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A comparison of methods used to measure the *in vitro* antimicrobial susceptibilities of *Mycoplasma* species of animal origin

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2010

SUMMARY

Title

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Antimicrobials are commonly used to treat mycoplasmosis in animals. In spite of this and the fact that antimicrobial resistance has been recorded for this group of bacteria there are no universally accepted *in vitro* means of testing for this resistance, nor is resistance testing for mycoplasmas a routine in most veterinary laboratories. So prior to testing for resistance to a number of mycoplasmas isolated from animals in South Africa it was necessary to compare different tests including broth and agar microdilution tests to find out which one would perform best.

Using the field strains *M. bovis*, *M. crocodyli*, *M. felis*, *M. gallisepticum* and *M. synoviae*, and the reference strains *M. gallisepticum* 56USDA, *M. gallisepticum* VaxSafe MG vaccine strain, *M. mycoides* T1/44 vaccine strain, and *M. mycoides* Y-*goat* (11706) broth- and agar-microdilution minimum inhibitory concentration (MIC) tests were performed using either modified Hayflicks or *Mycoplasma synoviae* media. Two different metabolism indicator systems were compared in the broth micro-dilution test (BrMIC) namely sugar fermentation (glucose or pyruvate) with phenol red (SFS) and evidence of reduction with resazurin (AlamurBlue®). It was also tested

whether amoxicillin and clavulanic acid (ACA) could be used in the tests to reduce problems associated with contamination.

Statistical analyses of the tests (repeatability and linear association) indicated that the BrMIC with SFS was the most reproducible method (pooled standard deviation = 0.14). The antimicrobial ACA was found to not to affect the MIC values (R^2= 0.976 to 0.996).

Furthermore one hundred forty two field strains including 93 *M. bovis,* 5 *M. synoviae,* 21 *M. gallisepticum*, 13 *M. bovirhinis,* 8 *M. crocodyli* and 6 *M. felis* were tested using the BrMIC+SFS with ACA method. Generally the mycoplasmas originating from poultry were resistant to commonly used antimicrobials and had higher MIC_{50} and MIC_{90} values than isolates originating from cattle, crocodiles and cats. It was found that most of the mycoplasmas were susceptible to doxycycline (tetracycline) and enrofloxacin with the exception of *M. gallisepticum* where 17.9% of strains were resistant to both. Resistance to tiamulin (100%) and tylosin (20 to 64%) was high for the poultry mycoplasmas. Most field isolates tested were resistant to erythromycin, nalidixic acid, florfenicol, norfloxacin, neomycin, sulphamethoxazole, trimethoprim and sulphamethoxazole/ trimethoprim combination, mostly resistant to norfloxacin and florfenicol.

It is concluded that BrMIC+SFS with ACA method is a reproducible method that reduces any problems with bacterial contamination. As observed with the poultry strains, it is quite clear that antimicrobial resistance is developing to commonly used antimicrobials such as tylosin, the related pleuromutilins, fluoroquinolones and tetracyclines. In species where antimicrobial therapy is applied routinely such as poultry and possibly feedlot cattle, it is recommended that MIC testing is done prior to any therapeutic interventions.

ACKNOWLEDGEMENTS

It is a particular pleasure to have the opportunity to thank all those who helped me in the research and preparation of this dissertation.

Sincere thanks are due to my supervisor Dr Jackie Picard for her meticulous and painstaking efforts as well as for her patience and forbearance; without her cheerful and ungrudging help this work would have never been brought to completion.

My sincere thanks Prof Moritz Van Vuuren for his support with editing of the dissertation as a co-supervisor.

I am well aware how hard it could have been to finish my lab work without technical skills I gained from Mr Johan Gouws who taught me kindly and patiently. My thanks also extended to Ms Debbie Landman for her laboratory support as her help saved lots of time.

My sincere debt is to my eldest brother Mohammed Kibeida who supported me financially, as without his support I could have been able to finish the degree.

To my mother, who gave me the motivation and a love of life.
And at the memory of my father, who taught me to do things with honesty for the benefit and sake of others before myself.

Last, and certainly not least, I am will most sincerely thank my colleagues Dr Habib Golezardy, Dr Akin Jenkins, Ms Mamohale Chaisi and Ms Ayesha Hashim for their emotional and motivational support whenever I was down during hard times.

ABBREVIATIONS

AB	AlamurBlue™
ACA	amoxicillin and clavulanic acid
AMIC	agar minimum inhibitory concentration
BrMIC	broth micro-dilution test
CA-SFM	Comité de l'Antibiogramme de la Société Française de Microbiologie
CBPP	Contagious bovine pleuropneumonia
CCPP	Contagious caprine pleuropneumonia
ccu/mP	colour changing units per milliliter
cfu	colony forming units
CLSI	Clinical and Laboratory Standards Institute
CRG	Commissie Richtlijnen Gevoeligheidsbepalingen
DIN	Deutsches Institut für Normung
ELISA	enzyme linked immunosorbent assay
EMEA	European Medicines Evaluation Agency
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
IFAT	indirect immunofluorescent antibody test
MCA	*Mycoplasma* Culture agar
MCB	*Mycoplasma* Culture broth
MIC	Minimum inhibitory concentration
*Mmm*LC	*Mycoplasma mycoides* subsp. *mycoides* Large colony variant
*Mmm*SC	*Mycoplasma mycoides* subsp. *mycoides* Small colony variant
MS	modified Frey's medium
NaOH	sodium hydroxide
NWGA	Norwegian Working Group on Antibiotics
OIE	Office International des Epizooties
PBS	phosphate buffered saline
SFS	sugar fermentation (glucose or pyruvate) with phenol red
SRGA	Swedish Reference Group of Antibiotics

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CHAPTER ONE

Introduction

Mollicutes which are considered to be the smallest self-replicating micro-organism, are known to cause a wide variety of infections in animals. Infections caused by mycoplasmas are usually mild in endemic populations but can be severe in immunologically naïve animals or those subjected to extreme stress (Minion, 2002). There are very few registered vaccines against mycoplasmosis in animals namely; the live attenuated T1/44 vaccine strain against contagious bovine pleuropneumonia (CBPP) and various live attenuated strains or bacterins against *M. gallisepticum* and *M synoviae* in poultry and bacterin vaccines against *M. hyopneumoniae* in pigs. With the difficulties associated with the use of these vaccines as well as the lack of vaccines for other mycoplasmal diseases, antimicrobials are increasingly being used to treat and prevent them (Gautier-Bouchardon et al., 2002). This has increased the risk of antimicrobial resistance development. Since mycoplasmas may not survive transport and are difficult to grow, the development and standardization of tests to detect this resistance has been slow. Currently, there are no internationally accepted standards regarding the methods, media used and breakpoints for the laboratory determination of resistance, making the comparison of results issued from different laboratories almost impossible (Waites et al., 2007).

Although antimicrobial resistance to mycoplasmas of animal and human origin have been reported in other parts of the world, there are currently no published results regarding the antimicrobial susceptibilities of the pathogenic *Mycoplasma* species isolated from diseased domestic animal in South Africa.

Literature review

1.1 Mycoplasmosis

1.1.1 Classification of Mycoplasmas

Mycoplasmas are the smallest micro-organisms that are capable of self- replication. They are considered as bacteria even though they lack a typical bacterial cell wall. They are classified as[1]:

- Class: Mollicutes
- Order: Mycoplasmatales
- Family: Mycoplasmataceae
- Genus: *Mycoplasma, Hepatoplasma, Ureaplasma, Mycoplasma,* unclassified Mycoplasmataceae, Environmental samples

Being fastidious and delicate micro-organisms, mycoplasmas require enriched media for growth. The basic medium is good quality beef infusion with supplements such as yeast extract. Additionally, *Mycoplasma* and *Ureaplasma* species require cholesterol and this is usually provided by adding 20 - 30% horse, calf, rabbit or pig serum to the culture medium (Quinn et al., 1994).

Several methods are used to characterize mycoplasmas, they include:

Biochemical and Enzymatic Methods

Tests that are used to detect specific enzymes and nutritional requirements of mycoplasmas have proven useful in their classification. These are used to differentiate mycoplasmas at the family and the genus level. Although rarely used in the diagnostic laboratory, biochemical tests namely: glucose fermentation; arginine and urea hydrolysis; phosphate activity; film and spot production; and the liquefaction of inspissated serum, have proven efficient to characterize most *Mycoplasma* isolates prior to immunological identification (Quinn et al., 1994).

[1] www.ncbi.nlm.nih.gov

Immunological Methods

Two different types of tests have been used to identify mycoplasmas. The first group includes procedures that use living mycoplasmas whose growth or metabolic function can be inhibited by specific antiserum, the so-called neutralization tests, *i.e.* growth inhibition and metabolic inhibition tests. The second group involves the identification of mycoplasmas by specific antibody reactions with whole organisms or their antigens, *e.g.* direct or indirect immunofluorescence tests and the enzyme linked immunosorbent assay (ELISA) (Goll, 1994). The lack of cross-reactivity of *Mycoplasma* species when using the neutralization tests are used as a criterion for speciation.

Molecular Techniques

Partial sequencing of the 16S rDNA amplicon is used to differentiate between the different *Mycoplasma* species. Furthermore, species-specific DNA probes have been developed for the identification for some species (McAuliffe et al., 2006). Strain variation has been determined using restriction fragment polymorphisms (Kokotovic et al., 1999).

1.1.2 Disease in Animals

Many mollicutes have been recognized in all animals and even some plant species. Among approximately the 200 known different species of mollicutes identified in animals, only a small number, mainly *Mycoplasma* species, are known as pathogens (Razin, 2006). The most important species of mycoplasmas and the diseases that they cause in domestic animals and farmed crocodiles are shown in Table 1.

Mycoplasmas are generally adapted to a specific host where they exhibit virulence and cause disease. Colonization of secondary (atypical) hosts is rare, where only mild or no disease was noted (Joachim, 2002). Other than the haemotropic mycoplasmas, mycoplasmas have a pronounced affinity for mucous tissues and consequently show a predilection for the respiratory system, mammary gland, urogenital tract and serous membranes. Disease in most animals tends to be chronic in nature with a high morbidity but relatively low lethality. In endemic herds, most infected animals tend to be clinically

Table 1: Major pathogenic mollicutes of animals (Joachim, 2002).

Animal Host/ Mollicutes species	Disease
Bovines	
M. mycoides subsp. *mycoides* SC	Contagious bovine pleuropneumonia (CBPP)
Mycoplasma sp. bovine group 7	Pneumonia and arthritis
M. bovis	Mastitis, pneumonia (calf), polyarthritis (calves), metritis, abortion, sterility
M. dispar	Pneumonia (calves)
M. californicum	Mastitis
M. canadense	Mastitis
M. bovigenitalium	Mastitis and genital disease
M. bovocculi	Conjunctivitis
M. wenyonii	Anaemia
Sheep and goats	
M. capricolum subsp. *capripneumoniae*	Contagious caprine pleuropneumonia (CCPP)
M. capricolum subsp. *capricolum*	Mastitis, arthritis
M. mycoides subsp. *capri*	Pneumonia, mastitis, arthritis, septicemia (goats)
M. mycoides subsp. *mycoides* LC	Pneumonia, mastitis, arthritis, septicemia (goats)
M. agalactiae	Infectious agalactia
M. ovipneumoniae	Atypical pneumonia (lambs)
M. conjunctivae	Infectious keratoconjunctivitis (sheep)
Wild caprine	
M. conjunctivae	Infectious keratoconjunctivitis (ibex, chamois)
Poultry	
M. gallisepticum	Chronic respiratory disease (chicken), sinusitis, infectious air sacculitis (turkey)
M. synoviae	Air sacculitis, arthritis, tendosynovitis
M. meleagridis	Air sacculitis, arthritis, sinusitis (turkeys)
Swines	
M. hyponeumoniae	Enzootic pneumonia
M. hyorhinis	Pneumonia, arthritis
M. hyosynoviae	Arthritis
M. suis	Anaemia
Horses	
M. felis	Pleuritis
M. equirhinis	
Dogs and cats	
M. cynos	Pneumonia
M. felis	Conjunctivitis, pneumonia (cats)
M. haemocanis	Anaemia (dogs)
M. haemofelis	Anaemia (cats)
Small rodents	
M. arthritidis	Arthritis (rat)
M. pulmonis	Respiratory & genital tract infection (rat, mouse)
Crocodiles	
M. crocodyli	Polyarthritis in crocodiles
M. alligatoris	Polyserositis and arthritis in crocodiles and alligators

normal carriers that only become diseased when stressed. These animals tend to be instrumental in the spread of disease to previously clean herds.

In poultry, *M. gallisepticum*, is a major pathogen that causes chronic respiratory disease in chickens and infectious sinusitis in turkeys, both of which result in insufficient growth and can in susceptible birds lead to death (Reinhardt et al., 2002). Various measures including the use of disease-free birds and vaccination have been applied in many countries in the hope of eradicating the disease. However, their haphazard application and difficulties associated with the detection of carrier birds often results in failure of these programmes. In those areas where infection remains endemic, measures to control the infection often rely heavily on the widespread use of antimicrobials where macrolides are a common drug choice for treatment of *M. gallisepticum* infection. However, resistance readily develops when these antimicrobials are used frequently (Gautier-Bouchardon et al., 2002).

In cattle the agent of CBPP, *Mycoplasma mycoides* subsp. *mycoides* SC (small colony) (MmmSC) is the most pathogenic where it is considered by the Office International des Epizooties (OIE) to be a major constraint to cattle farming in endemic areas of Africa. *Mycoplasma bovis* and to a certain extent *M. dispar*, which are commensals of the upper respiratory tract in cattle, have a global distribution and results in respiratory and joint disease in intensively reared calves and feedlot beef cattle (Thomas et al., 2003). *Mycoplasma bovis* is also an important cause of mastitis in dairy cows (Nicholas and Ayling, 2003). The pneumonic lesions associated with *M. bovis* infection appear as subacute or chronic suppurative bronchopneumonia with multiple foci of caseous necrosis, although some studies describe these nodular lesions as coagulative necrosis or abscesses (Farshid et al., 2002).

Sheep and goats are infected by a wide range of mycoplasmas among which the most important are *M. agalactiae* the primary cause of contagious agalactiae, *Mycoplasma capricolum* subsp. *capripneumoniae* the cause of contagious caprine pleuropneumonia, *M. mycoides* subsp. *mycoides* large colony type a possible cause of pizzle disease, *M. capricolum* subsp. *capricolum.* Other diseases include atypical pneumonia, caused by

M. ovipneumoniae and infectious keratoconjunctivitis ("pink-eye") caused by *M. conjunctivae* (DaMassa et al., 1991). Contagious agalactia affects both sheep and goats especially those used for milk production, which is characterized by mastitis, keratoconjunctivitis and arthritis. *M. agalactiae*, *Mmm*LC and *M. capricolum* subsp. *capricolum* have a similar if not identical clinical presentation. The disease caused by *M. agalactiae* is of considerable economic importance because of its high morbidity rather than high mortality (Loria et al., 2003). Contagious caprine pleuropneumoniae is one of the most serious fatal diseases of goats mostly reported in Africa. It has many similarities to contagious bovine pleuropneumoniae but is not transmissible to cattle. It has a high infectivity with high morbidity 100% with a mortality rate of 60-100% (Msami et al., 2001). *Mycoplasma conjunctivae* causes caprine and ovine conjunctivitis, keratoconjunctivitis, and can be isolated frequently from the eye and the nasopharynx. Hosts infected with this agent show lacrimation, conjunctival hyperemia, pannus, neovascularization, iritis, and keratitis (Belloy et al., 2003). *Mycoplasma capricolum* is primarily a goat pathogen but has also been encountered in sheep, in goats; *M. capricolum* is highly destructive, causing high morbidity and mortality (Monnerat et al., 1999). *Mycoplasma putrefaciens* can cause septicemia, pneumonia and mastitis in small ruminants that are predisposed by other diseases. It has received little concern till now even though it has been listed as one of the etiologic agents of the contagious agalactia syndrome by the World Organisation for Animal Health (Peyraud et al., 2003).

Many *Mycoplasma* species can cause diseases to swine, e.g., *M. hyponeumoniae*, *M. hyorhinis*, *M. hyosynoviae* and *M. suis*. Other mycoplasmas have been isolated from swine but their pathogenicity is questionable. *Mycoplasma hyopneumoniae* is an economically significant pathogenis that causes enzootic pneumonia in commercial pigs which is endemic in the majority of commercial herds and can predispose swine to subsequent respiratory bacterial infections. It is a major swine pathogen causing enzootic pneumonia, a chronic respiratory disease in pigs resulting in considerable economic losses (Boye et al., 2001). *Mycoplasma hyosynoviae* resides for long in the tonsils, probably the entire life of many pigs. It can cause a arthritis in grower and adult pigs resulting in growth retardation and inability to mate in boars (Boye et al., 2001). *Mycoplasma suis* belongs to the haemotrophic *Mycoplasma* group and causes severe infectious anemia in pigs with a fatal disease course (Hoelzle, 2007). Chronic *M. suis*

infections result in reproductive disorders in sows, growth retardation in piglets, and increased susceptibility to respiratory and enteric infections in feeder pigs.

Several species of mycoplasmas have been isolated from the respiratory tracts of both healthy and diseased horses, including *Mycoplasma equirhinis* and *M. felis*, without clear evidence of pathogenic significance. However, *M. felis* has been isolated from cases of pleuritis in horses and experimental infection with this organism induced pleuritis (Wood et al., 1997).

Mycoplasmas canis, *M. cynos* and *M. haemocanis* are the most important canine species, being associated with urogenital tract infections infertility, respiratory disease and anaemia, respectively (L'Abee-Lund et al., 2003).

1.1.3 Distribution of *Mycoplasma* spp. in Southern Africa

Other than the State controlled diseases *i.e.* CBPP and contagious caprine pleuropneumonia (CCPP), which are absent, it is known from laboratory data that all the other common causes (Table 1) of mycoplasmosis in animals, are present in this country (Table 2). Contagious bovine pleuropneumonia was eradicated from South Africa in 1924, but in southern Africa, still occurs in the northern part of Namibia, south-east Zambia, Angola and Tanzania (Thiaucourt et al., 2004). The disease is still widespread in eastern and western Africa and is in those countries considered as an important cause of economic losses in cattle. Contagious caprine pleuropneumonia which is present in many parts of eastern and northern Africa has never been identified in southern Africa. Three unique mycoplasma sequences were identified by sequencing of the 16S rRNA gene of the mycoplasma isolates obtained from the ostrich in the Northern and Western Cape Provinces. The three mycoplasmas species were named Ms01, Ms02 and Ms03 (Botes et al., 2005).

The Bacteriology Laboratory in the Department of Veterinary Tropical Diseases (DVTD) has from 1997 to 2007 isolated and identified mycoplasmas originating from several animals. These are listed in Table 2.

Table 2: **Mycoplasmas cultured and identified from specimens of animal origin in the DVTD from 1997 to 2007.**

Animal species	Sample	*Mycoplasma*	No of isolates
Cattle	Transtracheal washes and lungs	*M. bovis*	100
		M. bovigenitalium	35
		M. arginini	30
		Mycoplasma leachii	8
		Acholeplasma laidlawii	12
		Not identified	100
Crocodiles	Pericardial sac	*M. crocodyli*	2
	Joint fluid	*M. crocodyli*	14
		M. alligatoris	8
Ostriches	Nasal sinuses	Ostrich mycoplasma MS01 & MS02	14
Dorper Sheep	Prepuce	*Mycoplasma mycoides* subsp. *mycoides* LC	55
	Eye	*M. conjunctivae*	3
Chickens	Nasal sinuses and trachea	*M. gallisepticum*	25
	Joints	*M. synoviae*	1
Cat	Eye and nasal cavities	*M. felis*	5
Dog	Transtracheal washes	Unknown	3

1.2.4 Epidemiology

Since *Mycoplasma* species cannot survive for long time in the environment, their transmission depends mostly on the presence of the animal host where they are usually carried on the respiratory or genital mucosae. These carrier animals tend to be either those in the incubation stages, or those showing signs of disease and those that are sub-clinically affected. Occasionally recovered animals can shed them in persistent lesions e.g. lung sequestra as in CBPP and CCPP (Razin, 2006).

The three main important factors in transmission rate of most mycoplasmal infections are: closeness of contact, infection intensity, and the number of susceptible animals. Therefore transmission occurs most easily via animals nosing each other by the inhalation of respiratory, milk or urine aerosols in closely housed or trucked animals. Thus in cattle feedlots, intensively housed chickens and pigs, spreading *Mycoplasma* species is rapid (Whithear and Brwoning, 2004). Even under extensive sub-Saharan African farming conditions, spread of infection may be rapid due to infection being facilitated by the general practice of kraaling or penning the animals at night, and by the congregation of large numbers of animals at places such as watering troughs or markets. Direct and continuous contact between susceptible and diseased animals seems to be essential for transmission in such cases like CBPP (Thiaucourt et al., 2004) Mycoplasmas can also be transmitted venereally as in the case of *M. bovigenitalium* infection in cattle, *M. mycoides subsp. mycoides* LC in sheep and *M. canis* in dogs (Kanakas et al., 1999).

Because they lack a cell wall and are susceptible to desiccation and disinfectants mycoplasmas don't survive in the environment for long periods, hence the role of the environment in disease dissemination is of lesser importance. However, indirect dissemination can occur within a short period after excretion via food, water and some fomites (Christensen et al., 1994).

Environmental stress and pollutants such as ammonia and nitrites as well as concurrent disease increase susceptibility to mycoplasmosis. This may be through a detrimental

effect on the mucosal lining or through impaired macrophage function and activity of NK (natural killer) cells (Whithear and Brwoning, 2004).

1.2.5 Pathogenesis

Interactions between the pathogen, host and the environment determine the outcome of infections. The host has developed numerous ways of protecting itself while bacteria have developed many strategies to avoid or attack these host defenses. For a pathogen to succeed, it must have a way of entering its host, reaching their predilection side and adhere to it. It should invade the target tissue, and multiply. During this process, the successful pathogen must also evade the host defenses and cause some damage to the host. Finally it must be able to escape and transmit to fresh hosts. Such processes are challengeable to mycoplasmas which have a very limited number of genes and whose survival time outside the host is short (Whithear and Brwoning, 2004).

Mycoplasmas are considered as surface parasites, adhering to and colonizing the epithelial linings of the respiratory and urogenital tracts, rarely invading tissues (Razin, 1999). What might facilitate the direct contact of the mycoplasma membrane with that of its eukaryotic host is the lack of a cell wall creating a condition which, in principle, might lead to fusion of the two membranes, enabling the transfer or exchange of membrane components, and injection of mycoplasmal cytoplasmic content, including hydrolytic enzymes, into the host cell cytoplasm. This is further aided by the gliding motility these bacteria exhibit. Therefore, a significant percentage of genes in the minute mycoplasmal genomes are allocated to adhesion (Razin, 1999). Certain proteins found on the surface of some mycoplasmas also assist in adhesion, for example, *M. gallisepticum* where its primary attachment to the surface of the respiratory tract may be facilitated by the diffusely distributed VlhA heamagglutinin while in the case of *Mmm*SC, galactan is thought to be involved in surface adhesion (Nicholas and Bashiruddin, 1995).

Once colonization has happened infection may either spread locally as in the case of respiratory and urogenital tract infection, or penetrate epithelial barriers and spread haematogenously. The respiratory mycoplasmas such as *M. hyopneumoniae* are able to

affect ciliostasis in the respiratory tract, hence predisposing to deeper penetration of the respiratory tract as well as secondary bacterial invasion.

However, sometimes more generalized infection and even acute septicemia may occur as in *Mycoplasma mycoides* subsp*. capri* which has the pathogenic ability to cause septicemia, mainly in young goats (Thiaucourt et al., 2000). This may occur when host immunity is low or as a sequel to primary disease at another site (conjunctivitis, pneumonia, or mastitis).

Common consequences following a usually inapparent mycoplasmaemia are localization in and inflammation of serosal cavities or joints, manifesting as one or a combination of polyserositis, tendosynovitis, or arthritis. Infections leading to polyarthritis or polyserositis seem to persist and are always accompanied by chronic inflammatory processes. In chickens and turkeys *M. synoviae* appears as an inhabitant of the respiratory airways as a subclinical infection. Those strains with a specific tropism for synovial tissues tend to be more virulent (Kleven et al., 1975). On the other hand in cattle arthritis caused by *M. bovis* can follow a primary pneumonia in feedlot cattle or in calves suckling cows with *M. bovis* mastitis (Whithear and Brwoning, 2004).

There are only a few recognized virulence factors of mycoplasmas which include: hydrogen peroxide production, the carbohydrate capsule (Almeida and Rosenbusch, 1991; Tajima et al., 1982), the ability to utilize arginine from host cells and T-cell mitogens (Tu et al., 2005). Hydrogen peroxide and its superoxide derivatives seems to be generated as a product of the flavin-terminated electron transport chain common to most mycoplasmas (Brennan and Feinstein, 1969; Miles et al., 1991). They are responsible for the haemolytic activity of mycoplasmas *in vitro* and for their ciliostatic effect in tracheal organ cultures (Niang et al., 1998). Their action requires very close contact to host cells and is inhibited by host catalase. Recent studies of less-virulent strains of *M. mycoides* subsp. *mycoides* have suggested that the reduced virulence of these strains may be associated with a decreased capacity to generate hydrogen peroxide (Vilei and Frey, 2001). The ability of some mycoplasma to make use of crucial nutrients, such as arginine may also contribute to ciliostasis (Niang et al., 1998).

In addition, most diseases caused by mycoplasmas are associated with their ability to cause an excessive immunological response from the host (Whithear and Brwoning, 2004). The potent nucleases of mycoplasmas combined with superoxide radicals may be responsible for clastogenic effects observed in eukaryotic cells infected by some mycoplasmas (Rottem, 2003).

Another characteristic of mycoplasma infections is its ability to remain in the host in spite of an immunological response. Bacteria are thought to persist in the host by the formation of an adherent biofilm (McAuliffe et al., 2006). The ability of mycoplasmas to enter cells may contribute to resistance of mycoplasmas towards the immune system, as well as resisting antibiotic treatment (Winner et al., 2000). Furthermore, some *Mycoplasma* species, including poultry pathogens, have a remarkable ability to vary their major surface antigens, a process that is thought to help them to remain in their host by evading the immune response (Bradbury, 2005). These factors may explain the chronicity of mycoplasmal infections and the difficulties in eradicating mycoplasmas from infected tissue and cell cultures (Razin, 1999).

The interaction between mycoplasmas and their host immune system includes mycoplasma-mediated specific and non-specific immune reactions. Specific protective defense mechanisms involve the release of systemic as well as local anti-mycoplasmal antibodies of different classes and subclasses, stimulation of cell-mediated immunity, and opsonization and phagocytosis of organisms. Humoral immunity has a vital major role in defense against systemic dissemination of mycoplasmal lung disease. Mycoplasmas have been proven to affect the immune system by inducing mainly suppression, or polyclonal stimulation of B and T lymphocytes, increasing cytotoxicity of macrophages, induction of cytokines, natural killer cells and T cells, enhancing expression of cell receptors and activation of the complement cascade (Razin, 2006).

1. 2.6 Control strategies

Only those diseases that cause marked economic losses tend to be controlled or even eradicated. The control of mycoplasma infections is based upon:

- Mass vaccination and disease surveillance.

- Segregation of infected animals.
- Chemotherapy.
- For CBPP and CCPP a “Stamping out” policy is followed in disease-free countries such as South Africa.

Vaccination

Since mycoplasmas are considered as poor antigens, the control of these diseases with vaccination has met with limited success. It is found that for animals to become protected, they have to be exposed repeatedly to live attenuated vaccines. In fact *M. gallisepticum* and *M. synoviae* attenuated vaccines continue to circulate in the poultry population, so that the birds are permanently exposed to the vaccine strains (Razin, 2006). This practice has not been used for other animal species. However precaution must be exercised when using live *M. gallisepticum* F-strain vaccine which provides high level of protection in adult birds but may be pathogenic for chicks. Furthermore, there is always the risk of reversion to virulence (Razin, 2006). Inactivated vaccines are used against *M. hyopneumoniae* in pigs. In cattle where *Mmm*SC is endemic the live attenuated vaccine (T1/44 strain) that is repeatedly administered to improve the protective response. Thus, antimicrobial therapy is often resorted due to the limited range of vaccines available, difficulties in handling live vaccines and poor antigenicity of the vaccines (Ross and Young, 1993).

Chemotherapy

It is often necessary in the control of mycoplasmal infections to complement barrier measures by the use antimicrobial therapy. This will reduce economic losses and lateral and vertical transmission (Gautier-Bouchardon et al., 2002). The development of effective applicable therapeutic strategies depends upon an accurate and detailed understanding of the antibiotic resistance mechanisms as well as the antibiotic distribution within the animals and its mode of activity on the mycoplasmas. Their applications and limitations are discussed in the next section.

1.2 Antimicrobial Therapy

Antimicrobial drugs are chemical compounds that inhibit or abolish the growth of microorganisms, such as bacteria, fungi, or protozoans. They are used in animals, humans and plants to treat and prevent bacterial infections and to improve production efficiency in food-producing animals. The treatment of mycoplasmosis is based on aggressive and long term antimicrobial therapy (Hirsh, 2000). However, in most cases treatment is reported to be unsatisfactory (Nicholas and Ayling, 2003).

1.2.1 Classification of antimicrobials

At the highest level, antimicrobials can be classified depending on its mechanism of action as either bacteriocidal or bacteriostatic. Bacteriocidal drugs such as penicillin and streptomycin have a rapid lethal action and kill bacteria directly whereas bacteriostatics such as tetracycline and sulphanomides inhibit the growth of organisms. Bacteriostatic drugs depend upon the immune system to kill and remove the bacteria. However, in practice this classification is not always clear-cut. Most drugs are, in varying efficacy, both bacteriocidal and bacteriostatic (Yao and Mollering, 2007a).

Antimicrobials can also be classified according to their modes of action, which includes: interference with cell wall synthesis, *e.g.* penicillins, cephalosporins and fosfomycin; interference with cytoplasmic membrane, *e.g.* polymyxin; interference with protein synthesis *e.g*. tetracyclines, aminoglycosides and linezolid; interference with nucleic acid synthesis *e.g.* fluoroquinolones; and lastly inhibition of the metabolic pathway for folic acid synthesis *e.g.* sulphonamides and trimethoprim (Yao and Mollering, 2007a).

1.2.2 Antimicrobial classes used to treat *Mycoplasma* species infections

The use of antimicrobials in the treatment of mycoplasmosis in animals and humans is not as well documented as for the bacteria with cell walls. Mycoplasmas are considered to be sensitive to antimicrobial agents that affect RNA, DNA, protein synthesis, or the integrity of the cell membrane. Mycoplasmas are not affected by antimicrobials that target and interfere with the synthesis of folic acid (i.e., sulphonamides and trimethoprim) or that targets the cell wall such as the β-lactams and fosfomycin. This limits the range of antimicrobials available to treat infections. Antimicrobials that have

shown to be effective against most *Mycoplasma* species are tetracyclines, macrolides (erythromycin, clindamycin, tylosin and tiamulin), chloramphenicol, aminoglycosides, and fluoroquinolones (Renaudin and Bébéar, 1990). Antibiotics which are ineffective *in vitro* are likely to perform similarly *in vivo* while those with strong activities *in vitro* will not necessarily perform well in the field (Ayling et al., 2000). Furthermore, some antimicrobials are unable to penetrate granulomas and eukaryotic cells, where some mycoplasmas hide, making it a challenge to eradicate by antimicrobial treatment (Taylor-Robinson and Bebear, 1997). Chemotherapy, although officially discouraged in many African countries, is widely applied, some 69% of farmers in North Western Ethiopia confessed to treating CBPP cases with antibiotics recently in a survey G. Takele, unpublished cited by (Huebschle et al., 2006). This is despite the belief that treatment is largely ineffective and even counter-productive because of the risk of creating sub-clinical carriers (Thiaucourt et al., 2004).

Tetracyclines

Tetracyclines are usually considered the first line of treatment in mycoplasmosis. Tetracycline, which includes oxytetracycline and chlotetracyline, and the related antibiotics minocycline and doxycycline inhibits 30 S ribosomal subunits, preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor of A-site in the RNA-ribosome complex (Chopra and Roberts, 2001). Tetracycline is a broad-spectrum antibiotic with few side effects and relatively low toxicity. It is considered to be bacteriostatic and inhibits protein synthesis in susceptible microorganisms (Sanchez-Pescador et al., 1988). Doxycycline is considered more effective than the older tetracyclines because of its lipophilic nature allows it to better penetrate cells (Ayling et al., 2000).

Macrolides

Macrolides are generally bacteriostatic agents that inhibit bacterial RNA-dependent protein synthesis. They inhibit the synthesis of protein by binding reversibly to the 23S rRNA of the 50S ribosomal subunits of susceptible organisms, thereby blocking the translocation reaction of polypeptide chain elongation. By inhibiting the transpeptidation and translocation process, macrolides cause premature detachments of incomplete polypeptide chains. Generally they are bacteriostatic at standard dosage, but are bactericidal at the higher concentrations obtained in the lungs. Erythromycin is

occasionally used to treat certain mycoplasmal infections in animals, but is considered to be less effective than its derivatives tylosin and tilmicosin (Yao and Mollering, 2007a).

Pleuromutilins

Pleuromutilins are related to the macrolides and include tiamulin and valnemulin which are used predominantly in pigs. These agents act by inhibiting protein synthesis via binding to the 50S subunit of the bacterium and prevent elongation of peptide chains by interfering with peptidyl transfer, thereby suppressing protein synthesis (Robertson et al., 1988). Tiamulin and valnemulin have a remarkable activity against mycoplasma. Valnemulin proved to be effective in controlling *M. bovis* infection under field conditions (Stipkovits et al., 2001).

Aminoglycosides

Aminoglycosieds are bactericidal antibiotics that include streptomycin, dihydrostreptomycin, kanamycin, gentamicin, tobramycin, amikacin, and neomycin. They perform their action by penetrating the bacteria and binding to the 30S ribosomal subunit and cause a misreading of the genetic code, hence interrupting normal bacterial protein synthesis (Patrica, 2006). Some mycoplasmas are susceptible to aminoglycosides, for example, *Mycoplasma pneumoniae* is susceptible to streptomycin (Taylor-Robinson and Bebear, 1997).

Fluoroquinolones

Currently there are six fluoroquinolones marketed exclusively for use in veterinary medicine which include danofloxacin (cattle), enrofloxacin (cattle, dogs, cats and poultry), marbofloxacin (dogs), orbifloxacin (dogs and cats), difloxacin (dogs), and sarafloxacin (poultry) (Walker, 2000). Fluoroquinolones work through the inhibition of the enzymes DNA gyrase or topoisomerase IV, thus interfering with the supercoiling of bacterial chromosomal material. As a consequence, these agents are rapidly bactericidal against mycoplasmas (Sanchez-Pescador et al., 1988). Fluoroquinolone treatment tends to cause a clinical recovery from *Mycoplasma* infection in some species (Reinhardt et al., 2002).

1.2.3. Treatment of mycoplasmosis in animals

Poultry

In countries where the use of antimicrobials is less restricted, the use of antimicrobials that reach high concentrations in the respiratory and urogenital tracts such as tylosin, tiamulin and enrofloxacin are used (Stipkovits et al., 2001). In the USA enrofloxacin and tilmicosin are not approved for use in poultry, therefore tylosin and tetracyclines are used to treat mycoplasmosis as well as prevent egg transmission (Lockaby et al., 1998). Tylosin has been reported to be as effective as danofloxacin in controlling *M. gallisepticum* infection in broiler chickens (Jordan et al., 1993). Tetracyclines can be used in the treatment of chronic respiratory disease caused by *M. gallisepticum* and infectious synovitis caused by *M. synoviae* (Wang et al., 2001). Pleuromutilins have proved to be effective when administered in drinking water for controlling of *M. gallisepticum* infections (Gautier-Bouchardon et al., 2002).

Cattle

Tetracyclines, macrolides and the fluoroquinolones are usually used to treat mycoplasmal infections in cattle. In general, the antimicrobial susceptibility of mycoplasmas and ureaplasmas in decreasing order is tiamulin, tylosin and oxytetracycline, but individual susceptibility varies sufficiently for it to be necessary to carry out laboratory tests of susceptibility on each isolate (Nicholas and Ayling, 2003). For *M. bovis* many antimicrobials, including tylosin, oxytetracycline, lincomycin and oleandomycin have been used. However, while the isolates may be sensitive *in vitro*, the response is often unsatisfactory in affected animals (Radostits et al., 2007). Never the less, the massive and timely use of macrolide antibiotics, singly or in combination with other drugs, had a role in recovery of more than 90% of pneumonic calves (ter Laak et al., 1993). Although these antimicrobials resulted in a clinical cure, they did not eliminate mycoplasmas from the herd. Valnemulin has been effective for treatment of infected calves with *M. bovis* under field and experimental conditions (Stipkovits et al., 2001). The fluoroquinolones: danofloxacin, enrofloxacin and marbofloxacin are considered to be very effective against *M. bovis* (Radostits *et al*. 2007). An *in vitro* trial for tilmicosin and danofloxacin on clinical isolates of MmmSC showed that they were effective both in terms of mycoplasmastatic, and mycoplasmacidal activity respectively (Ayling et al.,

2000). Fluoroquinolones have also been used for treatment of *otitis media* and pneumonia caused by mycoplasmas (Rosenbusch et al., 2005b).

Pigs

Currently in South Africa, tylosin is most commonly used to control *M. hyopneumoniae* infections in commercial piggeries. In the absence of resistance, tetracyclines, including doxycycline can also be used to treat and prevent disease resulting from *M. hyopneumoniae* and *M. suis* infections (Maes et al., 2008). Tiamulin has been successfully used in field cases of *Mycoplasma* infections (Vicca et al., 2007). It has proved to have superior efficacy than tylosin in treating experimental mycoplasma but it has no effect in treating swine in the early stages of experimentally induced *M. hyponeumoniae* when administered orally (Ross and Young, 1993). Fluroquinolones have also been useful in treatment of *M. hyponeumoniae* infections (Maes et al., 2008).

Ovines

Application of a single injection of long-acting tetracycline simultaneously with topical tetracycline has been reported to be effective for treatment of ovine keratoconjuntivitis caused by *M. conjunctivae* (Hosie and Greig, 1995). Spiramycin and florfenicol has proved to be effective *in vitro* against *M. mycoides* subsp. *mycoides* LC infections (Kidanemariam et al., 2005). Enrofloxacin was tested among other antimicrobials for treatment of *Mycoplasma agalactiae* and has proved to be the most effective followed by lincomycin:spectinomycin (lincospectin), tylosin, tetracycline and spiramycin (Loria et al., 2003). Based on the results obtained by (Antunes et al., 2007), enrofloxacin and its metabolite ciprofloxacin (which is available for human use only) appeared to be effective *in vitro* against *M. mycoides* subsp. *mycoides* LC and *Mycoplasma capricolum* subsp. *capricolum*.

1.2.4. Antimicrobial resistance of the mycoplasmas

Factors contributing to resistance in* Mycoplasma *species

The introduction of antimicrobial agents in human clinical medicine and animal husbandry has been one of the most significant achievements of the 20th century. The

first antimicrobial agents were introduced in the 1930s, and a large number of new agents were discovered in the following decades. However, shortly after the introduction, resistance started to appear and in all known cases emergence of antimicrobial resistance has followed the introduction of new antimicrobial compounds (Curiale and Levy, 1982). In fact, antimicrobial resistance of micro-organisms that cause significant disease in humans and animals is considered by the World Health Organization (WHO), Food and Agricultural Organization (FAO) and Office des International Epizooties (OIE) to be an emerging problem that threatens the health of humans and animals alike (Aarestrup, 2004). The overuse or misuse of antimicrobials in veterinary and human medicine as well as in the environment is considered to be the primary cause of antimicrobial resistance development, similar to the pesticide resistance development in insects (Marily, 1996). One of the major factors in charge for development of resistance is the long term use of sub-therapeutic concentrations as well as the amount of antimicrobials used (Taylor-Robinson and Bebear, 1997). The evolutionary theory of genetic selection requirements is that as close as possible to 100% of the infecting organisms must be killed to avoid selection of resistance; if a small portion of the population survives the treatment and is allowed to multiply, the average susceptibility of this new population to the compound will be much less than that of the original population, since they have descended from those few organisms which survived the original treatment. This survival often results from an inheritable resistance to the compound which was infrequent in the original population but is now much more frequent in the descendants thus selected entirely from those originally infrequent resistant organisms. Evidence of this to oxytetracycline and tylosin has recently been reported in Europe (Thomas et al., 2003).

Soon after antimicrobial drugs have been readily available for human and veterinary medicine usage, it was recognized that decreased bacterial susceptibility could adversely affect clinical outcome (Paul et al., 2005). This is no less true for the mycoplasmas. Increasing resistance of mycoplasmas against tetracyclines (ter Laak et al., 1993) macrolides (Chirstensen et al., 1994) and quinolones (Bebear et al., 1999) has been reported both in animal and human species. Mycoplasmas are more resistant to chloramphenicol than the Gram-negative bacteria but have resistance level similar to those found in some Gram-positive cocci such as *Staphylococcus aureus* and

Enterococcus faecalis (Mahairas and Minion, 1989; Minion and Kapke, 1998). Mycoplasmas are inherently resistant to those antimicrobials that target the bacterial cell wall, namely the β-lactam antimicrobials.

Antibiotic resistance can be acquired as a result of gene mutation or the acquisition of new genetic material (Silletti and Lorian, 1986). As mycoplasmas have higher mutation rates than conventional bacteria, potentially they can more rapidly develop resistance to antimicrobials. Although, some information exists about the mechanisms involved in resistance in human *Mycoplasma* species to fluoroquinolones (Bebear et al., 1998), macrolides (Lucier et al., 1995), and tetracyclines (Blanchard et al., 1992) . There is scant data in the literature concerning the acquisition and mechanisms of antimicrobial resistance in veterinary mycoplasmas (Gautier-Bouchardon et al., 2002).

Tetracycline resistance is usually by over expression of the bacterial membrane efflux pumps. For example, high level-resistance has been associated with the presence of Tet M determinant which is always associated with a conjugative transposon and has a wide host range among urogenital bacteria of human origin (Marilyn and George, 1986). The Tet M determinant has also been detected in ureaplasmas and codes for the manufacture of proteins which binds to ribosomes, making them resistant to tetracycline both *in vivo* and *in vitro* (Roberts, 1990).

Among the macrolide-lincosamide-streptogramin B (MLS) family of antibiotics are erythromycin and tylosin (Yao and Mollering, 2007b). There is no cross resistance among these antibiotics (Quiros et al., 1988). Naturally occurring MLS-resistant strains of mycoplamas have been described for a variety of species including *M. pneumoniae* and *Ureaplasm urealyticum* (Mowles, 1988). Four different mechanisms of resistance have been proposed for bacteria: 1) lack of entrance into the cell, 2) chemical inactivation of the MLS antibiotic, 3) lack of binding to the ribosomal target, and 4) lack of an inhibitory response upon binding to the ribosome target. Many studies on field strains also support the hypothesis that considerable resistance to macrolides might be quickly selected in veterinary mycoplasmas by repeated treatments with tylosin against *M. gallisepticum* and *M. synoviae* (Aarestrup and Friis, 1998).

Fluoroquinolones are active against many bacteria including mycoplasmas. Mutation to the fluoroquinolones is a step-wise procedure usually starting with a mutation (base pair substitutions, deletions or insertions) in the g*yr*A gene which confers resistance to the fluoroquinolone precursor and nalidixic acid (David, 2000). Thereafter the mutations occur in *gyrA, gyrB, par*C or *par*E genes which confer resistance to the fluoroquinolones. This resistance is common and can result in cross-resistance to a wide range of fluoroquinolones (Wolfson and Hooper, 1989). Recently it has been ascertained that low-level resistance by the use of efflux pumps can also be carried in plasmids (Jacoby, 2005).

Mutational resistance to aminoglycosides is recorded for various *Mycoplasma* species and can be a multi-step or high-level-step type of resistance (Lee et al., 1987). Three types of antibiotic modifying enzymes have been found in both Gram-negative and Gram-positive bacteria, namely: acetyltransferases, phosphotransferases, and nucleotransferases. None of these enzymes have been revealed in *Mycoplasma*, but experimentally one transposon encoding for gentamicin resistance was introduced by transformation into *Mycoplasma* strains, which expressed them (Mahairas and Minion, 1989; Minion and Kapke, 1998).

Resistance to specific animal mycoplasmas

Increased resistance to antibiotics, particularly oxytetracycline, spectinomycin and tilmicosin, used for the treatment of *M. bovis* infections, have been reported in Europe (Ayling et al., 2000). Some *Mmm*SC strains have developed resistance to tylosin based on a broad range of MIC obtained against it (Ayling et al., 2005).
M. agalactiae strains have very poor susceptibility to nalidixic acid with MIC_{90} values >256 µg/mL and resistance to erythromycin (Antunes et al., 2007).

Therapeutic failures in the treatment of *M. gallisepticum* infections in chickens has been reported in various parts of the world, especially for the fluoroquinolones and macrolides (Reinhardt et al., 2002).

Acquired antimicrobial resistance of *M. hyopneumoniae* in pigs has been reported to tetracyclines (Aarestrup and Friis, 1998), and recently also to macrolides, lincosamides and fluoroquinolones (Vicca et al., 2007). Tylosin is no longer recommended for the therapy of *M. hyosynoviae* pneumonia in pigs due to the widespread use of this drug for therapy and growth promotion over the years (Aarestrup and Friis, 1998).

1.3 Antimicrobial Susceptibility Testing

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The antimicrobial susceptibility of bacteria can be tested quantitatively or qualitatively. Data resulting from quantitative methods can be related to actual concentrations of antimicrobial inhibiting the growth of bacteria, whereas qualitative methods categorise bacteria as susceptible, intermediate or resistant and are related to the clinical breakpoint concentration of the antimicrobial (Jorgensen, 1993).

Qualitative methods such as the Kirby Bauer disk diffusion method has been validated for testing the fast-growing veterinary pathogens as well as the fastidious Pasteurellaceae (CLSI, 2008). However, the Kirby Bauer method has not proven to be repeatable for obligate anaerobic bacteria and some of the fastidious bacteria such as *Mycoplasma* and *Campylobacter* species (Yan and Gilbert, 2004). Therefore only quantitative methods can be employed to test the antimicrobial susceptibility of *Mycoplasma* species (Hannan et al., 1989).

Several methods are available to quantify the *in vitro* antimicrobial resistance of bacteria and include broth and agar dilution tests as well as the ε-test (AB Biodisk, Solna, Sweden) (Hannan, 2000). All these methods measure the minimum inhibitory concentration (MIC) of the antimicrobial that is defined as the lowest concentration of an antimicrobial that inhibits visible growth or metabolism of a bacterium after its optimal incubation period *in vitro*. These methods have been well standardized for the fast-growing bacteria, obligate anaerobes and *Campylobacter* regarding media to be used, incubation type as well as inoculum density (Wolfson and Hooper, 1989). Antimicrobial susceptibilities are best done at physiological pH (7.2–7.4) to reflect physiological conditions. The MIC of many antimicrobial agents can be affected by testing at a lower

pH such as in cases of CO_2 incubation, as an example MICs of tetracycline are decreased whereas the MICs of macrolides, clindamycin, aminoglycosides and quinolones are increased (Evangelista et al., 2002). The inoculum size may also influence MIC results especially in the case of bacteriostatic drugs (Waites et al., 1997).

While the data necessary to establish these performance characteristics of all the tests used are well defined in human medicine and are collated by several organizations including the Clinical and Laboratory Standards Institute (CLSI), they are less well defined in veterinary medicine. Recently, the CLSI subcommittee on Veterinary Antimicrobial Susceptibility Testing has begun to further develop performance standards for antimicrobial susceptibility testing of veterinary pathogens. These performance standards include test methods, quality control guidelines, and interpretive criteria for veterinary antimicrobial agent (CLSI, 2008). Similar efforts on developing standards are developed by the British Society of Antimicrobial Chemotherapy as well as from the European Committee on Antimicrobial Susceptibility Testing (EUCAST), the European Medicines Evaluation Agency (EMEA) in Europe, Food and Drug Administration (FDA) in the USA, CA-SFM (Comité de l'Antibiogramme de la Société Française de Microbiologie) in France, DIN (Deutsches Institut für Normung) in Germany, CRG (Commissie Richtlijnen Gevoeligheidsbepalingen) in the Netherlands, NWGA (Norwegian Working Group on Antibiotics) in Norway, and SRGA (The Swedish Reference Group of Antibiotics) in Sweden (Kahlmeter et al., 2003). However, up to date, none of these organizations have considered the susceptibility testing of mycoplasmas.

1.3.1 Susceptibility testing for the mycoplasmas

Since there has been reduced response to antimicrobials in clinical mycoplasmosis, the need for an *in vitro* system to test the susceptibility of the mycoplasmas has increased. However these tests have not been standardized for mycoplasmas with each laboratory making use of its preferred media, inoculation density, incubation times and methods, making the comparison of data between laboratories difficult (Robertson et al., 1988). This problem is compounded by the fact that *Mycoplasma* species are variable in their

media requirements. Some also grow very slowly leading to the possibility that some antimicrobials may deteriorate before the endpoint is reached (Robertson et al., 1988).

Due to their technical difficulty in preparation, agar dilution methods are less used. They have, however, four major advantages over broth dilution methods namely: large numbers of strains can be easily tested at the same time by the use of an inoculum replicating apparatus; and microbial heterogeneity, including a mixture of susceptible and resistant organisms or a spontaneous resistant mutant can be readily detected by observing the bacterial growth on the agar surface. Agar dilution MICs tend to be lower than broth dilution MICs because in broth dilution tests a single surviving CFU (colony forming unit) can grow to produce visible turbidity. This single CFU may be resistant as a result of acquisition of new genetic material, by a mutation, or may represent a true mixture of resistant and susceptible cells (Lorian 1986). Furthermore the differences between the agar and broth dilution tests may vary with different microorganisms and antibiotic combinations (Lorian 1986).

The broth dilution test is preferred to the agar dilution test when testing for a wide range of antimicrobials as it is cheaper and easier to perform than the agar dilution test (Yan and Gilbert, 2004). It is also the method recommended by the *ad hoc* working group that established the "The International Research Program on Comparative Mycoplasmology", a branch of the International Organization for Mycoplasmology (Senterfit et al., 1986). In fact, most human clinical laboratories in North America use this method in an automated system (Gould, 2000). Since visible growth is not always possible to obtain for the mycoplasmas, the *in vitro* broth MIC tests have been amended to include an indicator system i.e. phenol red with 1% glucose in the case of glucose fermenters. Therefore the MIC definition has been modified to the lowest dilution which prevents a colour change in the media (Robertson et al., 1988).

A number of laboratory-associated factors influence the MIC values of antimicrobials when used in the testing of *Mycoplasma* species and include (Kenny and Cartwright, 1996):

- Some mycoplasmas will not grow in broth medium and therefore can only be tested using solid media.

- The culture media is complex and differs between *Mycoplasma* species, making it difficult to standardise a medium for all the species. This media may also negatively affect the action of certain antimicrobials.
- Mycoplasmas are small and often grow poorly resulting in no turbidity of liquid media and thus indicators of metabolism must be used in liquid media.
- Mycoplasmas usually require at least 5% CO_2 for growth, which can negatively affect pH indicators in the growth media as well as the action of certain antimicrobials.
- There is a prolonged incubation period where antimicrobials can lose their activity and some mycoplasmas may already be in the lag phase of growth.

In order to obtain the most optimum results the following recommendations should be adhered to (Hannan, 2000).

- Whether using a liquid or solid MIC assay system, it is essential that, to avoid obtaining falsely low MIC results, optimal media for growing specific mycoplasma species are used.
- The cultures must be pure.
- The mycoplasma inocula are carefully standardised. The recommended viable counts for inocula are 10^3 to 10^5 ccu/mP for liquid assays and 10^3 to 10^5 cfu/ plate for the agar dilution method.
- For incubation period and conditions, most mycoplasmas grow well between 35 ℃ and 37 ℃, hence a temperature of 36± 1 ℃ is recommended for both liquid and solid MIC assays, unless it is proved that optimal growth occurs outside the temperature range mentioned (Hannan, 2000). In solid MIC assays, the incubation atmosphere that is suitable for mycoplasmas is usually air enriched with 5 % CO_2.
- The growth phase of the organisms seems to be less important as lag phase cultures of *M. gallisepticum*, *M. synoviae*, *M. bovis* and *M. hyopneumoniae* have been shown to give very similar results to cultures in the logarithmic growth phase in liquid assays.
- Antimicrobials should be stored according to the manufacturer's recommendations and MICs determined in terms of their active base component, particularly when comparing results between laboratories.

- In liquid and solid MIC assays it is important that tests are carefully controlled, with growth, sterility, pH end-point (in liquid assays) and compound solvent controls and that standard reference mycoplasmas with established antibiotic sensitivity patterns are included in each experiment. For fluid assays all culturing is supposed to be done in standard air incubators to avoid the negative CO_2 can have on the pH indicators and on some antibiotics.
- In liquid MIC assays the microdilution plates must be adequately sealed to prevent exchange of gases between wells which might result in false colour changes and erroneous MIC endpoints and that the reading of liquid or solid MIC tests are carefully standardised.

Mycoplasmas remain a burden in animal health for its resistance for many antimicrobials that are widely used for tackling mycoplasma diseases. Despite their susceptibility to a variety of other broadspectrum antibiotics, most of which only inhibit their multiplication and do not kill them, they remain unsusceptible to penicillins and other antibiotics that act on bacterial cell wall. The tetracyclines have always been in the forefront of antibiotic usage, but macrolides are also widely used for respiratory tract infections. Still the extensive use of antimicrobials for treating mycoplasmal infection contributes for its resistance against these drugs as the case in some African countires. Furthermore, having no standard international protocols for antimicrobial susceptibility testing against mycoplasma remains a serious challenge for targeting mycoplasmal infections which made it difficult to compare results originating from different laboratories; hence this project aim is to evaluate different procedures in order to find an appropriate one including suitable growth indicators for testing mycoplasmas susceptibility.

Aims and Objectives

Problem statement

The antimicrobial susceptibility of *Mycoplasmas* species of animal origin is not commonly performed in South Africa. Similarly there is no data on the evaluation of any

antimicrobial susceptibility testing on *Mycoplasma* spp. of animal origin. Hence, this project aims were to determine a reliable and practical MIC method for *Mycoplasma* spp. of veterinary significance that can assist the veterinarians in South Africa in targeting the mycoplasmal infection to improve the animal health and welfare. Furthermore this test would be used to evaluate the antimicrobial resistance in mycoplasmas cultured from clinical specimens in South Africa.

Aims

1. Implement and standardize a method with a high level of reproducibility.
2. Assess the *in vitro* efficacy of antimicrobials commonly used to treat mycoplasmosis in animals
3. Determine the levels of resistance of *Mycoplasma* spp. to antimicrobials used to treat mycoplasmosis in animals.

Objectives

1. Select the antimicrobials to be tested.
   Compare the repeatability and ease of performance of 3 different MIC tests using six different mycoplasmas from untreated animals or reference strains. The methods used were: the microtitre broth method using tetrazolium dyes as an indication of bacterial oxidation; the microtitre broth method using glucose or pyruvate with a phenol red indicator to detect bacterial fermentation and the agar dilution test.
2. To find out whether amoxicillin-clavulanic acid which is used to eliminate contamination would significantly affect the results obtained in the broth dilution tests.
3. To determine the *in-vitro* MICs of antimicrobials against *Mycoplasma* species isolated from clinically ill animals in South Africa.
4. To measure the MIC_{50} and MIC_{90} of various animal *Mycoplasma* spp. to commonly used antimicrobials and compare the results obtained to known susceptible species in order to determine cut off values.

5. To compare these values to any break-point or cut-off values published in the literature.
6. Detect the level and pattern of resistance of *Mycoplasma* spp. to antimicrobials known to have activity against them.

Benefits arising from the project

1. Establish a reliable MIC test that can be used for the *in vitro* testing of resistance in *Mycoplasma* spp. that can be used for continuous surveillance of resistance in these bacteria.
2. More precise knowledge on antimicrobial resistance of mycoplasmas causing disease in animals in South Africa.
3. The data will assist clinicians in South Africa in antimicrobial therapy selection for a specific disease condition.
4. Capacity building of the researcher, researchers and technical staff in bacteriology within the Department of Veterinary Tropical Diseases at the Faculty of Veterinary Science, University of Pretoria.

CHAPTER TWO

Materials and Methods

2.1 Introduction

Antibiotics are commonly used to treat mycoplasmosis in animals. In spite of this and the fact that antimicrobial resistance has been recorded for this group of bacteria there are no universally accepted *in vitro* means of testing for this resistance, nor is resistance testing for mycoplasmas a routine procedure in most veterinary laboratories. Therefore, prior to testing for resistance to a number of mycoplasmas isolated from animals in South Africa it was necessary to compare different tests including broth and agar microdilution tests to determine which one would perform best and was the easiest to apply for testing field isolates. The project was divided into 2 phases: a phase that compared two *in vitro* antimicrobial susceptibility tests, the so-called "validation phase" and the MIC testing of mycoplasmas isolated from animals in South Africa.

2.2 Validation Phase

2.2.1 *Mycoplasma* strains

During the validation process 9 mycoplasmas known to be highly susceptible to antimicrobials were used, namely: *Mycoplasma bovis*[1] (B414/04); *Mycoplasma crocodyli* (clinical isolate) (B910/03)[1]; *Mycoplasma felis*[2]; *Mycoplasma gallisepticum*[1] (B758/08); *Mycoplasma gallisepticum* 56 *USDA*[3]*; Mycoplasma mycoides* subsp. *mycoides* Small Colony T1/44 vaccine strain[4]; *Mycoplasma mycoides* Y goat (11706)[5], *Mycoplasma*

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[2]Mycoplasma Experience Ltd. Phone: +44 (0) 1737 226662 Fax: +44 (0) 1737 224751.
[3] U.S. Department of Agriculture 1400 Independence Ave., S.W. Washington, DC 20250.
[4] Onderstepoort Biological Products. P/Bag X07 Onderstepoort 0110, South Africa. T: +27- 12-522-1500 F: +27-12-565-5260
[5] American Type Culture Collection (ATCC). P.O. Box 1549. Manassas, VA 20108. USA. Tel: (703) 365-2750. Fax: (703) 365-2700.
[6] Bioproperties. 36 Charter street. Ringwood VIC 3134. Australia. T: + 61398760567. F: +61398760556

synoviae[1] (B312/08) and *Mycoplasma gallisepticum* VaxSafe MG vaccine strain[6]. The mycoplasmas were stored as agar plugs in a -86 °C freezer[1]. *Staphylococcus aureus* ATCC 29213[2] and *s*[2] were also used as control strains to test whether the concentration of antimicrobials used were correct.

2.2.2 *Mycoplasma* species purification and identification

Frozen isolates were defrosted at room temperature and inoculated onto modified Hayflick's agar (Hayflick, 1965) medium for all the above-mentioned mycoplasmas except *M. synoviae* for which modified Frey's medium (MS) medium (cited by Kleven & Levensohn 1996) was used. Plates were incubated at 36±1°C in a 5% CO_2 in air incubator[3]. Since these media were used for all the studies, they shall be hence forth referred to as *Mycoplasma* Culture Broth (MCB) or Mycoplasma Culture Agar (MCA). Growth was checked daily with a stereo microscope[4] using the 40X lens until typical colonies resembling "fried eggs" appeared. A plug containing a single colony was sucked up using a tip of a sterile Pasteur pipette[5] and inoculated onto solid agar medium and incubated as previously described. The purity and identity of each culture was checked using a species-specific indirect immunofluorescent antibody test (IFAT) (Wood et al., 1997) as follows:

- Agar that contained a high density of single colonies from a fresh culture was cut into a 5 mm block and placed with 3 other blocks equidistant on a microscopic slide.
- The blocks were fixed to the slide by embedding them in a mixture of 65% paraffin[6] and 35% Vaseline[6].

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[2] American Type Culture Collection (ATCC) P.O. Box 1549. Manassas, VA 20108. USA. Tel: (703) 365-2750. Fax: (703) 365-2700.
[3] Heraeus Holding GmbH. Postfach 1561. D-63405 Hanau. Germany. Tel: +49 (0) 61 81 / 35-0. Fax: +49 (0) 61 81/ 35-35 50
[4] Nikon Corporation. Ohi Plant 6-3, Nishi-ohi 1-chome, Shinagawa-ku, Tokyo 140-8601 Japan. Tel: +81-3-3773-8973. Fax: +81-3-3773-8986
[5] Lasec SA (Pty) Ltd. 7 Kielboot Rd. Laser Park. Honeydew. Gauteng 2170. PO Box 1296. North Riding 2162.
[6] Johnson & Johnson (Pty) Ltd. PO Box 727. East London 5200. Tel: +27 (0)43 709 3211. Fax: +27 (0)43 745 2679

- A drop of phosphate buffered saline pH 7.2- 7.4 (PBS) was placed on each agar block to maintain the osmolarity of the cells.
- A species-specific rabbit polyclonal antiserum[1] dilution 1:50 was prepared in PBS and approximately 50 µl dropped on a block. The polyclonal antisera was prepared in-house by the use of a series of rabbit inoculations.
- The slides were incubated at room temperature for 30 minutes in a moist chamber[2] and washed 2×10 minutes in PBS using a syringe.
- One drop of 1:30 fluorescein-conjugated anti-rabbit globulin[3] was placed on each block.
- Blocks were incubated at room temperature for 30 minutes in the moist chamber and washed 2× 10 minutes in PBS.
- If all the colonies showed fluorescence using epifluorescent illumination and a 20X objective on a light microscope the culture was considered to be identified as expected and pure.

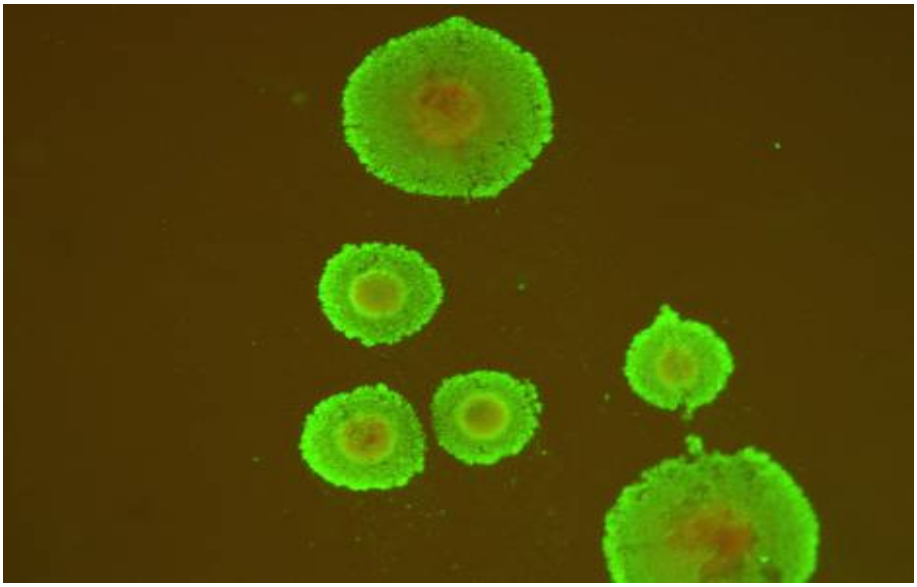

Figure 1: Indirect immunofluorescent antibody test (IFAT) showing fluorescent *Mycoplasma* colonies.

[1] Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, Univeristy of Pretoria, P/Bag X04, Onderstepoort, South Africa 0110. In-house manufactured antirabbit antibodies using type strains of *Mycoplasma* species.
[2] Orb Diagnostics, Cc. P.O.Box 763. Edenvale, 1610. Johannesburg. South Africa. TEL: +27(0)11452952- 23. FAX: +27 (0)11 452 952.
[3] www.bindingsite.com

2. 2.3 Standardization of the mycoplasma inocula

Once the cultures were determined to be pure and correctly identified, an agar block containing 5-10 colonies of each of the *Mycoplasma* isolates was cut out from the agar of each mycoplasma isolate and transferred into a tube containing 5 mℓ MCB and incubated for 3 to 7 days until visible growth was obtained. The tubes were vortexed and their optical density measured using a spectrophotometer[1] with a wave length of 540 nm.

A serial ten-fold dilution was done by transferring 0.1 mℓ from the broth culture to 0.9 mℓ MCB and consecutively repeating this process until 8 tubes had been inoculated, giving a dilution range of 10^{-1} to 10^{-8}. Using a single-channel pipette[2], 100 µℓ was taken from each dilution and inoculated on a separate MCA plate and spread using an alcohol, flame-sterilized, bent Pasteur pipette[3]. Plates were incubated at 36±1 ℃ in a 5% CO_2 in air incubator. Growth was checked daily until clear colonies were seen. Plates with dilutions that had well separated single colonies were counted under the stereomicroscope.

Since only the original growth of the mycoplasmas (not the dilutions) gave a repeatable optical density reading with the spectrophotometer and it was assumed that most mycoplasmas are of equal density, the optical densities of all the mycoplasmas were plotted in a single graph (Figure 1) in Excel Microsoft™ 2000 with $\log_{10}$ of the colony forming units (cfu/ mℓ) on the x-axis and the optical density on the y-axis. This graph was then used to calculate the dilution ratio required to get approximately 10^6 cfu/mℓ using the spectophotographic readings obtained from the broth cultures.

[1] Cecil Instruments Ltd. Milton Technical Centre. Cambridge CB24 6AZ . ENGLAND. Tel : +44 (0)1223 420821. Fax : +44 (0)1223 420475
[2] Biohit Oyj. Laippatie 1. 00880 Helsinki. Finland. Tel: +358 9 773 861. Fax: +358 9 773 86200 .
[3] Lasec SA (Pty) Ltd. 7 Kielboot Rd. Laser Park. Honeydew. Gauteng. 2170. PO Box 1296. North Riding. 2162

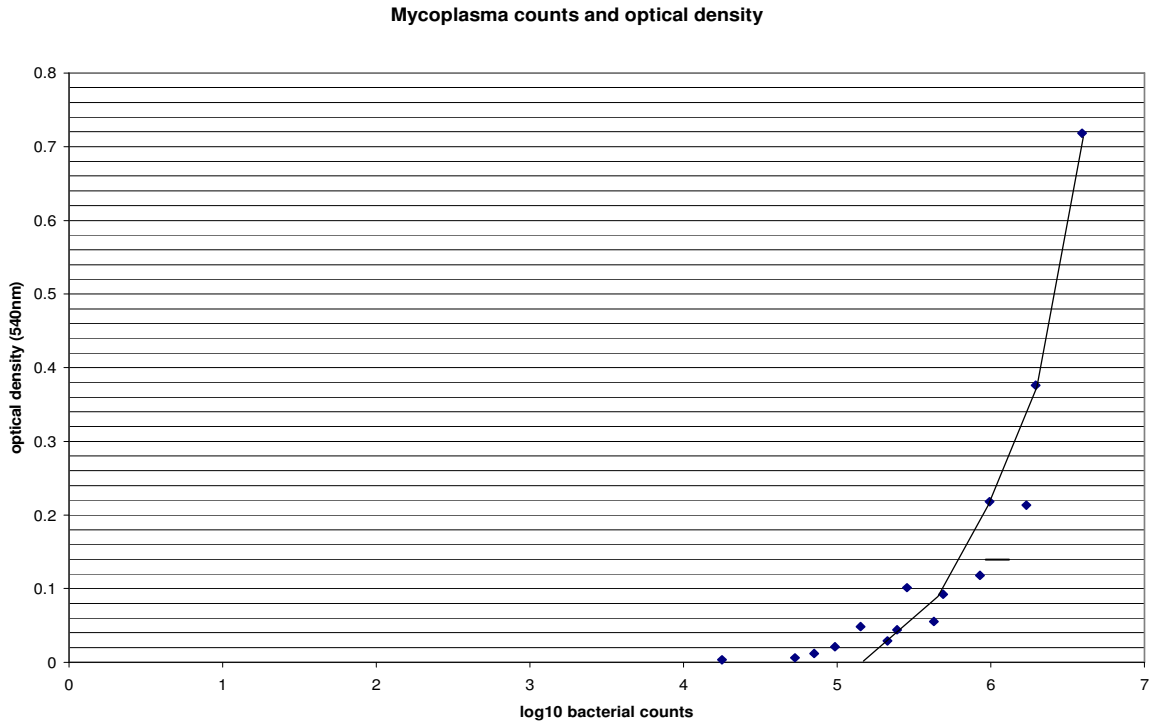

Figure 2: Colony forming units graph showing relationship between mycoplasmal count and optical density. The line represents the dilution range of 10^5 used.

2.2.4 Preparation of the antimicrobial agents

The MICs of the various mycoplasmas were tested for each of the antimicrobials listed in Table 3. The preparation of the stock solutions for all the selected antibiotics was done making use of guidelines of the Clinical Laboratory Standards Institute (CLSI, 2008). The following formula was used to determine the weight of antibiotics needed for the stock solution:

$$\text{Weight (mg)} = \frac{\text{Volume (mℓ) of stock solution x Concentration (µg/mℓ) wanted}}{\text{Potency of the drug (µg/mg)}}$$

The potency of the antimicrobial drug was calculated by using the purity of the product (on the certificate of analysis) and the relative molecular weight of the active molecule.

Once the antibiotic powders had been weighed on an analytical scale[1] they were diluted in 100mℓ of the diluent (table 3). This stock solution was designed to be 10 - 100 times the required concentration of the working solution to insure that the antimicrobial effects of any solvents would be negligible. The stock solutions were stored at -20℃ until required. A 1:10 working solution was made using sterile deionized water. Exceptions were enrofloxacin, norfloxacin and trimethoprim where a 1:100 working dilution was made.

Antimicrobial dilution ranges were obtained by carrying out doubling dilutions using the appropriate mycoplasma growth medium (Hannan 2000). The dilution ranges of the antimicrobials used are shown in Table 3.

Table 3: Preparation of the antimicrobial dilution ranges

Antimicrobial	**Solvent**	**Dilution range (µg/mℓ)**
Doxycycline	Water	0.5-64
Enrofloxacin	0.1N NaOH and water	0.0625-8
Erythromycin	95% ethanol	0.5-64
Florfenicol	95% ethanol	1-128
Nalidixic acid	0.1N NaOH[1] and water	1-128
Neomycin	Deionized water	1-128
Norfloxacin	0.1N NaOH and water	1-128
Sulfamethoxazole	0.1N NaOH, heated and water	16-2048
Tiamulin	Deionized water	0.5-64
Trimethoprim	0.1N acetic acid and water	0.96875-108
Tylosin	Deionized water	0.5-64
Trimethoprim/sulfamethoxazole (SxT)	No stock solution	0.96875-108

[1] Sartorius AG. Weender Landstrasse 94-108. D-37075 Goettingen, Germany. Tel: +49.551.308.0 Fax: +49.551.308.3289

* To avoid precipitation, the working solution of trimethoprim/sulphamethoxazole was made using a 1:1 ratio of the individual working solutions of trimethoprim/sulphamethoxazole.

2.2.5 The liquid MIC assay

The media used included Hayflick's and MS media including phenol red[1] 1% for *M. synoviae*, *M. gallisepticum* and *M. bovirhinis*; pyruvate[2] 1% for *M. bovis*, alamar blue[3] 10% was used with all isolates. A 1% powder combination of 0.5g amoxicillin[4] and 0.1g clavulanic acid[4] was used with all different indicators to reduce contamination. It was filter sterilized (0.22 µℓ filter[5]) and then added to one bottle of 100 mℓ serum[6], mixed well and then added to 500 mℓ Hayflick's broth.

Sterile, round-bottomed 96-well microtitire plates[7] were prepared by adding 200 µℓ of the working dilution of each antimicrobial alphabetically from row A1 to A12 using a single-channel pipette. Using a multi-channel pipette, 100 µℓ MCB was inoculated into each well starting at row B and ending with row H with the exception of well H12 (mycoplasma inoculum control as positive control). Using a multi-channel pipette, doubling dilutions of antimicrobials was carried out down the column starting with row A from which 100 µℓ was picked and inoculated in the next row till the last row with the exception of well H11 (medium sterility control as negative control). Plates were stored in a -80℃ freezer until needed.

Plates when required were taken out of the freezer and left to defrost at room temperature. Into each well of the sterile 96-well microtitre plate, 100 µℓ of the prepared

[1] BDH chemicals Ltd. Broom Road, Poole, Dorset BH12 4NN, UK. Tel: +44-(1202)-745520 Fax: +44-(1202)-738299.
[2] FLUKA. Chemika/Biochemika. Fluka Chemie AG. Industriestrasse 25. 9471 Buchs. Switzerland. Tel: 081/755 25 11. Fax: 081/756 54 49
[3] US Biological. P.O.BOX 261. Swa,scott, Massachusetts 01907. Tel: 800-520-3011. Fax: 781-639-1768.
[4] GlaxoSmithKline South Africa. Carisbrook Building. The Campus. 57 Sloane Street. Bryanston 2021. South Africa
[5] Gelman Sciences. 600 South Wagner Road. Ann Arbor, MI 48103-9019. Tel: 313-665-0651. Fax:313-913-6114.
[6] Highveld Biological (PTY) LTD. P.O.BOX 1456. Lyndhurst 2106. RSA. Tel: +27(011) 4430241
[7] Thermo Fisher Scientific, Roskilde Site, Kamstrupvej 90. Postbox 280. DK-4000. Roskilde. Tel: +45 4631 2000. Fax: +45 4631 2175

mycoplasma suspension was added starting at row B and ending at row H except H11. Plates were covered using a transparent, self-adhesive seal[7] (Nunc) and incubated at 37℃. The plates were examined daily for a colour change (from red to yellow with phenol red, & pyruvate indicators and dark blue to red with Alamar Blue).

The lowest antimicrobial concentration that showed no change in color was recorded as the minimum inhibitory concentration (MIC) and recorded on a data sheet that resembled the layout of the 96-well plates and were typed into a computer Excel Microsoft™ 2003 spread sheet.

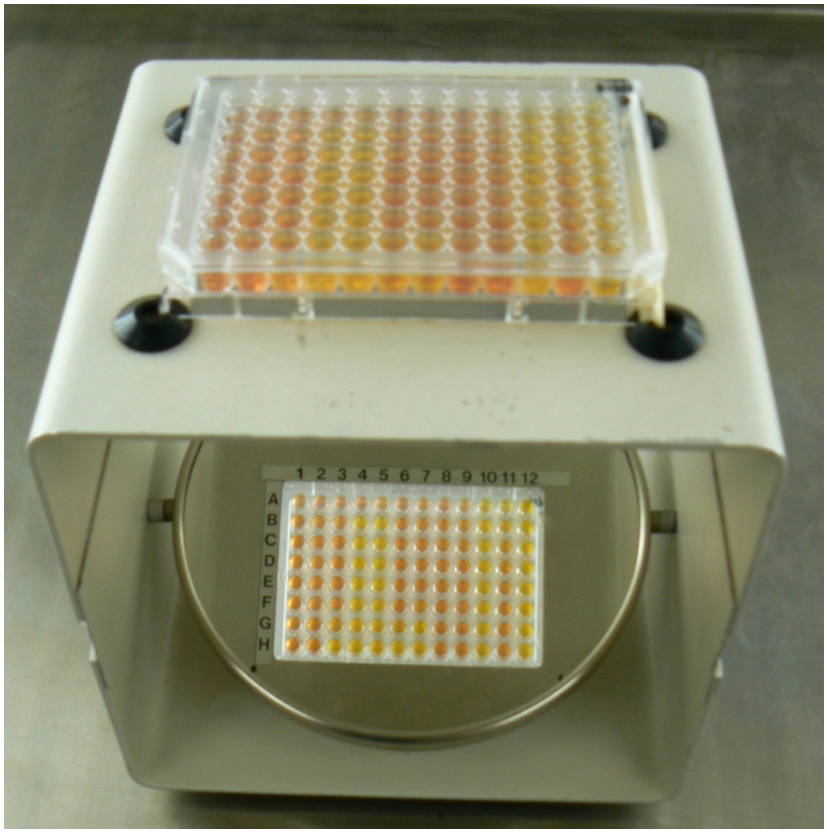

Figure 3: A 96 well plate showing a result of a broth microdilution assay*

*Row A: Highest concentration of antimicrobials ordered alphabetically
Row A-H: contains mycoplasma inocula
H11: negative control (broth)
H12: positive control (mycoplama inoculm)

2.2.6 Solid Agar MIC assay (modified method of Hannan et al. 1989)

Agar plates containing the same range of antimicrobial concentrations as for the broth MIC were prepared in 65 mm plastic Petri dishes[1]. Control antimicrobial-free plates were also prepared. With doubling dilution each antibiotic was prepared first in either Hayflick's or MS broth in 50mℓ Schott bottle. As some antimicrobials are heat-sensitive the dilutions have been made in molten agar in 50 mℓ Schott bottles[1] in a water bath set to a temperature of 56℃. Eight bottles with 5 mℓ sterile water with the exception of the first one filled with 9 mℓ were used to carry out doubling dilutions. 1 mℓ of each antibiotic was added to 9 mℓ sterile water and an amount of 5 mℓ was transferred to second bottle and a doubling dilution was carried out with the rest. A combination of 1:1 trimethoprim/sulfamethoxazole was used.

The agar bottle was left on a 90mm Petri dish filled with warm water to ensure its fluidity, then poured into 65 mm diameter Petri dishes and the working dilution for each antimicrobial was added quickly to it and mixed well and rapidly to ensure that the antimicrobial was distributed thoroughly within the agar and was then allowed to solidify at room temperature. A grid was drawn on the agar plates to allow 9 mycoplasma isolates to be inoculated per plate. Later an aliquot of 5 µℓ of each mycoplasma suspension was added to its allocated spot on the agar of each antimicrobial dilution, as well as to the control plate without antimicrobials. Once the inoculum droplets were absorbed, the plates were incubated at 36±1 ℃ in a 5% CO_2 jacketed incubator until visible colonies were observed on the antibiotic-free growth control plate using 40X magnification of the stereomicroscope. The same procedure with agar plates was done first in test tubes but contamination was an obstacle, hence working with plates was easier with less contamination and better results.

[1] Orb Diagnostics, CC. P.O.Box763, Edenvale, 1610, Johannesburg, South Africa. Tel: +27(0)11452952- 23. Fax: +27 (0)11 452 952 – 4.
[1] SCHOTT AG. Hattenbergstrasse 10. 55122 Mainz. Germany. Tel: +49 (0)6131/66-0. Fax: +49 (0)6131/66-2000

To record the results, the plates were checked for growth under a stereomicroscope using a 40X objective. The end-point (MIC) was considered as the lowest concentration of antimicrobial that caused inhibition of growth and size of the colonies compared with that on the control plate.

2.2.7 Controls

The following controls were applied to ensure that each test was working optimally:

The indirect immunofluorescence test was applied to all isolates to confirm and ensure its purity and identification (Section 2.1).

Each MIC test in the validation phase included reference mycoplasmas (e.g., the type strain of each species under investigation) with known susceptibilities against a range of antimicrobials, to confirm the validity of results.

Staphylococcus aureus ATCC 29213 and *Escherichia coli* ATCC 25922 were incubated with each batch of antibiotic dilutions by making use of the liquid microtitre method (CLSI-NCCLS, 1994). That was to ensure that the antibiotic dilutions were correct.

100 mℓ of each inoculum was spread onto 7% horse blood Columbia Agar (Oxoid, Basingstoke, England) and incubated at 37 ℃ to ensure that no bacterial contaminants were present.

2.2.8 Test repeatability of the liquid and agar MIC tests

The repeatability of all the tests was compared by repeating them and the controls daily for 5 consecutive days except for Alamar Blue which was repeated for two or three consecutive days for some. This was done using the same conditions and operator. The results were recorded and analyzed statistically in Excel Microsoft™ 2003.

2.2.9 Statistical analysis used to compare the different tests

The MIC where 50% and 90% all strains within a species were equal to or less than for a particular antimicrobial was calculated and recorded as the MIC_{50} and MIC_{90} respectively (Schwarz et al., 2010).

The log_2 of each result was calculated and used to determine the median and standard deviation of each result. The mean of the standard deviations of all the antibiotics tested was used to determine which test was the most repeatable. Linear regression was calculated based on the average of media results obtained to model the relationship between them. From the results obtained, it was determined which of the tests should be used for each species and what test will be used in the second phase of the project.

2.3 Testing of field isolates

The best test as ascertained in the validation phase was used together with the controls on approximately 142 different *Mycoplasma* strains isolated from animals with disease. These mycoplasmas included 93 *M. bovis*, 17 *M. gallisepticum*, 5 *M. synoviae*, 13 *M. bovirhinis,* 8 *M. crocodyli* and 6 *Mycoplasma felis*. The broth microdilution assay was used using phenol red and a 1 % sugar as an indicator system with the addition of 1g amoxicillin and 0.2 g clavulanic acid/500mℓ was applied for all these isolates. The same antimicrobials and concentration range used in the validation phase was tested. Results were recorded in a table that represented the 96-well plate. The MIC_{50} and MIC_{90} were calculated for all isolates. A percentage range distribution table was also calculated for the MICs based on published breakpoints as shown in (Table 4). Breakpoints are MIC cut-off values that are used to categorize a bacterial population into susceptible, intermediate and resistant categories**.** They breakpoints are used routinely in the clinical laboratory setting to guide clinical decision-making (Christopher. et al., 2008).

The percentage resistance for each antibiotic and mycoplasma species was determined using published data as a guideline (Table 5).

Table 4: The breakpoints of MICs of each antimicrobial agent.

Antimicrobial	**Breakpoint (µg/mℓ)**	***Mycoplasma* species**	**Published Source**
Doxycycline	≤8	*M. gallisepticum*	Hannan 2000
Erythromycin	≤4	*M. gallisepticum*	Hannan 2000
Enrofloxacin	≤4	*M. synoviae*	Hannan 2000
Florfenicol	≤2	*Pasteurella multocida*	CLSI 2008
Nalidixic Acid	≤2	Most mycoplasmas	Hirose *et al*., 2003
Neomycin	≤4	Most mycoplasmas	Hirose *et al*., 2003
Norfloxacin	≤2	Human mycoplasmas	Japanese Society of Chemotherapy, 1994)
Tiamulin	≤8	*M. hyorhinis*	Hannan 2000
Tylosin	≤8	*M. agalactiae*	Hannan 2000
Sulfamethoxazole	≤256	*Salmonella* Typhimurium	SVARM 2007
Trimethoprim-Sulfamethoxazole	2/38	*Staphylococcus* species	CLSI 2008
Trimethoprim	≤8	*Salmonella* Typhimurium	SVARM 2008

Key: **SVARM= Swedish Veterinary Antimicrobial Resistance Monitoring; CLSI= Clinical Laboratory Standards Institute**

CHAPTER THREE

Results

3.1 Introduction

In order to determine the best test the repeatability of the average standard deviations of a $\log_2$ transformed minimum inhibitory concentrations (MIC) of each of the five tests were compared. Furthermore linear regression was done on the means of the $\log_2$ transformed MIC. The broth microdilution test using phenol red and glucose or pyruvate as an indicator together with the inhibitor amoxicillin clavulanate was used for the field strains of mycoplasma.

Once the MIC values were determined for each field strain, a distribution table showing the frequency distribution of each antimicrobial within the tested range is depicted for each *Mycoplasma* species tested. Furthermore the percentage susceptibility based upon published breakpoint values as well as the MIC_{50} and MIC_{90} is shown.

3.2 Validation Phase

The MIC values for the repeatability tests as well as the different tests performed are shown in Appendix I for the following mycoplasmas: *Mycoplasma bovis*[4]; *Mycoplasma crocodyli* (clinical isolate)[1]; *Mycoplasma felis* MEVTD8[5]; *Mycoplasma gallisepticum*[1]; *Mycoplasma gallisepticum* 56 *USDA*[6]*; Mycoplasma mycoides* subsp. *mycoides* Small

[4] Bacteriology Lab, Dept of Vet Tropical Diseases, Fac Of Vet Science, Onderstepoort 0110, University of Pretoria, South Africa.
[5]Mycoplasma Experience Ltd. Phone: +44 (0) 1737 226662 Fax: +44 (0) 1737 224751.
[6] U.S. Department of Agriculture 1400 Independence Ave., S.W. Washington, DC 20250.

Colony T1/44 vaccine strain[4]; *Mycoplasma mycoides* Y goat (11706)[5], *Mycoplasma synoviae*[1] and *Mycoplasma gallisepticum* VaxSafe MG vaccine strain[6].

Staphylococcus aureus ATCC 29213 and *Escherichia coli* ATCC 25922 reference strains that were used as quality control isolates gave results within the range recommended by the CLSI M3-A3 document indicating that the concentrations of antimicrobials used were correct.

3.2.1 Repeatable Testing

The results from the repeat testing of the selected mycoplasmas were $\log_2$ transformed and the standard deviation calculated for each of the antibiotics tested. The average standard deviation was then calculated for each *Mycoplasma* strain tested and for each test (Table 5). The test showing the lowest average standard deviation for all of the *Mycoplasma* strains tested (Table 6) was considered to be the most repeatable test.

In the operator's hands the microdilution test using AlamurBlue™ as an indicator had the highest variation, thereafter the agar dilution test. The lowest standard deviations with similar results were the microbroth dilution tests with or without antibiotics where phenol red was used as an indicator of fermentation.

[4] Onderstepoort Biological Products. P/Bag X07 Onderstepoort 0110, South Africa. T: +27- 12-522-1500 F: +27-12-565-5260
[5] American Type Culture Collection (ATCC). P.O. Box 1549. Manassas, VA 20108. USA. Tel: (703) 365-2750. Fax: (703) 365-2700.
[6] Bioproperties. 36 Charter street. Ringwood VIC 3134. Australia. T: + 61398760567. F: +61398760556

Table 5: Comparison of the average standard deviations (to 2 decimal places) for each test and *Mycoplasma* species tested.

Test	*M. my T1/44*	*M. felis*	*M. crocodyli*	*M. bovis*	*M. gallisepticum 56 USDA*	*M. gallisepticum*	*M. Y goat*	*VAXSAFE*	*M . synoviae*	Standard deviation
BrMIC+ SFS	0.31	0.28	0.12	0.32	0.17	0.31	0.51	0	0.24	0.14
BrMIC+ SFS+ACA	0.39	0.16	0.08	0.24	0.13	0.34	0.3	0	0.15	0.13
BrMIC +AB	0	0.12	0.18	0.06	0	0	0.1	0.73	3.08	1.01
AMIC	0.93	1.54	1.05	1.62	0.97	0.28	0.99	0.6	0.36	0.46

Key: BrMIC+SFS- broth microdilution MIC with a sugar fermentation system; BrMIC+SFS+ACA - microdilution MIC with a sugar fermentation system with amoxicillin and clavulanic acid; BrMIC+AB - broth microdilution MIC with AlamurBlue™; AMIC – Agar MIC

Table 6: Repeatability of tests based on the standard deviation comparisons for *Mycoplasma* species.

	Doxycycline	Enrofloxacin	Erythromycin	Florfenicol	Nalidixic acid	Neomycin	Norfloxacin	Sulphomet-hoxazole	Tiamulin	Trimethro-prim	Tylosin	S+T	Pooled standard deviation
M. mycoides mycoides T1/44													
BrMIC+ SFS	0	0.72	0	1.10	0	0	0.45	0	0	0.89	0	0.55	0.31
BrMIC+ SFS+ACA	0	0.72	0	1.10	0.45	0.45	0.55	0	0	0.89	0	0.55	0.39
BrMIC +AB	0	0	0	0	0	0	0	0	0	0	0	0	0
AMIC	0	2.68	0	1.41	0	0	1.14	0	4	0.89	1	0	0.93
M. felis MEVTD8													
BrMIC+ SFS	0	0	0	0	0	0	1.10	0.5	0.90	0	0	0.84	0.28
BrMIC+ SFS+ACA	0	0	0	0	0	0	0.90	0.58	0	0	0	0.45	0.16
BrMIC +AB	0	0	0	0	0	0	0	0	0	1.41	0	0	0.12
AMIC	1.34	2.45	3.5	3.42	3.5	0	3.27	0	0	0	1	0	1.54
M. crocodyli (field isolate)													
BrMIC+ SFS	0	0	0	0.89	0	0	0	0	0	0	0	0.55	0.12
BrMIC+ SFS+ACA	0	0	0	0.55	0	0	0	0	0	0	0	0.45	0.08
BrMIC +AB	0	0	0	0	0	0	1.5	0	0	0	0	0.71	0.18
AMIC	0	1.48	4.35	1.10	0.45	0	0.58	0	0.71	0	3.95	0	1.05
M. bovis (field isolate)													
BrMIC+ SFS	0.89	0	0.90	0.71	0.84	0	0.45	0	0	0	0	0	0.32
BrMIC+ SFS+ACA	0	0	0	0.89	0	0	0.71	0	0	0.45	0.89	0	0.24
BrMIC +AB	0	0	0	0	0	0	0	0	0	0	0	0.71	0.06
AMIC	1.67	1.95	0.5	0.82	0.89	0	1.79	0	4	0	4.35	3.50	1.62
M. gallisepticum 56 USDA													
BrMIC+ SFS	**0**	0	0	0	0	0	1.10	0	0	0	0	0.89	0.17
BrMIC+ SFS+ACA	**0**	0	0	0	0	0	1.10	0	0	0.45	0	0	0.13
BrMIC +AB	**0**	0	0	0	0	0	0	0	0	0	0	0	0
AMIC	**0**	2.35	4.35	1.64	0	0	1.30	0	0	0	2	0	0.97

Key: BrMIC+SFS- broth microdilution MIC with a sugar fermentation system; BrMIC+SFS+ACA - microdilution MIC with a sugar fermentation system with amoxicillin and clavulanic acid; BrMIC+AB - broth microdilution MIC with AlamurBlue™; AMIC – Agar MIC

Table 6 continued: Repeatability of tests based on the standard deviation comparisons for *Mycoplasma* species.

	Doxycycline	Enrofloxacin	Erythromycin	Florfenicol	Nalidixic acid	Neomycin	Norfloxacin	Sulphometh-oxyzole	Tiamulin	Trimeth-roprim	Tylosin	S+T	Pooled standard deviation
M. gallisepticum (field isolate)													
BrMIC+ SFS	**0**	**0**	**0**	**0**	**0.55**	**0.55**	**1.10**	**0**	**0.55**	**0.45**	**0**	**0.55**	0.31
BrMIC+ SFS+ACA	**0**	**0**	**0**	**0**	**0.55**	**1.10**	**1.10**	**0**	**0.55**	**0**	**0**	**0.84**	0.34
BrMIC +AB	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	0
AMIC	**0**	**0.45**	**0.58**	**1.34**	**0**	**0**	**0.45**	**0**	**0**	**0**	**0.5**	**0**	0.28
M. mycoides Y goat													
BrMIC+ SFS	**0**	**0.72**	**1.79**	**2.24**	**0.45**	**0**	**0.89**	**0**	**0**	**0**	**0**	**0**	0.51
BrMIC+ SFS+ACA	**0**	**0.72**	**0.89**	**0**	**0.45**	**0**	**0.45**	**0**	**1.10**	**0**	**0**	**0**	0.30
BrMIC +AB	**0**	**0**	**1.16**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	0.10
AMIC	**1.34**	**0.62**	**4**	**3**	**0**	**0**	**2.41**	**0**	**0**	**0.45**	**0**	**0**	0.99
M. synoviae (*VAXSAFE* vaccine)													
BrMIC+ SFS	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	0
BrMIC+ SFS+ACA	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	0
BrMIC +AB	**0.5**	**1**	**0.816**	**0**	**1.5**	**1**	**1.26**	**0.58**	**0.5**	**0.5**	**0.5**	**0.58**	0.73
AMIC	**0.55**	**0.55**	**0.45**	**0.45**	**0.84**	**0.55**	**0.84**	**0**	**0.55**	**1**	**1**	**0.45**	0.60
M. synoviae (field isolate)													
BrMIC+ SFS	**0**	**0.45**	**0.89**	**0**	**0**	**0.55**	**0**	**0.45**	**0**	**0**	**0**	**0.55**	0.24
BrMIC+ SFS+ACA	**0**	**0**	**0.89**	**0**	**0**	**0**	**0**	**0.89**	**0**	**0**	**0**	**0**	0.15
BrMIC +AB	**3.46**	**5.51**	**0.58**	**1.16**	**4.04**	**4.58**	**4.73**	**4.62**	**2.31**	**2.52**	**0.5** **8**	**2.83**	3.08
AMIC	**0.84**	**1.01**	**0**	**0.71**	**0**	**0.89**	**0.89**	**0**	**0**	**0**	**0**	**0**	0.36

Key: BrMIC+SFS- broth microdilution MIC with a sugar fermentation system; BrMIC+SFS+ACA - microdilution MIC with a sugar fermentation system with amoxicillin and clavulanic acid; BrMIC+AB - broth microdilution MIC with AlamurBlue™; AMIC – Agar MIC

3.2.2. Test comparison

Simple linear regression analyses were done using Microsoft 2003™ Excel comparing two tests to each other in all the combinations. The median results of the $\log_2$ transformed MIC values of the repeat testing were used. The tests with an R^2 value of close to one and the lowest standard error were considered to have a close relationship (Table 7). Tests with the closest relationships were preferred when selecting a test for field strain MIC testing.

As shown in Table 5, the phenol red broth dilution tests with and without amoxicillin and clavulanic acid gave similar results with a strong association (R^2: 0.981 to 0.996) with a standard deviation of 0.008. The standard error of these tests for all the isolated tested was less than 1 (range 0.297 to 0.699).

With the exception of the two *M. synoviae* strains tested, the use of AlamurBlue® as an indicator of oxidation-reduction in the broth dilution tests gave similar results to the broth dilutions tests where phenol red and a sugar was used as an indicator of fermentation. The R^2 values varied from 0.990 to 0.957 with a standard deviation of 0.012. Although there was less association between these tests when the potentiated penicillin was added to the phenol red as indicator, the association was still excellent with a range in the R^2 values of 1 to 0.51 and a standard deviation of 0.0163.

When comparing the broth dilution tests with the agar dilution tests, the results were variable with excellent associations between *M. mycoides* Y *goat, M. gallisepticum* 56 USDA and *M. mycoides* T1/44 with R^2 values of 0.968, 0.958 and 0.994 respectively. However, the field strain of *M. gallisepticum* gave a very poor association with R^2 being 0.254. Thus even when the *M. synoviae* strains were excluded the standard deviation of these MICs were 0.258.

Table 7: Linear regression analyses of the medians of the log_2 transformed results.

	MSP+MSS	MSP + AB	MSP+Agar	MSS+AB	MSS+Agar	AB+Agar
M. mycoides mycoides T1/44						
R Square	0.981	0.981	0.994	1	0.980	0.980
Standard Error	0.699	0.699	0.378	0	0.702	0.702
P values	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
M. felis MEVTD8						
R Square	0.981	0.957	0.880	0.975	0.935	0.929
Standard Error	0.693	1.050	1.748	0.751	1.216	1.340
P values	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
M. crocodyli (field isolate)						
R Square	0.997	0.979	0.767	0.975	0.769	0.746
Standard Error	0.291	0.723	2.399	0.794	2.427	2.559
P values	0.0003	<0.0001	0.0002	<0.0001	0.0002	0.0003
M. bovis (field isolate)						
R Square	0.976	0.980	0.742	0.951	0.756	0.747
Standard Error	0.762	0.699	2.508	1.187	2.656	2.530
P values	0.0003	<0.0001	0.0002	<0.0001	0.0003	0.0003
M. gallisepticum 56 USDA						
R Square	0.986	0.990	0.958	0.977	0.928	0.947
Standard Error	0.592	0.515	1.035	0.775	1.378	1.229
P values	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
M. gallisepticum (field isolate)						
R Square	0.996	0.961	0.254	0.966	0.250	0.284
Standard Error	0.297	0.983	4.285	0.907	4.234	3.845
P values	<0.0001	<0.0001	0.0975*	<0.0001	0.0952	0.0745
M. mycoides Y *goat*						
R Square	0.984	0.966	0.968	0.955	0.962	0.956
Standard Error	0.639	0.929	0.904	1.036	0.953	1.019
P values	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
M. synoviae (*VAXSAFE* vaccine)						
R Square	0.996	0.257	0.409	0.270	0.405	0.743
Standard Error	0.301	4.017	3.583	3.946	3.562	1.474
P values	<0.0001	0.0925	0.0252	0.0261	0.0837	<0.0001
M. synoviae (field strain)						
R Square	0.996	0.289	0.458	0.287	0.462	0.840
Standard Error	0.301	4.130	3.605	4.159	3.612	1.358
P values	<0.0001	0.0712	0.0150	0.0150	0.0725	<0.0001

Key: MSP= MS medium + phenol red, MSS= MS medium + amoxicillin clavulanate, AB= AlamarBlue™.

*Shaded areas indicate those results where P>0.05

3.3 Testing of field isolates

A total of 142 stored field strains of *Mycoplasma* species were tested. The species and origin are shown in Table 8.

Table 8: Number and origin of field isolates tested.

***Mycoplasma* species**	**Origin**	**Number tested**
M. bovis	Bovine transtracheal aspirate (TTA)	93
M. bovirhinis	Bovine transtracheal aspirate (TTA)	13
M. synoviae	Chicken joint	4
	Chicken airsac	1
M. gallisepticum	Chicken nasal sinus	9
	Chicken joint	1
	Chicken trachea	7
M. crocodyli	Crocodile joint fluid	6
	Crocodile peritoneal fluid	1
	Crocodile pericardial fluid	1
M. felis	Cat nasal flush	6
Total		**142**

Tables 9 to14 depict the percentage MIC distribution for each dilution the MIC_{50} (median values) and MIC_{90} for *M. bovis*, *M. bovirhinis*, *M. synoviae*, *M. gallisepticum*, *M. crocodyli* and *M. felis* respectively. The percentage resistance also included in these tables was based on published breakpoints shown in Table 4.

Table 9 Percent MIC distribution for *M. bovis* field isolates obtained from clinically infected cattle (n=93).

Antimicrobials	% resistance	Concentration Range (µg/mP)																MIC50	MIC90
		0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	4096	
Doxycycline	0				95.6	2.2	2.2												0.5	0.5
Enrofloxacin	0	81.7	8.6	7.5	2.2														0.063	0.25
Erythromycin	100										3.2	96.8							>64	>64
Florfenicol	93.5					6.5	1.1	4.3	67.7	13.9	2.2	1.1	3.2						8	4
Nalidixic acid	100												100						>128	>128
Neomycin	100										1.1	2.2	96.7						>128	>128
Norfloxacin	100							4.3	26.9	62.3	6.5								16	16
Tiamulin	1.1				98.9				1.1										0.5	0.5
Tylosin	98.9							1.1		1.1	6.5	1.1	90.3						64	>64
Sulphamethoxazole	100														2.2	97.8			>2048	>2048

		Concentration Range (µg/mP) for trimethoprim and trimethoprim/ sulphamethoxazole								
		0.987	1.9378	3.875	6.75	13.5	27	54	108	
Trimethroprim	100								100	>108	>108
Trimethoprim-sulpha	100				2.1	1.1			96.8	108	108

The shaded areas indicate the concentration range tested for each substance. Bold vertical lines indicate the microbiological cut-off values defining resistance. MICs above the range are given as the concentration closest to the range. MICs equal to or lower than the lowest concentration tested is given as the lowest tested concentration.
Key: Dox=doxycycline; enr=enrofloxacin; flor=florfenicol; nal=nalidixic; neo=neomycin; nor=norfloxacin; sulfa=sulfamethoxazole; tia=tiamulin; trim=trimethoprim; tyl=tylosin; T-S=trimethoprim-sulfamethoxazole. Concentration range from 0.98675-216 is for trimethoprim.

Table 10: Percent MIC distribution for *M. bovirhinis* field isolates obtained from clinically infected cattle (n= 13).

Antimicrobials	% resistance	Concentration Range (µg/mP)																	MIC50	MIC90
		0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	4096		
Doxycycline	0				100														0.5	**0.5**
Enrofloxacin	7.7		23.1	46.2	23.1					7.7									0.25	**0.5**
Erythromycin	100							7.7				30.8	61.5						64	**>64**
Florfenicol	84.6					15.4		23.1	38.5					23.1					8	**>128**
Nalidixic acid	100								7.7				30.8	61.5					8-128	**>128**
Neomycin	100								15.4				38.5	46.2					8-128	**>128**
Norfloxacin	100							30.8	15.4	23.1	7.7	7.7		15.4					16	**64**
Tiamulin	0				76.9	23.1													0.5	**0.5**
Tylosin	38.5				46.2	7.7		7.7					38.5						0.5	**>64**
Sulphamethoxazole	100															15.4	53.8	30.8	2048	**>2048**

		Concentration Range (µg/mP) for trimethoprim and trimethoprim/ sulphamethoxazole									
		0.987	1.938	3.875	6.75	13.5	27	54	108		
Trimethroprim	100								7.7	92.3	
Trimethoprim-sulpha	100						15.4	38.5	46.2		

The shaded areas indicate the concentration range tested for each substance. Bold vertical lines indicate the microbiological cut-off values defining resistance. MICs above the range are given as the concentration closest to the range. MICs equal to or lower than the lowest concentration tested is given as the lowest tested concentration. Key: Dox=doxycycline; enr=enrofloxacin; flor=florfenicol; nal=nalidixic; neo=neomycin; nor=norfloxacin; sulfa=sulfamethoxazole; tia=tiamulin; trim=trimethoprim; tyl=tylosin; T-S=trimethoprim-sulfamethoxazole. Concentration range from 0.98675-216 is for trimethoprim.

Table 11 Percent MIC distribution for *M. synoviae* field isolates obtained from clinically infected chickens (n = 5).

Antimicrobials	% resistance	Concentration Range (µg/ml)																	MIC50	MIC90
		0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	4096		
Doxycycline	0				100														0.5	0.5
Enrofloxacin	0						100												2	2
Erythromycin	100											100							64	64
Florfenicol	40					60	40												2	1
Nalidixic acid	100												100						2	1
Neomycin	100										60	20	20						16	64
Norfloxacin	100													100					>128	>128
Tiamulin	100									50		50							16	64
Tylosin	20				80							20							32	32
Sulphamethoxazole	100																100		2048	2048

Concentration Range (µg/ml) for trimethoprim and trimethoprim/sulphamethoxazole

		0.987	1.938	3.875	6.75	13.5	27	54	108	MIC50	MIC90
Trimethroprim	0		100							0.969	0.969
Trimethoprim-sulpha	100							40	60	108	>108

The shaded areas indicate the concentration range tested for each substance. Bold vertical lines indicate the microbiological cut-off values defining resistance. MICs above the range are given as the concentration closest to the range. MICs equal to or lower than the lowest concentration tested is given as the lowest tested concentration.
Key: Dox=doxycycline; enr=enrofloxacin; flor=florfenicol; nal=nalidixic; neo=neomycin; nor=norfloxacin; sulfa=sulfamethoxazole; tia=tiamulin; trim=trimethoprim; tyl=tylosin; T-S=trimethoprim-sulfamethoxazole. Concentration range from 0.98675-216 is for trimethoprim.

Table 12. Percent MIC distribution for *M. gallisepticum* field isolates obtained from clinically infected chickens (n= 17).

Antimicrobials	% resistance	Concentration Range (µg/mP)																MIC50	MIC90
		0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	4096	
Doxycycline	17.6				70.6	11.8			5.9			11.8							0.5	64
Enrofloxacin	17.6	70.6	5.9				5.9		11.8	5.9									0.063	8
Erythromycin	94.1				5.9						5.9	5.9	82.4						>64	>64
Florfenicol	70.6					29.4	29.4		17.6	11.8			11.8						2	16
Nalidixic acid	100											5.9		94.1					>128	>128
Neomycin	76.4					11.8	11.8	5.9		5.9	11.8			52.9					>128	>128
Norfloxacin	64.7					29.4	5.9	11.8		11.8	23.5	5.9		11.8					32	>128
Tiamulin	100								5.9				94.1						8	64
Tylosin	64.7				35.3					5.9			58.8						>64	>64
Sulphamethoxazole	94.1								5.9						5.9	11.8	76.5		>2048	>2048

		Concentration Range (µg/mP) for trimethoprim and trimethoprim/sulphamethoxazole								
		0.987	1.938	3.875	6.75	13.5	27	54	108	
Trimethroprim	17.7	82.4						5.9	11.8	54	108
Trimethoprim-sulpha	88.2	11.8			5.9	5.9		5.9	70.6	13.5	108

The shaded areas indicate the concentration range tested for each substance. Bold vertical lines indicate the microbiological cut-off values defining resistance. MICs above the range are given as the concentration closest to the range. MICs equal to or lower than the lowest concentration tested is given as the lowest tested concentration.
Key: Dox=doxycycline; enr=enrofloxacin; flor=florfenicol; nal=nalidixic; neo=neomycin; nor=norfloxacin; sulfa=sulfamethoxazole; tia=tiamulin; trim=trimethoprim; tyl=tylosin; T-S=trimethoprim-sulfamethoxazole. Concentration range from 0.98675-216 is for trimethoprim.

Table 13. Percent MIC distribution for *M. crocodyli* field isolates obtained from clinically infected crocodiles (n= 8).

Antimicrobials	% resistance	Concentration Range (µg/mP)																MIC50	MIC90
		0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	4096	
Doxycycline	0			100															0.25	0.25
Enrofloxacin	0	100																	0.031	0.031
Erythromycin	100										25	75							32	128
Florfenicol	25				75		12.5	12.5											0.5	4
Nalidixic acid	100													100					256	256
Neomycin	75				12.5		12.5	12.5	12.5	12.5	12.5	25							8	64
Norfloxacin	62.5				37.5			37.5	12.5		12.5								4	32
Tiamulin	0			100															0.25	0.25
Tylosin	0			87.5			12.5												0.25	2
Sulphamethoxazole	100																	100	4096	4096

Concentration Range (µg/mPl) for trimethoprim and trimethoprim/sulphamethoxazole

		0.987	1.938	3.875	6.75	13.5	27	54	108		MIC50	MIC90
Trimethroprim	100									100	216	216
Trimethoprim-sulpha	100								100		108	108

The shaded areas indicate the concentration range tested for each substance. Bold vertical lines indicate the microbiological cut-off values defining resistance. MICs above the range are given as the concentration closest to the range. MICs equal to or lower than the lowest concentration tested is given as the lowest tested concentration.
Key: Dox=doxycycline; enr=enrofloxacin; flor=florfenicol; nal=nalidixic; neo=neomycin; nor=norfloxacin; sulfa=sulfamethoxazole; tia=tiamulin; trim=trimethoprim; tyl=tylosin; T-S=trimethoprim-sulfamethoxazole. Concentration range from 0.98675-216 is for trimethoprim.

Table 14. Percent MIC distribution for M. *felis* field isolates obtained from clinically infected cats (n= 6).

Antimicrobials	% resistance	Concentration Range (µg/ml)																	MIC50	MIC90
		0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	4096		
Doxycycline	0			100															0.25	0.25
Enrofloxacin	0	100																	0.031	0.031
Erythromycin	50			33.3		16.7							50						1	128
Florfenicol	83.3			16.7			50	33.3											2	4
Nalidixic acid	100													100					256	256
Neomycin	100										16.7	33.3		50					64	256
Norfloxacin	50				33.3		16.7	33.3	16.7										2	8
Tiamulin	0			100															0.25	0.25
Tylosin	16.7			66.7			16.7		16.7										0.25	8
Sulphamethoxazole	100																	100	4096	4096

		Concentration Range (ug/ml) for trimethoprim and trimethoprim/sulphamethoxazole																		
		0.987	1.938	3.875	6.75	13.5	27	54	108											
Trimethroprim	100									100									216	216
Trimethoprim-sulpha	100								100										108	108

The shaded areas indicate the concentration range tested for each substance. Bold vertical lines indicate the microbiological cut-off values defining resistance. MICs above the range are given as the concentration closest to the range. MICs equal to or lower than the lowest concentration tested is given as the lowest tested concentration.
Key: Dox=doxycycline; enr=enrofloxacin; flor=florfenicol; nal=nalidixic; neo=neomycin; nor=norfloxacin; sulfa=sulfamethoxazole; tia=tiamulin; trim=trimethoprim; tyl=tylosin; T-S=trimethoprim-sulfamethoxazole. Concentration range from 0.98675-216 is for trimethoprim.

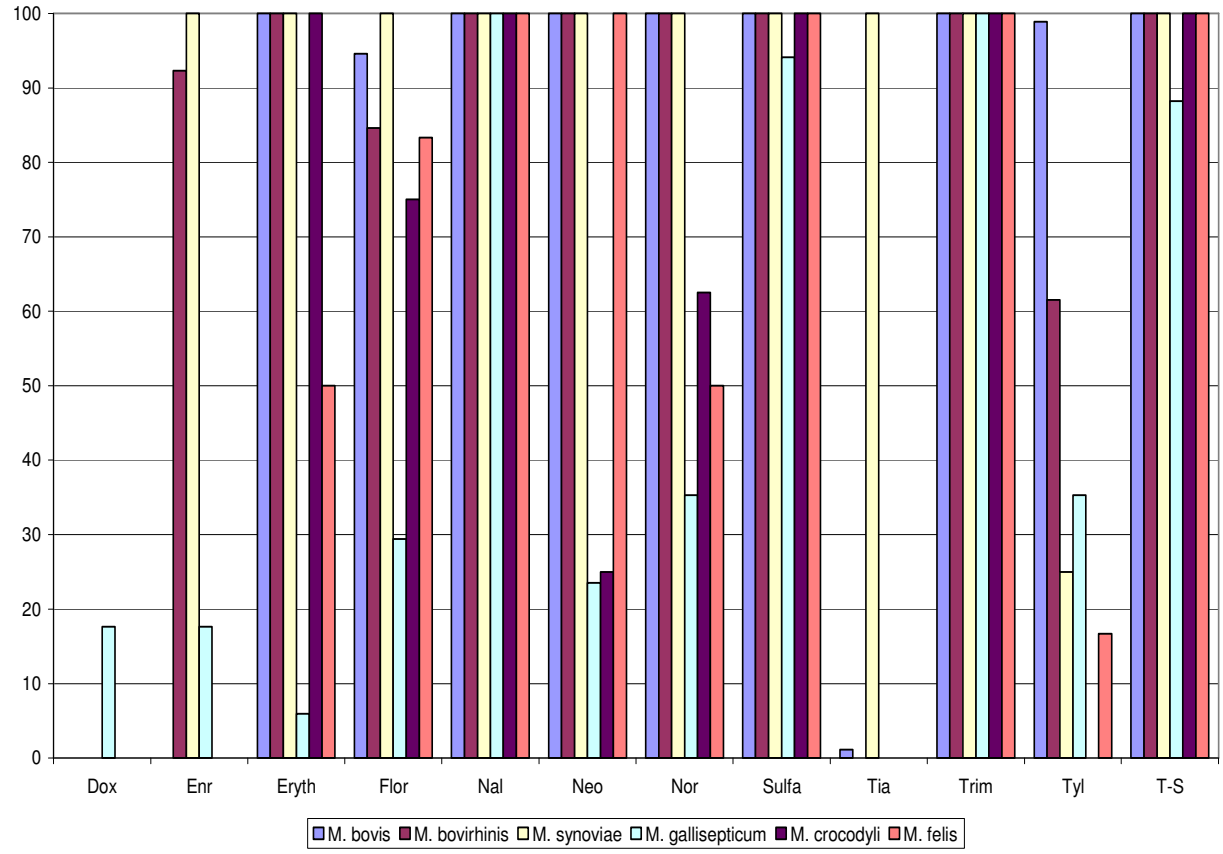

Figure 4: The percentage antimicrobial resistance distribution of field strains of *M. bovis, M. bovirhinis, M. synoviae, M. gallisepticum, M. crocodyli* and *M. felis*.

Key: Dox: doxycycline, Enr: enrofloxacin, Eryth: erythromycin, Flor: florfenicol, Nal: nalidixic acid, Neo: neomycin, Nor: norfloxacin, Sulfa: sulfamethoxazole, Tiam: tiamulin, Trim: trimethoprim, Tyl: tylosin, T-S: trimethoprim sulphamethoxazole

Table 9 shows that all the 93 strains of *M. bovis* tested were susceptible to doxycycline, and enrofloxacin with only one strain being resistant to tiamulin. Most strains of *M. bovis* were resistant to florfenicol and tylosin whilst all were resistant to nalidixic acid, norfloxacin and neomycin.

As depicted in Table 10, it was found that all the strains (n=13) of *M. bovirhinis* were susceptible to doxycycline and tiamulin. Only one strain was resistant to enrofloxacin and five strains were resistant to erythromycin. There was a high level of resistance of this mycoplasma to florfenicol and 100 % resistance to erythromycin, nalidixic acid, neomycin, norfloxacin, sulphamethoxazole and trimethroprim-sulphamethoxazole.

Although only a few strains of *M. synoviae* (n=5) tested, as shown on Table 11, they were susceptible only to doxycycline and trimethoprim with slight resistance to tylosin and almost half of them were resistant to florfenicol. All isolates were resistant to enrofloxacin, erythromycin, nalidixic acid, sulphamethoxazole, tiamulin, trimethoprim-sulphamethoxazole.

Table 12 shows that for the strains of *M. gallisepticum* (n=17) tested which were highly resistant to erythromycin, nalidixic acid, neomycin, norfloxacin, sulphamethoxazole, trimethoprim-sulfamethoxyazole, florfenicol, tiamulin and tylosin. Slight resistance was observed against doxycycline, enrofloxacin and trimethoprim.

A small number of *M. crocodyli* isolates (n=8) were tested as shown on Table 13 in which the isolates were very susceptible to doxycycline, enrofloxacin, tiamulin and tylosin. Whereas there was a high level of resistance against erythromycin, nalidixic acid, sulphamethoxazole, trimethoprim and trimethoprim-sulphamethoxazole and slight resistance to neomycin.

Table 14 shows that the strains of *M. felis* (n=13) tested were susceptible to doxycycline, enrofloxacin as well as tiamulin, while they were resistant to neomycin, sulfamethoxazole, trimethoprim and trimethoprim-sulphamethoxazole showed 100% resistance. Half of the isolate tested were resistant to both erythromycin and norfloxacin.

Figure 4 illustrates the percentage resistance of all the mycoplasmas tested to selected antimicrobials. Although there was variation in resistance patterns between the *Mycoplasma* species, generally most of the field strains were susceptible to tiamulin and doxycycline. With the exception of *M. bovirhinis* and *M. synoviae*, most field isolates were susceptibile to enrofloxacin. The same was not true for the older flouroquinone, norfloxacin. Unexpectedly high levels of resistance were encountered especially by the bovine mycoplasmas to tylosin. As expected, antimicrobial resistance to the sulphonamides, trimethoprim, neomycin and nalidixic acid were high, indicating that these antimicorbials should not be used to treat *Mycoplasma* infections.

CHAPTER FOUR

Discussion

4.1 The validation phase

Like any laboratory test used in diagnostics, it is important that antimicrobial susceptibility tests have a high level of precision. Precision is measured by the use of repeatability testing, inter-laboratory testing and whether the results obtained by the system are comparable to the results obtained by an acknowledged "gold standard" reference method (OIE, 2008). For MIC testing, it is often assumed that the lack of precision comes from factors such as variations in methodology between different operators and variations in the test materials, e.g. growth media components and other chemicals (Wexler, 1991). Aspects of test validation that were tested in this study included repeatability testing and inter-test comparison as well as the MIC testing of field strains. Inter-laboratory comparison was not performed.

As the tests used were not obtained commercially, it was important to use quality control strains to ensure that the test systems were functioning i.e. the antibiotics were in the correct concentrations (Jorgensen, 1993). As there was at the time of the study no *Mycoplasma* species recommended by the CLSI that could serve as quality control strains, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 reference strains were incorporated in each batch of tests to assure that the antimicrobials used were effective and that working dilutions were correctly prepared.

4.1.1 Repeatability

An important validation parameter to express precision is repeatability. Repeatability is defined as the variation in measurements taken by a single person or instrument on the same item and under the same conditions (OIE, 2008). For MIC tests, 95% of MIC values should fall within 3 $\log_2$ dilutions (CLSI, 2009). In this study the mean standard deviations of all the repeats within a species and then within a test was used to determine the variance. A low standard deviation indicates that the data

points tend to be very close to the same value while high standard deviation indicates that the data is spread out over a large range of values. The lower the mean standard deviation was, the more repeatable the test was considered to be. Although the repeatability of the micro-broth dilution tests were acceptable (Table 6) as standard deviations of greater than 1 (more than one $\log_2$ variation) were considered to be unacceptable, it was not the case for the agar microdilution tests (pooled standard deviation variation of 0.28 – 1.54). This was unusual as most of the literature reports that the agar dilution test provides the highest level of precision. In this study it was thought that technical difficulties resulted in the variation noted in these tests as the antimicrobial dilutions had to be made in molten agar which increased the risk of microbial contamination as well as the possibility of uneven mixing of antimicrobials. Hand and aerosol contamination was also more difficult to prevent. The presence of bacterial inhibitors was not tested in the agar dilution test. This may have resulted in more repeatable results. Furthermore, a stereo light microscope was used for colony identification in the agar dilution test, making the test more labour intensive. The good repeatability of the broth dilution tests were ascribed to the use of a standardized concentration of bacteria, the use of cloned mycoplasmas, a sensitive indicator system and the same person performing the tests (Taylor-Robinson and BeBear, 1997).

With the exception of *M. synoviae* in the microbroth test using AlamarBlue® as an indicator, the average standard deviation of the tests were similar. Although *M. synoviae* is known to reduce tetrazolium salts, the bacterium grows slowly and has proven in the media provided to be a weak reducer. Therefore the colour changes were difficult to assess with the naked eye.

4.1.2 Test comparison using linear regression

Regression analysis is a parametric statistical tool for the investigation of relationships between variables[7]. Linear regression analysis for methods was done to test the linearity of the relationship between the various broth and agar dilution methods where the closer to 1 a R^2 value is the greater the similarity of the test results and where the results are considered significant if $P<0.05$. These tests

[7] http://en.wikipedia.org/wiki/Linear_regression (Accessed 4/02/2010)

showed that the use of amoxicillin plus clavulanic acid as well as the indicator used made no statistical difference to the tests ($p<0.0001$ in all tests). The exception was when *M. synoviae* was tested using AlamurBlue® as an indicator of reduction (see below). Interestingly, for all the tests when the fast growing mycoplasmas (*M. mycoides mycoides* T1/44, *M. mycoides* Y *goat, M. felis* MEVTD8, *M. crocodyli, M. bovis, M. gallisepticum* 56 USDA) no statistical differences were observed for all the tests. This indicates that any of the tests used would give accurate results. For those mycoplasmas that proved to grow more slowly, the use of agar in the case of the field isolate of *M. gallisepticum* and AlamurBlue® in the case of *M. synoviae* gave very different results. Therefore for most mycoplasmas of animal origin, the test used can depend on the laboratory. However, one must carefully select the test system and validate it for the slow growers. It must be noted that Hannan, (2000) reported that the agar system was described as the method of choice for testing strains of *M. meleagridis* as most strains do not grow in broth while the broth dilution tests was described as the method of choice if small numbers of strains were to be tested. Field strains of *Mycoplasma meleagridis* which causes respiratory disease in turkeys was not available for testing in this study. Note that differences in the tests used have previously been recorded for other *Mycoplasma* spp. For instance Waites et al., (1991) found that the MIC_{50} and MIC_{90} of *Ureaplasma urealyticum* obtained in broth were consistently four-fold lower than those on agar. Ter Laak et al., (1991) found that the broth and agar dilution methods gave similar MIC results when they were applied on porcine mycoplasmas. Interestingly they found that the broth dilution test should not be read after two days after inoculation as the MIC values tended to be higher. The values did not change for the agar dilution test. This effect was most probably due to the fact that most antimicrobials have a static effect on mycoplasmas and that some lose their activity over time. This is especially true for the tetracyclines that are known to be oxidized by ultra-violet light in aqueous solution (Davis et al., 1975).

4.1.3 Effect of indicators

Since mycoplasmas are extremely small and grow slowly, the detection of visible growth by the visualization of cloudiness in the media or the presence of a bacterial pellet is often not possible. Therefore, indicators of growth when using broth cultures have to be used. All the indicators used are those that determine the presence of

metabolically active mycoplasmas whether it be those making use of sugar fermentation; protein hydrolysis; reduction of terazolium salts, Tween 80 hydrolysis or urea reduction (Devrieuse and Haesebrouk, 1991). The most popular to date are those based on sugar fermentation; glucose fermentation for most mycoplasmas or fermentation of another sugar such asprotein hydrolysis i.e. pyruvate for *M. bovis* or arginine (ter Laak et al., 1991). For most fermentative mycoplasmas a change from a red slightly alkaline to yellow acidic pH when either 1% glucose, and pyruvate which were fermented while arginine was hydrolysed and produced an alkaline colour change, which has proven to be a highly readable MIC (Hannan, 2000). It has, however, one drawback in that the test must be performed at a pH of 6.0 to 6.5. Therefore false MIC results may be obtained for antibiotics such as erythromycin which must function at a pH of 7.0 (Taylor-Robinson and BeBear, 1997). Furthermore a carbon dioxide-enriched atmosphere cannot be used for these tests as the carbon dioxide dissolves in the media to form the weak acid carbonic acid. This carbonic acid causes the medium to change to orange making it difficult to visualize the MIC value.

The use of a reducing agent such as resazurin (AlamarBlue®) was tested as it overcomes the problems associated with some of the mycoplasmas that are known to be poor fermenters and those such as *M. bovis* that don't ferment glucose. (AlamarBlue®) and other tetrazolium salts have been used with great success for the slow-growing mycobacteria as well as many aerobic Gram-positive and Gram-negative bacteria (Rosenbusch et al., 2005a). The redox indicator AlamarBlue® has been used extensively in mammalian cell culture cytotoxicity assays, fungal susceptibility assays and planktonic bacterial susceptibility assays (Pettit et al., 2005). This indicator which turns from blue to red when reduced by metabolically active bacteria was first reported by the Japanese Society for Chemotherapy (1981) as a possible colorimetric MIC method that could be used on mycoplasmas. However, its use as an indicator for mycoplasmas is limited as some mycoplasmas such as *M. agalactiae* and *M. bovirhinis* are found to be good reducers while others like *M. felis* and *M. bovigenitalium* are poor reducers (Kirchhoff et al., 1992). This was especially noted in this study for *M. synoviae* which didn't reduce it.

4.1.4 Effect of bacterial inhibitors

Due to the fact that *Mycoplasma* spp. are relatively slow-growing and only grow in highly enriched media, great care was taken to avoid the contamination of these cultures by faster growing and less fastidious bacteria found in the environment as well as on the skin and in respiratory aerosols of laboratory workers (Keceli and Miles, 2002). An essential consideration in designing isolation media for mollicutes is the inclusion of selective inhibitors to reduce the growth of cell-walled bacteria. Generally the following the beta-lactams penicillin, ampicillin and methicillin have been used as they prevent cross-linking of the peptidoglycans within the cells wall of bacteria and are therefore totally ineffective against the wall-less mycoplasmas (Hannan, 2000). Other antibacterials that have been used include polymixin B sulphate, sulphamethazine and bacitracin. Formerly the toxic thallium acetate and more recently Amphotericin B has been used as an additive of mycoplasma media to inhibit fungal growth,(Keceli and Miles, 2002).

Even though the researcher worked with pure colonies, sterile media and equipment, it was found that bacterial contamination was difficult to prevent. Furthermore, it has been found in this laboratory that the common antibacterials, namely ampicillin used to prevent bacterial contamination tended only to prevent the growth of some Gram-positive bacteria, the Gram-negative and resistant Gram-positive bacteria still grew well. Thus it was decided to use the broad-spectrum beta-lactam antibiotic amoxicillin plus clavulanic acid. As antimicrobials can act synergistically or antagonistically against each other, it is generally not recommended that antimicrobials are used to prevent bacterial contamination in an MIC test (Hannan 2000; CLSI, 2008; Nicholas et al., 2008). Therefore it was important that a comparison was done using the same indicator system, but a test with antimicrobials and one without antimicrobials. In this study it was found that tests gave similar results when using linear regression and that both were highly repeatable. This was similar to a study done by Whithear, et al. (1983) for testing sensitivity of fermentative avian mycoplasmas revealed that MIC values for erythromycin, spiramycin, streptomycin, tetracycline, tiamulin, and tylosin in media inoculated with combinations of 1,000 IU/ml penicillin G, 1,000 µg/ml ampicillin, or 1,000 µg/ml amoxicillin with 500 µg/ml thallium acetate were identical to those in control medium without inhibitors.

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4.2 Testing of Field Isolates

From the validation study, it was clear that any test could be used on fast growing mycoplasmas. Since a large number of field strains were to be tested, and one wanted to avoid bacterial contamination, it was decided to use the broth dilution sugar fermentation method with added amoxicillin plus clavulanic acid. This was unusual, with the exception of a few studies, most of the published antimicrobial susceptibility tests on mycoplasmas whether they be broth or agar dilution tests have not contained any bacterial inhibitors (Godinho, 2008).

4.2.1 Difficulties in storage and propagation of mycoplasmas

Not all the strains that were stored (approximated 200) could be recovered as freezing is lethal to many living systems (Mazur, 1970). Some mycoplasmas have been can survive less than one year at -20 ºC, less than one month at 4 ºC and only several days at 37 ºC (Lin and Kleven, 1982). Another study stated that mycoplasma strains survived for at least 10 months at -26 ºC, but changes were recognized in colony size and growth rate of cultures stored longer than 10 months (Kelton, 1964). In the experience of this laboratory storage of mycoplasmas at -20℃ tends to result in the rupture of the delicate cell membranes when they are defrosted (Mazur, 1970). Therefore, this storage method is not recommended. Even though the storage of culture plugs containing viable *Mycoplasma* -80 ºC which is more rapid than a household freezer and causes the formation of small crystals has proven to be an effective storage method, it is believed that the viability of these colonies decrease with time. At -65 ºC, Kelton (1964) showed that there was little loss in viable numbers from 12 months of storage, and no changes in the organisms were apparent. A study showed that some mycoplasma broth culture were viable after 13 years of storage at -70 ºC although there was up to 10^{4}-fold reduction in the titer of organisms was observed in some cultures Isolates were from different years with a maximum of around 8 years of storage.

4.2.2 Determination of breakpoints

The clinical breakpoint for an antimicrobial agent is the lowest concentration of the antimicrobial agent that a pathogen can be treated with (Baywater, 2006). These values are based on clinical, pharmacological and microbiological data and have to be individually determined for each antimicrobial agent, host species and pathogen (McGowen and Wise 2001). Therefore, these values are often not available for all pathogens, nor is there consensus in the literature for between different countries. For example, among the United States and 5 European countries the susceptibility MIC breakpoints for cefotaxime and ceftazidime varies from 1 to 8 mg/mℓ (Ferraro, 2001). These differences may be due to different dosing of antimicrobials in various countries or from use of different laboratory methods to determine antimicrobial susceptibility. In addition, some philosophical differences may exist among the various organizations and societies responsible for issuing these breakpoints (Ferraro, 2001). Clinical breakpoints are different to microbiological cut-off values in that the latter are determined by comparing the MIC ranges of resistant and susceptible populations. Most surveillance programmes make use of microbiologial cut-off values as these are easier to standardize. However, for diagnostic laboratories that wat to ensure that antimicrobial agents will be effective in the therapy of disease, the microbiological break-points are considered more useful. Therefore, it has become the habit of diagnostic laboratories to make use of published standards such as the Clinical Laboratory Standards Institute (CLSI) in the USA. These standards are usually updated every two years with separate publications for human and veterinary pathogens (CLSI, 2008).

Since *Mycoplasma* species vary widely in nutritional and cultural requirements there are no CLSI approved MIC breakpoint values for mycoplasmas of animals or humans as well as no standard methods for testing. Furthermore, to date, there is no international authority that publishes MIC breakpoints for the mycoplasmas and therefore these reference values have either been based on individual publications that have been ascertained for a specific *Mycoplasma* species or interpretative criteria used for the walled bacteria are used (Rosenbusch et al., 2005). However, the latter is not ideal as mycoplasmas tend to give lower MIC values than the walled bacteria for susceptible antimicrobials and it has been found that published

breakpoints for the mycoplasmas tend to be lower than that of the mycoplasmas. For example, the Japanese Society of Chemotherapy (1994) consider the breakpoints of MICs for tetracyclines, macrolides, aminoglycosides and fluoroquinolones as 1.0, 0.5–2.0, 2.0–4.0 and 1.0–2.0 µg/ml, respectively. These have been based on breakpoints for common human mycoplasmal pathogens (Hirose et al., 2003). The corresponding MIC breakpoints in the latest CLSI (2008) document are 8, 16, 8 and 5 µg/ml for tetracyclines, macrolides, aminoglycosides and fluoroquinolones respectively.

This made it difficult to determine whether a *Mycoplasma* strain was resistant or susceptible. Therefore, it was decided to use published breakpoints values as interpretative criteria in the following order of preference: mycoplasmas of veterinary importance; mycoplasmas of medical significance; or breakpoints recommended by the CLSI (2008). The values used are represented in Table 4.

Therefore there is a need to determine breakpoints, which have been defined as the relationship between MIC values and effective treatment dose for different antimicrobials against mycoplasmas in different host animals (Francoz et al., 2005).

4.2.3 Antibiotics used to treat mycoplasmas

It has long been reported that mycoplasmas are generally susceptible to antibiotics that inhibit protein synthesis while resistant to those that act on bacterial cell wall components (because of the absence of the latter) (Taylor-Robinson and Bebear, 1997), hence only those antimicrobials with possible efficacy against mycoplasmas were used in the project as shown in Table 8. Antibiotics that are generally used to treat mycoplasmal infections include tetracyclines, MLSK (Macrolide-lincosamide-streptogramin-ketolide) group, fluoroquinolones and aminoglycosides. Another class, the pleuromutilins, are exclusively used for treatment of animal mycoplasmas.

Tetracyclines such as oxytetracycline and doxycycline are usually used for treatment of poultry, bovine or swine mycoplasmal infections because they are affordable (Haines 2001). Doxycycline, minocycline and tigecycline are used to treat human

infections (Haines et al., 2001). Doxycycline has also been proved to be effective against human mycoplasmas as it is used for treatment of *Mycoplasma pneumoniae* (Taylor-Robinson and Bebear, 1997). In this study, it was found that the tetracyclines, represented by doxycycline, were 100 % effective against most of *Mycoplasma* field strains with the exception of *M. gallisepticum* where only 17.6% were susceptible. The latter could represent the extensive use in South Africa of tetracyclines in commercial poultry as food or water additives.

The MLSK are the antimicrobials of choice when treating mycoplasmal infections in both humans and animals. In humans erythromycin and josamycin are most often used (Waites et al., 1993). In animals the erythromycin derivatives tylosin, tilmicosin and tulathromycin are used to treat a wide range of animal mycoplasmas such as *M. bovis*, *M. canis*, *M. hyorhinis*, *M. hyponeumoniae*, *M. hyosynoviae*, *M. gallisepticum* and *M. mycoides mycoides*. In this study resistance to this group of antimicrobials for mycoplasmas that originated from food animals was unusually high; 100% to erythromycin in the case of *M. bovis, M. bovirhinis* and *M. synoviae* and 94.1 % for M. *gallisepticum*. The resistance to tylosin was lower i.e. 100% to *M. bovis* and 64.7% to *M. gallisepticum* but still of concern.

The pleuromutilins include tiamulin and valnemulin which are mainly dedicated for treatment of avian or swine mycoplasmosis as they show an outstanding efficacy in these species (Stipkovits et al., 2001). With the exception of the poultry mycoplasmas *M. gallisepticum* and *M. synoviae*, all the other mycoplasmas were highly susceptible to tiamulin (98.9 to 100%).

As the antimicrobial resistance to the macrolides has increased, as shown in this study, the third generation fluoroquinolones are increasingly being used to treat mycoplasma infections. In South Africa, fluoroquinolones registered for veterinary use are norfloxacin, danofloxacin, enrofloxacin, orbifloxacin and marbofloxacin. They are used to treat respiratory, urogenital tract, eye and joint infections in cattle, swine, birds, dogs and cats (Rosenbusch et al., 2005a). With the exception of a few *M. gallisepticum* strains most of the mycoplasmas were susceptible to enrofloxacin. The same was not true for norfloxacin an earlier generation fluoroquinolone, where the

levels of resistance were much higher. The first generation quinolone, nalidixic acid was not active against all the field isolates tested and this supports reported published data where it was found to be ineffective against mycoplasmas (Roberts, 1992).

Aminoglycosides are not usually used to treat mycoplasma infections. This was evidenced by the high MIC_{50} and MIC_{90} values shown by most mycoplasmas to neomycin. Among the aminoglycosides only spectinomycin that is used for animal mycoplasma even though it showed high MIC values comparing to other antimicrobials tested as shown with *M. mycoides* subsp. *mycoides* SC (Ayling et al., 2000).

4.2.4 Susceptibility of each isolate to the antibiotics associated with antimicrobial use in that species

By far the highest number of mycoplasmas in this study originated from feedlot cattle. The MIC_{50} and MIC_{90} of *M. bovis* respectively to tetracyclines were 0.5 µg/mP and 0.5 µg/mP respectively. These are higher than results from the E test method (0.023 µg/mP) (Gerchman et al., 2009) which could either be due to a test difference as or the fact that even though mycoplasma in cattle were 100% susceptible to tetracyclines, there may be an increasing trend to resistance as a result of tetracycline usage. Tetracyclines are commonly administered in the feed or water to prevent secondary bacterial infections of the respiratory tract during the adaptation phase on a cattle feedlot. *Mycoplasma bovis* was also susceptible to enrofloxacin and tiamulin and that agrees with the results found by (Thomas et al., 2003). Interestingly, *M. bovis* was highly resistant to the macolides erythromycin and tylosin. In South Africa, during the winter season respiratory infections in feedlot are nowadays usually treated with tylosin, tilmicosin or tulathromycin as tetracyclines are often considered ineffective. Therefore, even though tilmicosin and tulathromycin were not tested, the high MIC_{50} (64 µg/mP) and MIC_{90} (>64 µg/mP) values to the related tylosin are not surprising.

Interestingly *M. bovirhinis* which is predominantly a commensal in the respiratory tract of cattle was found to be more resistant to antimicrobials than the respiratory pathogen *M. bovis* as it was only fully susceptible to tetracyclines, tiamulin and norfloxacin, but not enrofloxacin. It has been reported to be susceptible to enrofloxacin, perfloxacin, erythromycin and lincospectin (Eissa et al., 1999). In this study all the strains were resistant to erythromycin which is contrary to what Eissa et al., (1999) reported. Most of the strains isolated originate from the period when tilmicosin was introduced into the feedlot. In the first three years of its introduction, this antimicrobial was extremely effective in the treatment of bovine respiratory disease and was therefore used extensively in spite of its high cost. Therefore, it would be expected that resistance directed to the use of tylosin, tilmicosin or tulathromycin would also result in cross-resistance of the parent molecule (Zanella et al., 1998).

Most poultry, especially layer farms in South Africa are endemic for *M. gallisepticum* associated sinusitis and some farms have evidence of synovitis due to *M. synoviae* infections. In South Africa, tylosin is the preferred antimicrobial for the treatment of mycoplasmas, therefore, it is not surprising that mycoplasmas originating from this domesticated species will have a high level of antimicrobial resistance to MLSK as evidenced by that the MIC_{50} of *M. gallisepticum* and *M. synoviae* to tylosin was >64 µg/mP and 32 µg/mP respectively. Unlike mycoplasmas from the other host species, it was found that the poultry mycoplasmas were highly resistant to tiamulin. Tiamulin is registered for use in poultry and pigs in South Africa, where it can be administered in the drinking water (Jordan et al., 1998).

Resistance of the poultry mycoplasmas to the tetracyclines was surprisingly low, especially as it is recorded in commensal *E. coli* originating from the intestinal tract of chickens in South Africa and is known to be the most commonly administered antimicrobial (SANVAD, 2008). Therefore, it is still possible that tetracyclines can be used to treat mycoplasmosis of poultry in South Africa (Gautier-Bouchardon et al., 2002).

Crocodile farming in South Africa for the production of hides and meat is fairly well developed. As young crocodiles tend to be in pens containing at between 50 and 200 crocodiles and are often handled to a few months before slaughter to ensure that their hides are of a high quality, it has been found that outbreaks of polyarthritis due to *M. crocodyli* infections are common. This results in many of farmers with this disease using antimicrobials to treat infections. Common antimicrobials used to treat these infections include tetracyclines, tylosin and enrofloxacin (J. Picard, pers. comm. 2009). In spite of this *M. crocodyli* was susceptible with MIC_{90} values of <2 µg/ml to doxycycline, enrofloxacin, tiamulin and tylosin with some 25 % resistant to florfenicol. The MIC results of all these antimicrobials similar to those reported by (Helmick et al., 2002).

Myoplasma felis most commonly causes conjunctivitis and rhinitis in cats and has been reported to cause pleuropneumonia in horses. Strains were fully susceptible to tetracyclines, enrofloxacin and tiamulin with some resistance to norfloxacin (50%) and tylosin (16.7%). As infections tend to be mild, antimicrobial treatment in these species is uncommon. Doxycycline is the drug recommended for treatment of *M. felis* infections (Liehmann et al., 2006). An unusual report of a human infection showed that doxycycline was effective against *M. felis* where a patient was treated with it and recovered (Bonilla et al., 1997).

Conclusion and Recommendations

It is therefore, concluded that even though all the MIC testing methods were comparable, BrMIC+SFS with ACA method is a reproducible method that reduces any problems with bacterial contamination. It must also be noted, that these tests should always be re-evaluated when testing a *Mycoplasma* species, especially the slow growing one, not previously tested in the laboratory.

The next step in the test validation process is to perform inter-laboratory comparisons.

As observed with the poultry strains, it is quite clear that antimicrobial resistance is developing to commonly used antimicrobials such as tylosin, the related pleuromutilins, fluoroquinolones and tetracyclines. In species where antimicrobial therapy is applied routinely such as poultry and possibly feedlot cattle, it is recommended that MIC testing is done prior to any therapeutic interventions.

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APPENDIX I

MICs obtained in the daily repeatablility testing

Test	Day	Doxycycline	Enrofloxacin	Erythromycin	Florfenicol	Nalidixic acid	Neomycin	Norfloxacin	Sulphamethoxazole	Tiam ulin	Trimethoprim	Tylosin	S	:T
M. gallisepticum 56 USDA														
BrMIC	**D1**	0.25	0.31	0.25	0.5	128	128	2	2048	0.25	108	0.5	256	13.5
+SFS	**D2**	0.25	0.31	0.25	0.5	128	128	2	2048	0.25	108	0.5	1024	54
	D3	0.25	0.31	0.25	0.5	128	128	2	2048	0.25	108	0.5	1024	54
	D4	0.25	0.31	0.25	0.5	128	128	4	2048	0.25	108	0.5	1024	54
	D5	0.25	0.31	0.25	0.5	128	128	0.5	2048	0.25	108	0.5	512	27
BrMIC	**D1**	0.25	0.31	0.25	0.5	128	128	0.5	2048	0.25	108	0.5	c	c
+SFS	**D2**	0.25	0.31	0.25	0.5	128	128	0.5	2048	0.25	108	0.5	1024	54
+ ACA	**D3**	0.25	0.31	0.25	0.5	128	128	0.5	2048	0.25	108	0.5	1024	54
	D4	0.25	0.31	0.25	0.5	128	128	2	2048	0.25	108	0.5	1024	54
	D5	0.25	0.31	0.25	0.5	128	128	2	2048	0.25	54	0.5	1024	54
BrMIC	**D1**	0.25	0.31	0.25	0.5	256	256	2	4096	0.25	54	0.5	1024	54
+AB	**D2**	0.25	0.31	0.25	0.5	256	256	2	4096	0.25	54	0.5	1024	54
AMIC	**D1**	0.25	1	0.25	4	256	256	32	4096	0.25	216	0.5	2048	108
	D2	0.25	0.13	0.25	0.5	256	256	16	4096	0.25	216	0.5	2048	108
	D3	0.25	0.25	64	0.5	256	256	8	4096	0.25	216	8	2048	108
	D4	0.25	8	32	4	256	256	4	4096	0.25	216	0.5	2048	108
	D5	0.25	0.31	c	0.5	256	256	4	4096	0.25	216	c	2048	108
M. gallisepticum														
BrMIC	**D1**	0.25	0.31	0.25	0.5	64	128	0.5	2048	64	108	0.5	1024	54
+SFS	**D2**	0.25	0.31	0.25	0.5	64	128	0.5	2048	64	108	0.5	1024	54
	D3	0.25	0.31	0.25	0.5	64	128	0.5	2048	64	108	0.5	1024	54
	D4	0.25	0.31	0.25	0.5	32	64	2	2048	32	108	0.5	512	27
	D5	0.25	0.31	0.25	0.5	32	64	2	2048	32	54	0.5	512	27
BrMIC	**D1**	0.25	0.31	0.25	0.5	32	128	2	2048	64	108	0.5	512	27
+SFS	**D2**	0.25	0.31	0.25	0.5	32	128	2	2048	32	108	0.5	512	27
+ ACA	**D3**	0.25	0.31	0.25	0.5	64	128	0.5	2048	32	108	0.5	1024	54
	D4	0.25	0.31	0.25	0.5	64	32	0.5	2048	64	108	0.5	1024	54
	D5	0.25	0.31	0.25	0.5	64	32	0.5	2048	64	108	0.5	256	13.5
BrMIC	**D1**	0.25	0.31	0.5	2	128	128	2	2048	64	54	0.5	512	27
+AB	**D2**	0.25	0.31	0.5	2	128	128	2	2048	64	54	0.5	512	27
AMIC	**D1**	0.25	8	64	4	256	256	256	4096	0.5	216	128	2048	108
	D2	0.25	8	64	4	256	256	128	4096	0.5	216	64	2048	108
	D3	0.25	4	128	4	256	256	256	4096	0.5	216	64	2048	108
	D4	0.25	8	128	4	256	256	256	4096	0.5	216	128	2048	108
	D5	0.25	8	c	0.5	256	256	256	4096	0.5	216	128	2048	108

Key: BrMIC+SFS- broth microdilution MIC with a sugar fermentation system; BrMIC+SFS+ACA - microdilution MIC with a sugar fermentation system with amoxicillin and clavulanic acid; BrMIC+AB - broth microdilution MIC with AlamurBlue™; AMIC – Agar MIC; c - contamination

Test	Day	Doxycycline	Enrofloxacin	Erythromycin	Florfenicol	Nalidixic acid	Neomycin	Norfloxacin	Sulphamethoxazole	Tiam ulin	Trimethoprim	Tylosin	S	:T
M. synoviae														
BrMIC	**D1**	<0.5	2	>64	4	>128	64	32	>2048	<0.5	>108	<0.5	2048	108
+SFS	**D2**	<0.5	2	64	4	>128	32	32	2048	<0.5	>108	<0.5	1024	54
	D3	<0.5	2	>64	4	>128	32	32	>2048	<0.5	>108	<0.5	1024	54
	D4	<0.5	2	>64	4	>128	64	32	>2048	<0.5	>108	<0.5	2048	108
	D5	<0.5	1	32	4	>128	32	32	>2048	<0.5	>108	<0.5	2048	108
BrMIC	**D1**	<0.5	2	>64	4	>128	64	32	>2048	<0.5	>108	<0.5	2048	108
+SFS	**D2**	<0.5	2	32	4	>128	64	32	1024	<0.5	>108	<0.5	2048	108
+ ACA	**D3**	<0.5	2	>64	4	>128	64	32	>2048	<0.5	>108	<0.5	2048	108
	D4	<0.5	2	>64	4	>128	64	32	>2048	<0.5	>108	<0.5	2048	108
	D5	<0.5	2	>64	4	>128	64	32	>2048	<0.5	>108	<0.5	2048	108
BrMIC	**D1**	2	0.25	16	16	>128	32	>128	2048	4	27	64	c	c
+AB	**D2**	>64	>8	32	64	>128	>128	64	2048	64	>108	64	1024	54
AMIC	**D3**	2	0.13	16	16	2	<1	<1	<16	64	6.75	>64	64	3.86
	D1	32	>8	>64	64	>128	>128	>128	>2048	>64	>108	>64	2048	108
	D2	32	>8	>64	64	>128	64	128	>2048	>64	>108	>64	2048	108
	D3	64	8	>64	128	>128	128	64	>2048	>64	>108	>64	2048	108
	D4	64	4	>64	32	>128	>128	>128	>2048	>64	>108	>64	2048	108
	D5	>64	4	>64	64	>128	>128	>128	>2048	>64	>108	>64	2048	108
M. synoviae VAXSAFE														
BrMIC	**D1**	0.25	1	32	0.5	256	64	16	1024	0.25	216	0.25	512	27
+SFS	**D2**	0.25	1	32	0.5	256	64	16	1024	0.25	216	0.25	512	27
	D3	0.25	1	32	0.5	256	64	16	1024	0.25	216	0.25	512	27
	D4	0.25	1	32	0.5	256	64	16	1024	0.25	216	0.25	512	27
	D5	0.25	1	32	0.5	256	64	16	1024	0.25	216	0.25	512	27
BrMIC	**D1**	0.25	1	32	0.5	256	64	16	1024	0.25	216	0.25	512	27
+SFS	**D2**	0.25	1	32	0.5	256	64	16	1024	0.25	216	0.25	512	27
+ ACA	**D3**	0.25	1	32	0.5	256	64	16	1024	0.25	216	0.25	512	27
	D4	0.25	1	32	0.5	256	64	16	1024	0.25	216	0.25	512	27
	D5	0.25	1	32	0.5	256	64	16	1024	0.25	216	0.25	512	27
BrMIC	**D1**	16	0.25	32	4	32	16	64	256	64	216	64	256	13.5
+AB	**D2**	16	0.25	32	4	32	64	64	512	64	108	64	128	6.88
	D3	16	0.25	16	4	128	16	32	512	64	216	64	256	13.5
	D4	32	1	64	4	256	16	256	256	128	216	128	c	c
AMIC	**D1**	64	2	32	64	64	128	128	4096	32	54	32	2048	108
	D2	64	4	32	64	64	128	64	4096	32	54	32	2048	108
	D3	64	4	32	64	128	256	128	4096	64	216	128	2048	108
	D4	32	2	16	64	256	256	256	4096	64	108	64	2048	108
	D5	32	4	32	128	128	128	256	4096	64	216	128	1024	54

Key: BrMIC+SFS- broth microdilution MIC with a sugar fermentation system; BrMIC+SFS+ACA - microdilution MIC with a sugar fermentation system with amoxicillin and clavulanic acid; BrMIC+AB - broth microdilution MIC with AlamurBlue™; AMIC – Agar MIC; c - contamination

Test	Day	Doxycycline	Enrofloxacin	Erythromycin	Florfenicol	Nalidixic acid	Neomycin	Norfloxacin	Sulphamethoxazole	Tiam ulin	Trimethoprim	Tylosin	S	:T
***Mycoplasma mycoides* Y goat**														
BrMIC	**D1**	0.25	0.31	0.25	0.5	128	128	2	2048	0.25	108	0.5	1024	54
+SFS	**D2**	0.25	0.13	0.25	0.5	128	128	2	2048	0.25	108	0.5	1024	54
	D3	0.25	0.31	0.25	0.5	128	128	4	2048	0.25	108	0.5	1024	54
	D4	0.25	0.31	0.25	0.5	128	128	2	2048	0.25	108	0.5	1024	54
	D5	0.25	0.13	4	16	64	128	8	2048	0.25	108	0.5	1024	54
BrMIC	**D1**	0.25	0.13	1	0.5	64	128	4	2048	0.25	108	0.5	1024	54
+SFS	**D2**	0.25	0.13	0.25	0.5	128	128	4	2048	0.25	108	0.5	1024	54
+ ACA	**D3**	0.25	0.31	0.25	0.5	128	128	2	2048	1	108	0.5	1024	54
	D4	0.25	0.31	0.25	0.5	128	128	4	2048	1	108	0.5	1024	54
	D5	0.25	0.31	0.25	0.5	128	128	4	2048	1	108	0.5	1024	54
BrMIC	**D1**	0.25	0.13	1	0.5	128	128	4	2048	0.25	54	0.5	512	27
+AB	**D2**	0.25	0.13	1	0.5	128	128	4	2048	0.25	54	0.5	512	27
	D3	0.25	0.13	0.25	0.5	128	128	4	2048	0.25	54	0.5	512	27
AMIC	**D1**	2	0.25	0.25	128	256	256	16	4096	0.25	216	0.5	2048	108
	D2	0.25	0.31	0.25	2	256	256	8	4096	0.25	216	0.5	2048	108
	D3	0.25	0.13	0.25	2	256	256	256	4096	0.25	216	0.5	2048	108
	D4	0.25	0.25	64	4	256	256	64	4096	0.25	216	0.5	2048	108
	D5	0.25	0.13	c	0.5	256	256	4	4096	0.25	108	0.5	2048	108
***Mycoides mycoidues* SC T1/44**														
BrMIC	**D1**	0.25	0.13	0.25	2	128	128	8	2048	0.25	54	0.5	512	27
+SFS	**D2**	0.25	0.13	0.25	2	128	128	4	2048	0.25	27	0.5	512	27
	D3	0.25	0.31	0.25	0.5	128	128	8	2048	0.25	108	0.5	1024	54
	D4	0.25	0.31	0.25	0.5	128	128	8	2048	0.25	108	0.5	1024	54
	D5	0.25	0.31	0.25	0.5	128	128	8	2048	0.25	108	0.5	1024	54
BrMIC	**D1**	0.25	0.13	0.25	2	128	128	8	2048	0.25	108	0.5	512	27
+SFS	**D2**	0.25	0.13	0.25	2	128	128	8	2048	0.25	54	0.5	512	27
+ ACA	**D3**	0.25	0.31	0.25	0.5	128	128	4	2048	0.25	27	0.5	1024	54
	D4	0.25	0.31	0.25	2	128	128	4	2048	0.25	108	0.5	1024	54
	D5	0.25	0.31	0.25	0.5	64	64	4	2048	0.25	108	0.5	1024	54
BrMIC	**D1**	0.25	0.31	0.25	0.5	128	128	8	2048	0.25	108	0.5	1024	54
+AB	**D2**	0.25	0.31	0.25	0.5	128	128	8	2048	0.25	108	0.5	1024	54
AMIC	**D1**	0.25	0.13	0.25	2	256	256	32	4096	64	54	0.5	2048	108
	D2	0.25	0.13	0.25	0.5	256	256	16	4096	0.25	216	0.5	2048	108
	D3	0.25	0.13	0.25	0.5	256	256	8	4096	0.25	216	2	2048	108
	D4	0.25	8	0.25	4	256	256	4	4096	0.25	216	0.5	2048	108
	D5	0.25	0.13	0.25	0.5	256	256	8	4096	c	216	c	2048	108

Key: BrMIC+SFS- broth microdilution MIC with a sugar fermentation system; BrMIC+SFS+ACA - microdilution MIC with a sugar fermentation system with amoxicillin and clavulanic acid; BrMIC+AB - broth microdilution MIC with AlamurBlue™; AMIC – Agar MIC; c - contamination

Test	Day	Doxcycline	Enrofloxacin	Erythromycin	Florfenicol	Nalidixic acid	Neomycin	Norfloxacin	Sulphamethoxazole	Tiam ulin	Trimethoprim	Tylosin	S	:T
Mycoplasma bovis														
BrMIC	**D1**	0.25	0.13	0.25	2	64	256	4	2048	0.25	27	0.5	512	27
+SFS	**D2**	1	0.13	1	4	128	256	4	2048	0.25	27	0.5	512	27
	D3	0.25	0.13	0.25	2	128	256	4	2048	0.25	27	0.5	512	27
	D4	0.25	0.13	0.25	2	256	256	8	2048	0.25	27	0.5	512	27
	D5	0.25	0.13	0.25	1	256	256	4	2048	0.25	27	0.5	512	27
BrMIC	**D1**	0.25	0.031	0.25	2	256	256	4	2048	0.25	27	2	512	27
+SFS	**D2**	0.25	0.031	0.25	0.5	256	256	4	2048	0.25	27	0.5	512	27
+ACA	**D3**	0.25	0.031	0.25	0.5	256	256	8	2048	0.25	27	0.5	512	27
	D4	0.25	0.031	0.25	0.5	256	256	2	2048	0.25	27	0.5	512	27
	D5	0.25	0.031	0.25	0.5	256	256	4	2048	0.25	13.5	0.5	512	27
BrMIC	**D1**	0.25	0.13	0.25	2	64	256	4	2048	0.25	108	0.5	512	27
+AB	**D2**	0.25	0.13	0.25	2	64	256	4	2048	0.25	108	0.5	1024	54
AMIC	**D1**	4	1	64	8	256	256	16	4096	64	216	128	2048	108
	D2	1	1	64	2	256	256	256	4096	0.25	216	0.5	2048	108
	D3	0.25	4	64	4	256	256	256	4096	0.25	216	0.5	2048	108
	D4	0.25	8	128	4	256	256	256	4096	0.25	216	64	c	c
	D5	1	0.25	c	c	64	256	256	4096	c	216	c	16	0.97
M. crocodyli														
BrMIC	**D1**	0.25	0.13	0.25	2	128	128	4	2048	0.25	108	0.5	512	27
+SFS	**D2**	0.25	0.13	0.25	2	128	128	4	2048	0.25	108	0.5	512	27
	D3	0.25	0.13	0.25	2	128	128	4	2048	0.25	108	0.5	512	27
	D4	0.25	0.13	0.25	2	128	128	4	2048	0.25	108	0.5	1024	54
	D5	0.25	0.13	0.25	0.5	128	128	4	2048	0.25	108	0.5	1024	54
BrMIC	**D1**	0.25	0.13	0.25	2	128	128	4	2048	0.25	108	0.5	1024	54
+SFS	**D2**	0.25	0.13	0.25	4	128	128	4	2048	0.25	108	0.5	1024	54
+ACA	**D3**	0.25	0.13	0.25	4	128	128	4	2048	0.25	108	0.5	1024	54
	D4	0.25	0.13	0.25	2	128	128	4	2048	0.25	108	0.5	1024	54
	D5	0.25	0.13	0.25	2	128	128	4	2048	0.25	108	0.5	512	27
BrMIC	**D1**	0.25	0.13	0.25	2	256	256	4	2048	0.25	108	2	1024	54
+AB	**D2**	0.25	0.13	0.25	2	256	256	4	2048	0.25	108	2	512	27
AMIC	**D1**	0.25	4	0.25	2	256	256	32	4096	64	216	128	2048	108
	D2	0.25	0.5	0.25	0.5	256	256	16	4096	128	216	0.5	2048	108
	D3	0.25	1	64	2	256	256	16	4096	c	216	16	2048	108
	D4	0.25	2	32	2	256	256	32	4096	c	216	0.5	2048	108
	D5	0.25	0.31	c	0.5	128	256	32	4096	c	216	c	c	c

Key: BrMIC+SFS- broth microdilution MIC with a sugar fermentation system; BrMIC+SFS+ACA - microdilution MIC with a sugar fermentation system with amoxicillin and clavulanic acid; BrMIC+AB - broth microdilution MIC with AlamurBlue™; AMIC – Agar MIC; c - contamination

Test	Day	Doxycycline	Enrofloxacin	Erythromycin	Florfenicol	Nalidixic acid	Neomycin	Norfloxacin	Sulphamethoxazole	Tiam ulin	Trimethoprim	Tylosin	S	:T
Mycoplasma felis														
BrMIC	**D1**	0.25	0.31	0.25	0.5	128	128	0.5	2048	0.25	108	0.5	2048	108
+SFS	**D2**	0.25	0.31	0.25	0.5	128	128	0.5	2048	0.25	108	0.5	2048	108
	D3	0.25	0.31	0.25	0.5	128	128	2	1024	0.25	108	0.5	512	27
	D4	0.25	0.31	0.25	0.5	128	128	2	2048	0.25	108	0.5	1024	54
	D5	0.25	0.31	0.25	0.5	128	128	0.5		1	108	0.5	1024	54
BrMIC	**D1**	0.25	0.31	0.25	0.5	128	128	0.5	1024	0.25	108	0.5	1024	54
+SFS	**D2**	0.25	0.31	0.25	0.5	128	128	2	1024	0.25	108	0.5	512	27
+ACA	**D3**	0.25	0.31	0.25	0.5	128	128	2	2048	0.25	108	0.5	512	27
	D4	0.25	0.31	0.25	0.5	128	128	2	2048	0.25	108	0.5	512	27
	D5	0.25	0.31	0.25	0.5	128	128	2	c	0.25	108	0.5	512	27
BrMIC	**D1**	0.25	0.13	0.25	2	256	256	2	2048	0.25	54	0.5	512	27
+AB	**D2**	0.25	0.13	0.25	2	256	256	2	2048	0.25	216	0.5	512	27
AMIC	**D1**	2	16	0.25	128	256	256	128	4096	0.25	216	0.5	1024	108
	D2	0.25	1	0.25	0.5	256	256	64	4096	0.25	216	0.5	1024	108
	D3	0.25	0.25	0.25	16	256	256	32	4096	0.25	216	2	1024	108
	D4	0.25	1	32	4	256	256	4	4096	0.25	216	0.5	1024	108
	D5	0.25	8		0.5	256	256	0.5	4096	0.25	216	c	1024	108

Key: BrMIC+SFS- broth microdilution MIC with a sugar fermentation system; BrMIC+SFS+ACA - microdilution MIC with a sugar fermentation system with amoxicillin and clavulanic acid; BrMIC+AB - broth microdilution MIC with AlamurBlue™; AMIC – Agar MIC; c - contamination

APPENDIX II

MICs values (µg/mP) of field strains of animal *Mycoplasma* spp.

Identification	Optical Density	Doxycycline	Enrofloxacin	Erythromycin	Florfenicol	Nalidic Acid	Neomycin	Norfloxacin	Sulphamethoxazole	Trimethoprim	Tiamulin	Tylosin	S:T
M. gallisepticum													
B730/09	0.147	64	8	>64	128	>128	>128	16	>2048	108	>64	>64	>2054/108
B1072/08	0.056	64	8	>64	128	>128	>128	32	1024	108	>64	>64	2054/108
B642/05	0.048	<0.5	<0.06	>64	8	>128	>128	<1	>2048	<0.97	>64	16	>2054/108
B311/05	0.059	<0.5	>8	>64	16	>128	>128	>128	>2048	<0.97	>64	>64	>2054/108
B943/06		<0.5	<0.06	<0.5	<1	64	<1	<1	>2048	<0.97	8	<0.5	>2054/108
B313/05	0.118	8	<0.06	>64	8	>128	2	64	1024	<0.97	>64	>64	256/13.5
B1028/07	0.078	1	<0.06	>64	16	>128	>128	<1	>2048	<0.97	>64	>64	>2054/108
MG (A)	0.077	1	<0.06	>64	8	>128	>128	32	>2048	<0.97	>64	>64	>2054/108
B733/05	0.114	<0.5	<0.06	>64	2	>128	16	32	>2048	<0.97	>64	>64	>2054/108
B716/05	0.024	<0.5	0.13	64	<1	>128	2	16	>2048	<0.97	>64	>64	>2054/108
B758/08	0.134	<0.5	2	>64	2	>128	>128	32	>2048	<0.97	>64	>64	>2054/108
B943/06	0.176	<0.5	<0.06	<0.5	2	>128	>128	<1	>2048	<0.97	>64	<0.5	513/27
B283	0.061	<0.5	<0.06	>64	<1	>128	<1	4	512	<0.97	>64	<0.5	>2054/108
B1513	0.067	<0.5	<0.06	>64	2	>128	32	>128	<16	>108	>64	>64	<1.88/0.099
K4503	0.113	<0.5	<0.06	>64	2	>128	>128	2	>2048	<0.97	>64	<0.5	>2054/108
B8973	0.078	<0.5	<0.06	32	<1	>128	32	<1	>2048	<0.97	>64	<0.5	<0.099
B230	0.062	<0.5	<0.06	>64	<1	>128	4	4	>2048	<0.97	>64	<0.5	>2054/108
M. synoviae													
B2214/07	0.171	<0.5	2	64	<1	>128	16	32	2048	<0.97	64	<0.5	>2054/108
B2128/07	0.143	<0.5	2	64	2	>128	32	32	2048	<0.97	64	<0.5	>2054/108
B2182/07	0.171	<0.5	2	64	<1	>128	16	32	2048	<0.97	64	64	2054/108
B434/08	0.131	<0.5	2	64	2	>128	64	32	2048	<0.97	64	<0.5	2054/108
B312/08	0.125	<0.5	2	64	<1	>128	16	32	2048	<0.97	64	<0.5	>2054/108

Identification	Optical Density	Doxycycline	Enrofloxacin	Erythromycin	Florfenicol	Nalidic Acid	Neomycin	Norfloxacin	Sulphamethoxazole	Trimethoprim	Tiamulin	Tylosin	S:T
M. bovirhinis													
B1121/05	0.171	0.25	0.125	128	0.5	128	128	8	4096	0.25	216	4	>2054/108
B1126/07-1	0.188	0.25	0.5	64	4	128	128	4	2048	1	216	0.25	2054/108
B1126/07-4	0.246	0.25	0.25	128	8	256	256	8	2048	1	216	0.25	2054/108
B1159/05-6	0.127	0.25	0.125	64	128	128	128	16	1024	0.25	216	1	1026/54
B1258/07-13	0.222	0.25	0.125	64	4	256	128	4	4096	0.25	216	0.25	>2054/108
B1258/07-10	0.105	0.25	0.25	4	128	8	8	64	1024	1	108	128	2054/108
B1338/07-11	0.127	0.25	0.5	128	8	256	256	256	2048	0.25	216	128	>2054/108
B839/06-2	0.348	0.25	0.25	128	8	256	256	32	2048	0.25	216	128	>2054/108
B1295/05-8	0.153	0.25	0.5	128	8	256	256	16	2048	0.25	216	0.25	1026/54
B1126/07-3	0.177	0.25	128	128	128	256	256	256	4096	0.25	216	128	>2054/108
B1295/05-2	0.148	0.25	0.25	128	4	256	256	4	4096	0.25	216	0.25	>2054/108
B1295/05-3	0.187	0.25	0.25	64	0.5	128	128	4	2048	0.25	216	0.25	2054/108
B1195/05-4	0.166	0.25	0.25	128	8	256	8	16	2048	0.25	216	128	2054108
M. bovis													
B414/04-4	0.083	<0.5	0.13	>64	4	>128	>128	8	>2048	<0.5	>108	>64	>2054/108
B844/06-14	0.171	<0.5	<0.06	>64	8	>128	>128	8	>2048	<0.5	>108	>64	>2054/108
B940/06-8	0.181	<0.5	<0.06	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B1315/06-4	0.123	<0.5	<0.06	>64	16	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B1315/06-2	0.123	<0.5	<0.06	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B1379/06-7	0.102	<0.5	0.25	>64	8	>128	>128	32	>2048	<0.5	>108	>64	>2054/108
B1258/07-6		<0.5	<0.0	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
BB1295/05-6	0.165	<0.5	<0.06	>64	16	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B844/06-2	0.13	<0.5	<0.06	>64	8	>128	>128	8	>2048	<0.5	>108	>64	>2054/108
B1243/05-1	0.105	<0.5	<0.06	>64	4	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B1358/07-2		<0.5	<0.06	<0.06	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B1075/06-5	0.165	<0.5	<0.06	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B824/06-13	0.149	<0.5	<0.06	>64	8	>128	>128	8	>2048	<0.5	>108	>64	>2054/108
B1451/07-5	0.141	<0.5	<0.06	>64	8	>128	>128	8	>2048	<0.5	>108	>64	>2054/108
B338/07-4	0.166	<0.5	<0.06	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108

Identification	Optical Density	Doxycycline	Enrofloxacin	Erythromycin	Florfenicol	Nalidic Acid	Neomycin	Norfloxacin	Sulphamethoxazole	Trimethoprim	Tiamulin	Tylosin	S:T
***M. bovis* continued**													
B1452/07-2	0.157	<0.5	<0.06	>64	8	>128	>128	8	>2048	<0.5	>108	>64	>2054/108
B944/06-13	0.149	<0.5	0.5	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B844/06-12	0.148	<0.5	<0.06	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B1075/06-7	0.166	<0.5	<0.06	>64	8	>128	>128	8	>2048	<0.5	>108	>64	>2054/108
B940/06-2	0.142	<0.5	0.25	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B944/05-10	0.171	<0.5	<0.06	>64	8	>128	>128	4	>2048	<0.5	>108	>64	>2054/108
B824/06-4	0.143	<0.5	<0.06	>64	>128	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B1561/07-2	0.143	<0.5	<0.06	32	<1	>128	>128	8	>2048	<0.5	>108	>64	>2054/108
B1268/06-4	0.143	<0.5	<0.06	>64	4	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B1453/07-4	0.153	<0.5	<0.06	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B11/03-1	0.078	<0.5	0.13	>64	4	>128	>128	16	>2048	<0.5	>108	32	>2054/108
B1042/05-2	0.138	<0.5	<0.06	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B315/04	0.138	<0.5	<0.06	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B1268/06-4	0.133	<0.5	<0.06	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B844/06-3	0.138	<0.5	<0.06	>64	<1	>128	>128	8	>2048	<0.5	>108	32	>2054/108
B910/05-3	0.157	<0.5	0.5	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B824/06-15	0.188	<0.5	<0.06	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B844/06-2	0.138	<0.5	<0.06	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B844/06-5	0.169	<0.5	<0.06	>64	8	>128	>128	8	>2048	<0.5	>108	>64	>2054/108
B844/06-3	0.134	<0.5	<0.06	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B962/05-8	0.134	<0.5	<0.06	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B844/06-4	0.147	<0.5	<0.06	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B844/06-8	0.134	<0.5	<0.06	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B844/06-1	0.132	<0.5	<0.06	>64	16	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B1258/07-8	0.132	<0.5	<0.06	>64	8	>128	>128	32	>2048	<0.5	>108	32	>2054/108
B824/06-3	0.142	<0.5	<0.06	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B1338/07-7	0.133	<0.5	<0.06	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B1258/07-1	0.14	<0.5	<0.06	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B862/05-3	0.14	<0.5	<0.06	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B1338/07-2	0.139	<0.5	<0.06	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B1159/05-1	0.164	<0.5	<0.06	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108

Identification	Optical Density	Doxycycline	Enrofloxacin	Erythromycin	Florfenicol	Nalidic Acid	Neomycin	Norfloxacin	Sulphamethoxazole	Trimethoprim	Tiamulin	Tylosin	S:T
***M. bovis* continued**													
B1248/05-2	0.138	<0.5	<0.06	<0.06	16	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B1243/05-7	0.107	<0.5	<0.06	>64	<1	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
31/10/03	0.09	<0.5	<0.06	>64	<1	>128	>128	8	>2048	<0.5	>108	>64	>2054/108
B824/06-9	0.138	<0.5	<0.06	>64	8	>128	>128	8	>2048	<0.5	>108	>64	>2054/108
B940/06	0.138	<0.5	<0.06	>64	8	>128	>128	8	>2048	<0.5	>108	>64	>2054/108
B1758/07-8	0.152	<0.5	0.25	>64	8	>128	64	8	>2048	<0.5	>108	>64	>2054/108
B940/06-5	0.166	<0.5	<0.06	>64	16	>128	32	8	>2048	<0.5	>108	>64	>2054/108
B844/06-6	0.122	<0.5	<0.06	>64	<1	>128	>128	16	>2048	<0.5	>108	32	>2054/108
B1258/07-3	0.15	<0.5	<0.06	>64	16	>128	>128	8	>2048	<0.5	>108	>64	>2054/108
B844/06-15	0.093	<0.5	<0.06	>64	<1	>128	64	16	>2048	<0.5	>108	16	>2054/108
B1453/07	0.138	<0.5	<0.06	32	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B824/06-8	0.138	<0.5	0.25	>64	8	>128	>128	8	>2048	<0.5	>108	32	>2054/108
B12/03	0.078	<0.5	0.25	>64	8	>128	>128	8	512	<0.5	>108	4	>2054/108
B1194/07-2	0.081	<0.5	<0.06	>64	8	>128	>128	4	>2048	<0.5	>108	>64	256/13.5
B944/05-11	0.081	<0.5	<0.06	>64	32	>128	>128	16	>2048	<0.5	>108	>64	256/13.5
B1058/07-8	0.076	<0.5	0.13	>64	8	>128	>128	8	>2048	<0.5	>108	>64	>2054/108
30/05/06	0.167	<0.5	<0.06	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B13/03	0.081	<0.5	<0.06	>64	2	>128	>128	8	>2048	<0.5	>108	64	>2054/108
B844/06-4	0.172	<0.5	0.13	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B962/05-6	0.076	1	0.13	>64	>128	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B940/06-10	0.164	<0.5	<0.06	>64	c	>128	>128	c	>2048	<0.5	>108	>64	>2054/108
B1758/07-2	0.161	<0.5	<0.06	>64	16	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B1295/05-9	0.141	<0.5	0.1254	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B1159/05-8	0.135	<0.5	<0.06	>64		>128	>128	4	>2048	<0.5	>108	>64	>2054/108
B910/05-1	0.162	2	0.1254	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B1075/06-2	0.154	1	0.1254	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B1159/05-2	0.172	<0.5	<0.06	>64	16	>128	>128	16	>2048	<0.5	>108	32	>2054/108
B839/06-3	0.122	<0.5	<0.06	>64	32	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B844/06-4	0.138	<0.5	<0.06	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B824/06-2	0.123	<0.5	0.25	>64	c	>128	>128	16	>2048	8	>108	>64	>2054/108
B1768/07-5	0.146	<0.5	<0.06	>64	16	>128	>128	16	512	<0.5	>108	>64	>2054/108

Identification	Optical Density	Doxycycline	Enrofloxacin	Erythromycin	Florfenicol	Nalidic Acid	Neomycin	Norfloxacin	Sulphamethoxazole	Trimethoprim	Tiamulin	Tylosin	S:T
***M. bovis* continued**													
B1453/07-5	0.121	<0.5	<0.06	>64	8	>128	>128	8	>2048	<0.5	>108	>64	>2054/108
B1338/07-10	0.137	<0.5	<0.06	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B315/04	0.095	<0.5	<0.06	>64	8	>128	>128	32	>2048	<0.5	>108	>64	>2054/108
B824/06-4	0.128	<0.5	<0.06	>64	8	>128	>128	8	>2048	<0.5	>108	>64	>2054/108
B1159/06-5	0.136	<0.5	<0.06	>64	8	>128	>128	8	>2048	<0.5	>108	>64	>2054/108
B942/05-5	0.141	2	0.25	>64	16	>128	>128	16	>2048	<0.5	>108	>64	513/27
B844/06-15	0.138	<0.5	<0.06	>64	16	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B844/06-3	0.172	<0.5	<0.06	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B1453/07-2	0.132	<0.5	<0.06	32	8	>128	>128	32	>2048	<0.5	>108	>64	>2054/108
B315/06	0.088	<0.5	<0.06	>64	8	>128	>128	32	>2048	<0.5	>108	>64	>2054/108
B944/05-11	0.118	<0.5	<0.06	>64	64	>128	>128	32	>2048	<0.5	>108	>64	>2054/108
B1194/07-2	0.116	<0.5	<0.06	>64	8	>128	>128	4	>2048	<0.5	>108	>64	>2054/108
B1159/05-1	0.211	<0.5	<0.06	>64	>128	>128	>128	8	>2048	<0.5	>108	>64	>2054/108
B1338/07-9	0.155	<0.5	<0.06	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B1451/07-3	0.189	<0.5	<0.06	>64	8	>128	>128	16	>2048	<0.5	>108	>64	>2054/108
B1561/07-2	0.164	<0.5	<0.06	>64	8	>128	>128	8	>2048	<0.5	>108	>64	>2054/108