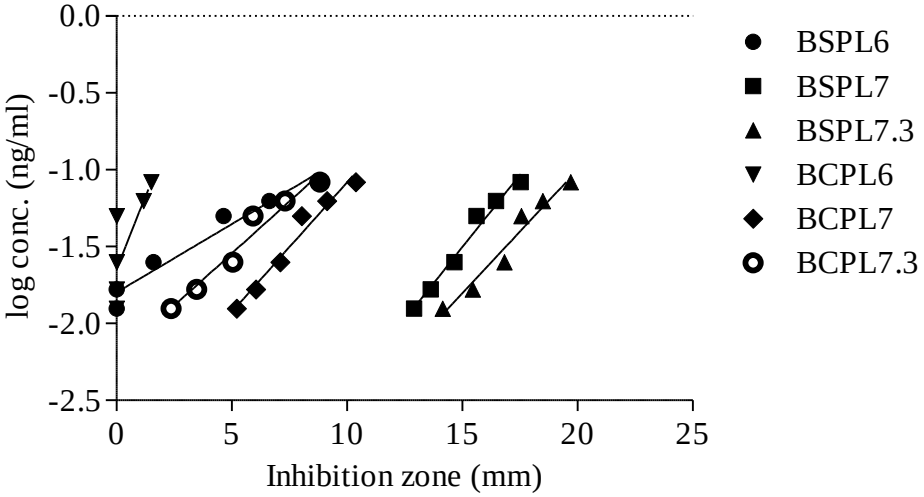

Figure 5: Linear regression curves of PEN G spiked liver on plates I-VI

R^2: BSPL6=0.9548, p<0.0008; BSPL7=0.9774, p<0.0002; BSPL7.3=0.9579, p<0.0007, BCPL6=0.6284, p<0.0600; BCPL7=0.9654, p<0.0005; BCPL7.3=0.9592, p<0.0006. The curves were used to calculate LOD of PEN G in chicken liver on plates I-VI.

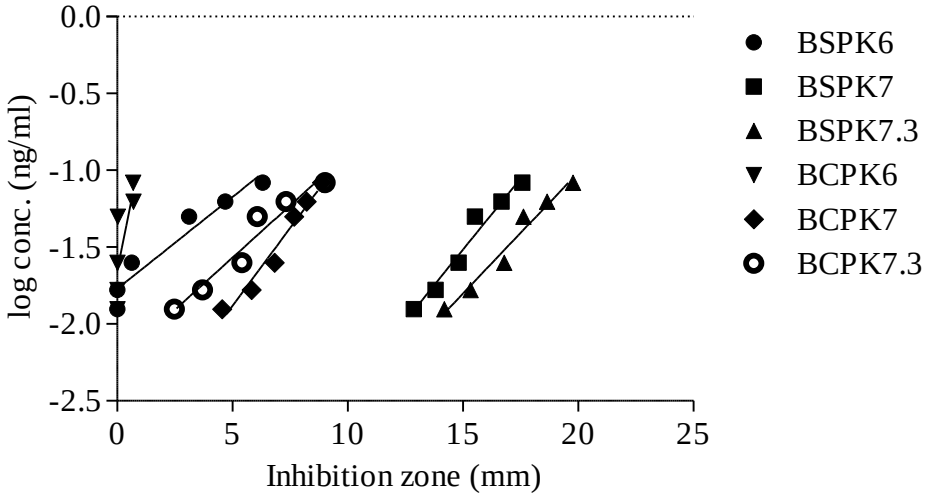

Figure 6: Linear regression curves of PEN G spiked kidney on plates I-VI

R^2: BSPK6=0.9275, p<0.0020; BSPK7=0.9632, p<0.0005; BSPK7.3=0.9685, p<0.0004; BCPK6=0.6292, p<0.0597, BCPK7=0.9647, p<0.0005; BCPK7.3=0.9462, p<0.0011. The curves were used to calculate LOD of PEN G in chicken kidney on plates I-VI.

CHAPTER FIVE

DISCUSSION

Microbiological inhibition tests are used for preliminary screening of foods for antibiotics residues so as to select samples which probably contain one or more analytes and which should be investigated further with more sophisticated immunochemical and/or chromatographic methods. Screening tests should be simple, cheap and fast (Okerman *et al*., 1998). A plate test consists of a layer of inoculated agar, with samples applied on top of the layer or in wells in the agar (Okerman *et al*., 1998; Pikkemaat *et al*., 2009). Screening methods are based on the use of sensitive bacteria and *B. cereus* and *B. subtilis* are normally incorporated (Koenen-Dierick *et al*., 1995; Pikkemaat *et al*., 2008). Bacterial growth turns the agar into an opaque layer, which yields a clear growth-inhibited area around the sample if it contains antimicrobial substances. The presence of antimicrobial residues in foods is of particular concern in developing countries, because legislation regarding MRLs for marketed products is often lacking and violation of withdrawal occurs regularly (Shitandi and Sternesjo, 2001).

The range of pH permitting growth of *B. cereus* in laboratory media has been reported to be 4.9 to 9.3 (Goepfert *et al*., 1972). However no *B. cereus* growth was found at pH 5 and 8. This could be partly contributed by the comparatively low inoculum of 10^5 cfu/ml used in this study. An inoculum of 10^8 cfu/ml was used in previous studies. As pH values drift away from pH 7, more concentrated inoculum is needed to give greater probability of initiating growth. It has also been reported that the effects of pH on growth of *B. cereus* vary with strain and growth medium used (Raevuori and Genigeorgis, 1975) and this could be another factor that resulted in this variation. The growth of *B. subtilis* is also greatly influenced by culture pH. In this study, no bacterial growth was observed at pH 5 and 8. These findings are in accordance with previous studies which reported reduction in bacterial growth at extreme pH values which prevent growth by affecting enzymes involved in metabolism (Cheong, 2006).

The pH of the test medium is an important factor influencing the detection limits of most antibiotics. The pH influences the permeability of bacteria to antibiotics, the stability and activity of enzymes which inactivate antibiotics and the stability and kinetics of certain antibiotics whereas changes in ionization of the antibiotic may be the crucial factor (Abraham and Duthie, 1946; Todt and McGroarty, 1992; Corkill *et al*., 1994; Lakaye *et al*., 2002). Samples spiked with OTC produced larger zones of inhibition as the pH was decreased from 7.3 to 6. This was in line

with other previous studies that indicated that decreasing pH increases the activity of tetracyclines (Korkeala *et al*., 1983; Myllyniemi *et al*., 1999; Myllyniemi *et al*., 2000; Jevinova *et al*., 2003; Przeniosło-Siwczyńska and Kwiatek, 2007). The detection limits in both liver and kidney for OTC were below the respective MRLs. However the LODs were lower on plates IV (pH6), V (pH 7) and VI (pH7.3) than on plates I (pH 6), II (pH7) and III (pH7.3) in both liver and kidney. Plate V gave the lowest LOD for OTC spiked kidney fluid.

The activity of PEN G decreased on a *B. subtilis* plate when pH was decreased as has been reported previously (Schwartz, 1965). The results were not consistent for PEN G on *B. cereus* seeded plates where the zones of inhibition were larger in plate V than at both plates IV and VI. This may be attributed to the fact that both *B. cereus* and *B. subtilis* have penicillinase whose activity is enhanced at low pH. Additionally, it has been shown that penicillin acts as an inducer of this enzyme (Pollock, 1950; Pollock, 1961; Knox and Smith, 1962). It has also been reported that the mechanisms of the effect of pH on antimicrobial activity are inconsistent from drug to drug and also based on the sensitivity or resistance of the test microorganisms to various antibiotics (Amsterdam, 1996; Karraouan *et al*., 2009). PEN G was detected below the MRLs on plates I, II, III, V and VI. The LODs were lowest on plates II, III, and V in both kidney and liver. The LOD was above the MRL on plate IV in both liver and kidney being highest in kidney fluid. Plate III gave the largest zones of inhibition for PEN G for both kidney and liver and the LODs were also optimal, meaning that this plate was the most sensitive to residues of PEN G. However these results were at variance with those reported by Popelka *et al*., (2005). This difference can be attributed to different sample preparation and matrix effects.

Incurred samples obtained from routine monitoring programs have been used to evaluate the performance of a method (Currie *et al*., 1998; Myllyniemi *et al*., 1999; Myllyniemi *et al*., 2000; Myllyniemi *et al*., 2001; Okerman *et al*., 2004). However, such an approach is very much limited by the availability of these samples. It is also impossible to produce incurred samples from different animal species with a specified concentration of specific residue. This has resulted in validation of most microbiological methods using antibiotic standard solutions and hence potential matrix effects are neglected. It is generally expected that the presence of matrix components has a negative effect on the sensitivity of an assay (Okerman *et al*., 1998; Pikkemaat *et al*., 2007). However Myllyniemi *et al*., (2000) showed that incurred kidney samples containing PEN G or OTC at their MRLs caused wider inhibition zones compared to standard solutions of

corresponding concentration. Fortifying extracted matrix fluid with the analyte or analytes at the required concentration may give more realistic results although tissue binding is not taken into account (Cantwell and O'Keeffe, 2006; Pikkemaat *et al*., 2009). In this study chicken liver and kidney were spiked with known concentrations of PEN G and OTC.

Kidney and liver are commonly used for screening slaughter animals for the presence of antibiotic residues (Hassan *et al*., 2007; Shahid *et al*., 2007; Pikkemaat *et al*., 2008; Pikkemaat *et al*., 2009). Oxytetracycline spiked kidney fluid produced larger zones of inhibition compared to liver spiked with the same antibiotic. This is because the OTC MRL in kidney lies substantially higher than the MRL in the liver and therefore was spiked with slightly higher antibiotic concentration. Penicillins and OTC are rapidly absorbed from the gastrointestinal tract of chicken due to their high tissue penetrating ability (Alhendi *et al*., 2000) and the maximum mean concentrations are found in the kidney followed by the liver (Anadon *et al*., 1994; Serrano *et al*., 1999; Alhendi *et al*., 2000). This is due to the fact that these organs are involved in storage, metabolism and the elimination of the drug (Warner *et al.*, 1990; Hassan *et al*., 2007). Kidney produced the lowest LOD for OTC and this was on plate V. Although chicken liver and kidney have equal MRLs for PEN G, inhibition zones produced by kidney and liver spiked with PEN G were at variance. However, the LODs were generally comparable in both liver and kidney. The presence of naturally occurring growth inhibiting compounds in kidney causes non-specific inhibition (Pikkemaat *et al*., 2008). This may have led to the variability in sizes of inhibition zones produced by both OTC and PEN G in kidney and liver. However, some high molecular weight naturally occurring growth inhibiting compounds in kidney, such as lysozymes, were removed by the centrifugation step.

Penicillin G and OTC were microbiologically detected in both liver and kidney samples. The detection capability of the plates seeded with *B. cereus* for OTC was better than that of the plates seeded with *B. subtilis*. This was in agreement with previous studies which reported that plates seeded with *B. cereus* were more specific for tetracyclines (Okerman *et al*., 2001; Okerman *et al*., 2004). *Bacillus subtilis* detected OTC below the MRL although PEN G produced largest inhibition zones on this plate. This was in line with previous observations which indicated that *B. subtilis* detect penicillins and tetracyclines as well as other antibiotic families but it is most sensitive for β-lactams detection (Okerman *et al*., 1998; De Wasch *et al*., 1998; Jevinova *et al*., 2003; Okerman *et al*., 2004; Popelka *et al*., 2005; Karraouan *et al*., 2009). In this study, *B.*

cereus and *B. subtilis* showed differential sensitivity and hence selectivity to OTC and PEN G respectively. Comparatively, the LODs for OTC and PEN G were lowest on plates seeded with *B. cereus* and *B. subtilis* respectively. However, this selectivity for these antibiotics was only observed with the usually low residue concentrations as very high concentrations also produced growth inhibition zones on the more selective plates. Okerman *et al*., (2004) also reported similar results.

Screening tests are designed to be easy inexpensive and time efficient. The results from this study reveal that both PEN G and OTC residues can be detected microbiologically in poultry tissues by the two test organisms. However, operational characteristics of each test were different. The tests differed not only in sensitivity and LODs but also in selectivity. Plate III can be used to effectively assay for residues of PEN G in chicken kidney or liver. Plate V is efficient for assay of OTC residues in chicken liver or kidney. These plates gave optimal LOD for these antibiotics.

A combination of these two plates can therefore be sufficient for the assay of PEN G and OTC. Chicken kidneys are small and more often than not are not removed with other offals. They therefore remain in the chicken meat after slaughter. Analysis of chicken liver using these two plates is sufficient for the detection of level antibiotic residues in chicken meat. This approach also offers the advantage of not requiring specialist microbiology facilities or expertise in order to perform the analysis. It is technically simple and can be performed in most laboratories.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The following conclusions were drawn from the study:

1. The pH of the growth media influences the growth of test organisms *B. cereus* and *B. subtilis* on Mueller Hinton agar. These organisms grow optimally at pH 6 to7.3.
2. The pH of the growth media, type of organ, antibiotic concentration and the test organism affect to a larger extent the detection of OTC and PEN G antibiotic residues.
3. Oxytetracycline and PEN G can be detected below the MRLs by *B. cereus* and *B. subtilis.* However the LODs are affected by pH of growth media, the type of organ and the test organism.
4. Plate III (*B. subtilis*; pH 7.3) and V (*B. cereus*; pH 7) can be used effectively for routine screening for residues of PEN G and OTC respectively in chicken kidney or liver. However, due to the small size of chicken kidney, assay of residues of these antibiotics can sufficiently be performed using chicken liver.
5. The described method offers the advantage of not requiring specialist microbiology facilities or expertise in order to perform the analysis. It is technically simple and can be performed in most laboratories.

6.2 Recommendations

1. The findings of this study indicate the feasibility of identifying antibiotic residues in kidney and liver samples using a microbiological method. Therefore the described method can be applied in routine monitoring of antibiotic residues in the Kenyan poultry industry.
2. The described method offers a good basis for developing improved resolution antibiotics residues screening test. Moreover, further studies should be performed to validate the method using other antibiotic groups.

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APPENDIX

Appendix 1: Plate composition and incubation conditions

Test organism	Test plate	pH	Antibiotic target	+ve control (µ/l)	Sample	Cfu/ml	Medium	Incubation temp/ time °C/h
B. subtilis	I	6		600µg/ l	Liver	10^5 spores	MHA	30°C/16h
	II	7	OTC	600 µg/l	Liver	10^5 spores	MHA	30°C/16h
	III	7.3		600µg/l	Liver	10^5 spores	MHA	30°C/16h
	I	6		50 µg/l	Liver	10^5 spores	MHA	30°C/16h
	II	7	PEN G	50 µg/l	Liver	10^5 spores	MHA	30°C/16h
	III	7.3		50 µg/l	Liver	10^5 spores	MHA	30°C/16h
	I	6		1200µg/l	Kidney	10^5 spores	MHA	30°C/16h
	II	7	OTC	1200µg/l	Kidney	10^5 spores	MHA	30°C/16h
	III	7.3		1200µg/l	Kidney	10^5 spores	MHA	30°C/16h
	I	6		50µg/l	Kidney	10^5 spores	MHA	30°C/16h
	II	7	PEN G	50µg/l	Kidney	10^5 spores	MHA	30°C/16h
	III	7.3		50µg/l	Kidney	10^5 spores	MHA	30°C/16h
B. cereus	IV	6		600µg/l	Liver	10^5 spores	MHA	30°C/16h
	V	7	OTC	600µg/l	Liver	10^5 spores	MHA	30°C/16h
	VI	7.3		600µg/l	Liver	10^5 spores	MHA	30°C/16h
	IV	6		50µg/l	Liver	10^5 spores	MHA	30°C/16h
	V	7	PEN G	50µg/l	Liver	10^5 spores	MHA	30°C/16h
	VI	7.3		50µg/l	Liver	10^5 spores	MHA	30°C/16h
	IV	6		1200µg/l	Kidney	10^5 spores	MHA	30°C/16h
	V	7	OTC	1200µg/l	Kidney	10^5 spores	MHA	30°C/16h
	VI	7.3		1200µg/l	Kidney	10^5 spores	MHA	30°C/16h
	IV	6		50µg/l	Kidney	10^5 spores	MHA	30°C/16h
	V	7	PEN G	50µg/l	Kidney	10^5 spores	MHA	30°C/16h
	VI	7.3		50µg/l	Kidney	10^5 spores	MHA	30°C/16h