DETERMINATION OF THE SPECIFICITY AND NATURE OF *Mycoplasma mycoides* subsp. *mycoides* ADHERENCE TO THEIR HOST CELLS

MARTIN KIOGORA MWIRIGI

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university or for any other award.

MARTIN KIOGORA MWIRIGI

Signature..... Date..22-11-2010

This thesis has been submitted for examination with our approval as University of Nairobi supervisors.

Prof. H. Ochanda
University of Nairobi
School of Biological Sciences
P.O.Box 30197 00100
Nairobi, Kenya

Signature..... Date..22nd November, 2010

Dr. J. Naessens
International Livestock Research Institute
P.O.Box 30709 00100
Nairobi, Kenya

Signature..... Date..22 November 2010

DEDICATION

To

My dear wife Wangui and son Mutembei for their patience and endurance during the preparation of this thesis. Your encouragement was exceptional.

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NOMENCLATURE

During the course of this thesis, the Mycoplasma Nomenclature Committee changed the nomenclature of a number of mycoplasma species (Manso-Silvan *et al,* 2009), and in particular mycoplasma from the mycoides cluster, some of which are the subject of this thesis. The names used in this thesis are the new names, as required, and the table below compares the old and new names. M. stands for Mycoplasma.

Old name	**New name**
M. mycoides subsp. *mycoides* Small Colony type	*M. mycoides* subsp. *mycoides*
M. mycoides subsp. *mycoides* Large Colony type	*M. mycoides* subsp. *capri*
M. mycoides subsp. *Capri*	
M. capricolum subsp. *capricolum*	*M. capricolum* subsp. *capricolum*
M. capricolum subsp. *capripneumoniae*	*M. capricolum* subsp. *capripneumoniae*
Mycoplasma sp. bovine serogroup 7	*M. leachii sp. nov.*

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACRONYMS/ABBREVIATIONS

BBE	Bovine Bronchial Epithelial
CBPP	Contagious Bovine Pleuropneumonia
CCC	Colour Changing Units
CPS	Capsular Polysaccharide
DMEM	Dulbecco's Minimum Essential Medium
DMSO	Dimethylsulphoxide
DVS	Department of Veterinary Services
ECaNEp	Embryonic Calf Nasal Epithelial
FAO	Food and Agriculture Organisation
FACS	Fluorescence-activated cell sorting
FC	Flow Cytometry
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
GLpO	L-α-glycerophosphate
ILRI	International Livestock Research Institute
KARI	Kenya Agriculture Research Institute
MAb	Monoclonal Antibody
OIE	World Organization for Animal Health
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase Chain Reaction
PPLO	Pleuropneumonia-like Organism
USAID	United States Agency for International Development

ABSTRACT

Mycoplasma mycoides subsp. *mycoides* is the etiological agent of contagious bovine pleuropneumonia (CBPP). The disease is a significant constraint to cattle production throughout most of Sub-Saharan Africa. The contribution of cytoadherence and molecular factors involved in pathogenesis of *M. mycoides* subsp. *mycoides* is poorly understood. This study aimed at providing data about cytoadherence of *M. mycoides* subsp. *mycoides* on primary cultured bovine bronchus epithelial (BBE) cells and compared adherence rates to different cell lines using flow cytometry. In this assay, *M. mycoides* subsp. *mycoides* was grown in "pleuropneumonia - like organism" (PPLO) medium and incubated with BBE cells at 37°C. The BBE cells were then stained with polyclonal antibodies to mycoplasma shown to adhere specifically to the BBE cells. The binding was dependent on both the concentration of the mycoplasmas and time and up to 70-99% of epithelial cells were positive. The specificity of mycoplasma adherence was examined using three different cell lines; bovine skin fibroblast (BY 122 ISF pSV neo), bovine testicular endothelial cells (282 BTVE pSV neo and baby hamster kidney (BHK) cells. Adherence rate of the three cell lines were significantly lower than that of bovine bronchus epithelial cells. Inhibition of adherence was observed upon pre-incubation of mycoplasma with a monoclonal antibody against capsular polysaccharide (PK-2). Monoclonal antibody against a surface protein L-α-glycerophosphate oxidase (GlpO) failed to inhibit significantly the adherence to BBE cells. Proteolysis of the BBE cells surface using proteinase K partially inhibited binding of the *M. mycoides* subsp. *mycoides* to the BBE cells. Treatment of the BBE cells using glycosidase F to cleave the carbohydrate on the surface did not show significant inhibition. The significant reduction of adherence rate by PK-2 directed against an epitope on capsular polysaccharide located on the

surface of *M. mycoides* subsp. *mycoides*, suggests involvement of mycoplasma carbohydrates in adherence to the primary culture of BBE cells. The results also indicate that at least some proteins on the BBE cell surface are involved in *M. mycoides* subsp. *mycoides* adherence and may be a receptor for a mycoplasma ligand.

CHATER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

1.1.1 Background

Mycoplasma mycoides subsp. *mycoides* is the aetiological agent of Contagious Bovine Pleuropneumonia (CBPP), a severe infectious disease of cattle (Lloyd *et al.,* 1971).

M. mycoides subsp. *mycoides* is a bacterium of the class *Mollicutes* in the phylum Firmicutes. Phylogenetic classification has grouped *M. mycoides* with five closely related and highly pathogenic mycoplasmas into the *M. mycoides* cluster (Manso-Silvan *et al.*, 2009) that include; *Mycoplasma capricolum* subsp. *capricolum*, *Mycoplasma capricolum* subsp. *capripneumoniae*, *M. mycoides* subsp. *capri*, *M. mycoides* subsp. *mycoides* and *Mycoplasma leachii sp. nov.*.

CBPP is an insidious pneumonic disease of cattle sometimes referred to as lung sickness. Clinically, CBPP is manifested by anorexia, fever and respiratory symptoms such as dyspnoea, cough and nasal discharges. The forms in which the disease occurs include; acute, subacute and chronic. This lung disease is transmitted by direct contact between infected and susceptible hosts (Mariner *et al.*, 2006).

The disease is a significant constraint to cattle production throughout most of sub-Saharan Africa. It represents a major threat to raising cattle, particularly in Africa, where it creates great economic losses in regions of endemicity (Provost *et al.,* 1987, FAO, 2003). An annual economic loss due to this disease in sub-saharan Africa has been estimated to about US $ 2

Billion (Masiga *et al.*, 1998). CBPP is the only bacterial disease included in the A-list of the World Organization for Animal Health (OIE, 2001) of prioritized communicable animal diseases. Thus, from a global socioeconomic perspective, it is the most important bacterial epizootic.

1.1.2 The biology of mycoplasma

Mycoplasmas are widespread in nature as parasites of mammals, reptiles, fish, arthropods, and plants (Razin and Barile, 1985 in Razin and Jacobs, 1992). None of them are free-living; most are surface parasites, adhering to the epithelial lining of respiratory or urogenital tracts, rarely invading tissues (Razin *et al.*, 1998). The mycoplasmas may be considered as the smallest prokaryotes (Razin and Jacobs, 1992) and probably evolved from gram-positive bacteria having lost most of their genome and the genes for a lot of metabolic processes and thus are dependent on a parasitic lifestyle (Razin *et al.*, 1998).

Specificity to host and tissue is exhibited, probably reflecting their nutritionally exacting nature and obligate parasitic mode of life. However, there are numerous examples of the presence of mycoplasmas in hosts and tissues different from their normal habitats (Razin, 1992). *M. mycoides* subsp *mycoides* has been shown to be significantly more cytotoxic for bovine endothelial cells than caprine and porcine, suggesting that the cytotoxicity reflects specificity for the bovine species (Valdivieso-Garcia *et al.*, 1989).

The mycoplasmas have a minimum set of organelles: a plasma membrane, ribosomes and a highly coiled circular double-stranded DNA molecule, the typical prokaryotic genome (Razin, 1999). The genome sizes of *Mycoplasma* species range from 580 kb for *M. genitalium* to 1,380 kb for *M. mycoides* subsp. *capri* (Caporale *et al.*, 1996). A significant percentage of genes in the minute mycoplasmal genomes is devoted to adhesion, some of

these genes code for membrane proteins, exposed at least partially on the membrane surface, acting as adhesins (Razin, 1999). Carbohydrates are components of mycoplasma membranes found in form of glycolipids, lipopolysaccharides, polysaccharides, and glycoproteins. *M. mycoides* subsp. *mycoides* has been shown to be covered by a capsule made of galactan (Buttery *et al.*, 1976).

1.1.3 Distribution of CBPP in Africa, Europe and Asia

In Africa CBPP is found in an area south of the Sahara, from the Tropic of Cancer to the Tropic of Capricorn and from the Atlantic to the Indian Ocean. The map bellow illustrates the spread of CBPP as reported in the OIE from 1990s to 2003.

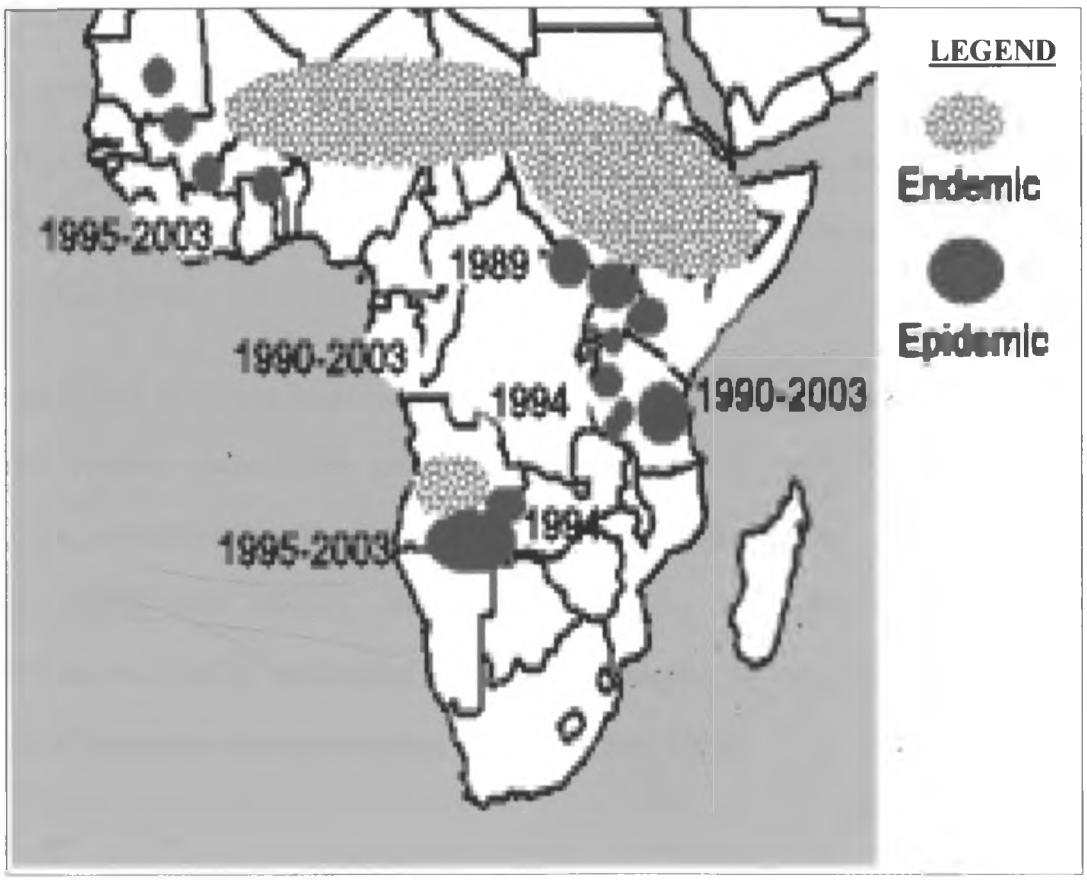

Figure 1. Distribution of Contagious Bovine Pleuropneumonia from 1990 to 2003 in Sub-Saharan Africa (FAO, 2003).

CBPP was eradicated from much of Europe during the early part of the 20th century, but outbreaks of the disease have occurred in parts of southern Europe during most decades of the century (Nicholas *et al.,* 1996). Seropositive animals have been reported in southern France on a few occasions between 1980 and 1984. In Italy the disease reappeared in 1990 but was eliminated in 1993 (FAO, 1997). In Portugal, the disease reappeared in 1983; Following the implementation of an eradication programme the number of cases have declined rapidly, from 2818 in 1996, 64 in 1997, 12 in 1998 with a single case in 1999 (OIE, 2001). In Asia, CBPP has been reported recently in India, Bangladesh and Myanmar as well as in the Middle East (FAO, 1997). Eradication of CBPP in the USA was achieved in 1892 and Australia in 1972 by stamping out of infected herds (Provost *et al.*, 1987).

1.1.4 Control of CBPP

Local quarantine, antibiotic treatment, destruction of whole herds when infected as well as vaccination are the current strategies used in control of CBPP. Of these, vaccination is the most widely used in most African countries since it is thought to be the most economical (Tulasne *et al.*, 1996).

The vaccine used in current immunization efforts is the live *M. mycoides* subsp. *mycoides* attenuated vaccine strains T1/44 and T1sr, both derived from strain T1 obtained by consecutive sub-cultivation. These vaccines have been shown to have drawbacks, for example, exhibit poor efficacy, stability and possibility of reversion to virulence (Rweyemamu *et al* 1995). Vaccinations also cause circumscribe swelling (Willems'reaction) at the site of inoculation leading to evasion by farmers (Sori, 2005).

According to Thiaucourt *et al.,* (1998 and 2002), control of CBPP requires highly efficient diagnostic tests that detect *M. mycoides* subsp. *mycoides* together with vaccine strategies that

are able to reduce the infectious pressure in infected areas by preventing the pathogen from achieving host colonization, and multiplication. Understanding of the mechanism of the pathogenic process, especially the early interaction between the organism and the host is therefore essential and could be used to prevent colonization and infection of the respiratory airways epithelium (Kelly *et al* 1999 and Ofek *et al* 2003). It is also a prerequisite to designing of a safe and efficient vaccine against *M. mycoides* subsp. *mycoides* (Pilo *et al.*, 2007).

In this study immunofluorescence was used in locating the *M. mycoides* subsp. *mycoides* that had adhered onto the primary bovine bronchial epithelial (BBE) cells. Immunofluorescence has been used in investigations to locate mycoplasma in pathogenicity studies in both animals and in organ cultures and it is one of the recommended serological tests for characterization of new species of *Mollicutes* (Whitcomb *et al.*, 1995 in Bradbury, 1998).

The adherence of *M. mycoides* subsp. *mycoides* to BBE cells and inhibition using different antibodies was also demonstrated using Flow Cytometry (FC). FC is a sensitive technique which can be both quantitative and qualitative (Gunasekera *et al.*, 2000). FC has been used to detect several bacteria with a combination of fluorescence stains, antibodies, or Oligonucleotide probes (Cheek *et al.*, 1997, Clarke and Pinder, 1998 and Gunasekera *et al.*, 2000).

1.2 Literature review

1.2.1 Mycoplasma induced Pathogenesis

The molecular mechanisms of *Mycoplasma mycoides* subsp. *mycoides* pathogenicity and its virulence factors are still not well understood. Surface lipoproteins are expected to play a role as triggers in mechanisms of pathogenicity, since they are known to have a central role in interactions between mycoplasmas and eukaryotic cells, particularly with respect to adhesion (Pilo *et al.*, 2007). The carbohydrate containing components on the cell surface of mycoplasmas may play an important role in the interaction of the parasite with the cell membrane of its host (Razin, 1978). It has been proposed that the capsule of *Mycoplasma dispar* plays a role in the pathogenesis of bovine pneumonia in calves by participating in the attachment process on the host (Gourlay and Howard 1978 in Almeida and Rosenbusch 1991).

Several molecular mechanisms may contribute to the pathogenicity and/or virulence of *M.mycoides* subsp. *mycoides* (Pilo *et al.*, 2006). These mechanisms may permit mycoplasma to specifically adhere to host tissue, evade the host's immune defense, enable persistence and dissemination in the infected animal, exert a cytotoxic effect, and cause inflammation and disease symptoms (Vilei *et al.*, 2007). The loss of any of these mechanisms can lead to attenuation or loss of virulence.

The intimate association of the mycoplasmas with their host cell surface provides a nutritional advantage to the parasites. The attached mycoplasmas not only enjoy a higher concentration of nutrients adsorbed onto their host cell membrane but may also utilize the fatty acids and cholesterol of the host membrane itself (Razin, 1999). The attachment of

mycoplasmas to the surface of host cells may induce damage through interference with membrane receptors or alter transport mechanisms of the host cell (Debey and Ross, 1994).

M. mycoides subsp. *mycoides* synthesizes a capsular polysaccharide that form a slime layer (Gourlay and Thrower, 1968 in Lloyd *et al.*, 1971) that comprises 10 per cent of the dry weight of cells in young cultures (Lloyd *et al.*, 1971). The capsular polysaccharide, composed of 6-Ob- D-galactofuranosyl-D-galactose has been shown to increase the virulence of the strongly attenuated *M. mycoides* subsp. *mycoides* vaccine strain KH3J when added to the inoculum prior to experimental infections of cattle (Hudson *et al.*, 1967). It also induces lesions in joint and kidney and prolong mycoplasmaemia in infected cattle (Lloyd,*et al.*, 1971). March *et al.*, (1999) showed that a strain of *M. mycoides* subsp. *mycoides* that produces low amounts of capsular polysaccharide was much more sensitive to growth inhibiting antisera than strains that produced larger amounts of polysaccharide. The capsular polysaccharide therefore seems to play a role in the capacity of persistence and dissemination of *M.mycoides* subsp. *mycoides* in the infected host.

Glycerol metabolism plays a key role in virulence of *M. mycoides* subsp. *mycoides* (Pilo *et al.*, 2005, Bischof *et al* 2008). The L-α-glycerophosphate oxidase (GlpO) of *M. mycoides* subsp. *mycoides* is a membrane protein that participates in the cytotoxicity of *M. mycoides* subsp. *mycoides* (Pilo *et al.*, 2005). Recombinant GlpO lacking six amino acids (Gly12-Gly13-Gly14-Ile15-Ile16-Gly17) as demonstrated by Bischof and others (2009), is devoid of glycerophosphate oxidase activity. Pilo and others (2005) have shown using cell culture of embryonic calf nasal epithelial (ECaNEp) cells that H_2O_2 is translocated into eukaryotic host cells causing cytotoxicity and cell death. Thus, an efficient translocation of H_2O_2 into host cells is a prerequisite for the cytotoxic effect and requires an intact adhesion mechanism to

ensure a close contact between mycoplasmas and host cells. Inside the host cells, H_2O_2 acts as a powerful mediator of cell injury, which then leads to inflammatory processes (Bischof *et al* 2008).

Currently, a few lipoproteins from *M. mycoides* subsp. *mycoides* which have been characterized in detail include; Lpp A (Monnerat *et al.*, 1999), LppB (Vilei *et al.*, 2000), LppC (Pilo *et al.*, 2003 and LppQ (Abdo *et al.*, 2000). Most of them are major antigens and are readily detected in the serum of infected cattle on immunoblots. The role of these lipoproteins in virulence is still unclear.

1.2.2 Mycoplasma colonization of host cells

The mycoplasma's ability to adhere to the surface of epithelial cells is a prerequisite for colonization and maintenance of infection (Razin and Jacob, 1992). *M. mycoides* subsp. *mycoides* remain tightly attached to the surface of epithelial cells but do not penetrate them (Razin *et al.,* 1998, Pilo *et al.*, 2005). However Baseman *et al.*, 1995 have demonstrated other mycoplasmas including *M. penetrans*, *M. pneumoniae* and *M. genitalium* to be intracellular.

A possible adhesion mechanism in *M. mycoides* subsp. *Mycoides*, particularly on calf epithelial cells, could be the partial fusion of the surface exposed epitopes with the host cell wall (Pilo *et al.,* 2005). The lack of a cell wall in mycoplasmas may facilitate the direct contact of the mycoplasma membrane with that of its eukaryotic host, creating a condition which, in principle, could lead to fusion of the two membranes, enabling the transfer or exchange of membrane components, and injection of the mycoplasmal cytoplasmic content, including hydrolytic enzymes, into the host cell cytoplasm (Razin, 1999).

1.2.4 Adhesins on the mycoplsama

Mycoplasmal cell components responsible for attachment termed as adhesins (Razin and Jacobs, 1992), play a crucial role in the primary steps employed by mycoplasmas while interacting with their host eukaryotic cells using specific mammalian membrane receptors. Since mycoplasmas do not seem to secrete toxins that could act over long distances, adhesion is particularly important in mycoplasmal virulence and host specificity (Razin *et al.,* 1998).

Mycoplasma pneumoniae is the most extensively studied system with respect to the adhesins. Two adhesins have been described to be involved in the attachment of *M. pneumoniae* to host cells: P30 (32 kDa) and the major adhesin P1 (170 kDa), (Vu *et al.,* 1987). Electron microscopy showed that elongated *M. pneumonia* cells adhered to the host cell membrane through their terminal structure (Razin and Jacobs, 1992).

Involvement of the proteins in adhesion to host epithelial cells is evidenced by studies on MgPa, a surface protein of *M. genitalium* and P1 and P116 of *M. pneumoniae* (Svenstrup *et al.,* 2002). In some species, highly reiterated proteins are involved in cytoadherence and individual proteins undergo high-frequency phase variation (Behrens *et al.,* 1996). Each protein may recognize a different host-cell receptor and this may be a mechanism by which mycoplasma undergo tissue tropism and adapt to a new environment (Dybvig and Voelker, 1996 in Pilo *et al.,* 2007).

Several adhesins have also been identified in various Mycoplasma species (Sachse *et al.,* 1996; Noormohammadi *et al.,* 1997; Razin *et al.,* 1998; Kitzerow *et al.,* 1999; Sachse *et al.,* 2000; Minion *et al.,* 2000; Fleury *et al.,* 2002; Belloy *et al.,* 2003). However specific adhesins have not yet been detected in *M. mycoides* subsp. *mycoides.*

1.2.5 Nature of host binding receptors

The animal cell surface is covered by various forms of glycoproteins, glycolipids and proteoglycans (Karlsson, 1986; Rostand and Esko, 1997). Many pathogenic microorganisms have exploited this cell surface glycoconjugates as receptors for attachment, an essential process for establishment of colonization and production of toxic effects (Zhang *et al.*, 1994). The chemical nature of the receptors on the eukaryotic cell surface responsible for mycoplasma attachment has been established for only a few of the mycoplasma species showing adherence properties. The available evidence indicates that the host cell receptor sites for attachment are composed of sialoglycoconjugates (Sobeslavsky *et al.*, 1968; Gabridge *et al.*, 1977; Banai *et al.*, 1978; Feldner *et al.*, 1979; Banai *et al.*, 1980; Glasgow and Hill, 1980). The carbohydrate moiety of the glycoprotein, which serves as a receptor for *M. pneumoniae* on human erythrocytes, has been identified as having a terminal NeuAc(_2–3)Gal(1–4)GlcNAc sequence (Roberts *et al.*, 1989). Sialic acid-free glycoprotein and glycolipid receptor for *M. pneumoniae*, has also been isolated (Geary *et al.* 1990, Krivan *et al* 1989).

1.2.6 Anti-adhesion therapy and immunity

Anti-adhesion therapy and anti-adhesin immunity aim to reduce contact between host tissues and pathogens, either by prevention or reversal of adhesion of the infectious agent. Prevention of adhesion at an early stage following the exposure of the host to pathogens can in some cases prevent the disease (Ofek *et al.*, 2003). Five proteins associated with a protective immune response namely, MSC_1046 (LppQ), MSC_0271, MSC_0136, MSC_0079, and MSC_0431 may be important candidates in the development of a novel subunit vaccine against CBPP (Hamsten, 2010).

Receptor analogs as agents for anti-adhesion therapy would be used against pathogens that bind to animal cells via carbohydrate-specific adhesins (lectins). In these cases, the receptor analogs are saccharides that are structurally similar to those of the glycoprotein or glycolipid receptors for the adhesion and therefore act by competitive inhibition (Ofek and Sharon, 1977). Adhesin analogs as anti-adhesive agents may be used to prevent infections (Kelly *et al.*, 1999), inhibition is based on the assumption that the isolated adhesion molecule, or an active synthetic or recombinant fragment, binds to the receptor and competitively blocks adhesion of the bacteria.

Anti-adhesion therapy has an advantage over conventional antibiotic treatment in that bacteria would encounter difficulties in developing resistance (Thomas and Brooks, 2003) and are mild and safer (Karlsson, 1998) compared with the present chemotherapy approaches. The major drawback of anti-adhesion therapy is that most pathogens possess more than one type of adhesin, so that during the infectious process the population of pathogens may express more than one of these adhesins. Other factors like adhesion-receptor interactions such as hydrophobic and other non-specific interactions under different shear-forces also play important roles in adhesion (Ofek *et al.*, 2003).

Adhesin-based vaccines are used to prevent symptomatic infections by blocking adhesion. This is achieved either by active or passive means. Although active anti-adhesin immunity is expected to prevent infection by stimulation of secretory IgA on mucosal surfaces, significant amounts of serum IgG also appear to reach these surfaces, such as the gut, oral cavity and even the urinary tract (Robbins *et al.*, 1995). In passive immunity, the target host is treated with anti-adhesin antibodies made in another host (Moon and Bunn, 1993).

"The future of anti-adhesion therapy will depend on better knowledge of the properties and specificities of bacterial adhesins and on the development of appropriate agents that block adhesion. Such agents could consist of combinations of powerful carbohydrate inhibitors targeting a variety of distinct bacterial surface adhesins, or non-specific and/or broadly reactive inhibitors, such as cranberry constituents, that target multiple adhesins. Once such compounds become available, they could become drugs of choice for the management of a number of infectious diseases" (Ofek *et al.*, 2003).

1.2.7 Justification

Mycoplasma mycoides subsp. *mycoides*, the aetiological agent of CBPP, is considered the most pathogenic of the *Mycoplasma* species that infect cattle. Its virulence is probably as a result of a coordinated action of various components of an antigenically and functionally dynamic surface architecture (Pilo *et al.*, 2007).

The search for new CBPP vaccines has become a major issue for African countries that are facing an increase in outbreaks. The rationale for this search is based on a better understanding of the mycoplasma virulence mechanisms and immune responses (Thiaucourt *et al.*, 2003).

Understanding of the mechanism of the pathogenic process, especially the early interaction between the organism and the host is essential and could be used to prevent colonization and infection of the respiratory airways epithelium (Kelly *et al.*, 1999 and Ofek *et al.*, 2003). It is also a prerequisite to designing of a safe and efficient vaccine against *M. mycoides* subsp. *mycoides* (Pilo *et al.*, 2007).

Definition of adhesion epitopes may open the way for constructing *M. mycoides* subsp. *mycoides* vaccine. The injection of the live attenuated whole cell component of *M. mycoides* subsp. *mycoides* has been shown to have drawbacks that include: possibility of reversion to virulence and circumscribe swelling (Willems' reaction) at the site of inoculation.

This project aimed to study the mechanism of attachment and to characterize surface components of *M. mycoides* subsp. *mycoides* involved in that process. This was to be achieved by; demonstrating significance of capsular polysaccharide and proteins on the *M. mycoides* subsp. *mycoides* in adherence, illustrating specificity of adherence to different cell lines and determining the nature of receptors on the host cells participating in the adhesion process.

1.2.8 Hypothesis

Adherence of Mycoplasma to epithelial and other cells in the lungs is a necessary condition for infection and pathology. An understanding of the adhesins and specificity of the attachment between Mycoplasma and host cells may allow us to devise ways of preventing this interaction and its pathological consequences. I speculate that *in vitro cell* culture and live mycoplasma can be used as a model to study interaction between *M. mycoides* subsp. *mycoides* and mammalian cells.

1.2.9 Objectives

1.2.9.1 General objective

The objective of this study was to identify the specificity and nature of the adherence between *Mycoplasma mycoides* subsp. *mycoides* and bovine cells.

1.2.9.2 Specific objectives

1) To develop an assay to measure *Mycoplasma mycoides* subsp. *mycoides* adherence to mammalian cells.
2) To determine host specificity to bovine bronchial epithelial cell adherence with different cell lines (bovine testicular vein endothelial cells (282 BTVE pSV neo), bovine skin fibroblast (BY 122 ISF pSV neo) and baby hamster kidney (BHK)).
3) To determine adhesins on the mycoplasma and nature of receptors on the primary bovine bronchial epithelial cells.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

The main biological agents used in this study were *M. mycoides* subsp. *mycoides* (B237) obtained from Kenya Agricultural Research Institute (KARI, Muguga), bovine testicular vein endothelial cells (282 BTVE pSV neo), bovine skin fibroblast (BY 122 ISF pSV neo), baby hamster kidney (BHK) obtained from International Livestock Research Institute (ILRI), primary bovine bronchus epithelial (BBE) cells and peripheral blood mononuclear cells (PBMCs). Polyclonal monospecific antibodies against *M. mycoides* subsp. *mycoides* were produced in rabbits at ILRI. Ascitic fluid of monoclonal antibodies PK2 (specific for a capsular carbohydrate epitope (Kiarie *et al.,* 1996) and Mulosh 1 & 2 (specific for GlpO) were provided by KARI, Biotechnology Centre.

Other antibodies and their specificities used in these studies include; CC31 (bovine CD45R), IL-A88 (bovine MHC class I), IL-A19 (bovine MHC class I), A136 (bovine WC11), IL-A118 (bovine CD44), IL-A96 (bovine WC9), IL-A163 (bovine WC9) and B.1.1.66 (bovine MHC class I).

2.2 Establishment of an assay to measure mycoplasma adherence

2.2.1 *M. mycoides* subsp. *mycoides* preparation for the assays

The mycoplasma for use in adherence assays were cultured in "pleuropneumonia - like organism" or PPLO medium (Becton Dickinson, Park, U.S.A.) according to (Rodwell *et al.,* 1975). *M. mycoides* subsp. *mycoides* (B237) were grown at 37 °C for 48 h. When the medium changed from red to orange, indicating the exponential growth phase (Clausen *et al.,* 2001), a concentration of approximately 10^9 mycoplasmas/ml was reached. The mycoplasmas were

harvested by centrifugation at 3,500 rpm(Allegra^{TM} 25R centrifuge, Germany) for 30 min at 4 °C, washed once in Dulbecco's minimal essential medium (DMEM, ICN biochemicals, Costa, CA,USA). To minimize the number of self-aggregation of *M. mycoides* subsp. *mycoides*, the suspension was sheared through a 27-gauge needle five times and adjusted to a concentration of approximately 10^9 colour changing units ml^{-1} before adhesion to host cells.

Estimation of the concentration of the viable mycoplasma was done by determination of color changing units (CCU). This was done by making serial 10-fold dilutions of mycoplasma (B237) in PPLO medium. The broth base medium was supplemented with phenol red as the pH indicator (starting pH, 6.65). The mycoplasma were then grown in a humidified tissue culture incubator set at an ambient 5% CO_2 concentration at 37 °C. The maximal dilution which produced a color change in the broth medium was determined when color change was no longer changing from red to orange. The viability titers were expressed in CCU. The harvesting of the mycoplasma for each experiment was done at 10^9 Mycoplasmas per ml.

2.2.2 Primary culture of bovine bronchial epithelial cells

2.2.2.1 Collection of bovine bronchial epithelial cells

Bovine bronchial epithelial (BBE) cells were established from cattle slaughtered in Dagoretti abattoirs using the protease digestion technique as described by (Thomas *et al.* 2003) with some modifications. Briefly, the bronchus was collected from the right cranial lobe of adult cattle and placed in Alsevers solution (Sigma-Aldrich Corp. St. Louis, MO, USA). This was followed by immersion of the bronchus into 0.1% protease XIV (w/v) (Sigma Chemicals Co., St Louis, USA) at 4 °C overnight. Digestion was then stopped by adding Dulbecco's minimal essential medium containing 10% inactivated foetal bovine serum (Hyclone Laboratories Inc.), 200 IU of penicillin per ml, 150 µg/ml of streptomycin, 2mM L-glutamine and 0.15M

2-mercaptoethanol. Cells were then collected by gently pipetting the inside bronchus using (Ca^{2+} and Mg^{2+}) Dulbecco's phosphate- buffered saline (pH 7.2) (PBS) and filtered through a sterile gauge before a first centrifugation (200g, 10 min). They were then re-suspended in 15ml of medium with 10% inactivated FBS, filtered through a 100μM Falcon membrane (Becton Dickinson, Park, USA) to disperse clumps of cells, and incubated at 37 °C in 5% CO_2 for 1hour in T75 tissue culture flasks (Costar, Corning Incorporated, NY, USA) to eliminate adhering and contaminating fibroblasts or macrophages. The non-attached cells were then collected by centrifugation and tested for viability by trypan blue (Sigma Chemicals Co.) exclusion test.

2.2.2.2. Cultivation of primary BBE cells

After the trypan blue exclusion test, cells were seeded in T25 tissue culture flasks (Costar, Corning Incorporated, NY, U.S.A.). Cultivation of the BBE cells was done as described by Mwangi and others, 1998. Briefly, the cells were checked for confluency under a microscope. Once confluent, the culture medium was drawn out using a 5ml stripette and poured out into a waste solutions beaker. About 5ml of PBS-EDTA 1X sterile solution was dispensed into the flask to rinse the cells, and then poured into a waste solution beaker in the hood. The cells were then detached by trypsinising with 3ml of trypsin-EDTA (Gibco Life Technologies) solution for 5-10 min. Detachment was then observed under a microscope. The flask was pounded 3 times on a hard surface to allow further detachment of adherent cells from the flask. This was followed by addition of 7ml of culture media into the flask and the cell suspension was transferred into a 20ml universal bottle. This was followed by centrifugation at 1,800 rpm (Allegra™ 25R centrifuge, Germany) for 5 minutes. The supernatant was poured into a waste solution beaker, while the universal bottle was shaken to detach the pellet from the bottom of the bottle. Using a 10ml stripette, 9ml of the culture medium was added to

suspend the cells. The cell suspension was then seeded into the T75 cell culture flask (Costar, Corning Incorporated, NY, USA) to which 5 ml of culture medium had been added before, to give a total volume of 14ml. The flask was then incubated at 37 °C in a humified Heraeus incubator in 5% carbon dioxide concentration. The cells were maintained in complete DMEM with 10% inactivated foetal bovine serum, 200 IU of penicillin per ml, 150 µg/ml of streptomycin, 2mM L-glutamine and 0.15M 2-mercaptoethanol and used for *Mycoplasma mycoides* subsp. *Mycoides* Small Colony infection. The BBE cells used in the experiments were used at between passages 4 and 10. The uniformity of cell monolayer was observed microscopically before each adherence assay and cells were checked for the absence of mycoplasma contamination by culturing in PPLO.

2.2.3 Resuscitation and growth of BY122 ISF pSV neo, BHK and BTVE 282 pSV neo.

The cell lines BY122 ISF pSV neo (bovine skin fibroblast cells), BHK (baby hamster kidney cells) and BTVE 282 pSV neo (bovine testicular vein endothelial cells) had been preserved in a freezing solution of 10% dimethylsulphoxide (DMSO) and 10% DMEM growth media in sealed cryovials while immersed in liquid nitogen. The DMEM contained 20% heat inactivated fetal calf serum (FCS). Cryovials containing the cells were picked from the liquid nitrogen tanks and thawed in water bath at 37° for about 1 minute. The cells were then dispensed into 20 ml-universal bottle containing 10 ml of complete DMEM. This was followed by centrifugation at 1800 rpm (AllegraTM 25R centrifuge, Germany) for 5 minute after which the supernatant was poured off and the cells re-suspended in 5ml culture media. The re-suspended cells were then transferred into a labeled T25 culture flask and incubated at 37°C in a humidified Heraeus incubator with 5% CO_2. Growth of the cells was checked 24 hours later after adhering at the bottom of the flask.

2.2.4 Viable cell counts of primary bovine bronchial epithelial cells

Viable cell counts were done using a hemocytometer. Briefly, a 1:10 dilution of the cells was prepared in 0.4% trypan blue suspension. The mixture was allowed to stand for 5 min at 15 to 30 °C (room temperature) and a small amount transferred to the hemocytometer. Under the microscope, non-viable cells were stained blue, while viable cells excluded the dye and remained colourless.

2.2.5 Indirect Immunofluorescent Microscopy

Indirect Immunofluorescent Microscopy was done as described by Henrich, 1993 with modifications. Briefly, BBE Cells (5×10^4 to 10×10^4 cells per ml) were grown till adherence on glass coverslips in 6 well plates (Costar, Cambridge, Mass.) for 2-3 days at 37 °C until they were 20% confluent. Prior to the assay, the medium was removed and replaced by DMEM without supplements. Whole mycoplasma (2×10^7 CCU/ml) were added and incubated for 2 h at 37 °C, after which BBEC were washed twice with prewarmed DMEM. Cytoadherent mycoplasma were incubated with rabbit sera (diluted 1:100). After two washes with DMEM, the cells were then fixed for 16 h with 4-paraformaldehyde. Bound anti-mycoplasma antibodies were detected with DyLight 488 Donkey anti-rabbit conjugate (Thermo Fisher Scientific Inc., Baltimore, USA) diluted 1: 500. During incubation with the conjugate, BBEC were counterstained with 0.005% Evans Blue for 60 min. After three washes in DMEM, the coverslips were air dried and mounted on slides for microscopy with an anti-fade solution (p-phenylamine dihydrochloride (Sigma Chemicals Co., St Louis, USA) 1µg/ml in PBS and glycerol 90%, pH 9.0). An immunonofluorescent microscope (Axio Imager, Carl Zeiss AG, Gottingen, Germany) was used to examine the slides. Photographs of the cytoadherent cells or proteins were taken at a magnification of X400.

2.2.6 Flow Cytometric Binding Assay Development

2.2.6.1 Detection of *M. mycoides* susp. *mycoides* by Flow cytometry

Bovine Bronchial Epithelial Cells were grown in 500 µl minimal essential medium (DMEM) to a confluent state in 24-well plates to reach approximately 10^5 cells per well. Prior to the assay, the medium was removed and replaced by 500 µl of DMEM without antibiotics. After this, 200 µl of *M. mycoides* susp. *mycoides* (approximately 10^8 CCU/ml) was transferred onto each BBE cell monolayer and incubated for 2 h at 37 °C. After removal of excess liquid, the BBE cells were washed thrice with 500 µl of prewarmed DMEM to remove nonadherent mycoplasma. The cells were then detached using PBS-EDTA buffer by incubating for 10 min. and washed twice by centrifuging at 1000 rpm (AllegraTM 25R centrifuge, Germany) for 5 minutes. Staining of the cells was then done through immunofluorescence as described by Cheek *et al.,* (1997) with modifications. Briefly, cells were suspended at $2x10^7$ cells/ml in DMEM supplemented with 0.01% sodium azide and 2% FCS (FACS medium). Fifty µl of cells were added to 96 well round-bottom plates. Then 50µl of rabbit polyclonal sera at a dilution of 1:100 was added to the wells and incubated for 1 h on ice. After 1 h, 100µl of FACS medium was added to each well and centrifuged at 200 x g for 3 minutes. The medium was removed by flicking out, fresh medium was added after the cells were re-suspended by shaking then centrifuged again. This procedure was repeated twice. After the final wash, the medium was removed and cells re-suspended by shaking. This was followed by addition of 50 µl of polyvalent Donkey anti-Rabbit IgG (H+L) conjugated to DyLight^{TM}488 (Jackson ImmunoReaserch Laboratories, INC., USA) at a dilution of 1:200 and cells incubated for 30 minutes on ice. Cells were washed 3 times and suspended in 100µl of FACS medium. Cells were then transferred into a 5ml-falcon tube and analyzed by flowcytometry (FACS canto II, Becton Dickinson Co.,San Jose, USA) according to the manufacturer's instructions.

2.2.6.2 Analysis using Flow Cytometry

Analysis of flow cytometry data was done as described by Syfrig and others (1997). Three thousand signals were acquired and the results presented as dot plots. To calculate the percentage of positive cells, a threshold was set on the fluorescence signal, such that control samples without *M. mycoides* susp. *mycoides* would have 0% fluorescencent positive cells. This threshold was kept the same for all samples of an experiment and the percentage of fluorescent positive cells, or mycoplasma-bound cells was determined as the percentage of cells above the threshold. Percentage of inhibition of *M. mycoides* susp. *mycoides* binding by Monoclonal antibodies (MAb) was then calculated as the percentage of positive cells of the sample without MAb minus the percentage of positive cells of the sample containing the inhibitory MAb, divided by the percentage of positive cells of the sample without MAb, and the figure multiplied by 100.

2.3 Specificity of *Mycoplsama mycoides* subsp. *mycoides* to mammalian cells determination

2.3.1 Specificity between pathogen and host cells

Specificity was achieved through comparison of adherence rates between bovine bronchial epithelial cells with other cells using flow cytometry. Bovine cells (BBE, 282 BTVE pSV neo, and BY 124 ISF pSV neo) and baby hamster kidney cells (BHK) at 2 x 10^5 cells/well were grown in a 24 well plate until they were confluent. The cells were then infected with *M. mycoides* subsp. *mycoides* at a concentration of 10^8 CCU/ml. The assay was then performed as in section 2.2.7.1.

2.4. Adhesins identification by inhibition of *Mycoplasma mycoides* susp. *mycoides* attachment to BBE cells

2.4.1 Inhibition by monoclonal antibodies PK-2 and GLPO

Antibodies specific for mycoplasma epitopes were screened for their capacity to inhibit cytoadhesion to BBE cells. Two monoclonal antibodies against *M. mycoides* susp. *mycoides* that could be obtained: PK-2 (Kiarie *at al.,* 1996) detects a carbohydrate epitope on *M. mycoides* susp. *mycoides*, and Mulosh1 detects *M. mycoides* susp. *mycoides* L-α-glycerophosphate oxidase (GlpO), a surface protein (Pilo *et al.,* 2005). In the inhibition assay, mycoplasma cells were preincubated for 1 h at 37 °C with monoclonal antibodies which were used at the same dilution as in the adherence assay. After that, BBE cells were incubated with the preincubated mycoplasmas for 2 h at 37 °C. The assay was then done as described above. The capacity of the MAb to inhibit adherence was examined on the basis of reduction in percentage of BBE cells that had mycoplasma attached. Mycoplasma infected bovine epithelial cells without antibody served as positive controls.

2.4.2 Effect of capsular polysaccharide on adherence capability

The capsular polysaccharide of *M. mycoides* subsp. *mycoides* provided by KARI, Biotechnology centre was diluted 1:100 in DMEM media. 200µl of the dilutions were pre-incubated with confluent BBE cells in 24 well plate for 2 h and washed. This was followed by addition of 200µl (10^9CCU/ml) of *M. mycoides* subsp. *mycoides* .·The assay was then performed as in section 2.2.7.1.

2.5 Determination of the nature of host cell receptor(s)

2.5.1 Effect of proteinase K treatment on BBE cells

Bovine Bronchial Epithelial cells were treated with Proteinase K to determine whether the receptors were protein in nature. The activity of proteinase K (Qiagen) was first tested by demonstrating its ability to cleave surface proteins on peripheral blood mononuclear cells (PBMC) (Syfrig *et al.,* 1997). The PBMC were obtained from bovine as described previously (Goddeeris and Morrison, 1988). Blood was collected from jugular vein into a syringe containing an equal volume of Alsever's solution and mixed gently. The blood was then layered on a Ficoll Paque solution and centrifuged at 2000 rpm (AllegraTM 25R centrifuge, Germany) for 30 minutes at room temperature with brakes of the centrifuge switched off. PBMC from the interface were aspirated using a sterile pipette, transferred onto a sterile tube and topped up with warm Alsever's solution. This was followed by centrifugation at 1200 rpm (AllegraTM 25R centrifuge, Germany) for 10 minutes at room temperature. The PBMC were then washed three times with Alsever's solution by centrifuging at 800 rpm (AllegraTM 25R centrifuge, Germany) at room temperature to remove platelets. The pellet of PBMC was then resuspended in DMEM media. One million cells per ml PBMC were added to 0.5 mg proteinase K per ml in DMEM. The mixture was incubated at 37°C for 2 h. The cells were subsequently washed twice with 10% fetal bovine serum in DMEM. Viability of the cells was more than 70% at this stage. Then, 50µl of the digested PBMC were transferred onto a 96 plate with round bottom, 50µl of anti-bovine CD45R MAb CC31 was added and incubated for 1 h on ice. Cells were washed 2X with FACS medium, and 50µl of a secondary antibody (anti mouse conjugated with FITC) was added and left to incubate for 30 minutes. After 2X washes in FACS medium the cells were suspended in 100 µl and analyzed using FACSCanto.

After confirmation of the activity 200µl of proteinase K (0.5 mg/ml) was added to a confluent monolayer of BBE cells in a 24 well plate and incubated for 2h at 37 ^{0}C. After incubation the reaction was stopped by adding 10% FBS in DMEM into each well, which was then washed. The BBE cells were then incubated with *M. mycoides* subsp. *mycoides* for 2 h followed by staining of the cells through flow cytometry as in section 2.2.7.1.

2.5.2 Treatment of BBE cells with N-glycosidase F

To determine if N (asparagines)-linked carbohydrate chains of glycoprotein were involved as receptors in *M. mycoides* subsp. *mycoides* adherence to BBE cells N-glycosidase F (Boehringer mannheim GmbH, Germany) were used. 200µl N-Glycosidase (1U) were added to a confluent monolayer of BBE cells in a 24 well plate and incubated for 2h at 37 ^{0}C. After incubation the reaction was stopped by adding 10% FBS in DMEM into each well, which was then washed. The BBE cells were then incubated with *M. mycoides* subsp. *mycoides* for 2 h followed by staining of the cells through flow cytometry as in section 2.2.7.1.

2.5.3 Blocking adherence using monoclonal antibody against BBE cells

2.5.3.1 Screening of monoclonal antibodies

Monoclonal antibodies towards epitopes on the BBE cells were also used to study the nature of receptors on the BBE cells. The monoclonal antibodies were first screened to see if they recognized epitopes on the BBE cells. Briefly, BBE cells were grown in 24 well flask until they were confluent. Monoclonal antibodies to different specificities were added into the wells at a dilution of 1:500 and incubated for 1 h. They included IL-A88 and IL-A19 (BoMHC class I), B1.1.G6 (human α-microglobulin) IL-A136 (BoWC11), IL-A96 and IL-A118 (BoCD44), IL-A136 (BoWC9, which is possibly identical to CD9). The surface bovine leukocyte molecules are described by Naessens *et al.*, (1997). This was followed by three

washes using warm DMEM. The cells were detached by incubating with warm PBS/EDTA for 10 minutes at 37°C. Then 50µl of BBE cells were transferred onto 96 round bottomed well plate. A 1:500 dilution of anti-mouse FITC was added and incubated for 30 minutes. Detection of bound monoclonal antibodies was done by flow cytometry.

2.5.3.2 Inhibition of *M. mycoides* subsp. *mycoides* binding to BBE cells by MAb against BBE cells

Monoclonal Antibodies IL-A88 (IgG2a), IL-A19 (IgG2a), A136, 4D11, A96, A163 and B.1.1.66 were each added to confluent growing BBE cells in 24 well tissue culture flask and incubated for 1 h at 37°C. The cells were washed in warm PBS and then incubated with *M. mycoides* subsp. *mycoides* , stained, and analysed on the FACScan as described in section 2.2.7.

3.6 Statistical analysis

In each of the assays all samples were tested in duplicate or triplicate. Results are expressed as mean values with Standard Deviations. P values were calculated using non-parametric Mann-Whitney test.

CHAPTER THREE

RESULTS

3.1 Primary culture of Bovine Bronchial Epithelial (BBE) Cells

Primary BBE cells were established from bronchus tissue recovered from adult cattle slaughter in a local abattoir. The BBE cells were simple squamous epithelial cells. The epithelial cells attached to a tissue culture flask and appeared flattened and polygonal in shape. The cells were large and had a prominent, protruding nucleus characteristic of epithelial cells (Plevis *et al.*, 1993). The BBE cells grew until a confluent monolayer was formed and maintained tight contact with neighboring cells (Figure 2).

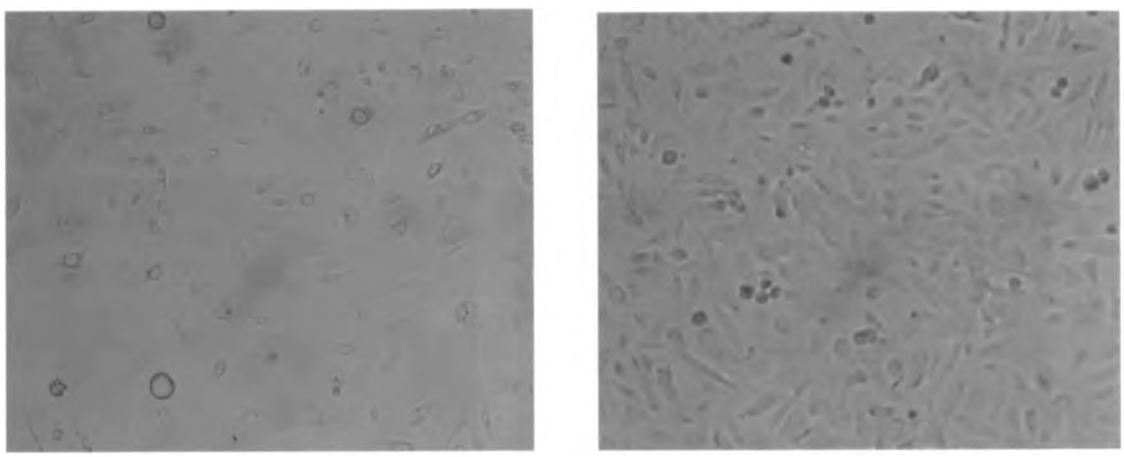

Figure 2. Growing of Bovine Bronchial Epithelial (BBE) cells. Left photograph: BBE cells growing in a 25 cm^2 tissue culture flask after 2 days. Right photograph: Confluent monolayer of BBE cells, after 3 days. (Magnification X100)

3.2 Adherence of *Mycoplasma mycoides* subsp. *Mycoides* to BBE cells

3.2.1 Indirect immunofluorescence microscopy

Visualization of adherence of *Mycoplasma mycoides* subsp. *Mycoides* to BBE cells using indirect immunofluorescence microscopy is as shown in figure 3. Bovine Bronchial Epithelial Cells derived from a bovine bronchus, were used as host epithelial cells in the adhesion assays. Polyclonal rabbit antibody against whole *M. mycoides* subsp. *Mycoides*, strongly detected cytoadhering organisms on the BBE Cells. Fluorescent spots were identified as shown by arrows in figure 3, indicating that the cells of *M. mycoides* subsp. *Mycoides* were clustered on the BBE Cells. Controls included (1) BBE cells without mycoplasma, but treated in the same way with antiserum and conjugate, (2) cells mixed with mycoplasma, but treated with pre-immune serum and conjugate, and (3) cells mixed with mycoplasma, and treated with conjugate only.

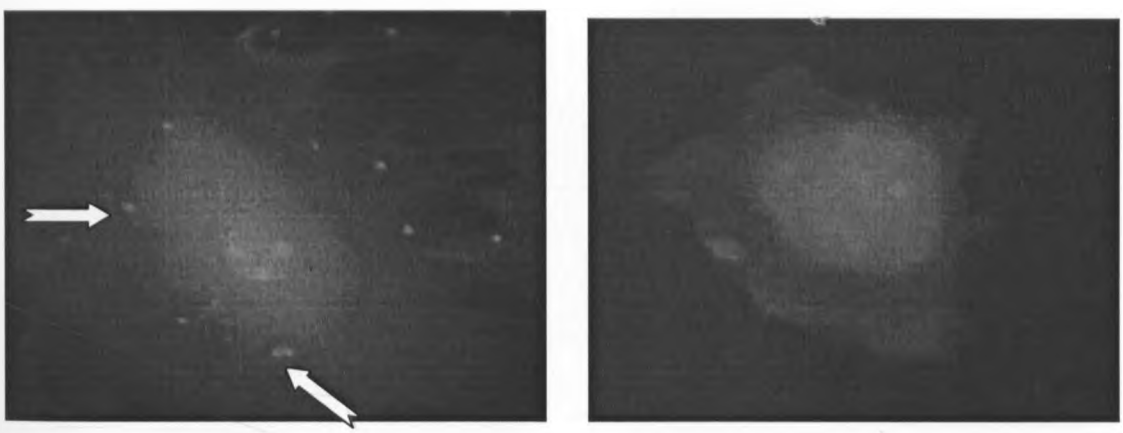

Figure 3. Immunofluorescent staining of *M. mycoides* subsp. *mycoides* bound to Bovine Bronchial Epithelial cells. Left photo: host cell with adherent *M. mycoides* subsp. *mycoides*, detected by specific rabbit antisera. The bound rabbit Abs were visualized by fluorescent DyLight 488-conjugated Affinipure Donkey Anti-Rabbit IgG (H+L). Right photo: negative control, BBE cells not mixed with mycoplasma and treated as in left panel. Arrows indicate bright fluorescent patches of mycoplasma clusters on the surface of the epithelial cell. Counterstain: Evans Blue. Total magnifications, X 400

3.2.2 Indirect immuno flow cytometry

A flow cytometric assay was developed to measure the adherence rate of *M. mycoides* subsp. *mycoides* to BBE cells. The mycoplasma SC bound specifically to the BBE cells. Results showed that BBE cells that had bound *M. mycoides* subsp. *mycoides* were easily distinguished from those that had no mycoplasma. A dot plot of DyLight 488 fluorescence versus forward scatter (FSC-A) of BBEC showed *M. mycoides* subsp. *mycoides* adhered onto the surface (Fig 4). The data was acquired on a four-decade Logarithmic scale. The percentage of BBE cells that had no bound *M. mycoides* subsp. *mycoides* were always less than 5%. However, the cells below the threshold were not negative for mycoplasma, as they did not have the profile of the negative cells (Fig. 4a, 4b and 4c). They are the tail of the distribution just below the set threshold and are epithelial cells with a lower fluorescence and thus with less mycoplasma attached to their surface.

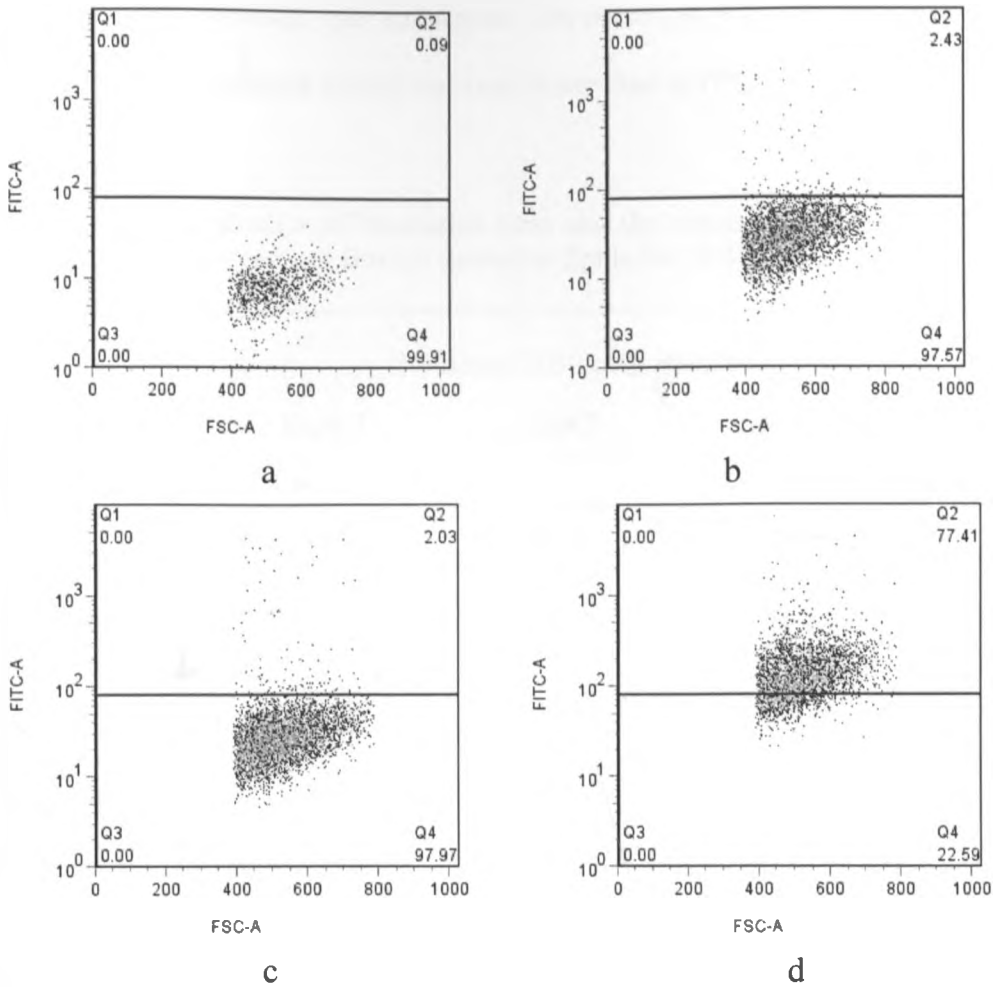

Figure 4. Flow cytometric analysis of attachment of *M. mycoides* subsp. *mycoides* to Bovine Bronchial Epithelial (BBE) cells (a) BBE cells only; (b) BBE cells + non-specific serum; (c) BBE + mycoplasma + non-specific serum (d) BBE cells + mycoplasma + *M. mycoides* subsp. *mycoides* -specific rabbit serum.Vertical axis: fluorescence intensity. Horizontal axis: forward light scatter.

3.2.2.1 Kinetics of adherence to BBE cells

The effect of incubation time and mycoplasma concentration was used to standardize the assay conditions (Table 1 and 2). The data in table 1 and 2 were used to generate the graph in figure 5 and 6 respectively. Using a *M. mycoides* subsp. *mycoides* inoculum of 10^9 CCU/ml, adhesion occurred in a linear fashion. Adherence of *M. mycoides* subsp. *mycoides* to BBE cells increased linearly until a plateau was reached after 120 minutes. Binding activity of *M.*

mycoides subsp. *mycoides* was temperature dependent, at 4°C adherence was about (16% BBE cells had mycoplasma bound) six times lower than at 37°C.

Table 1. Binding kinetics of incubation time and the adherence capacity of *M. mycoides* subsp. *mycoides* to Bovine Bronchial Epithelial (BBE) cells.

	(%) Bound BBE cells attached to B237			
Time (minutes)	Expt. 1	Expt.2	Expt.3	Mean and SD
5	4.68	4.86	5.03	4.83±0.17
60	65.43	61.12	56.84	60.79±4.3
120	96.39	96.93	90.16	96.59±0.7
160	95.96	97.63	97.47	97.29±1.7

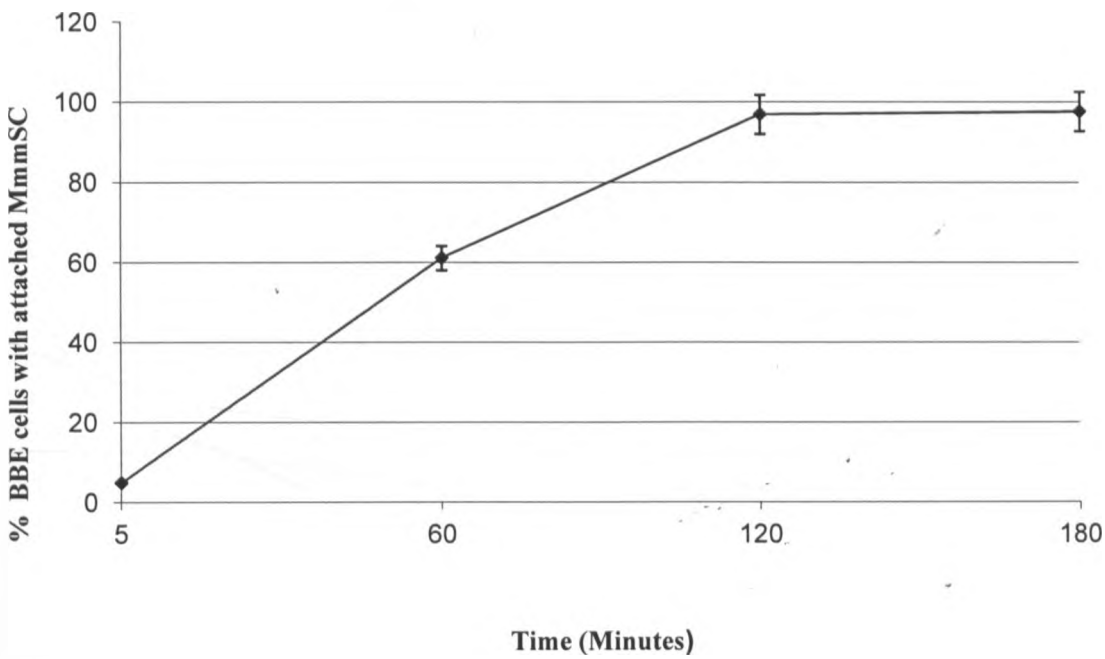

Figure 5. Binding kinetics of incubation time and the adherence capacity of *M. mycoides* subsp. *mycoides* to Bovine Bronchial Epithelial (BBE) cells.

The degree of binding was also influenced by the number of *M. mycoides* subsp. *mycoides* (Fig. 6). Low binding was observed with $2x10^5$ mycoplasma/10^7 BBE cells but fluorescence increased with increasing dose of mycoplasma until a maximum was reached at $2x10^9$ mycoplasma/10^7 BBE cells. Saturation of 10^7 BBE cells was observed with more than 10^9 CCU of *M. mycoides* subsp. *mycoides*. This suggests that each cell can bind up to 200 mycoplasma organisms, provided all mycoplasma adhered. This should be further confirmed using other techniques, such as real time PCR.

Table 2. Attachment of *M. mycoides* subsp. *mycoides* to Bovine Bronchial Epithelial cells depending on the number of added *M. mycoides* subsp. *mycoides*

Mycoplasma Concentration (Log)	Expt. 1	Expt. 2	Mean and SD
1.3	3.1	4.3	3.7 ±0.8
2.3	2.6	3.6	3.1 ±0.7
3.3	3.2	4.2	3.7 ±0.7
4.3	5.1	4.1	4.1 ±0.7
5.3	9.5	10.4	9.9 ±0.6
6.3	21.2	29.5	25.3 ±5.8
7.3	40	50.4	45.2 ±7.3
8.3	74.7	70.2	72.4 ±3.2
9.3	99.2	98.9	99.0 ±0.2
10.3	99.4	99.5	99.4 ±0.1

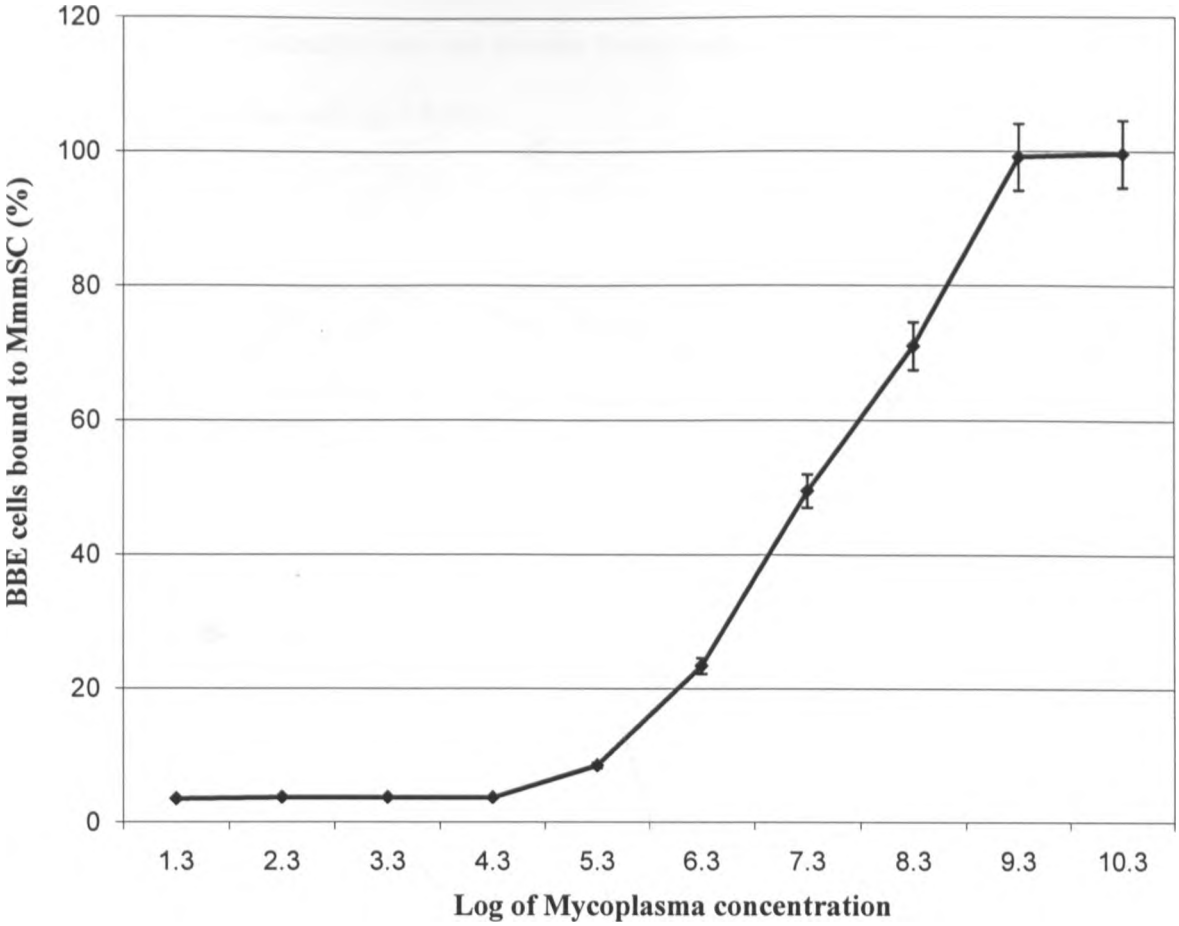

Figure 6. Attachment of *M. mycoides* subsp. *mycoides* to Bovine Bronchial Epithelial cells depending on the number of added *M. mycoides* subsp. *mycoides* ($2x10^1$ to $2x10^{10}$ CCU). The values ploted represent mean values from two independent tests.

3.3 Adherence rates to different cell lines

The capacity of *M. mycoides* subsp. *mycoides* to adhere to different cell types was assessed by a comparative binding study using a number of different cell lines: bovine skin fibroblasts (BY 122 ISF pSV) neo, bovine endothelial cells (BTVE 282 pSV) neo and hamster kidney cells (BHK).

The results shown (Table 3 and depicted in figure 7) that the adherence rate to bovine fibroblasts, bovine endothelial cells and hamster kidney cells were significantly lower than those to bovine epithelial cells ($p < 0.001$).

Table 3. Comparison between adherence rate (%) of *M. mycoides* subsp. *mycoides* to Bovine Bronchial Epithelial (BBE) cells cells, Baby Hamster Kidney Cells (BHK), Bovine Skin Fibroblast (BY 122 ISF pSV neo) and Bovine Testicular Vein Endothelial (BTVE 282 pSV neo) cells. The given adherence rate represent the mean value ± standard deviation from two independent sets of experiments.

	% of Bovine Bronchial Epithelial cell attached to *Mycoplasma mycoides* subsp. *Mycoides* SC			
Cells	Expt.1	Expt.2	Expt.3	Mean and SD
BBE	79.4	90	89.7	86.3 ±6.0
BHK	48	37	42	42.3 ±5.5
BY 122 ISF pSV neo	39.87	60	50.935	50.2 ±10.1
BTVE 282 pSV neo	16.5	25	19.75	20.4 ±4.3

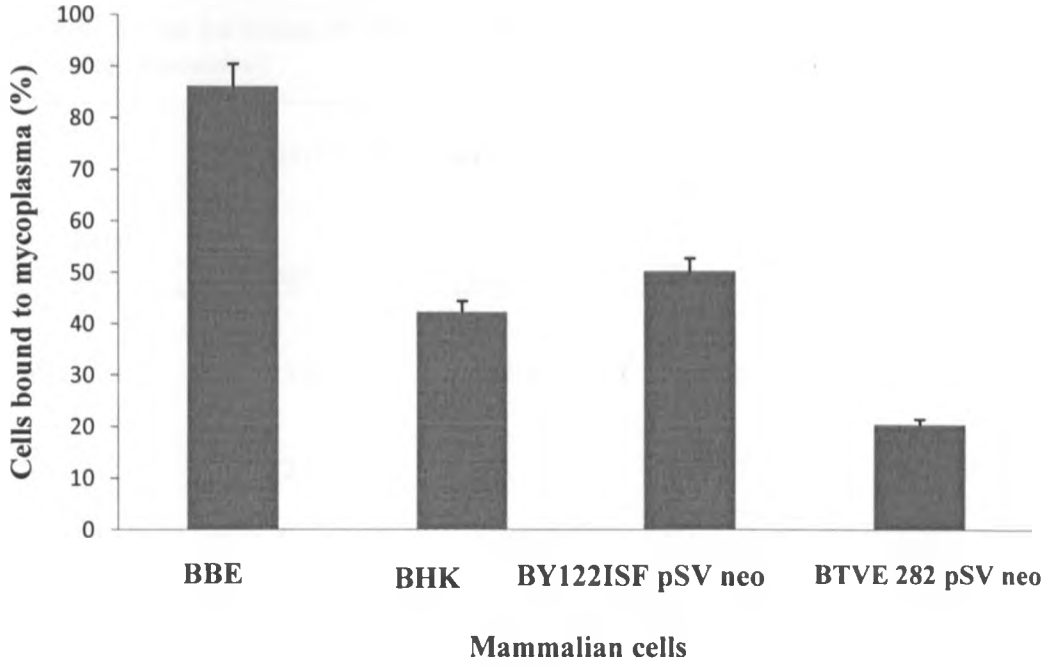

Figure 7. Comparison of adherence capacity between Bovine Bronchial Epithelial (BBE), Baby Hamster Kidney (BHK), Bovine Skin fibroblast (BY122 ISF pSV neo and Bovine Testicular Vein Endothelial (BTVE 282 pSV neo) cells. The data are mean value ± standard deviation from three independent sets of experiments.

3.4 Inhibition of adherence by monoclonal antibodies (MAbs) and rabbit sera

The adhesion inhibition assay examined whether the monospecific antibodies against two mycoplasma antigens (PK-2 against capsular polysaccharide and Mulosh 1 against GlpO) were able to block *M. mycoides* subsp. *mycoides* from binding to the BBE cells. Dilutions (1:100) of ascetic fluid containing high concentrations (not exactly determined at this stage) of the antibodies were incubated with *M. mycoides* subsp. *mycoides* before infection of the BBE cells to test their adherence inhibition effect. The inhibitory effect of MAb PK-2 on *M. mycoides* subsp. *Mycoides* adherence is shown in Table 4 and Figure 8. The data was used to generate the chart in figure 6. A MAb that is not specific for *M. mycoides* subsp. *Mycoides* (ILA-11) was used as a negative control. The cytadherence of the mycoplasma was detected with polyclonal antibodies against whole cells of *M. mycoides* subsp. *mycoides*.

Table 4. Attachment inhibition of monoclonal antibodies specific to *Mycoplasma mycoides* subsp. m*ycoides* .

Monoclonal antibodies	% Bovine Bronchial Epithelial cells with attached *Mycoplasma mycoides* subsp. *Mycoides*			
	Expt. 1	Expt. 2	Expt. 3	Mean and SD
PK-2	43.63	37.5	58.36	46.4±10.7
Mulosh 1	82.59	76.15	82.37	80.3±6.4
ILA 11	74.8	64.77	69.785	69.7±5.0
No MAb	86.83	77.78	94.97	86.5±8.5

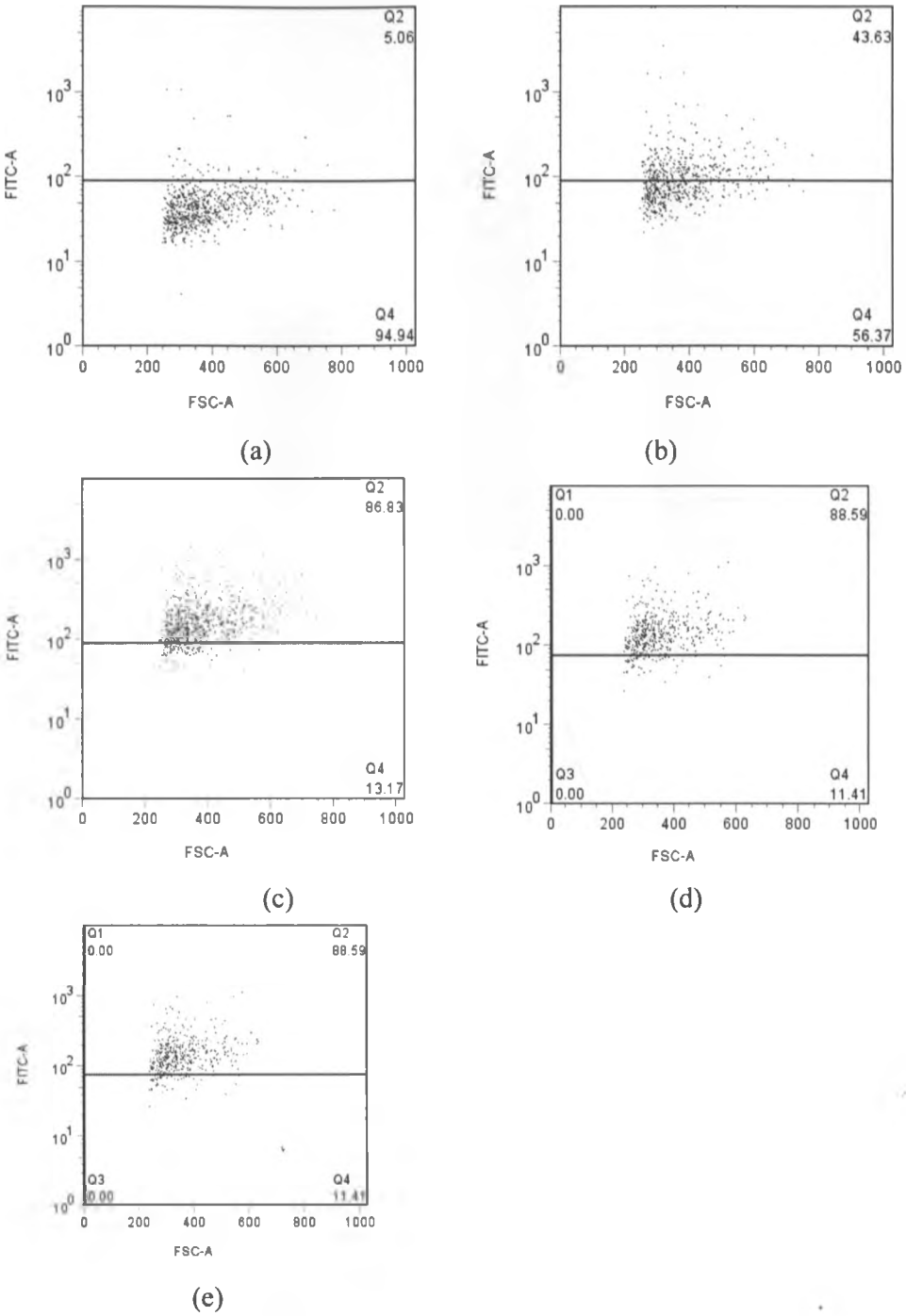

Figure 8. Flow cytometric analysis of inhibition of mycoplasma mycoides susp. Mycoides binding to Bovine Bronchial Epithelial (BBE) cells by monoclonal antibodies (MAb) ((a) BBE cells without mycoplasma; (b) BBE + mycoplasma (pre-incubated with PK -2 + rabbit specific serum (c) BBE cells + mycoplasma (pre-incubated with mulosh 1) + specific rabbit serum (d) BBE cells + Mycoplasma (pre-incubated with ILA 11) + Specific rabbit serum and (e) BBE cells + Mycoplasma (no MAb). Vertical axis: fluorescence intensity. Horizontal axis: forward light scatter.

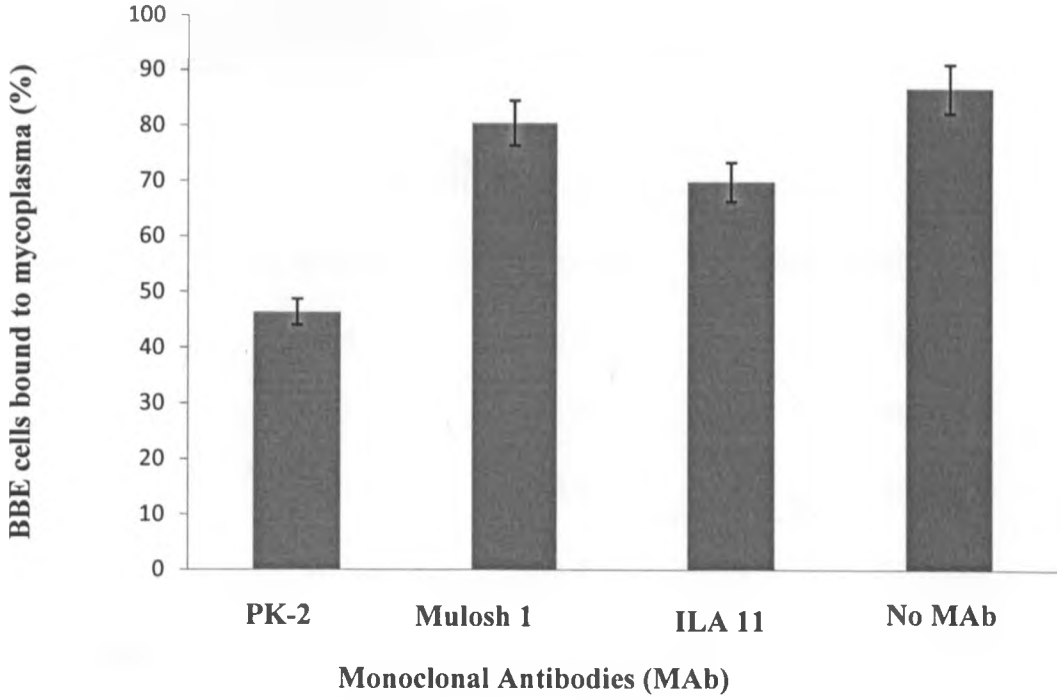

Figure 9. Inhibition of *Mycoplasma mycoides* subsp. *Mycoides* adherence to Bovine Bronchial Epithelial (BBE) cells by monoclonal antibodies (MAb) PK-2 and Mulosh 1. MAb ILA 11 was used as control. The data presented is mean value of two independent tests.

M. mycoides subsp. *Mycoides* pre-incubated with the non specific antibodies served as the negative control. MAb PK-2 reduced *Mycoplasma mycoides* subsp. *Mycoides* binding by 55.16%.

3.5 Effect of capsular polysaccharide on adherence ability

Following the positive results of inhibition assay by the PK-2, speculation was that the inhibition resulted from interference with the *Mycoplasma mycoides* subsp. *Mycoides* capsular polysaccharide. Therefore the effect of the capsular polysaccharide was further evaluated by pre-incubating purified polysaccharide with BBE cells. No detectable inhibition was observed (Table 5).

Table 5. Effect of pre-incubation of capsular polysaccharide (CP) with Bovine Bronchial Epithelial cell Adherence assays. Mean values and standard deviation from test done in triplicate.

	% of BBE cells bound to Mycoplasma			
Dilutions of CP	1st reading	2nd reading	3rd reading	mean and SD
1:10	97.4	98.8	98.4	98.2±0.7
1:100	99.3	98.96	98.5	98.9±0.4
1:1000	98.1	98.8	97.4	98.1±0.7
No CP	90.2	95.8	96.4	94.1±3.5

3.6 The nature of nature of receptor(s)

3.6.1 Blocking using Antibody against BBE cells

Blocking studies using monoclonal antibodies directed towards BBE cells epitopes. Antibodies that recognized epitopes on the BBE cells, IL-A88 (bovine MHC class I), IL-A19 (bovine MHC class I), IL-A136 (bovine WC11), IL-A118 (bovine CD44), IL-A96 (bovine WC9), IL-A163 (bovine WC9) and B.1.1.66 (bovine MHC class I), were tested. They were shown to be incapable of blocking the binding of *M. mycoides* subsp. *Mycoides* (Table 6).

Table 6. Blocking of *M. mycoides* subsp. *Mycoides* binding to the Bovine Bronchial Epithelial (BBE) cells epitopes by monoclonal antibodies specific bovine cells epitopes.

Monoclonal antibodies	Antigen	% BBE cells bound to mycoplasma			Mean and SD reduction in attachment (%)
		1st reading	2nd reading	Mean	
No blocking antibody		96.77	98.7	97.735	
IL A88	MHC class I	89.37	94.37	91.87	5.99 ±2.2
IL A19	MHC class I	96.03	93.77	94.9	2.82 ±2.9
IL A136	BoWC15	94.74	96.5	95.62	2.15 ±0.03
A96	BoWC9	87.86	90.5	89.18	8.94 ±0.2
A163	BoWC9	88.16	92.12	90.14	7.78 ±0.7
B.1.1.66	MHC class I	86.67	88.33	87.5	11.07 ±0.4

3.6.2 Treatment of BBE cells with Proteinase K and N-Glycosidase F

In order to investigate whether the binding receptor was located on a surface protein, BBE cells were treated with proteinase K before testing their capacity to bind *M. mycoides* subsp. *Mycoides*. The activity of proteinase K was confirmed as shown in the figure 10, as it removed the epitope for MAb CC31. Proteinase K was shown to reduce the amount of *M. mycoides* subsp. *Mycoides* bound to the BBE cells after treatment (Table 7). N-Glycosidase F (PNGase F) treatment of the BBE cells was used to determine if the mycoplasma receptors are N (asparagine)-linked carbohydrate chains of glycoproteins. The activity of PNGase F was not determined during the assay. N-Glycosidase reduced binding of *M. mycoides* subsp. *Mycoides* to BBE cells minimally (Table 7).

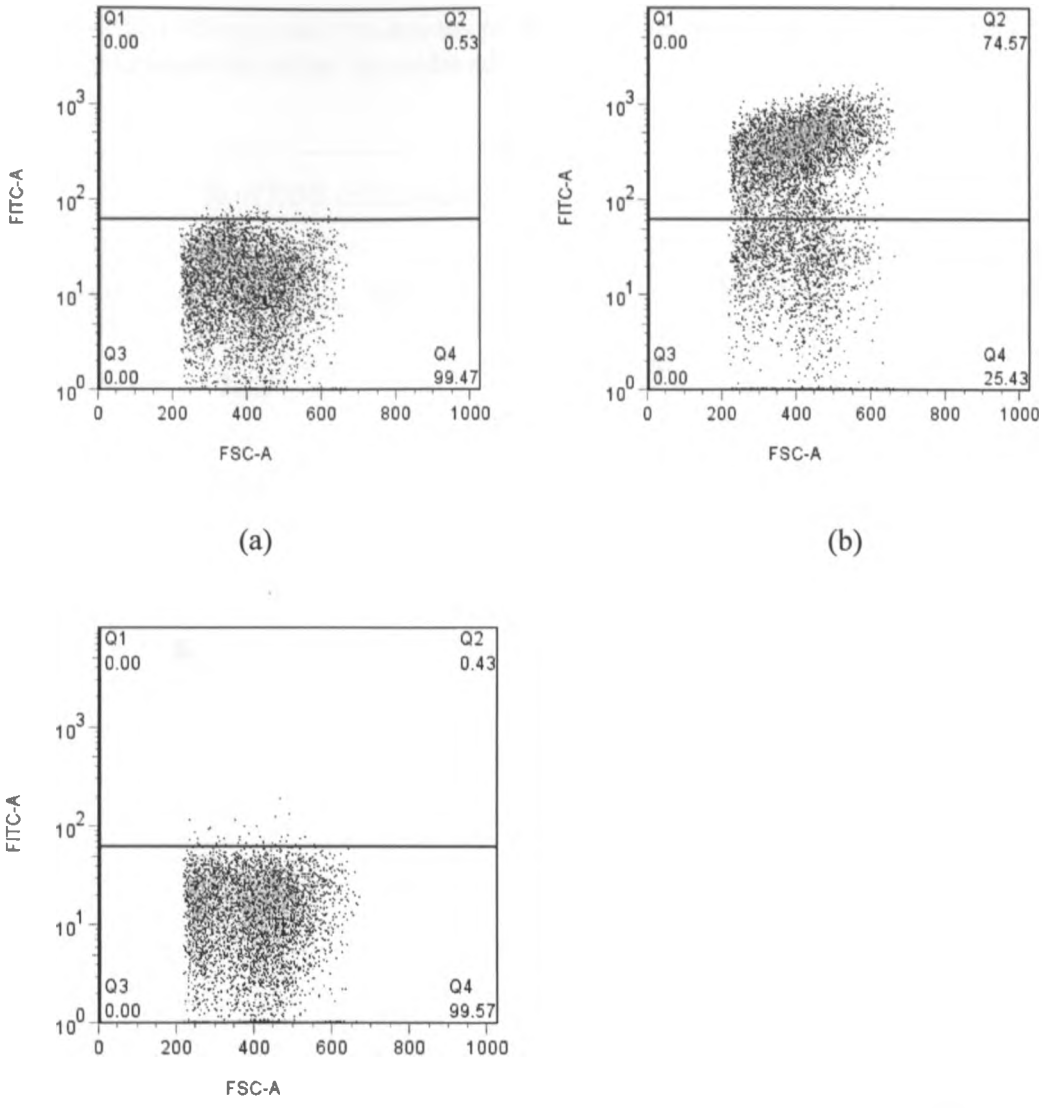

Figure 10. Flow cytometric analysis showing enzymatic activity of proteinase K. a) Control; Peripheral Blood Mononuclear Cells (PBMC) incubated with secondary antibody only, b) PBMC stained with CC31 (anti-CD45R) and c) PBMC treated with Proteinase K and stained with CC31.

Table 7. Effect of enzymatic pre-treatment of the Bovine Bronchial Epithelial (BBE) cells on *Mycoplasma mycoides* subsp. *mycoides* adherence.

	% of BBE cells attached to mycoplasma				
Treatment	Expt.1	Expt.2	Expt. 3	Mean	Mean and SD reduction of attachment (%)
No treatment	72.2	78.6	70.4	73.4	
Proteinase K	50.6	52.4	49.5	50.5	31.2 ±1.9
N-glycosidase	60.7	74.9	60.8	64.9	11.5 ±5.9

CHAPTER: FOUR

DISCUSSION, CONCLUSION AND RECOMMENDATION

4.1 Discussion

Colonization of epithelial surface is usually the initial step in the process of infection by mycoplasma. It has been demonstrated that *Mycoplasma mycoides* subsp. *Mycoides* (*M. mycoides* subsp. *Mycoides)* adhere to bovine epithelial cells (Bischof *et al.*, 2008) but the mycoplasmal membrane adhesins that mediate adhesion remains unidentified.

To facilitate this study of adherence of *M. mycoides* subsp. *Mycoides* to host cells primary cultures of Bovine Bronchial Epithelial cells were established. The primary cell culture enabled the creation of a model system resembling the *in vivo* situation more closely than a permanent tissue culture cell line.

In this study, flow cytometry was used to develop an immunofluorescent assay to measure the *in vitro* binding of *M. mycoides* subsp. *mycoides* to the BBE cells. The flow cytometric technique represents a rapid and reproducible technique to quantify and compare mycoplasma adherence (Cheek *et al.*, 1997). The low standard error value between sets of experiments underlines the reproducibility of the experiments. Binding was detected by labeling mycoplasma with fluorescencent antibody and analyzing the epithelial cells in a flowcytometer. In the standardization steps of the assay conditions, time and concentration of mycoplasma were varied (Figure 5 and 6). Saturation of host receptors was achieved by approximately 200 mycoplasma per BBE cell compared to 300 per ECaNEp (embryonic calf nasal epithelial) cell (Bischof *et al.*, 2008). The assay performed well and easy discrimination of the mycoplasma bound cells and cells that had no mycoplasma was made. Reduction in

adherence at low temperatures (4°C) may have been caused by death of mycoplasma or because the binding event requires energy.

Immunofluorescence microscopy was performed to demonstrate surface attachment of *M. mycoides* subsp. *Mycoides* SC on BBE cells (Figure 2). The assay also analyzed the suitability of the polyclonal antibodies for detecting the mycoplasma adhering to the BBE cells for adhesion detection assay in surface exposure assay.

The ability of *M. mycoides* subsp. *Mycoides* to adhere to bovine skin fibroblast (BY 122 ISF pSV neo), bovine endothelial (BTVE 282 pSV neo) and baby hamster kidney (BHK) cells was verified. The same conditions were used for growth and maintenance of all the cells including BBE cells, to rule out differences due to *in vitro* culture. As the greatest amount of mycoplasmal adsorption was seen with BBE cells and low adhesion in BY 122 ISF pSV neo, BTVE 282 pSV neo and BHK cells it appears that binding of *M. mycoides* subsp. *Mycoides* to BBE cells is specific. This kind of specificity has also been described in other mycoplasmas (Razin *et al.,* 1998). *M. mycoides* subsp. *Mycoides* has also been shown not to adhere to caprine endothelial cells (Valdivieso-Garcia *et al.,* 1989). However, there seems to exist some non-specific adherence to other cells, which may be mediated by other receptor-adhesin combinations.

To confirm the role of capsular polysaccharide (CPS) of *M. mycoides* subsp. *Mycoides* in attachment to the epithelial linings of the cattle bronchus, monoclonal antibodies (MAbs) specific to GlpO and CPS were used to study their ability to influence mycoplasmal attachment to host cells. Monoclonal antibody PK-2 was able to reduce *M. mycoides* subsp. *Mycoides* from binding to the BBE cells by 55%. The ability of this MAb to significantly

reduce *M. mycoides* subsp. *Mycoides* adherence indicates involvement of the corresponding antigen peptidoglycan in the adhesion process. Inhibition of 100% was not achived, either because the affinity of the MAb was weak compared to the affinity between cell receptor and mycoplasma ligand or because the number of receptors are very high. If 200 mycoplasma can bind to one cell, there are likely to be a lot of receptor sites on each cell and inhibition of all of these sites becomes very difficult. Alternatively, additional binding ligands/receptors may exist and confuse the data. Capsular polysaccharide is assumed to give the mycoplasma physico-chemical resistance against the host immune defense, but have also been shown to be involved in pathogenic mechanisms (Frey *et al.*, 2003). Increased capsule presence and attachment to host cells in other capsulated pathogenic mycoplasmas such as *M. hyopneumoniae* (Tajima and Yagihashi, 1982) and *M. gallisepticum* (Tajima *et al.*, 1982) has been shown.

The effect of capsular polysaccharide that had been extracted (Kiarie *et al.*, 1996) from *M. mycoides* subsp. *Mycoides* failed to inhibit attachment to BBE cells (Fig. 7). Inhibition by saccharides may fail because the affinity at individual sites is often low (Karlsson, 1998), making a univalent ligand inefficient in interfering with the natural multiplicity.

MAb (Mulosh 1) against L-α-glycerophosphate oxidase (GlpO), a protein that is located on the membrane (Pilo *et al.*, 2005) failed to produce significant reduction of *M. mycoides* subsp. *Mycoides* SC adherence to BBE cells. The result indicates that GlpO does not participate in *M. mycoides* subsp. *Mycoides* SC adherence to BBE cells. Although H_2O_2 translocation into host cells requires an intact adhesion mechanism that ensures intimate interaction between mycoplasma and host cells (Bischof *et al.*, 2008), GlpO might not be

having a key role. However, it is also possible that the MAb recognizes an epitope located outside the ligand structure that mediates interaction with the target cell.

The role of surface proteins of BBE cells as receptors was determined by treating the BBE cells with Proteinase K and was shown to reduce adherence of *M. mycoides* subsp. *Mycoides* minimally. The Proteinase K used had been shown to cleave surface protein on the peripheral blood mononuclear cells (PBMCs) (Fig. 10). The site of cleavage by proteinase K is the peptide bond adjacent to the carboxyl group of aliphatic and aromatic amino acids with blocked alpha amino groups. The enzyme was used because of its broad specificity. The measured reduction of adherence by 30 percent represents a partial inhibition, indicating a significant contribution to attachment of non-proteinaceous receptors and, perhaps, proteinase K-resistant proteins. Partial reduction in adherence of Mycoplasma bovis to BBE cells by trypsin (Thomas *et al.,* 2003), has been shown.

Treatment of BBE cells with N-Glycosidase F showed no significant reduction in adherence (Table 4) by *M. mycoides* subsp. *Mycoides*. This probably indicates that the host receptor may not be N (asparagines) – linked carbohydrate chains of glycoproteins. Or else the enzyme did not work properly in the experiment, as way of testing its activity was not available.

Inhibition by MAbs against a number of bovine cells surface antigens did not show any reduction in the adherence of *M. mycoides* subsp. *Mycoides* to BBE cells (Table 3). This indicates that the MAb used in the assay did not recognize receptors on the epithelial cells. The MAbs used included; IL-A88 (bovine MHC class I), IL-A19 (bovine MHC class I),

A136 (bovine WC11), IL-A118 (bovine CD44), IL-A96 (bovine WC9), IL-A163 (bovine WC9) and B.1.1.66 (bovine MHC class I).

4.2 Conclusion

The flow cytometric assay established for this thesis work provides a useful tool for investigating host-parasite adherence. The adherence assay is a useful *in vitro* model for study of *Mycoplasma mycoides* subsp. *Mycoides* attachment mechanism.

The fact that the antibodies, produced against capsular polysaccharide in this in-vitro study, reduced the number of attached mycoplasmas suggests a protective function of the humoral immune defense system in vivo. This suggests that immune responses to the carbohydrate epitope might have a protective effect.

M. mycoides subsp. *Mycoides* showed host cells specificity by having highest % of adherence to BBE cells. The ability of *M. mycoides* subsp. *Mycoides* to adhere to bovine skin fibroblast (BY 122 ISF pSV neo), bovine endothelial (BTVE 282 pSV neo) and baby hamster kidney (BHK) cells was verified. This suggests that the host cells may have unique receptors.

4.3 Recommendations

Further studies should be carried out on the following areas;

I. Characterization of the capsular polysaccharide to determine the specific structures involved in attachment.
II. Inhibition by more monoclonal antibodies targeting surface epitope on *Mycoplasma mycoides* subsp. *Mycoides*
III. Treatment of host cells with enzymes that cleave carbohydrate on the BBE cells.

IV. Detailed comparison of cytoadherence mechanism among different strains of *Mycoplasma mycoides* cluster.

4.4 Limitations of the study

Some of the limitations included;

1. Maintaining exact number of mycoplsama and mammalian cells in each assay was a challenge.
2. Testing of the N-Glycosidase activity was not achieved

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APPENDICES

Apendix 1

1.1 PPLO Broth Base

BBL™ Mycoplasma Agar Base (PPLO Agar Base)
Approximate Formula* Per Liter
Beef Heart, Infusion from (solids) ..... ….2.0 g
Pancreatic Digest of Casein .....7.0 g
Beef Extract .....3.0 g
Yeast Extract .....3.0 g
Sodium Chloride .....5.0 g
Agar ..... 14.0 g

Difco™ Mycoplasma Supplement
Approximate Formula* Per 30 mL Vial
Yeast Extract .....0.01 g
Horse Serum, Desiccated ..... 1.6 g

Dissolve 21 g of the powder in 700 mL of purified water. Mix thoroughly. Autoclave at 121°C for 15 minutes. Cool medium to 50-60°C. Aseptically add 300 mL Difco Mycoplasma Supplement to the medium. Mix well. Test samples of the finished product for performance using stable, typical control cultures.