CHARACTERISATION, PATHOGENICITY AND ANTIBIOTIC RESISTANCE OF *MYCOPLASMAS* INVOLVED IN VULVOVAGINITIS IN GOATS.

BY

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ABSTRACT

Vulvovaginitis is an inflammation of the vulva and vagina caused by bacteria, fungi, viruses, parasites and allergy. The condition results in reduction in mating ability, infertility, abortion and death of the affected animals with resultant economic loss to the livestock industry. Although mycoplasmas have been isolated from cases of vulvovaginitis, their role as sole causative agents and in the pathogenicity of the disease have not been investigated in goats in Nigeria. The aim of this study was to characterise mycoplasma isolates from cases of vulvovaginitis and also and determine the pathogenicity of mycoplasma-induced vulvovaginitis in goats.

Two hundred and twenty-one vaginal swabs were obtained from identified cases of vulvovaginitis in goats aged 8-11 months from markets in and around Lagos metropolis. Samples were analyzed bacteriologically and mycoplasmotologically. Mycoplasmas were identified biochemically using standard procedures and conventional polymerase chain reaction with specific primers. Antisera raised in rabbits using 0.5ml of 4.0 x10^7 CFU/ml of selected *Mycoplasma* species including *M. bovis, M. capri, M. capricolum* and *M. arginini* were used to group the mycoplasmas serologically by growth inhibition method. Sensitivity of the isolated *Mycoplasmas* and other bacteria to aminoglycosides, fluoroquinolones, cephalosporins and nitrofurans was carried out. Pathogenicity of the isolated *M. bovis, M. capri, M. capricolum and M. arginini* was evaluated using 2ml inoculum containing 4.0 x 10^7 CFU/ml of each isolate per vulva to reproduce vulvovaginitis over a six week experimental period. Four goats were used for each experimental group and the control group. Animals were observed for symptoms of vulvovaginitis such as hyperemia, vulva swelling and vaginal mucus discharges. Post-mortem gross and histopathological examination was carried out and findings reported. Other data were analysed using descriptive statistics.

Two hundred and fifty-seven bacterial isolates were recovered from the 221 field samples as follows: *Mycoplasma capricolum* (1.6%), *Mycoplasma arginini* (1.6%), *Mycoplasma capri* (1.2%), *Mycoplasma bovis* (0.78%), *Ureaplasma* species (1.2%), *Acholeoplasma* species (0.8%). Other bacteria were *Escherichia coli* (35.4%), *Streptococcus* species (29.2%) and *Staphylococcus* species (28.4%). Mycoplasma isolates were confirmed with the production of specific 280bp bands. Isolated *Mycoplasma* species and other bacteria were sensitive to ciprofloxacin and nitrofurantoin. Six different combinations of antibiotic resistant patterns were observed with amoxicillin, norfloxacin and ampicillin having the highest level of resistance (100.0%) and nitrofurantoin the least (33.0%). Clinical symptoms, which included hyperemia, swollen vulva, vaginal mucus discharges with pyrexia (38.9 – 39.6^0 C), were first observed in the *M. bovis*- infected group on day three, ten and thirteen. Mortality was recorded on days 30, 33, 38 and 41 post- inoculation in the *M. bovis, M. capricolum, M. arginini* and *M. capri*- infected groups, respectively. *M. bovis* produced the most severe lesions marked by lymphoid necrosis of the vulva tissue, diffuse hyperaemia in the lung alveolar septa and massive alveolar infiltration with neutrophils while the mildest lesions were observed in the *M. arginine*-infected group.

Vulvovaginitis was reproduced in goats with all the *Mycoplasma species* as the major infective agent and *Mycoplasma bovis* as the most pathogenic. Ciprofloxacin and nitrofurantoin were the most effective antibiotics against Mycoplasmas and other bacteria isolated.

Keywords: Vulvovaginitis, *Mycoplasma species*, Antibiotic sensitivity

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CERTIFICATION

I certify that this study has been carried out by Ohiomah John Izebere in the Department of Veterinary Microbiology and Parasitology , University of Ibadan, Ibadan, Nigeria.

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DEDICATION

This project work is dedicated to the Almighty GOD, my FATHER and CREATOR who is the pivot on whom my entire destiny revolves.

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John O. Izebere
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CHAPTER ONE

1.0 Introduction

Vulvovaginitis is the term used to describe the inflammation of the vagina and/or vulva. These inflammatory conditions sometimes refered to as vulvitis or vaginitis can be caused by Bacteria, Viruses, Fungi, or Parasites. In addition, vulvovaginitis can be caused by low estrogen levels (called "atrophic vaginitis") or any type of allergy or irritation, and also by response of the genital organs to spermicidal products, condoms and soaps (Tirumani, 2006). It has been observed that vulvovaginitis causes vaginal discharge, irritation and itching in affected animals as a result of the sloughing of the epithelial lining of the vagina which is sometimes accompanied with mucopurulent discharges (Tirumani and Samra, 2006). Investigation carried out in ewes and does shows that the common pathogens isolated from vulvovaginitis include: *Mycoplasma* sp, *Ureaplasma* sp. *Mycobacterium* sp. *Trichomonas* sp. and *Candida* sp (Chima, *et al*., 1986, Tirumani and Samra, 2006).

1.1 Bacterial vulvovaginitis

Bacteria are the most common cause of vaginitis. The occurrence of bacterial vaginosis is difficult to determine, however, occurrence in females is about 41% (Tirumani and Samra, 2006). Bacterial vaginosis is not considered a sexually transmitted disease although it can be acquired by sexual intercourse. (Tirumani and Samra, 2006). Animals suffering from bacterial vaginosis have very high numbers of bacteria such as *Trichomonas fetus, Mycoplasma species*, *Lactobacillus species* and *Campylobacter species.* These bacteria can be found at numbers 100 to 1000 times greater than those found in the healthy vagina. (DaMassa, *et al;* 1986).

1.2: Viral Vulvovaginitis:

The recorded case of viral vulvovaginitis is the infectious pustular vulvovaginitis of cattle and it is caused by the same virus that causes infectious bovine rhinotracheitis viz *Bovine herpesvirus-1* (Blood and Rodostis, 1989). In general, the respiratory and vaginal infections behave as separate and distinct disease entities however, the syndromes may occur together in individual animals. Infectious pustular vulvovaginitis is an acute contagious disease characterized by inflammation, necrosis and pustule formation on the mucosa of the vulva and vagina (occasionally, similar lesions may occur on

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the skin of the penis and prepuce). The syndrome is frequently transmitted by coitus. Initially, there is hyperemia of the vaginal and vulval mucosa and focal hemorrhages in lymphoid follicles of the submucosa. Subsequently, pock-like lesions (2 to 3 mm in diameter) which are slightly elevated, pale, soft and friable, replace the hemorrhage over the lymphoid follicles. The affected epithelium becomes necrotic, ulcerates or erodes. Microscopically, intranuclear inclusion bodies may be observed in epithelial cells early in the disease. The disease usually subsides in 10-12 days (Tirumani and Samra, 2006).

1.3 Fungal vulvovaginitis

Candida species are most often implicated as the causative agents in the vulvovaginitis caused by fungi. This condition is also called "candida vulvovaginitis," "candidal vaginitis," "monilial infection," or ‘vaginal yeast infection’ and accounts for twenty to twenty-five (20-25%) percent of the vaginitis cases (Tirumani and Samra, 2006). In 80-90% of the cases, candida vulvovaginitis is caused by an overgrowth of the yeast *Candida albicans*. The remaining cases are caused by other species of *Candida*. It is not known what causes the yeast overgrowth, however, it has been postulated that antibiotics treatment can inadvertently kill normal bacteria in the vagina and cause an overgrowth of *candida* (Tirumani and Samra, 2006). Candida vulvovaginitis is not considered a sexually transmitted disease because *Candida* species are commonly found in the healthy vagina (Tirumani, 2006). Vaginal yeast infections tend to occur more frequently in females that are pregnant, (probably due to the release of estrogen which contains glycogen—a substrate on which candida thrives). It also occurs in diabetic, in excess dietary imbalance, in excess birth control pills and antibiotics intake (Tirumani and Leber, 2009).

1.4: Parasitic vulvovaginitis

Parasitic vulvovaginitis also sometimes called “trichomoniasis” or ‘trich’ accounts for 15-20% of the cases of vaginitis in females (human and animals) (Tirumani and Sumra, 2006). Trichomoniasis in sheep and goats is caused by the protozoan parasite *Trichomonas ovis*, the infection often results in abortion in the ewe and doe (Ferron *et al*; 1962). Trichomoniasis is a sexually transmitted disease. This means that the disease is passed from person-to-person or animal-

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to- animal only by sexual contact. Trichomoniasis occurs in both man and animals and is caused by an infection with the single-celled parasite *Trichomonas vaginalis* in man and *Trichomonas ovis* in goats (Ferron*, et al;* 1962, Tirumani and Leber, 2009).

1.5 Mycoplasmas

Mycoplasmas are bacteria associated with vulvovaginitis in goats (Cottew, 1984; DaMassa, *et al;* 1992). They belong to the Order Mycoplasmatales, Family Mycoplasmataceae and the Class Mollicutes. They have been associated with diseases in animals and human, resulting in economic losses, death, infertility, abortion and low birth weight and reduced litter sizes in animals (DaMassa, *et al;* 1992). Mycoplasmas are free-living, cell wall-less prokaryotes that are small in size (0.125 – 0.250 µm) and can pass through bacteriological filters (0.45µm), having unusually small genome with low Guanine and Cytocine (G+C) content ratio (23:40.) and show unusual nutritional needs. In place of a rigid cell wall is a triple - layered unit (peptidoglycan) membrane and this property makes the organism to be highly pleomorphic and completely resistant to penincillins (Okwoli, 2007).

Mycoplasmas reside primarily on the mucus surfaces of the respiratory and/or urogenital tracts but may cause invasive disease in immunocompromised hosts or under other special circumstances (Waites, 2002). Mycoplasmas have been isolated from wild cat (Hill, 1972), chamois (Nicolet & Freund, 1975), ground squirrel (Langford, 1977), hedgehog (Tan *et al.,* 1971), elephant (Clark *et al.,* 1980) and several species of wild birds (Koshimizu *et al.,*1978; Shimizu *et al.,* 1979). According to Chima, *et al;* (1986), *Mycoplasma capricolum, Mycoplasma mycoides subsp. mycoides* (small colony type), *Mycoplasma bovis, Mycoplasma mycoides subsp. capri, Mycoplasma arginini* have been associated with diseases of the genital tracts and are known to cause irritations, discharges and abortions. Other *Mycoplasma species* associated with diseases of the respiratory tracts include *Mycoplasma agalactiae, Mycoplasma arginini, Mycoplasma oculi, Mycoplasma mycoides subsp. capri*, (Waites *et al;* 2001). Some of these Mycoplasma organisms that have the oropharynx and respiratory tracts as their primary sites are occasionally found in the urogenital tracts due to oro-genital contact (Goulet *et al*, 1995: Taylor-Robinson and Furr, 1997). It is on record that mycoplasma was first isolated from cattle with contagious bovine pneumonia in France but was then called the pleuropneumonia organism (Nocard and Roux, 1898), however, other subsequent isolation of mycoplasma include: *Mycoplasma agalactiae* associated with granular vulvovaginitis in goats,

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(Cottew 1984), *Mycoplasma arginine* isolated from ovine keratoconjunctivitis (DaMassa, *et al*; 1992), and also from joints of septicemic goats (DaMassa 1992). *Mycoplasma capricolum* is associated with polyarthritis of goats (Cordy and Adler, 1960), Mastitis in cows (Taoudi and Kirchoff, 1986), from septicemic disease in alpine ibex (Schweighardt, *et al;* 1989) and from vulvar scabs of ewes in the United Kingdom (Jones *et al*, 1983).

1.6 Ureaplasmas

Ureaplasmas which are mycoplasmas possessing urease, were initially isolated from man (Howard and Pocock, 1983). Amongst human isolates a number of distinct serotypes exist which are at present included in a single species, *Ureaplasma urealyticum* (Shepard*, et al;* 1974). Ureaplasma isolates from cattle, (Taylor-Robinson, *et al.,* 1967), ovine (Livingstone, *et al.,* 1978) are also serologically heterogenous. Due to their minute size, distinctive characteristics and morphology of their agar colonies, these organisms were called T-(tiny) form PPLO (pleuropneumonia – like organisms) or T-mycoplasmas. *Ureaplasma urealyticum* (a human specie) is found in the genitals of women (Okwoli, 2007), *Ureaplasma diversum* is found in the genitals of cattle (Taylor-Robinson *et al,* 1978; LeGrand *et al,* 1995) and from cattle with pneumonia (Howard and Gourley, 1982), *Ureaplasma felimum* and *Ureaplasma cati* from the oral cavity of cats (Harasawa *et al*, 1990), *Ureaplasma gallorale* from chicken (Koshimizu *et al*, 1978) and *Ureaplasma diversumi* from the vagina of sheep with clinical vulvovaginitis (Doig and Ruhnke, 1977). The genital mycoplasmas are acquired mainly by adults through contamination of the environment especially their gazing places and by calves when they come in direct contact with the infected dam at birth, by drinking contaminated milk, nose-to-nose contact with other infected or carrier calves, by ingestion of the organism from contaminated nipples and buckets, and also through the environment (Leadley, 2010).

Consequent to their being common commensals of urogenital tracts of adult ewes and does, Mycoplasmas and Ureaplasmas are fast gaining recognition as important opportunistic pathogens associated with a range of animal disorders including:

- Granular vulvovaginitis in goats - *Mycoplasma agalactiae*,(Cottew ,1984),
- Ovine keratoconjunctivitis - *Mycoplasma arginine* (DaMassa, *et al;* 1992),

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- Polyarthritis of goats – *Mycoplasma capricolum* (Cordy and Adler, 1960),
- Mastitis in cows - *M. mycoides sub. mycoides, M. capricolum, M. arginini* and *M. bovis* were isolated from mastitis in cow (Taoudi and Kirchoff, 1986; Amosun. 2011; Ph.D thesis).
- Vulvar scabs of ewes *Mycoplasma agalactiae* (Jones *et al*,1983b),
- Genitals of cattle - *Ureaplasma diversum* (Taylor-Robinson *et al*, 1978; LeGrand *et al,* 1995),
- Pneumonia in Cattle – *M. bovis.* (Howard and Gourley, 1982),
- Oral cavity of cats - *Ureaplasma felimum* and *Ureaplasma cati* (Harasawa *et al*, 1990),
- Semen of Bull – *Mycoplasma capricolum* (Breard and Poumarat, 1988),
- Lungs of Chicken - *Ureaplasma gallorale* (Koshimizu *et al*, 1978),
- Respiratory disease in horses – *M. felis, M.subdolum* (wood, *et al;* 2007).
- Toxic epidermal necrolysis in calves - *Mycoplasma bovis* (Senturk, *et al*; 2012).

In Nigeria, few studies have reported cases of genital mycoplasmas in goats. A study by Chima *et al*, (1995) showed an increase in the prevalence of vulvovaginitis in sheep and goat in Nigeria. In an ealier study, Chima*, et al* (1986) characterized four different species from 55 mycoplasma strains isolated from sheep and goats viz: *Acholeplasma laidlawii* (5*), Mycoplasma agalactiae* (7), *Mycoplasma bovis* (2) and *Mycoplasma bovigenitalium* (38) but three (3) strains could not be classified. There is also less information as it relates to the characterization of the Mycoplasmas causing vulvovaginitis in goat in Nigeria (DaMassa, *et al;* 1992).
Vulvovaginitis is a fairly common condition among goats in some areas of Nigeria and studies carried out in sheep and goats have shown that *Mycoplasma agalactiae*, *Mycoplasma capricolum, Ureaplasamas*, and ovine/caprine group 11 *of Al-Aubaidi* (1972) have been isolated from clinical cases of vulvovaginitis (Chima *et al*, 1986).

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JUSTIFICATION OF STUDY

- Vulvovaginitis infection affects mating and breeding processes thus resulting in reduction in the population of goats in Nigeria, this invariably leads to economic losses such as :
  – loss of availability of leather and skin from Sokoto red, Kano brown and Bornu white breeds of goats.
  - decrease in protein supply from these animals.

- Consequence to the development of mycoplasmology all over the world, scientists in this field have been able to demonstrate that there is increase in the rate of mycoplasmal infection in livestock.

- Few studies have characterised the *Mycoplasmas* of goats in Nigeria.

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OBJECTIVES:

This study focuses on the isolation, identification and characterization of the *Mycoplasmas* involved in Vulvovaginitis in goats in Lagos State of Nigeria.

The intention therefore is:-

1. To isolate, identify and characterize the mycoplasmas associated with vulvovaginitis in goats using biochemical and serological assays.

2. To determine the antibiotic susceptibility patterns of the mollicutes and other bacteria isolated by applying commonly used antibiotics.

3. Confirmation of the identities of the mycoplasma isolates using molecular procedures (PCR).

4. Determination of the pathogenicity of identified isolates as causative agents of vulvovaginitis.

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CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Historical background

The Mycoplasma is a bacterial microorganism. It belongs to the class Mollicutes, in the Order Mycoplasmatales and Family Mycoplasmataceae. Before the 1930's, mycoplasmas were considered to be viruses because they are so small in size that they passed through filters of size 0.45µm which normally blocks the passage of ordinary bacteria (Razin, 1992). Indeed, mycoplasmas are small enough to penetrate the surface of agar medium and as a consequence they grow into the agar resulting in the classical "fried-egg"-type colonies seen when viewed microscopically (Taylor-Robinson, 1995). Mycoplasmas are free-living, cell wall-less prokaryotes. They have unusually small genome with low Guanine and Cytocine (G+C) content ratio (23:40) and show unusual nutritional needs. In place of a rigid cell wall there is a triple-layered peptidoglycan unit membrane and this property makes the organisms to be highly pleomorphic and completely resistant to the penicillins. Mycoplasma was first isolated from cattle in france with contagious bovine pneumonia and was then called the pleuropneumonia organism (Nocard and Roux, 1898). The mycoplasma organism was grown in broth containing little amount of rabbit serum contained in colloidal sacs inserted into the peritoneal cavity of rabbit. The growth of this organism was quite successful but did not help in studying their morphology. However, Dujardin-Beaumetz, 1900 (cited in Edward and Freundt, 1956), succeeded in growing the organism on solid medium and described the colony as having a dark centre and light periphery because the colonies grew down into the agar as a result of their small size. Bordet, 1910 and Borrel *et al*, 1910(cited in Edward and Freundt, 1956), gave detailed descriptions of the morphology and staining characteristics of mycoplasmas after subjecting the organisms to prolonged treatment with Giemsa stain.

Most researchers depended on their morphology and characteristics to evolve a nomenclature for mycoplasmas hence organisms with similar characteristics to the first isolate were generally called pleuropneumonia-like organisms (PPLO). However, the name *Astericiccus mycoides* was first given to the organism of bovine, named pleuropneumonia (Borrel *et al*, 1910). The generic name *Asterococcus* was rejected because it had previously been used to name a genus of algae. Another generic name *Borrelmyces* was suggested for these organisms (Turner, 1935). This name was later rejected because it was actually Bordet and not Borrel that first described the morphology of the

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organism (Sabin, 1941) and also because *Borrelmyces* suggests a relationship with fungi (Edward, 1955). The first valid generic name given to the organism was *Mycoplasma* (Nowak, 1929), and it was finally accepted because of all the suggested names only *Mycoplasma* drew attention to the plastic nature of the organism- a property generally accepted for the organism (Edward, 1955). Several Order names like Paramycetales or Anulomycetales (Sabin, 1941). Borrelmycetales (Turner, 1935); Mollicutales (Edward, 1954); Mycoplasmatales (Freundt, 1955) and Pleuropnemoniales (Tulusne and Brislou, 1955) with their corresponding family names- "ceae" were proposed. The Order Mycoplasmatales with family Mycoplasmayaceae was however approved. The name Mollicutes was proposed for the class of the order mycoplasmatale (Edward and Freundt, 1967), and has since been accepted.

Various forms of classification have been used to separate the order into different families and genera. Firstly, based on their dependence on sterol for growth, it was split into two families (ICSB, 1972);

- Mycoplasmataceae- dependent on sterol for growth
- Acholeplasmataceae- not dependent on sterol for growth.

This was later reviewed and the order was divided into three families:- Mycoplasmataceae, Acholeplasmataceae and Spiroplasmataceae with Thermoplasma and Anaeroplasma as genera of uncertain origin (ICSB,1979). In 1984, non-sterol requiring family Acholeplasmataceae was elevated to the ordinal rank of Acholeplasmatales (Freundt, 1984) and the anaerobic genus was also placed in a family Anaeroplasmataceae. Mycoplasmas have also been divided into two broad groups on the basis of their fermentation behaviours- fermenters and non-fermenters of glucose. The non-fermenters usually metabolize arginine or urea (Lin, 1985).

The term Mycoplasma is widely used to refer to any member of the class mollicutes and "mycoplasma" as a word was derived from the Greek words *mykes,* for fungus and *plasma,* for something formed or molded (Hardison, 1997). Razin *et al, (*1998) reported more than 180 species in the class mollicutes which comprises 4 orders namely Mycoplasmatales, Entomoplasmatales, Acholeplasmatales and Anaeroplasmatales. It also comprises of 5 families – Mycoplasmataceae, Entomoplasmataceae, Spiroplasmataceae, Acholeplasmataceae and Anaeroplasmataceae.

Mollicutes are very small organisms ranging in size from 125 – 250nm. Brooks *et al,* (1996) reported that the smallest genome size of mollicutes is a little more than twice the genome size of

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certain large viruses and Razin and Freundt (1984) reported their ability to pass through membrane filters of 450nm pore diameter. Mollicutes are models for describing the minimum metabolism necessary to sustain independent life because of their size and apparently limited metabolic potential (Pollack *et al*, 1997). They resemble larger viruses in size but can be grown (although slowly) on artificial media that is highly enriched with nucleic acids, serum and yeast extract. The mean generation time for many mycoplasmas including ureaplasmas is 1 to 3 hours and for some 1 to 6 hours and 10 to 14 days to form colonies (Razin & Freundt, 1984). The colony size of mycoplasma is very small with a peculiar "fried - egg" colony shape. The mollicutes cannot be studied by the usual bacteriologic methods because of the small size of their colonies, the plasticity / flexibility and delicacy of their individual cells and their poor staining with aniline dyes (Brooks *et al*, 1996). Mollicutes that are incriminated in disorders of animals are only from the family of Mycoplasmataceae and from the two genera – *Mycoplasma* and *Ureaplasma*. With rare exception, mycoplasma species produce diseases that are of multi-factorial origin. The syndrome produced depends on environmental conditions, the genetic predisposition of the host animal and to a lesser extent the nature of the infecting microbe (Okwoli, 2007).

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2.2 Taxonomy of the Class *Mollicutes*

The taxonomic position of the class *Mollicutes* and their major characteristics as reported by Oshima and Nishida (September 2007) is as shown below inclusive of table 1:

Order: *Mycoplasmatales*

Family: *Mycoplasmataceae*

Genus: *Mycoplasma* (102 recognized species) Genus: *Ureaplasma* (6 recognized species).

Order: *Entomoplasmatales*

Family: *Entomoplasmataceae*

Genus: *Entomoplasma* (5 recognized species) Genus: *Mesoplasma* (12 recognized species)

Family: *Spiroplasmataceae*

Genus: *Spiroplasma* (33 recognized species)

Order: *Acholeplasmatales* Family: *Acholeplasmataceae*

Genus: *Acholeplasma* (13 recognized species)

Order: *Anaeroplasmatales* Family: *Anaeroplasmataceae*

Genus: *Anaeroplasma* (4 recognized species) Genus: *Asteroleplasma* (1 recognized species)

Undefined Taxonomic Status: Phytoplasma- The defining phenotype of members of the Class Mollicutes is the absence of any type of cell wall structure. Cell wall genes appear to have been lost during the degenerate evolution of mycoplasmas from Gram-positive eubacteria, and mycoplasmas are bounded only by a single cell membrane.

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Table 1. Properties of the Class Mollicutes

Genus	Effect of Oxygen	Cholesterol Required	Genome Size	Habitat
Acholeplasma	Facultative anaerobe	No	1,5001,650kb	Animals, some plants and insects
Anaeroplasma	Obligate anaerobe	Yes	1,5001,600kb	Bovine and ovine rumens
Asteroleplasma	Obligate anaerobe	No	1,500kb	Bovine and ovine rumens
Entomoplasma	Facultative anaerobe	Yes	790-1,140kb	Plants and insects
Mesoplasma	Facultative anaerobe	No	870-1,100kb	Plant and insects
Mycoplasma	Facultative anaerobe	Yes	580-1,350kb	Animals and humans
Phytoplasma	--	--	640-1,185[a]	Plants and insects
Spiroplasma	Facultative anaerobe	Yes	780-2,200kb	Plants and insects
Ureaplasma	Facultative Anaerobe	Yes	760-1,170kb	Animals and humans

These genome sizes are based on DNAs in phytoplasma-infected plant and insect tissues
Source: Oshima and Nishida (September 2007).

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The medical and agricultural importance of members of the genus *Mycoplasma* and related genera has led to the extensive cataloging of many of these organisms by culture, serology and small sub-unit rRNA gene and whole genome sequencing. A focus in the sub-discipline of molecular phylogenetics has both clarified and confused certain aspects of the organization of the class *Mollicutes* (Johansson and Petterson, 2002). Originally the popular name "mycoplasmas" has commonly denoted all members of the class Mollicutes. The name "Mollicutes" is derived from the Latin mollis (soft) and cutes (skin) (Johansson and Petterson, 2002), and all of these bacteria do lack a cell wall and the genetic capability to synthesize peptidoglycan. Now Mycoplasma is a genus in Mollicutes. Despite the lack of a cell wall, many taxonomists have classified *Mycoplasma* and it's relatives in the phylum *Firmicutes*, consisting of low G+C Gram-positive bacteria such as *Clostridium, Lactobacillus*, and *Streptococcus* based on 16S rRNA gene analysis (Johansson and Petterson, 2002). The order *Mycoplasmatales* contains a single family, Mycoplasmataceae, comprising two genera: *Mycoplasma* and *Ureaplasma*. Historically, the description of a bacterium lacking a cell wall was sufficient to classify it to the genus *Mycoplasma* and as such it is the oldest and largest genus of the class with about half of the class' species (107 validly described), each usually limited to a specific host and with many hosts harboring more than one species, some pathogenic and some commensal (Oshima and Nishida, 2007). A limiting criterion for inclusion within the genus *Mycoplasma* is that the organism has a vertebrate host. The type species, *M. mycoides*, along with other significant mycoplasma species like *M. capricolum*, is evolutionarily more closely related to the genus *Spiroplasma* in the order *Entomoplasmatales* than to the other members of the *Mycoplasma* genus. This and other discrepancies will likely remain unresolved because of the extreme confusion that change could engender among the medical and agricultural communities. A study of 143 genes in 15 species of *Mycoplasma* suggests that the genus can be grouped into four clades: the *M. hyopneumoniae* group, the *M. mycoides* group, the *M. pneumoniae* group and a *Bacillus-Phytoplasma* group (Oshima and Nishida, 2007). The *M. hyopneumoniae* group is more closely related to the *M. pneumoniae* group than the *M. mycoides* group.

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2.3 Animal mycoplasmas

Mycoplasmas often times have been isolated from domestic and laboratory animals and they are important economically in agricultural and in biomedical research (Simecka, 1992). Mycoplasmal infections are often associated with diseases of the lungs, genitourinary tract, joints and other tissues including the epidermis (skin) (Senturk, *et al*; 2012). These diseases have been shown to cause significant disease problems in commercial and experimental animals. One of the most economically important mycoplasmal diseases in goats is contagious caprinepleuropneumonia (CCPP) often caused by *Mycoplasma capricolum* subsp. *capripneumoniae* (MacOwan and Minette, 1979). Other reported causative agents of CCPP include *Mycoplasma mycoides subsp. capri* (Ojo, 1976.), *Mycoplasma mycoides* subsp. *mycoides* (villalba *et al*; 1992) and *Mycoplasma capricolum* (Da Massa, *et al*; 1992.). In addition, Ikheloa *et al*; (2004) isolated *Mycoplasma arginini, Mycoplasma bovis* and *Mycoplasma capricolum* from the pneumonic lungs of goats in Nigeria.

Vulvovaginitis is a fairly common condition among goats in some areas of Nigeria and studies carried out in sheep and goats have shown that *Mycoplasma agalactiae*, *Mycoplasma capricolum, Ureaplasamas*, and ovine/caprine group 11 *of Al-Aubaidi* (1972) have been isolated from clinical cases of vulvovaginitis (Chima *et al*, 1986). Mastitis due to mycoplasma has been reported virtually in all mammals but it is of greatest economic importance in cows, goats and sheep (Bushnell, 1984). *Mycoplasmas bovis* is the most common species of mycoplasma associated with mastitis in dairy cattle (Moroney, 2007). Other causes of bovine mastitis include *Mycoplasma bovigenitalium, Mycoplasma bovirhinis, Mycoplasma califonicum, Mycoplasma canadense* and *Mycoplasma dispar* (Goulay, 1981; Jasper *et al.,* 1987 and Gonzalez *et al*., 1988). Mycoplasma mastitis in sheep and goats is usually caused by *Mycoplasma agalactiae* but other mycoplasmas such as *Mycoplasma capricolum* and *Mycoplasma mycoides subsp mycoides* (LC) are also incriminated (Cottew, 1984). *Mycoplasma bovis* was isolated from cases of Toxic epidermal necrolysis in three 2 months old calves from their joint lesions, tracheobronchial aspiration fluid and skin samples (Senturk, *et al*; 2012). Various isolations of mycoplasmal organisms made from the genital tract of animals suggests that they could cause very harmful effects, for instance Tortschanoff, *et al;* (2002) reported that *Mycoplasma subdolum* play a significant role in the genital disorder of horses, Chima, *et al;* (1995) isolated *Mycoplasma bovigenitalium* and *Mycoplasma arginini* from the vaginal tract of an infertile

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sheep with mucopurulent vaginal discharge in Nigeria and Sweta, *et al;* (2002) reported that besides clinically recognisable genital infections in diseased goats, latent colonization of the genital tract in asymptomatic carriers is often observed. The diseases caused by *Mycoplasma species* may lead to economic loss in the livestock industry in particular and losses to the nation's foreign exchange in general.

2.4 Plants and insects mycoplasmas

Plant pathogenic mycoplasmas were discovered by electron microscopy in 1967, long after the discovery and culture in 1898 of the first pathogenic mycoplasma of animal origin - *Mycoplasma mycoides* (Razin, 1978; Bove 1981). Plant pathogenic mycoplasmas are responsible for several hundred diseases and they are in two genera: the *phytoplasmas* and the *spiroplasmas* (Bove 1981). These two genera contain microbes which have a dual host cycle in which they can replicate in their leafhopper or psyllid insect vectors as well as in the sieve tubes of their plant hosts (Gasparich, 2010). Major distinctions between the two genera are that most spiroplasmas are cultivable in nutrient rich media, possess a very characteristic helical morphology, and they are motile, while the phytoplasmas are resistant to in-vitro cultivation and exhibit a pleiomorphic or filamentous shape (Gasparich, 2010). The phytoplasmas represent the largest group of plant pathogenic Mollicutes. Only three plant pathogenic spiroplasmas are known today. *Spiroplasma citri*, the agent of citrus stubborn was discovered and cultured in 1970 and shown to be helical and motile (Mello*, et al*; 2006). *Spiroplasma kunkelii* is the causal agent of corn stunt (Casati, *et al*; 2006). *Spiroplasma phoeniceum*, responsible for periwinkle yellows, was discovered in Syria (Garnier*, et al;* 2001). There are many other spiroplasmas associated with insects and ticks. Plant pathogenic mycoplasmas are restricted to the phloem sieve tubes which circulates the photosynthetically-enriched sap, the food for many phloem-feeding insects (aphids, leafhoppers, psyllids, etc.). Interestingly, phytopathogenic mycoplasmas are very specifically transmitted by leafhoppers or psyllid species (Garnier, *et al;* 2001).

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Table 2: Mycoplasmas of Goats and Sheep: Summary of the infections with associated diseases, pathogenecities and geographical distribution of the more important species.

Mycoplasmas	Hosts	Sites infected commonly	Others	Disease asdociated	Pathogenecity	Reported occurences	Remarks
M. agalactiae	S,G	Udder	Joints,eyes, lungs ug tract	Arthritis, keratoconjunctivitis, granular vulvovaginitis	P	France, Greece Italy, spain USA,	Affects both S& g, Live and dead vaccines used
M. arginini	S,G and many others	Resp. tract	Ug tract, joints and eyes	Pneumonia, arthritis, vulvovaginitis	P	Ubiquitos	Not pathogenic per see in resp. tract
M. caprocolum	S,G	Joints	Udder, lungs ug tract	Severe polyarthrutus, mastitis, pneumonia	P	Austria, france, india, spain, UK,USA, Zimbabwe	True importance of this virulent organism not known
F38 species	G	Resp. tract	Internal organs due to septicemia spread	CCPP	P	Kenya, Sudan	F38 IS associated with and experimentally replaces classical CCPP.
M. conjunctinae	S, G, chamose	Eyes	Resp tract occationally	Keratoconjunctivitis	IP	Probably ubiquitos	Pathogenic for both S and G
M.m. Capri	G, rare in sheep, calves	Resp. tract	Udder, joints, eyes	CCPP, Agalactiae, polyarthritis, keratoconjunctivitis	P	France, Greece, Italy, Portugal Africa.	Respiratory form seen more in kids
M. m. mycoides	G, but mainly adults	Resp. tract	Joints	CCPP-like disease, polyarthritis	P	Africa, New Guinea	Causative agent of CCPP,
M.ovopneumonia	S,G	Resp, tract	Eyes, ug tract	Chronic pneumonia	lp	Probably ubiquitos	Predisposing agent for P. hemolytica infection in sheep
M. putrifaciens	G	Udder	Ears	Mastitis	lp	Austrlai, france, USA.	Full importance not yet known
A.laidlawii	Many	Ug tract	Resp. tract	None		ubiquitos	Any association with disease due to secondary infection
Oculi	G,S, Pigs, horses, cattle	Eyes	Ug tract, lungs	keratoconjunctivitis	lp	India, Japan UK, USA.	Early experimental evidenceof severe pathogenecity
Ureaplasmas	S,G	Ug tract	Very rarely uterine disease	Vulv ovaginitis	lp	Probably ubiquitos	At least 3 serotypes are known

KEY:
CCPP-Contagious caprine pleuropneumonia
Lp—lightly pathogenic
Resp--- Respiratory
G- goats, S-sheep,
ug--urogenital
P--pathogenic

SOURCE: DaMassa, *et al*; 1992: Mycoplasma of goats and sheep.

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2.5. *Mycoplasma* species

2.5.1 *Mycoplasma agalactiae*

This important mycoplasma causes natural disease in both goats and sheep. It occurs primarily in Medi-terranean countries but is also reported from many other areas of the world, (Jones, 1987). The agent has been noted on only 2 occasions in the United States (Jasper and Dellinger, 1979). *Mycoplasma agalactiae* disease is often described as "contagious agalactia of sheep and goats," a particularly unfortunate designation because:

1) There is no compelling evidence indicating that the disease is particularly contagious among mature goats or sheep.

2) The term "agalactia" suggests that only the female is affected when actually both sexes are susceptible.

3) Many authors now include other mycoplasmas as causes of "contagious agalactia," i.e., *Mycoplasma capricolum, Mycoplasma mycoides* ssp. *capri, Mycoplasma mycoides* ssp. *mycoides* (caprine or large-colony biotype), and *Mycoplasma putrefaciens,* because all can produce mastitis leading to agalactia (DaMassa, *et al*; 1992).

The disease caused by *Mycoplasma agalactiae* is of considerable economic importance because of high morbidity rather than high mortality. Clinical disease can be unapparent, mild, acute, or chronic, (Cottew, 1985). In the female, acute clinical signs often are first noticed after freshening at the beginning of lactation, (Zavagli, 1951). Usually, there is general malaise, fever, and mastitis that lead to decreased milk yield and agalactia. Large numbers of the organism can be shed in the milk, and the blood can contain the organism for a short time. Severe keratoconjunctivitis may develop. Often, the organism settles in the joints, causing arthritis that may involve one or several joints (polyarthritis). Although experimental evidence is lacking, suckling kids may acquire the infection by ingesting colostrum or milk containing the organism. The dam can easily become infected by the introduction of even a few organisms into the teat canal either as a result of unsanitary procedures either in the milking parlor or during hand milking.

Similar infections of the udder via the teat canal occur with *Mycoplasma capricolum* (DaMassa, *et al,* 1984) and with caprine strains of *Mycoplasma mycoides* ssp. *mycoides* and *Mycoplasma putrefaciens (*DaMassa and Porter, 1987). *Mycoplasma agalactiae* has also been associated with cases of granular vulvovaginitis in goats (Singh, *et al*, 1975). Lung lesions are not a normal feature

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of *Mycoplasma agalactiae* infection, although outbreaks of pleurisy among goats from which the mycoplasma was isolated have been reported (Cottew, 1979).

2.5.2 Mycoplasma arginini

This mycoplasma occurs in goats and sheep and can be isolated from various anatomical sites of other hosts (Jones, *et al*, 1983b). Most often the organism is considered non-pathogenic. Jones, (1985), observed that, *M. arginini* was not mastitogenic when injected into the lactating mammary gland of goats, but it did persist in sheep udders at high titer for at least 9 days, causing lacteal neutrophilia without alteration in milk consistency and/or appearance. *Mycoplasma arginini* has been isolated from cases of ovine keratoconjunctivitis (Leach, 1970). Cottew, 1979, reported this organism's frequent occurrence in pneumonic sheep lungs, mouth, and esophagus. *Mycoplasma arginini* has also been isolated from the joints of goats dying of experimental septicemia caused by either *M. mycoides* ssp. *mycoides* (caprine or large-colony biotypes) or *M. capricolum* (DaMassa, *et al;* 1992).

2.5.3 Mycoplasma bovirhini

Little information is available concerning the isolation of *M. bovirhinis* from goats. Cottew, (1970) however, reported the isolation of this organism from goats on at least one occasion, which represents the only known isolation of *M. bovirhinis* from goats.

2.5.4 Mycoplasma bovis

This mycoplasma has occasionally been isolated from the lungs of goats. Ojo and Ikede, (1976), recovered from a diseased caprine lung in Nigeria, an isolate representing *Mycoplasma agalactiae* ssp. *bovis.* This mycoplasma has since been elevated to species rank and is now known as *Mycoplasma bovis.* Since *Mycoplasma bovis* is often associated with mastitis in cattle, a study was carried out by Ojo, (1976) to examine the pathogenicity of that agent following its introduction into the teat canal of lactating goats. Pyrexia was evident by the third day postinoculation, and the infected gland was swollen, firm, and pale. By the fifth day postinoculation, the gland had increased in size to about 4 times normal. Abnormal milk secretion, showing occasional clots, was evident by the first

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day \postinoculation; milk changed to a yellow serous fluid after 3-5 days. Counts of the mycoplasma attained a titer of 4 x 10^7 colony-forming units (CFU)/ml of milk. Histologically, a purulent exudate was found in the lactiferous duct and sinus and in the interlobular ducts.The epithelial lining remained intact, but neutrophils and macrophages were seen in the lamina propia of the lactiferous sinus and in the connective tissue around the interlobular ducts and within the alveoli. No appreciable systemic reaction (other than pyrexia) was noticed, and the mycoplasma was not recovered from other sites at necropsy five days postinoculation. DaMassa, *et al;* (1992) asserted that *Mycoplasma bovis* has been identified on three occasions from the lungs of goats at necropsy. *Mycoplasma bovis* has recently been isolated from Toxic epidermal necrolysis cases from three 2 months old calves in Turkey (Senturk*, et al;* 2012). Isolates of *M. bovis* were gotten from the bacteriological cultures of the joint, tracheobronchial aspiration fluid and skin samples of the infected calves.

2.5.5 Mycoplasma capricolum

Mycoplasma capricolum is primarily a goat pathogen but has also been encountered in sheep, cows, and alpine ibex *(Capra ibex ibex)* (DaMassa, *et al;* 1992). In goats, *M. capricolum* is highly destructive, causing high morbidity and mortality. It was described first as a virulent form of "PPLO" from kids in California, (Cordy, *et al,* 1955), the primary clinical sign was a severe arthritis (polyarthritis) affecting nearly every diarthrodial joint. Cordy and Adler, (1960) parenterally inoculated this organism and caused high morbidity and mortality in sheep and pigs used. The agent was later named *M. capricolum (*Tully*, et al*, 1974). *Mycoplasma capricolum* causes acute to peracute disease when inoculated parenterally (Taoudi, *et al*, 1987) or when given orally (DaMassa*, et al*, 1984). The organism initiates a definite, progressive pathologic process best described as septicaemia with severe joint localization (Cordy, *et al;* 1955). Pyrexia is a cardinal sign in kids but may be of short duration and go unnoticed in adults. Hot, swollen, painful joints can occur within 3 days of exposure, causing inability to stand. Upon necropsy, the lymph nodes of kids dying of acute disease are swollen and diffusely reddened and may show discrete hemorrhages. In chronic cases, joints may be only moderately enlarged, moist, firm, and infrequently reddened. Epicardial, myocardial and endocardial petechiae and fibrinous pericarditis and peritonitis can be present. Fibrin deposits can also be found in the scrotal cavity and covering the liver. Prominent lung changes are not

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a common feature of outbreaks, but reports describe collapsed lungs with a rubbery consistency and patchy, depressed, red areas of consolidation have been reported (DaMassa, *et al*, 1984). Fibrinous adhesions between lung lobes can occur. Kidneys may show medullary edema, hyperemia, pink amorphous material in the tubules, and a degeneration of the convoluted tubules. Hepatic necrosis, conjunctivitis, and/or keratoconjunctivitis have been observed. Cellulitis is common at the site of experimental inoculation. In all but acute cases, severe fibrinopurulent polyarthritis affects nearly all joints, which are surrounded by congestion and edema. Fibrin masses can be found within the joints, and hemorrhagic erosions of the articular cartilages can occur. In a study by DaMassa, *et al,* 1987b, 37 young kids acquired a lethal *M. capricolum* infection by the ingestion of 1 x 10^5 CFU/ml in the milk, and kids in close confinement with infected penmates also quickly acquired the infection. Septicemia (mycoplasmemia) was present as early as 24 hours after oral exposure, and the blood of all kids was positive for *M. capricolum* by the fifth day postinoculation, with counts as high as 1 x 10^6 CFU/ ml. DaMassa, *et al,* (1991) in a latter study, found out that the primary histologic change was fluid leakage from the vasculature, with moderate –to- marked leukocyte exudation. All kids had diffuse interstitial pneumonia, with high-protein edema fluid in the alveoli and diffuse monocyte-macrophage accumulation in the alveolar walls and alveoli.

Many kids had histiocytic meningitis over most areas of the brain, and one kid had an acute vessel wall necrosis. Spleens had moderate necrosis of sinusoidal cells. Fibrin and neutrophil exudation were present in the joint cavity, and necrosis of the synovial lining and foci of coagulative necrosis in the collagenous stroma surrounding the joints were found. Diffuse neutrophilic infiltration was found in the hepatic sinusoids and in the glomeruli. Two kids showed fibrinous or fibrinleukocyte thrombi in the lungs, liver, and meningeal vessels.

Experimentally, mastitis is easily produced by the introduction of *M. capricolum* into the teat canal, which can lead to agalactia and death (DaMassa, *et al*, 1984). As many as 1 x 10^8 CFU/ml can be shed in the milk, and the infection spreads from 1 udder half to the other. Udders harden, and the supramammary lymph nodes become enlarged. Does that receive small numbers of the organism into the teat may become mastitic but not agalactic and can shed small numbers of the organism for about 4 months. The *M. capricolum* -infected udder contains a pronounced neutrophilic exudate within the ductal system, and there is a progressive epithelial cell necrosis of the acini (DaMassa*, et al*; 1984). DaMassa and Porter, (1987), later observed that there was interstitial pneumonia, acute

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fibrinopurulent arthritis, and vascular thrombosis in various organs. Thrombi, composed of fibrin or fibrin and leukocytes, were seen in adrenal glands, heart, meninges, lung, liver, and spleen. Unlike kids, no menningitis was observed in adults. Two goats had neutrophilic epicarditis, with extension into the heart muscle. Glomerulonephritis was present in most adult goats. Necrosis of lymphoid follicles and multifocal necrosis of the sinusoids of the spleen were noted in one animal.

Epidemiologically, many adult does probably become infected by the infusion of the organism into the teat canal, probably because of unsanitary milking procedures either in the milking parlor or during hand milking (as with caprine strains of *Mycoplasma mycoides* ssp. *mycoides* and *Mycoplasma putrefaciens)(*DaMassa and Porter, 1987*).* Horizontal transmission of *Mycoplasma capricolum* among adult goats has not been demonstrated, although young kids readily acquire the infection from diseased parents. Other than the 1955 epidemic in kids, (Cordy, *et al*, 1955), only sporadic outbreaks of *M. capricolum* disease has occurred. Also, an organism that most probably represents *M. capricolum* or an *M.capricolum-* like mycoplasma was responsible for an incidence of severe mastitis in the Central Valley of California that resulted either in death or culling of about 200 lactating does (DaMassa, *et al*; 1992). In that outbreak, kids were not affected, probably because they were fed with heat-treated colostrum or milk replacer. Other outbreaks of natural disease attributable to *Mycoplasma capricolum* include diseases in sheep in Zimbabwe (Swanepoel, *et al*, 1977) and a septicemic disease in lambs in the United States that was initiated by an organism closely related to *Mycoplasma capricolum (*DaMassa*, et al;* 1987a). This agent has also been isolated from cases of mastitis in cows (Taoudi and Kirchoff, 1986), from bull semen, (Bread and Poumarat, 1988) from septicemic disease in alpine ibex (Schweighardt, *et al*, 1989), and also from vulvar scabs of ewes in the United Kingdom (Jones, *et al,* 1983). *Mycoplasma capricolum* is a heterogeneous mycoplasma and antisera to several isolates may be necessary to identify all the strains. In the United States, isolates similar to the type strain (California Kid) of *Mycoplasma capricolum* are now considered unusual mycoplasmas and are being replaced by serologic types such as *Mycoplasma* sp.GM262G (DaMassa, *et al;* 1992).

However, strain GM262G is considered a strain of *M. capricolum,* based on:

1) Its weak reactions with polyclonal antiserum to the type strain of *Mycoplasma capricolum,*

2) A definite but delayed ability (4-6 weeks) to hydrolyze arginine, and

3) Possession of the enzyme or-nithine transcarbamylase.

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Isolates similar to strain GM262G cannot often be identified serologically using polyclonal antisera to the type strain of *M. capricolum* but do respond variably with polyclonal antiserum to *Mycoplasma* sp. F38. *Mycoplasma capricolum* and *Mycoplasma* sp. F38 share a DNA homology of about 80% (Christinsen and Erno*,* 1982).

2.5.6 Mycoplasma conjunctivae

Mycoplasma conjunctivae causes caprine and ovine conjunctivitis/keratoconjunctivitis and can be isolated frequently from the eye and the nasopharynx (Barille, 1973). Hosts infected with this agent show lacrimation, conjunctival hyperemia, pannus, neovascularization, iritis, and keratitis (Cottew, 1979). Chamois can also become infected and show more severe clinical symptoms, leading to total blindness (Nicolet and Freundt, 1975). The disease is usually mild, lasting upto one week or, in more severe cases, almost a month. Laboratory experiments show some cases of keratoconjunctivitis presumably caused by *Mycoplasma conjunctivae,* which was isolated from the eyes of both goats and sheep that recovered without treatment (DaMassa, *et al;* 1992). In an experimental study, cloned cultures of *Mycoplasma conjunctivae* given subconjunctivally caused injection of the vessels of the bulbar and palpebral conjunctiva and increased lacrimation (Trotter, *et al,* 1977). The clinical disease was similar to natural outbreaks among goats. Clinical signs were generally moderate and transient and recurred in some sheep. Grossly, there was increased lacrimation, an increase of lymph follicles of the third eyelid, hyperemia of the conjunctiva, and corneal opacity. In some cases, a rim of vessels was present that extended from the limbus into the cornea. The microscopic lesions included conjunctivitis of the upper, lower, and third eyelids characterized by extensive subepithelial infiltration of mononuclear cells, hyperemia, endothelial swelling, and a greater than average number and size of lymph follicles.

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2.5.7 Mycoplasma gallinarum

Mycoplasma gallinarum has been isolated at least once from sheep and goats (Cottew, 1979). *M. gallinarum* has been isolated twice from goat placentas recovered in the barnyard in the United States of America (DaMassa, *et al;* 1992). The significance of these isolations is unknown, because the mycoplasma may have been acquired by contamination from fowls.

2.5.8 Mycoplasma mycoides ssp. capri

This mycoplasma, long believed to be the etiologic agent of contagious caprine pleuropneumonia (CCPP), is now considered by many investigators to be either unusual or rare, although another report (Jones and Wood, 1988) revealed that the agent may be prevalent in Oman. The type strain (PG3) of *M. mycoides* ssp. c*apri* is serologically distinct from *M. mycoides* ssp. *mycoides* (large colony strains Y and GM12 and small-colony types such as PG1). High-resolution, 2-dimensional gel electrophoresis has shown, however, that strains of *M. mycoides* ssp. *capri* are more closely related to large-colony types than to small-colony types of *Mycoplasma mycoides* ssp. m*ycoides (* Rodwell and Rodwell, 1978). Significant cross-reactions between some strains of *M. mycoides* ssp. *capri* and *M. mycoides* ssp. *mycoides* can occur with some antisera, but some strains of *M. mycoides* from goats are difficult to assign to either subspecies because they share serological properties with both species.

Progressively, more information is available concerning the pathogenicity of *Mycoplasma mycoides* ssp. *capri,* both from natural and experimental cases. Agnello*, et al;* (2012) isolated *Mycoplasma mycoides ssp capri* from severe outbreaks of polyarthritis in kids which were characterized by lethargy, inability to move, inappetance, septicaemia and respiratory disease in Sicily, Italy. *Mycoplasma mycoides* ssp. *capri* given endobronchially initiated a pleuropneumonia in goats that closely resembled the disease initiated by caprine strains of *M.m.* ssp. *mycoides* (Ojo, 1976*).* Infected animals developed high fever and were unable to eat. Gross lesions were confined to the lungs, pleura, and pericardium. Lung lobes showed various degrees and stages of hepatization, with dilated interlobular septa. The pleura of the affected lung was often covered with fibrin and adhered to the diaphragm, chest wall, and pericardium. Excess thoracic fluid and fibrinous pericarditis was common. The associated lymph nodes, especially the bronchial, mediastinal,

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mandibular, and retropharyngeal, were enlarged, edematous, and hemorrhagic. The important histopathologic lesions included edema of the lung, congested alveolar septa, acute pyogenic bronchopneumonia, and acute purulent pleurisy. *Mycoplasma mycoides* ssp. *capri* has also been isolated from natural cases of caprine mastitis (Perreau*, et al;* 1972). Misri, *et al*, (1988) experimentally inoculated the organism into the teat canal of goats and this resulted initially in decreased milk production and progressed to an agalactic syndrome. At necropsy, there was purulent material in the inoculated udder side, and the supramammary lymph node on the affected side was enlarged. Initial histopathologic changes included a diffuse purulent mastitis with marked infiltration of neutrophils within the luminal acini. These lesions progressed to a chronic interstitial mastitis with atrophy of the glandular parenchyma and subsequent replacement by fibrosis. As with infections caused by *M. putrefaciens,* the organism did not spread to the opposite half of udder. Body temperature and appetite remained normal. In another study (Ojo, 1976), Nigerian strain goats infected with *M. mycoides.* ssp. c*apri* did not develop either arthritis or mastitis.

2.5.9 Mycoplasma mycoides ssp. mycoides (large-colony or caprine biotypes)

Mycoplasma mycoides ssp. *mycoides* is best known as the causative agent of contagious bovine pleuropneumonia (CBPP), a highly destructive disease of cattle that was eradicated from the United States in 1892 (DaMassa*, et al;* 1992). Cottew and Yeats, (1978), asserted that mycoplasmas of goat origin serologically indistinguishable from the bovine type of *M. m.* ssp. *mycoides* (strain PG1) have caused severe disease in goats. Based principally on morphologic and cultural characteristics *Mycoplasma mycoides* ssp. *mycoides* is divided into large colony (LC) and small-colony (SC) types. In general, LC types (also called caprine biotypes by some authors) have more robust cultural characteristics than do SC (bovine) biotypes. LC types grow to a significantly larger diameter on agar, grow more extensively in liquid broth, rapidly digest inspissated serum and casein more rapidly, and survive for a longer period at 45°C than do SC types (Cottew and Yeats, 1978). According to Valdivieso-Garcia and Rosendal, (1982), exceptions do occur but these parameters have been useful for separating the small colony from the large colony types. The small colony types of *M. mycoides* ssp. *mycoides* are almost exclusively found in bovines. On 3 occasions, however, SC types designated strains O, P, and Vom have been recovered from goats in New Guinea, Sudan, and Nigeria

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respectively (Cottew and Yeats, 1978, Cottew, 1979). Conversely, LC types are almost exclusively found in goats but have been recovered from cattle in Australia (Cottew and Yeats, 1978) France (Perreau and Bind, 1981) and India (Kapoor*, et al*, 1989). In one instance, this mycoplasma has been associated with natural disease in sheep in Nigeria (Okoh and Ocholi*,* 1986). *Mycoplasma mycoides* ssp. *mycoides* is a member of the *Mycoplasma mycoides* cluster, a group of mycoplasmas that are notable pathogens of cattle, goats, and sheep and that share serologic, genomic, and antigenic characteristics (Cottew and Yeats*,* 1987). Six mycoplasmas make up the *M. mycoides* cluster: *M. m.* ssp. *mycoides* (LC), *M. m.* ssp. *mycoides (SC), M. m.* ssp. *capri, M. capricolum,* and two undetermined mycoplasmas designated bovine serogroup 7 and *Mycoplasma* sp. F38. These six organisms currently pose the most prominent taxonomic problems within the genus *Mycoplasma.* Caprine strains of *M. m.* ssp. *mycoides* are the dominant disease-producing mycoplasmas affecting milk production in goats worldwide. These strains are particularly important and are widespread pathogens in the goat population of the United States and many other countries (DaMassa, *et al;* 1992).

Ojo, (1976) observed that the gross and histological lung lesions in goats caused by caprine strains of *M. mycoides* ssp. m*ycoides* were remarkably similar to those caused by *M mycoides* ssp. *capri.* In other studies, pulmonary changes depended on the route of inoculation of the agent, it causes CCPP when given endobronchially, but causing only pleuritis, mild interstitial pneumonia, and pulmonary edema when given intravenously (Kassali and Ojo, 1981). In goats infected endobronchially with a Canadian strain of *M. m.* ssp. *mycoides,* all the six animals died due to mycoplasmal septicemia within one week of exposure. At necropsy, there was cellulitis in the ventral neck region and pulmonary edema, and in 4 goats there was excess serosanguinous fluid in the pleural cavity. All goats had acute pneumonia that ranged from localized areas in the hilar region to scattered areas 2 x 2 cm in all lobes. Fibrin was seen covering areas of the lung. One goat had pale and distended interlobular septa. Hyperemic and edematous pulmonary lymph nodes were found in all goats. Excess turbid synovial fluid was present in the carpal and hock joints of most goats (Rosendal, 1983). Histologically, all goats showed congested and edematous lungs, and early stages of necrosis were observed in the pneumonic tissue. Proteinaceous fluid, exfoliated cells, macrophages, and neutrophils were seen in the alveolar spaces. The lungs of two goats had arterial and arteriolar vasculitis with necrosis of the vessel wall and thrombosis. In one goat, there was

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necrosis of the interlobular septa, and in 4 goats there was necrosis in the periarteriolar lymphatic tissue in the spleen. One goat had focal necrosis in the adrenal cortex.

In many areas of the world where goats milk are kept, mastitis and arthritis initiated by *M. m.* ssp. *mycoides* are common (DaMassa, *et al* 1986; Rapoport and Bar-Moshe*,* 1984). Some strains of this mycoplasma that are known to produce arthritis and mastitis in European and North American milk goats, do not however, elicit those manifestations in African goat breeds (Adetosoye and Ojo, 1990). In milk goats, the clinical signs caused by caprine strains of *M. m.* ssp. *mycoides* include arthritis or polyarthritis, conjunctivitis, lymphadenitis, peritonitis, pericarditis, mastitis, septicemia, some form of pneumonia (interstitial, fibrinous), and pyrexia (DaMassa, *et al*, 1984). In the United States, disease outbreaks caused by *M.m.* ssp. *mycoides* have produced 90% morbidity and mortality in the new kid crop. Cellulitis at the site of experimental inoculation is common (DaMassa, *et al*, 1984).

Young kids readily acquire *Mycoplasma mycoides* ssp. *mycoides* by many routes, including oral (DaMassa, *et al*, 1984). Oral transmission occurs because does infected with this agent are often mastitic and routinely shed large numbers of this pathogen in their mammary secretions. With strains of low virulence, however, infection via the oral route may not occur. DaMassa, *et al,* (1987b) experimentally innoculated a one-time oral dose of 1 x 10^6 CFU/ml of *M. m.* ssp. *mycoides* (caprine strain GM 12) and this was sufficient to induce clinical mycoplasmosis (n = 37) terminating in fatal septicemia in 73% (37/ 51) of the inoculated kids. The disease was contagious; 8/14 non-inoculated control kids (57%) kept in close confinement with affected kids developed mycoplasmosis and died. The organism could be isolated from the blood of the affected kids as early as 24 hours after ingestion of the organism, with counts as high as 1 x 10^8 CFU/ml. Counts of the organism in the urine reached 1 x 10^6 CFU/ml. Pyrexia as high as 42.3˚ C could be detected in about 95% of the affected kids. Hot, swollen joints occurred within 4 or 5 days after oral exposure. At necropsy, all affected kids had a fibrinopurulent polyarthritis and areas of consolidation in the lungs. Excess pleural fluid and fibrinous adhesions between the lungs and the thoracic wall also were noticed. Atelectasis and areas of patchy to diffuse red consolidation, often overlayed with a fibrinous exudate, were seen in one or more lung lobes. Lung lesions included bronchiectasis and extensive pulmonary edema. Subcutaneous periarticular tissues often contained a fluid that was reddish and gelatinous. An exudate was frequently seen extending proximally and distally into the tendon sheaths (tenosynovitis). The joint spaces contained exudate ranging from variable amounts of stringy yellow fluid to copious

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amounts of a fibrinopurulent deposit. Erosions of the articular cartilages were seen in kids surviving for seven days. Four of the 37 kids had a generalized peritonitis. The kidneys, liver, and spleen were congested and enlarged, and the gall bladder was distended with bile. Pericarditis was also found. All kids had congestion of the meningeal vessels. In about 50% of the kids, the regional lymph nodes of the lungs and the prescapular and mesenteric ileocecal lymph nodes were enlarged 2-3 times. Histologic changes in tissue samples from the major organ systems were limited primarily to an acute vascular phenomenon. There was diffuse fluid leakage into the alveoli of the lungs, with a modest accumulation of leukocytes in the alveolar walls. In older kids necropsied at 48 and 72 days of age, the leukocytes found in the alveolar walls were macrophages. In younger kids necropsied at 20 and 12 days of age, the leukocytes were primarily neutrophils. Acute thrombi were seen occasionally in various organs. Meningeal, pleural, and peritoneal surfaces often had evidence of acute vascular leakage, with an early minimal perivascular accumulation of leukocytes. Meningeal vascular fluid leakage with a slight perivascular leukocyte accumulation were the only changes present in the brain and spinal cord (DaMassa, *et al*, 1992).

2.5.10 Mycoplasma ovipneumoniae

Mycoplasma ovipneumoniae plays a role in disease of goats and sheep. This mycoplasma can be isolated frequently from the lung, trachea, and nose and occasionally from the eyes of sheep with pneumonia but can also be found in the respiratory tract of healthy sheep. Sheep inoculated intravenously and one-day old lambs infected by aerosol developed an interstitial pneumonia (St. George and Horsfall, 1973). Significant microscopic lesions in lambs sacrificed between 1 and 56 days postinoculation consisted of proliferation of the alveolar walls and of the epithelium of the terminal bronchioles. Atelectasis was present. The organisms were not recovered at necropsy (Black, *et al;* 1988). Later studies have suggested that *M. ovipneumoniae* and *Pasteurella hemolytica* biotype A serotypes act synergistically to produce a chronic disease (atypical pneumonia) principally affecting lambs up to 12 months of age (Jones, 1989). Mortality rarely exceeds 10%, even under experimental conditions, but considerable economic loss due to unthriftiness can occur. Goats can also harbor *M. ovipneumoniae,* and growing evidence incriminates this mycoplasma in goat disease (Jones, 1989). Goltz, *et al*, (1986), infected young goats with *M. ovipneumoniae* and they developed

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pneumonic signs. The organism was recovered, but not regularly, from the infected goats. In a later study by Mohan and Obwolo, (1990) the experimental infection of goats and sheep with *M. ovipneumoniae* resulted in similar disease patterns. The thymus was enlarged, but there were no clinical signs or gross lesions in the lungs, trachea, or pleural lymph nodes; however, animals examined at later periods following intratracheal inoculation showed patchy areas of lung collapse. Histopathologic changes in the lungs included mild pneumonia, alveolar collapse, and a thickening of the interalveolar septa with neutrophils and mononuclear cells. The interstitial pneumonia was more prominent late in the experiment. No *Pasteurella* sp. or other bacteria of importance were isolated. *Mycoplasma ovipneumoniae* displays an uncharacteristic morphology on solid medium containing the usual concentration of agar (1.5-2.0%). Colonies do not have the “fried egg” appearance because they lack the central downgrowth that gives most mycoplasma colonies that morphology. Rather, the colonies on such agar appear granular. On medium with lower agar concentrations, the normal appearance is re-established (DaMassa, *et al,*. 1992).

2.5.11 Mycoplasma putrefaciens

Tully in (1974), described this mycoplasma and for its characteristic of producing in its growth medium (particularly liquid medium) a strong odor of putrefaction gave the name Mycoplasma putrefaciens to this organism. The organism was probably first isolated and designated as strain “KS” from the joints of goats in California in 1956 (Adler*, et al*, 1956). In 1980, this mycoplasma was identified as a cause of caprine mastitis leading to agalactia (Adler, *et al*, 1980). Often, there are no clinical signs other than mastitis and agalactia.This mycoplasma does not appear to induce pyrexia The organism remains localized on the affected udder side only, without spreading to the opposite udder half, and confers immunity to the affected udder half only (Brooks, *et al*, 1981). Young kids that were given infected milk orally, intranasally, intramuscularly, or intraperitoneally exhibited no abnormal clinical signs. Pyrexia did not occur, and the organism could not be isolated from the blood. No published studies attest to mycoplasmemia in *M. putrefaciens* -infected goats or to the establishment of disease in young kids given the organism orally. DaMassa*, et al;* (1992), however, recovered the organism from the blood, sporadically in low numbers. Kids nursing on a dam that was shedding large numbers of the GM499 strain developed severe arthritis affecting principally the

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carpal, hock, and stifle joints. Aspirates of affected joints confirmed the presence of *M. putrefaciens* (DaMassa, *et al;* 1992).

For several years, *M. putrefaciens* was thought to only cause mastitis leading to agalactia. In 1987, however, *M. putrefaciens* caused a severe outbreak of mastitis and arthritis/polyarthritis in a herd located in the Central Valley of California, requiring the destruction of 700 goats (DaMassa, *et al,* 1987a). The milk of nearly 400 lactating does contained almost pure cultures of *M. putrefaciens,* up to 1 x 10^9 CFU/ml. At postmortem examination, the joints of both the adults and kids contained a fibrinopurulent discharge. *Mycoplasma putrefaciens* was isolated in pure cultures and in large numbers from joints, tissues, and fluid not previously known to harbour the organism: brain, kidneys, lungs, lymph nodes, uterus, and urine. Bulk milk tank samples emitted an odour of putrefaction, with counts of *M. putrefaciens* up to 1 x 10^7 CFU/ml. The outbreak was milkborne and was initiated by the infusion of the pathogen into the teat canal by poor hygiene in the milking parlor and by feeding raw colostrum containing large numbers of the organism to kids. In 14-day-old kids infected with this strain of *M. putrefaciens,* the prominent histopathologic lesions were acute fibrinopurulent exudation into the joint cavities and synovial lining necrosis (DaMassa, *et al,* 1992). In older kids necropsied at 21 and 63 days of age, a lymphocytic-plasmacytic infiltration of connective tissue was found in joints having intact synovial linings. In 2 lactating does necropsied during the outbreak, the udder showed severe mastitis, which included neutrophilic exudation, mild fibrosis, and an occasional lymphoid nodule. The mastitis was more severe at the alveolar level than in the ducts (DaMassa, *et al,* 1992).

2.6 Undetermined species of mycoplasma

a**). Serotype 5** representative strain *Mycoplasma sp*. HRCG145 - Isolated from a caprine foot (DaMassa, *et al;* 1992).

b). **Serotype 7** representative strain *Mycoplasma sp*. A1343 – Isolated from the lung of a goat in West Virginia (Erno, *et al*., 1978).

c**). Serotype 11** representative strain *Mycoplasma sp* 2D – Isolated from Vaginal and preputial swabs from sheep in Texas, USA (Livingston and Gauer, 1983).

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d). Serotype 16 representative strains: *Mycoplasma* sp. F38, G69, SGPl.

Mycoplasma sp. F38 often reacts serologically with polyclonal antiserum to the type strain of *M. capricolum,* and there is a high degree of DNA relatedness between the two agents (Christiansen and Erno, 1982). *Mycoplasma* sp. F38 is now considered the etiologic agent of “true” or “classical” CCPP. In Kenya, CCPP has been initiated by at least two distinct mycoplasmas.

i). In one study by MacOwan and Minette (1979), designated as the “chronic” form, there was no spread of the disease among mature goats and the causative agent was *M. mycoides* ssp. *mycoides* (strain F30).

ii). In another case “acute” CCPP caused by strain F38, there was widespread dissemination of the disease Contagious caprine pleuropneumonia by contact with goats and this was initiated by *Mycoplasma* sp. F38 or an F38-like mycoplasma (MacOwan, 1984). Generally, pathologic changes are confined to the lung, which may develop pale yellow foci approximately 4-5 mm (DaMassa *et al*, 1992). Lesions may occur in any part of the lung, or they can coalesce so that the entire lobe or lung becomes solid. Chronically affected lungs can show a fibrinous pleuritis with occasional adhesions to the chest wall. Unlike *M. capricolum* and LC strains of *M. mycoides* ssp. *mycoides,* the subcutaneous or intramuscular inoculation of *Mycoplasma* sp. F38 does not elicit local cellulitis and subcutaneous edema at the point of inoculation. *Mycoplasma* sp. F38 does not appear to cause pathology in animals other than goats; (Harbi*, et al*.,1983), however, it has been isolated from the milk of mastitic cows in India, (Kumar and Garg*,* 1991) and a culturally, serologically,and biochemically similar organism has been isolated from sheep in Kenya (Litamoi, *et al;* 1990). Thus far, *Mycoplasma* sp. F38 (or an F38-like mycoplasma) has been isolated in Chad, India, Kenya, Oman, South Yemen, Sudan, Tunisia and Turkey. Additionally, an F38 polysaccharide was detected by latex agglutination in the sera of Mali goats (Adetosoye and Olanisebe, 1985; DaMassa*, et al.*. 1992).

e). **Serotype 17** representative strains: *Mycoplasma* sp. G, GM274B, GM790A.

Serotype 17, represented by *Mycoplasma* sp. G, was first isolated from the external ear canals of goats in Australia (Cottew and Yeats*,* 1978). Since then, a serologic type indistinguishable from *Mycoplasma* sp. G (designated GM274B) has been isolated on many occasions from the external ear canals of goats in the United States (DaMassa, unpublished data). Serotype 17 has usually been

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isolated from the external ear canal of goats, although isolation from a retropharyngeal lymph node of that species (Cortew and Yeats, 1978) and isolation of another similar serologic type (GM790A) from spleen, lung, and mastitic milk of two goats in the United States have been reported (DaMassa, *et al*., 1991).

It is also reported that this mycoplasma has been isolated occasionally from the nasal tracts of goats and once from the external ear canal of a young calf in the USA because the calf was housed in close contact with a small herd of young goats known to harbor strain GM274B in their ears (DaMassa, *et al*; 1992).

f). **Serotype 19** representative strains: *Mycoplasma* sp. U, GM623.

In a survey of the external ear canals of goats in Australia, an additional isolate was described and designated *Mycoplasma* sp. U (Cottew and Yeats, 1982). No information is available concerning its prevalence or pathogenicity. On one occasion, an isolate serologically similar to *Mycoplasma* sp. U was isolated from the external ear of a goat in California. The isolate was designated GM623 (DaMassa, *et al;* 1992).

g**). Serotype 20** representative strains: *Mycoplasma* sp. V, GM257A**.**

Mycoplasma sp. V was first isolated from the external ear canals of goats in Australia (Cottew and Yeats, 1982). In the United States, a serologic type represented by strain GM257A is identical with *Mycoplasma* sp. V and is a frequently isolated mycoplasma whenever the external ears of goats are cultured. It has also been isolated from pneumonic lung of goat in Nigeria by Ikheloa *et al,* 2004; however it's pathogenicity, if any, has not been established. This mycoplasma is easily distinguished from many other known mycoplasmas by the production of an intense black pigment on agar, rendering some, particularly, older colonies black. Pigmentation is not seen in liquid media nor is it evident when colonies are young or are crowded on agar; it develops best when colonies are widely separated. Preliminary data suggests that pigmentation develops only on agar supplemented with thallous acetate (DaMassa, *et al.,* 1992).

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2.7 Acholeplasma

2.7.1 *Acholeplasma axanthum*

In one report (Jones*, et al.,* 1983b), *A. axanthum* was isolated from vulvar scabs of ewes in the United Kingdom, representing the only isolation of this mycoplasma from sheep or goats. Its pathogenicity, if any, has not been established.

2.7.2 Acholeplasma granularum

Two (2) isolates of *A. granularum,* one from a goat and the other from sheep, are the only known isolations of these mycoplasmas from these hosts. The significance of these isolations is unknown. (Cottew, 1984).

2.7.3 Acholeplasma laidlawii

This organism is ubiquitous and is not specific to sheep or goats. However on many occasions, *A. laidlawii* has been isolated from, the respiratory tract of sheep and goats (DaMassa, *et al.,* 1992). In one study, however, the inoculation of this organism into the teat canal of lactating goats resulted in clinical mastitis leading to 90% milk loss in most goats tested and agalactia in others. Milk abnormality was observed only in the inoculated udder half and persisted for 19 days postinoculation (the longest period tested). In goats necropsied on the third day postinoculation, the udder and the supramammary lymph node on the inoculated side were slightly enlarged. Goats necropsied at subsequent intervals had udders that were smaller and had mammary lymph nodes that were larger than those of the uninoculated side (Singh*, et al.,* 1990). Histologically, mastitis was present only in the inoculated udder half. Acute diffuse purulent mastitis characterized by marked exudation of neutrophils into the luminal acini began on the third day postinoculation. Within one week, the mastitis became less purulent, with a decrease in the number of neutrophils in the lumina of the acini. The acinar epithelial cells were vacuolated and exfoliated, and there was mild fibrosis and an infiltration of lymphocytes in the interstitial tissue. By days 18 and 19 postinoculation, the mastitis was chronic and characterized by a severe parenchymal infiltration of lymphocytes and plasma cells. A marked fibrosis replaced the glandular parenchyma. The supramammary lymph nodes on the affected udder side showed acute to chronic lymphadenitis, with marked hyperplasia of lymphoid tissue (Singh, *et al*., 1990).

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2.7.4 Acholeplasma oculi

This organism has been isolated from cases of conjunctivitis or keratoconjunctivitis in goats, sheep, and other animals. It has also been isolated from pigs, as a cell culture contaminant, from horses and camels, and from cows and calves. Only one investigation has supported a pathogenic role for *A. oculi.* In a natural outbreak of the disease in goats in Minnesota, there was severe keratoconjunctivitis; (Al-Aubaidi, *et al.,* 1973) upon reinoculation of the agent, mild to severe conjunctivitis, pneumonic lesions, and death in some goats resulted (DaMassa, *et al*., 1992).

2.8 Ureaplasma

Ureaplasmas were formerly known as T-mycoplasmas. They were recognized and identified first in primary agar cultures of urethral exudates from man (Shepard, 1954). They were called "T-form colonies of PPLO" because of their minute size, distinctive characteristics, and morphology of their agar cultures as described by Shepard (1956). However, with the development of improved agar culture, identification based on the above criteria was less reliable since T colonies were no longer "tiny" (Shepard and Lunceford, 1978). The utilization of certain distinctive biochemical properties of T- mycoplasmas, in addition to morphology, size and staining reaction of agar colonies, subsequently provided reliable means of identification and characterization of the T- mycoplasma group (Shepard, *et al;* 1966; Shepard 1967). The most unique and distinquishing biochemical property of the T-mycoplasma group is the production of urease with resultant ability to hydrolyze urea with the production and accumulation of ammonia (Purcell, *et al.*, 1966; Shepard and Lunceford, 1967). This property is specific for T-mycoplasmas as no other species of mycoplasma produce urease. The term "T strain" of mycoplasma was originally intended to be a temporary designation for this unique group of organisms but with the increasing interest in its possible role in diseases of human and animals it became necessary to abandon this temporary tag. Shepard *et al*, (1976) thereby proposed a separate genus "Ureaplasma"-based on its ability to hydrolyze urea. Several animal species have been isolated (Okwoli, 2007). However the genus *U. urealyticum* contains two distinct genomic biotypes-T960 and

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Parvo. Jacobs*, et al.,* (1994) reported that the "parvum" biovar composed of smaller genome cell while the "T960" biovar are composed of larger genome cells.

Ureaplasma species have been cultivated from both goats and sheep, but there is a general lack of specific information on these organisms from these hosts, and little is known about their role, if any, in disease. However, strains of these organisms have been isolated from the urogenital tract and from urine of goats and sheep (Livingston and Gauer, 1983). In one study, it was observed that sheep with granular vulvitis contained ureaplasmas in the vulva, and inoculation of the organisms into healthy sheep resulted in granularity and hyperemia of the vulva (Doig and Ruhnke, 1977). According to Gregory, et al; (2012), seven species of ureaplasma are recognized and they are: human (*U. urealyticum* and *U. hominis*), bovine (*U. diversum*), canine (*U. canigenitalium*), feline (*U. felimum* and *U. cati*) and avian (*U. gallorale*).

Thus far, the *Ureaplasma* species of sheep and goats have not been characterized, and their serologic relationships have not been ascertained.

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2.9: Cell wall morphology and ultrastructure of mycoplasma

Mycoplasmas lack a rigid cell wall and the chemical precursors of cell wall peptidoglycan (Freundt, 1955). Electron microscopy of the ultrastructure of mycoplasmatales reveals that the cells possessed a triple-layered limiting membrane which is 75-100A thick and appear symmentrical in some strains and assymentrical in others (Domermuth *et al;* 1964). They further reported that in close contact with cell surface was an electron dense material which they tentatively interpreted to be a capsule substance. This capsule substance explains the various morphologic entities that mycoplasma exhibits. Their dominating shape is a sphere, but many mollicutes display other entities such as pear-shaped cells, flask-shaped cells with terminal tip structures, filaments of various lengths, and helical filaments. The ability to maintain such shapes in the absence of a rigid cell wall has long indicated the presence of a cytoskeleton in mycoplasmas (Razin; 1978). Some of the flask-shaped *Mycoplasma* species are capable of gliding on solid surfaces. The mechanism of this peculiar gliding motility is still unknown (Kirchhoff, 1992.). Mycoplasmas are Gram negative organism which stain weakly by this method (Shepard, 1957). Giemsa staining is the staining technique of choice. In Giemsa-stained

smear of clinical exudates and fluid cultures, the organisms frequently appear with one side pointed (Shepard and Lunceford, 1974). The organism's basic mode of replication in a liquid medium is believed to consist of a simple budding process, in different directions producing either coccal, diplococcal, small aggregates, or multidirectional, filamentous elements which continue to multiply, producing growth aggregates or colonies in fluid and in agar cultures. Hence on staining, short bacillary elements or "filaments", annular forms, or signet ring forms and bipolar `elements can often be observed, depending upon the strain, age of the organisms and method of examination (Shepard, 1967). This highly pleomorphic nature of mycoplasma, has been attributed to the absence of rigid cell wall (Lin, 1985; Joklik, *et al*; 1996). Pleomorphism in mycoplasma has been reported to be due to unfavourable culture condition as coccal and diplococcal forms were initially obtained while fragmenting filaments and budding forms were later encountered (Robertson, *et al*. 1993). This was due to the fact that genome replication is not necessarily synchronized with cell division (Joklik *et al.,* 1996). Manilof and Morowitz (1972) reported that the diameter of the smallest viable mycoplasma is 0.33mm. The size and shape of the mycoplasma cells are dictated by the plane of the section hence the cell diameters are usually measured at different stages of colony morphogenesis (Meloni *et al.,*1981) and electron microscopy of fixed thin-sectioned cells displayed progressive changes that were interpreted by Robertson*, et al,*(1993) as elongation, invagination and separation. Black *et al.*, 1972, reported that in addition to ribosomes, there are vacuoles-like structures in the interior of the cells. Colonially, the "fried egg" appearance of mycoplasma species is a well accepted fact by many authors and Meloni *et al.*, (1981) reported that it is caused by the central portion of growth penetrating downwards into the agar and the spreading of peripheral growth at the surface.

2.10 Pathogenicity of mycoplasma

Pathogenic mycoplasmas have a flask- or club- like cell shape structure with a protruding tip or bleb that attaches to eucaryotic cells serving as an attachment organelle. Meng and Pfister (1980) observed that these attachment organelles are cytoskeleton-associated proteins. The cytoskeleton-like structure is thought to function in modulating cell shape and to participate in cell division, gliding motility, and the proper localization of adhesins. The molecular basis of mycoplasma pathogenicity remains largely elusive. The clinical picture of mycoplasma infections in humans and animals is more

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suggestive of damage due to host immune and inflammatory responses rather than to direct toxic effects by mycoplasmal cell components (Okwoli, 2007). An inciting review discusses the role of mycoplasmas in disease pathogenesis, referring also to the variety of diseases of unknown etiology that have been linked to *mycoplasmas* (Baseman and Tully, 1997). These include the possible role of *mycoplasmas* as cofactors in AIDS pathogenesis, the Gulf War Syndrome, and other diseases of unexplained etiology such as the chronic fatigue syndrome, Crohn's disease, and various arthritides. Specific protective defense mechanisms include the production 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. The specific reactions elicited by invading mycoplasmas, essential for resistance and protection against mycoplasma infections, have also been shown to play a role in the development of lesions and exacerbation of mycoplasma induced diseases (Biberfeld,1985; Cassell *et al*,1985; Cole *et al*,1985; Howard and Taylor, 1985). In addition to eliciting anti-mycoplasmal immune responses, mycoplasmas exert a wide range of nonspecific immunomodulatory effects upon cells making up the immune system. *Mycoplasmas* affect the immune system by inducing either suppression or polyclonal stimulation of B and T lymphocytes; inducing cytokines; increasing the cytotoxicity of macrophages, natural killer cells, and T cells; enhancing the expression of cell receptors; and activating the complement cascade. The ability of *mycoplasmas* to immunomodulate host immune responsiveness contributes to their pathogenic properties, enabling them to evade or suppress their host defense mechanisms and establish a chronic, persistent infection (Baseman and Tully, 1997).

2.11 Identification and isolation of mycoplasma

In vitro cultivation of mycoplasmas has been a major drawback to research and laboratory diagnosis of most *mycoplasma* infections. Only a few of the *mycoplasmas* existing in nature have been cultivated so far and for a period of about 30 years, none of the phytoplasmas infecting insects and plants was cultivated in vitro in an axenic culture (Kirkpatrick and Smart, 1995). Some reasons adduced are that some of the cultivable mycoplasmas grow very poorly and slowly on the best mycoplasma media available (Razin. 1994), and also genetically there is remarkable scarcity in the *mycoplasmas* of the genes involved in biosynthetic pathways (Fraser *et al*; 1995. Himmelreich*, et al;*

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1996). To overcome the assimilative deficiencies of these *mycoplasmas,* complex media are used for their cultivation and such media are usually based on beef heart infusion, peptone, yeast extract, and serum with various supplements (Razin. 1991b). The use of these complex undefined growth media has interfered with the molecular definition of mycoplasmal metabolic pathways, genetic analysis, preparation of mycoplasmal antigens free of serum components, etc. Continual efforts to replace the serum component, with the aim of reaching a defined growth medium, have been made. Serum has been shown to provide, among other nutrients, fatty acids and cholesterol (required for membrane synthesis) in an assimilable nontoxic form. Efforts to replace the serum component with albumin, fatty acids, and cholesterol solubilized in Tween 80 or with liposomes made of phospholipids and cholesterol free fatty acid toxicity (Razin, 1978) have been partially successful (Okwoli, 2007). Therefore to improve the chances of in vitro cultivation of fastidious mollicutes a coculture with eucaryotic cell lines (cell - assisted growth) was adopted. This has resulted in the successful cultivation of the Colorado potato beetle spiroplasma (Konai *et al.,* 1996), and subsequently obtaining the primary cultures of this spiroplasma on cell-free media under a low-redox, enhanced CO_2 atmosphere and at a pH lower than 7.0 (for most mollicutes, the initial pH is adjusted to a slightly alkaline value), conditions which imitate those obtained in the insect cell cocultures (Konai *et al.,*1996). Also, the mycoplasmas grown in the cell culture (indicated by PCR monitoring) could be subsequently subcultured in a cell-free medium (Jensen *et al.,* 1996). The above method, as complex as it is, requiring "heroic" efforts, is certainly inadequate for routine cultivation of fastidious *mycoplasmas*, leaving the door open for the application of molecular techniques, such as PCR, for detection and identification of fastidious or uncultivable *mycoplasmas* (Razin. 1994)

2.12 Susceptibility of mycoplasma to antibiotics

Mycoplamas are not inhibited by antibiotics such as the penincillins that act on the cellwall of microorganism since they lack a rigid cell wall. However, they are susceptible to a variety of other broad spectrum antibiotics, most of which only inhibit their multiplication and do not kill them (Taylor-Robinson & Bebear, 1997). According to Hannan, (1995), mycoplasmas have been known to be susceptible to the following antibiotic groups: Macrolides (Erythromycin, Clarithromycin, Azithromycin etc); Aminoglycosides (Streptomycin, Neomycin, Gentamicin etc), Aminocyclitol

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(Spectinomycin etc), Tetracyclines (Tetracycline HCl, Doxycycline HCl, Minocycline HCletc), Lincosamides (Lincomycin HCl, Clindamycin HCl etc), Fluoroquinolones (Ciprofloxacin HCl, Ofloxacin, Nodoxacin etc), Diterpines (Tiamulin etc), Beta-lactan antibiotics (Ampicillin etc), others (Chloramphenicol, Nitrofurantoin, Rifampicin, Puromycin, Mupirocin, Novobiocin etc). The tetracyclines inhibits protein synthesis and they have always been at the forefront of antibiotic usage, particularly for genital infections, but macrolides are also widely used for mycoplasmal respiratory tract infections (Okwoli, 2007). Erythromycin has been reported as the drug of choice for treating neonatal infection of mycoplasmas not involving the central nervous system (Waites *et al.*, 1993). However in their study of the effect of prophylactic erythromycin in preventing vertical transmission of *U. urealyticum,* Ogasarawa and Goodwin (1999) also reported that prophylactic erythromycin does not decrease vertical transmission of the organism but may decrease the incidence of histologic chorioamnionitis and increase the latency period. *Mycoplasmas* have been found to be resistant to erythromycin, trimethoprim, sulphonamides and rifampicin (Taylor-Robinson, 1990). Antunes, *et al,* (2007) determined the efficacy of 15 antimicrobial agents against 37 *Mycoplasma putrefaciens* isolates by the minimum inhibition concentration method (MIC) and observed that the most effective antimicrobial classes were the fluoroquinolones, the tetracyclines, the lincosamide lincomycin, and the macrolides in that order. This stands as a definite report of decreased susceptibility to the macrolides, lincomycin, and the tetracyclines in *M. putrefaciens* strains. According to Leaflet and Rosenbusch (1998), 36 *Mycoplasma bovis* isolates from cases of pneumonia and 9 from cases of pneumonia and arthritis in a Feedlot Cattle were tested for susceptibility to antimicrobials currently used in cattle with respiratory disease (ampicillin, tilmycosin, spectinomycin, tylosin, lincomycin, tetracycline, ceftiofur, and erythromycin). Among the isolates from cases with pneumonia, resistance to more antimicrobials was shown in recent isolates than in isolates from earlier years. They also opined that cases of mycoplasmal pneumonia in feedlots may be very hard to treat with conventional antibiotic therapy. However, in-vitro susceptibility data suggests that tetracycline and lincomycin may provide good responses but similar cases involving arthritis are likely to be unresponsive to therapy (Leaflet and Rosenbusch, 1998). Outcomes of Mycoplasmal infection (M. pneumonia infection of lower respiratory tract) with antibiotics (Macrolides (MLs), tetracyclines (TCs) and fluoroquinolones (FQs) as drugs of choice for *M. pneumoniae* infection, has resulted in resistance to MLs (Principi and Esposito, 2012), and they further asserted that resistant strains are being

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increasingly detected. In their opinion macrolide prescription is needed in countries in which the incidence of ML-resistant *M. pneumoniae* is low, but in countries in which ML-resistant *M. pneumoniae* strains are very common, the replacement of a macrolide by a tetracycline or fluoroquinolone should be considered depending on the severity of the disease. A number of cases treated with ineffective antibiotics have shown similar outcomes to those observed in patients infected by susceptible strains. This seems to indicate that there is no need to change macrolide use systematically in the case of mild to moderate disease, but other antibiotics should be prescribed if the symptoms persist or there are signs of a clinical deterioration (Principi and Esposito, 2012).

2.13 Predisposing factors to mycoplasma infection.

In western Nigeria, goat production is mostly associated with communal and rented pastures. The caprine production in Nigeria consists of a semi-extensive system of production, and it is possible that risks of transmission are higher. Adverse climatic conditions, as well as other stress factors, may influence disease dynamics and the susceptibility of the animals to mycoplasmal infections. Other factors, such as high population density, previous overpasturing, movement of infected domestic flocks (DaMassa *et al*, 1992; Kinde *et al*, 1994), and the presence of chronic or systemic diseases that compromise the immune system ((DaMassa *et al*, 1992; Corrales *et al,* 2004) also may affect disease epidemiology. Another predisposing factor to mycoplasma infection is the period of lactation and seasons of the year, and both risk factors (season and reproductive phase) are associated with a higher frequency of infection (*Mycoplasma spp* was isolated from a mare during estrus). The association between lactation and infection by *M. agalactiae* is commonly observed in domestic flocks, which is consistent with intramammary transmission (Bergonier *et al,* 1997). The geographic distribution of *M. agalactiae* also may be influenced by transhumance, which involves the transferring of livestock from one grazing ground to another (as from lowlands to highlands) with the changing of season. Transhumance occurs due to the regional pasture and climatic conditions and can increase the probability of domestic and wild flocks sharing pastures in summer and winter. According to Bergonier *et al*, (1997) two major factors govern the evolution of the prevalence of *Mycoplasma agalactiae* in domestic goats: the physiologic status of the females and the movement of animals linked to transhumance, which promotes multiple contacts and stress. As the domestic animals and wildlife share pastures during transhumance, increased transmission to wildlife may occur. Such a relationship has been previously demonstrated with the transmission of *Mycoplasma conjunctivae*

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between domestic and wild small ruminants that share habitat in the Alps (Belloy *et al,* 2003). In her study, Amosun, (2011) found four different species of Mycoplasma organism (*Mycoplasma mycoides sub. mycoides, Mycoplasma capricolum, Mycoplasma arginini* and *Mycoplasma bovis)* as incriminated in clinical cases of bovine mastitis influenced by transhumance.

In domestic caprines, genetic factors linked to some breeds of dairy goats can influence susceptibility to intramammary infections (Barillet *et al*, 2001; Rupp and Boichard 2003). Okwoli, (2007) discovered that *Ureaplasma urealyticum* and *Mycoplasma hominis* are additional bacteria that might cause urogenital infections and consequently infertility in females (animals inclusive) due to the stress of pregnancy.

There is little published information on the potential effects of gender as a risk factor for *M. agalactiae*. Domestic goats of both sexes can be infected at the same frequency (Madanat *et al,* 2001) but morbidity is most often associated with pregnant and lactating females rather than males (Ruffin, 2001); this probably relates to changes in immunologic competence caused by physiologic and hormonal changes associated with reproduction (Real *et al* 1994). Lactating females often have morbidity rates between 10% and 90% (Bergonier *et al*, 1997). *Mycoplasma agalactiae* is transmitted orally (Hasso *et al,* 1994), and based on this transmission route, differences in infection rates between sexes should not occur at young ages (Jones, 1987). Other factors that may affect the epidemiology of *Mycoplasma agalactiae* in domestic goat populations include population imbalances characterized by a sex- and age-ratio disparity favoring males, subadults, and old animals (Chirosa *et al,* 2001).

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2.14 Lists of different disease cases where *Mycoplasmas* and *Ureaplasmas* have been isolated in various animals:

* Granular vulvovaginitis in goats - *Mycoplasma agalactiae* (Cottew, 1984)

*Ovine keratoconjunctivitis - *Mycoplasma arginine* (DaMassa, *et al;* 1992)

*Polyarthritis of goat – *Mycoplasma capricolum* (Cordy and Adler, 1960).

*Mastitis in cows - *M. mycoides spp. mycoides, M. capricolum, M. arginini* and *M. bovis* (Taoudi and Kirchoff, 1986; Amosun. 2011, Ph.D Thesis).

*Vulvar scabs of ewes (sheep) *Mycoplasma agalactiae* (Jones *et al*, 1983b).

*Genitals of cattle - *Ureaplasma diversum* (Taylor-Robinson *et al*, 1978; LeGrand *et al,* 1995)

*Pneumonia in Cattle – *Mycoplasma bovis.* (Howard and Gourley, 1982),

*Oral cavity of cats - *Ureaplasma felimum* and *Ureaplasma cati* (Harasawa *et al*, 1990)

*Semen of Bull – *Mycoplasma capricolum* (Breard A, *et al*; 1988)

*Lungs of chicken - *Ureaplasma gallorale* (Koshimizu *et al*, 1978).

*Respiratory disease in horses – *M. felis, M.subdolum.* (Wood *et al.* 2007)

*Toxic epidermal necrolysis in calves - *Mycoplasma bovis,* (Senturk, *et al*; 2012)

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2.15: Goat Breeds in Nigeria.

2.15.1. Goats

The only published characterisation of the traditional varieties of goat in Nigeria is by Ngere *et al.* (1984). Three main varieties of goat are recognised in Nigeria, the Sahel or Desert or West African long-legged goat, the Sokoto Red and the West African Dwarf.

2.15.1a*: Sahel or Desert goat*

The Sahelian or Desert goat is found along the northern border of Nigeria, particularly in Borno, where it is often known as 'Balami', although this name has not been adopted as it would lead to confusion with the better-known sheep race, Balami. Mason (1988) uses 'Sahel', which seems appropriate, as this race is distributed from Senegal to Sudan. In Nigeria, the Sahel goat is generally the variety preferred by pastoralists. The distribution of Sahel goats in Nigeria is shown in Map 1.

Sahel goats are very similar in appearance to the sheep with which they are often herded. The coat is white or dappled, the ears are pendulous and the legs are notably longer than other breeds. Wilson, *et al;* (1984) studied the productivity of goats and traditional management in the Republic of Niger and also included a valuable table of comparative data from other studies. Wilson and colleagues also studied the productivity of Sahel goats in the Republic of Mali (Wilson, 1987; Wilson and Sayers, 1987).

2.16.1b. Sokoto Red goat

The Sokoto Red, Kano Brown or Maradi goat (Maradi is a *Département* of the Niger Republic) is probably the most widespread and well-known type in Nigeria (Haumesser, 1975). It is the usual village goat in the northern two-thirds of the country although it is less common with transhumant pastoralists. The distribution of Sokoto Red goats in Nigeria is shown in Map 2. Ngere *et al*. (1984) argue that populations of the Sokoto Red spread south and east from Sokoto through the savanna belts giving rise to the Kano Brown and, further east, to the Sahel types of Borno State. This type of historical speculation is difficult to accept without more detailed evidence. The most complete overview of the breed is Robinet's (1967) comprehensive survey which integrates data from Nigeria and Niger. The Sokoto Red is the only Nigerian breed for which there is a record of systematic attempts to stabilise a particular type (Bourn *et al*, 1994). Henderson (1929), reviewing the

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work of the Veterinary Service in Sokoto Province, described how, in 5 years, 219,688 non-red male goats were castrated resulting in the replacement of non-red skins by the more valuable red in the local markets.

The Sokoto Red goat was the source of 'Morocco leather' known in Europe from the medieval period onwards. It acquired this name because it was transported across the Sahara by caravans controlled by Moroccan merchants. The Sokoto Red is still known for its suitability for fine leather. However, their skins do have coarse, thinly-spaced outer hairs, small sweat and wax glands and they lack excess fat. Alaku and Moruppa (1983) found that Sokoto Red goats slaughtered in the driest months suffered a 55% reduction in skin weight, making it 4.9% of the total body weight.

2.16.1c. West African Dwarf goat

Although the West African Dwarf (WAD) goat is found in 'many local types' Ngere *et al*., (1984); account differentiates them. Although they are stereotypically said to be native to the forest belts, their presence in Borno State and in adjacent Republics of Cameroon and Chad suggests that they were far more widespread until recently. They correspond to the West African Grassland Dwarf described for Cameroon by Ndamukong *et al*. (1989). Indeed, like Muturu cattle, they may once have been the main race of goat over most of Nigeria. Just as the zebu has replaced the muturu, so the WAD goats have been driven to remoter areas in the savannahs. There is a strong association between the diffusion of the Red Sokoto goat and Islam, so for example, in southern Sokoto state, the non-Islamised populations still retain WAD goats while most Hausa villages have exclusively Sokoto Red goats. The distribution of WAD goats in Nigeria is shown in Map 3.

Goats are not native to West Africa, so the WAD goat must originally have evolved from a longlegged type, probably ancestral to today's Sahel goat (Ngere *et al*., 1984). The WAD goat is usually black, although patched, pied, and occasionally all-white animals can be seen, even on the coast. Although Chang and Landauer (1950) argue that the WAD goat is proportionately dwarf, Epstein (1971) points out that the distorted forms and extremely short legs do suggest achondroplasy. This small size is probably an adaptation to the goats' environment though the nature of the selective force is unknown. The WAD goats in the semi-arid zone resemble Sokoto Red goats in their body

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proportions. Paradoxically, physiological experiments have shown that the WAD goat is not particularly adapted to high ambient temperatures (Montsma *et al*., 1985). High temperatures 30°C and relative humidity 60% cause a reduction in food intake. The WAD goat is believed to be trypanotolerant because it thrives in tsetse areas, but there have been no critical studies of this belief. It is unfortunate that there is no direct record of the breeds castrated, but presumably they were a mixture of Sahel types, WAD types and their crosses.

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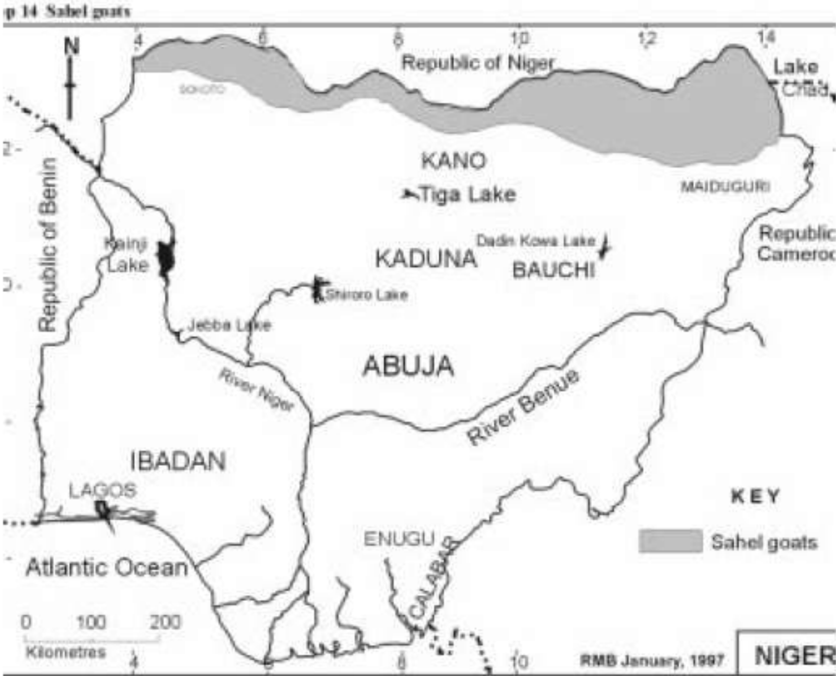
Map1: Sahel goat distribution in Nigeria.



source: Ngere, *et al*,1984; Animal Genetic Resources Information.

Map 2: Sokoto Red goat distribution in Nigeria.

source: Ngere, *et al*,1984; Animal Genetic Resources Information.

Map 3: West African Dwarf goat distribution in Nigeria.

source: Ngere, *et al*,1984; Animal Genetic Resources Information.

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2.16 **Mycoplasma and ureaplasma involvement in vulvovaginitis of goats**.

Mycoplasmas and ureaplasmas are economically important in the agricultural as well as in biomedical research because of their frequent isolation from every domestic and laboratory animal (Simecka, *et al*; 1992). Mycoplasmal infections are associated with diseases of the lungs, genitourinatory tract, joints and other tissues and these diseases have been shown to cause significant problems in commercial and experimental animals. The most important economic mycoplasmal disease of goats is contagious caprinepleuropeumonia (CCPP) with *Mycoplasma capricolum subsp. capripneumoniae* as the classical causative agent (MacOwan & Minette, 1979). Other causative agents of CCPP include *Mycoplasma mycoides subsp. capri* (Ojo, 1976.), *Mycoplasma mycoides subsp. mycoides* (villa *et al., 2011*) and *Mycoplasma capricolum* (DaMassa, *et al;* 1992.). Ikheloa, *et a*l; (2004) also isolated *Mycoplasma arginini, Mycoplasma V, Mycoplasma bovis* and *Mycoplasma capricolum* from the pneumonic lungs of goats in Nigeria. *Mycoplasma* ovine/caprine serogroup 11 was first isolated from an outbreak of granular vulvovaginitis (GVV) in sheep in Australia (Cottew, 1979). Subsequently, this organism was isolated from spontaneous cases of GVV in sheep in India (Tiwana 1982). Also Kumar *et al*; (1992) observed that there was hydropic degenereation of the epithelial cells lining the mucosa of vulva and vagina which indicated that *Mycoplasma ovine* / caprine serogroup 11 causes damage to the epithelial cells. This observation led to the conclusion that *M. ovine*/caprine serogroup 11 is pathogenic for female genital tract of sheep and it produces granular vulvovaginitis, lymphocytic cervicitis, endometritis and oophoritis (Kumar *et al*; 1992). The gross and microscopic lesions in the vulva, vagina, cervix and uterus observed by Kumar *et al*., (1992) were almost similar but different in severity to those observed by other workers in -experimentally induced GVV in sheep due to *Ureaplasmas* (Doig and Ruhnke, 1977), and also in Granular vulvovaginitis (GVV) in goats due to *Mycoplasma agalactiae* (Singh *et al*., 1975), *Acholeplasma laidlawii* and *Acholeplasma oculi* (Gupta *et al*., 1990).

Razin, (1978), observed that *mycoplasmas* have special affinity for secretory epithelial surfaces, where they get intimately attached to sialic acid receptors and other receptors present on host cells, thereby causing damage to host cells by various mechanisms. The infiltration of large number of lymphocytes and plasma cells in the epithelial, sub-epithelial, muscular and serosal layers and also around the blood vessels in the genital tract of the infected lambs indicates that strong cell-mediated responses are directed against the invading *Mycoplasma ovine/caprine* serogroup 11

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organisms. In like manner, Kumar *et al.,* (1992), also found out that local infection with *Mycoplasma ovine/caprine* serogroup 11 remains confined to the genital tract and does not result in systemic reaction, their findings agrees with Barile (1973) who asserted that most *Mycoplasma* pathogens were not highly invasive, but they confine themselves to epithelial surfaces and produce localized infections. DaMassa *et al* (1992), in their work sited the findings of others as they relate to diseases caused by Mycoplasmas in sheep and goats such as: *Mycoplasma agalactiae* has been associated with cases of granular vulvovaginitis in goats (Singh *et al.,* 1975), *Mycoplasma capricolum* is associated with the natural outbreak of disease of sheep in Zimbabwe (Swenepoel *et al*, 1977) and a septicemic disease in lambs in the United States that was initiated by an organism closely related to *Mycoplasma capricolum* (DaMassa *et al*, 1987b*),* also from vulvar scabs of ewes in the United Kingdom (Jones, *et al;* 1983b). Gourlay *et al*, (1973), isolated *Acholeplasma axanthum* from the vulvar scabs of ewes in the United Kingdom, which has no established pathogenicity (DaMassa *et al*., 1992). Experimentally also, Trichard *et al,* (1993) reproduced clinical ulcerative balanoposthitis and vulvovaginitis in 14 sheep and goats infected with *a Mycoplasma mycoides mycoides* LC field strain, isolated from the Straussheim Dorper stud in South Africa. This study encompassed series of field observations, therapeutic trials and experimental investigations that resulted in the isolation of a wide range of bacteria and various *mycoplasma species.*

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CHAPTER THREE

3.0 ISOLATION AND MICROSCOPIC IDENTIFICATION OF MYCOPLASMA ISOLATES.

3.1 Introduction.

Mycoplasmas are cell associated microorganisms, their proper isolation therefore will depend on a variety of factors such as proper specimen collection, the material used for the collection and the type of samples collected (Okwoli, 2007). The preference of swabs to collection of samples over other methods of collection was reported by Waites (2002). Isolation techniques employed can also play a part in isolation rates as some techniques have been found to be more sensitive than others. Morphologically, most mycoplasmas have a characteristic colonial 'fried egg' appearance on solid media. This feature is best detected by means of stereomicroscopy and most authors have used this method in identifying genital mycoplasmas (Okwoli, 2007). Various factors have been observed to affect the size and appearance of colonies and they include the species of *mycoplasma,* the constituent and degree of hydration of medium used, the agar concentration, the atmospheric conditions and the age of culture (Taylor – Robinson, *et al;* 1988; Okwoli, 2007). There is a wide size range observed for mycoplasmas, for example bovine colonies may exceed 2mm in diameter while colonies of ureaplasmas have 15 -60µm as diameter size (Taylor – Robinson and Williams, 1988). Colony growth is generally visible after 24hours and maximum size is reached after 48hours of incubation at 36°C (Shepard, *et al.*, 1978).

Microscopic studies revealed that mycoplasma cells stain poorly by the Grams method (Duguid, *et al*, 1978). Consequently, various staining techniques including – Giemsa stain, Dienes stain, Cresyl fast violet or fluorochroming with acridine orange have been employed (Duguid, *et al*; 1978). Razin and Freundt (1984) reported that examination of methanol-fixed *mycoplasma* organisms stained with Giemsa was preferable to the Gram stain.

This work deals with the collection, processing of samples and isolation of *mycoplasmas* and other bacteria from clinical specimens from goat vulvovaginitis.

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3.2 MATERIALS AND METHODS

3.2.1 Animals: Two hundred and twenty one (221) samples (140 mucus and 81 bloody discharges) were collected aseptically from the vulva of goats which had clinical vulvovaginitis in farms in Lagos.

3.2.2 Sampling: Two (2) samples were collected from lateral and posterior vaginal fornices of each animal using commercially prepared sterile swab sticks and were placed in a commercially prepared ice pack and transported to the Microbiology laboratory of the Department of Veterinary Microbiology and Parasitology in the Faculty of Veterinary Medicine, University of Ibadan, Ibadan.

3.2.3 Media: The following media were used for the isolation and cultivation of mycoplasmas, ureaplasmas and acholeplasmas.

3.2.3.1 Mycoplasma broth medium: Mycoplasma broth medium was prepared as shown below:

Mycoplasma broth base	25g
Distilled water	75ml

These were sterilized by autoclaving at 121^0C for 15 minutes. It was allowed to cool to room temperature and the mycoplasma supplements were added:

Mycoplasma supplement G contains the following:

a). Horse serum	20ml
b). Yeast extract (25% w/v)	10.0ml
c). Thallous acetate	25.0mg
d). Penicillin	20,000IU

3.2.3.2 Mycoplasma Agar medium:

Content :- Mycoplasma agar base	35gm
Distilled water	65ml

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These were sterilized by autoclaving at 121^0C for 15 minutes and on cooling, the supplements were aseptically added as in the liquid medium.

Mycoplasma supplement G contains the following:

a). Horse serum 20ml

b). Yeast extract (25% w/v) 10.0ml

c). Thallous acetate 25.0mg

d). Penicillin 20,000IU

The agar is initially boiled to dissolve and then cooled to room temperature; add the constituted mycoplasma supplement G, mix properly and then 15ml was delivered each into the glass petri dishes and then spread evenly.

3.2.4 Atmosphere of cultivation: The mycoplasma agar plates were incubated under increased carbondioxide atmosphere while the mycoplasma broth was incubated in air.

3.2.5 Temperature of growth: All cultures including those on solid media and broth were incubated at 37^0C.

3.2.6 Processing of samples

3.2.6.1. Inoculation and incubation of Broth Media

Immediately samples arived the microbiology laboratory, inoculations into broth were done. One swab was inoculated into sterile mycoplasma broth and was incubated at 37^0C in air for 72 hours while the second swab was inoculated into sterile tryptose soy broth and incubated at 37^0C for 24 hours for the isolation of other bacteria.

3.2.6.2 Smears: Smears were made from each of the swab sample on clean glass slides and stained by Giemsa technique (Alluoto, *et al.,* 1970) for the detection of *Mycoplasma sp* and Gram stain for the isolation of other bacteria.

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3.3 Inoculation and incubation of solid media

A liquid to solid culture technique was employed for the inoculation of the solid media as follows:- The incubated liquid media were inoculated unto the solid media as follows: the tryptose soy broth (TSB) was inoculated unto Blood and McConkey agar for the isolation of other microorganisms in the samples while the incubated mycoplasma broth was inoculated unto the mycoplasma agar after a 10 fold dilution was made. The Blood and McConkey agar were incubated at 37°C for 24hrs while the mycoplasma agar were incubated under increased carbondioxide atmosphere in a candle jar at 37°C for upto 11 days.

(a) **Other Microorganisms**: After 24hrs of incubation of the Tryptose soy broth (TSB) culture they were sub-cultured separately onto Blood agar (BA) and McConkey agar. These agar plates were incubated overnight at 37°C inorder to facilitate the isolation of other organisms. They were biochemically studied according to Barrow and Feltham (1995).

(b) Microscopy: After the overnight incubation, colonies on these plates were observed using the Light Microscope at X100 objective and recorded. Discrete colonies observed were stained by the Grams stain for further identification.

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3.4 Isolation and morphological identification

3.4.1 **Mycoplasma** - After 72hrs of incubation of the mycoplasma broth culture, they were sub-cultured onto the corresponding mycoplasma agar plates. The agar plates were incubated at 37°C under increased carbon dioxide atmosphere using a candle jar, and were examined as from the third day. Plates without growth were re-incubated for further observation, and any plate that did not show any growth after 11 days of incubation was discarded.

3.4.2 - **Microscopy:-** After 3 days of incubation, the mycoplasma agar plates were examined using a dissecting microscope of X40 objective to identify mycoplasma colonies with typical "fried egg" or nipple shaped appearance with an elevated central spot. The plates were examined up to the 11th day and suspected colonies were noted and subsequently cloned and identified.

3.4.3 **Cloning of isolates** - This is a process of preventing the continous growth of a micro organism through several passages in liquid culture devoid of antibiotics or inhibiting substances (penicillin or thallium acetate), thus inhibiting the genetic codon from replicating. This process was performed on every isolate with "fried egg" appearance. Using a sterile scapel blade, the portion of the agar medium containing the isolates was cut and inoculated into mycoplasma broth and incubated. On the third day of incubation, the mycoplasma broth was sub-cultured unto its corresponding mycoplasma agar medium and was also incubated. When growth was seen, this procedure was repeated three times to get the organism in the pure form and avoid the L- form bacteria. The cloned isolates were left on the last broth stage and stored in the freezer (-20°C) for further tests.

3.5 Digitonin sensitivity test.

Digitonin sensitivity testing was performed on organisms with the fried egg appearance. Digitonin sensitivity was introduced to test for the sterol requirement of the cloned mycoplasma isolates. Digitonin disc were prepared by sterilizing punctured filter paper disc (6.0mm in diameter)in the hot air oven at over 160°C for 1.15hrs .On cooling they were soaked with 0.02ml of 1.5 % (w/v) ethanoic solution of digitonin (Sigma Chemical Co. St. Louise, USA) and allowed to dry overnight at 37^{0}C under aseptic conditions.

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Method.

Digitonin sensitivity testing was done according to the method described by Freundt *et al.*, (1973.)

All Suspected isolates were inoculated respectively into their respective well-dried agar plates using the running drop technique (Okwoli, 2007). A digitonin disc was placed on the centre of each inoculum and the plates were subsequently incubated at 37^0C under increased carbondioxide atmosphere for 3 days. Presence of zone of inhibition/clearance indicated that the test organism was either a *Ureaplasma spp or Mycoplasma spp* while no zone or clearing showed that it was a non-cholesterol (*Mycoplasma*) organism (Razin, *et al;* 1998).The size of the diameter of the zone of inhibition was measured and recorded as shown in Table 4.

3.6 Urease test.

*Mycoplasma*s can be classified according to whether they ferment glucose, hydrolyse arginine or hydrolyze urea. It has been observed that the hydrolysis of urea is unique as a conclusive test for ureaplasmas (Waites *et al*, 2001).

Method.

The suspected 18 isolates (13 *Mycoplasma,* 3 *Ureaplasma* and 2 *Acholeplasma*) were inoculated onto urea agar slopes and incubated at 37^0C under increased carbondioxide atmosphere for 72hrs. The presence of discolouration from pale amber to intense red or deep amber on examination after 3 days indicates that the urea has been hydrolysed. Three out of the eighteen tested samples were positive for this test.This is a positive test for the detection of *Ureaplasma spp*. This is shown on plate 8.

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SOME FIELD CASES FROM WHICH SAMPLES WERE TAKEN:

Plate 1: Swollen and Hyperemic vulva seen in a goat on the field.

Plate 2: Red and Inflamed vulva seen in a goat on the farm.

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3.7 **Results**

A total of eighteen swab samples were examined for their morphological appearance on mycoplasmal agar plates. Using the eye-piece X100 and X40 dissecting microscopic magnification, most colonies observed have serrated edges on agar plates and appear as shinning, slightly raised central portion and milky in colour. However, in some plates, more than one type of colony size, shape and colour was observed. The different sizes observed is adduced to the presence of other mollicutes in the samples cultured as genital swab samples is believed to contain multiples of microorganisms. The digitonin test showed that two of the 18 isolates were negative as there was no zone of inhibition seen in the reaction with the digitonin dics (Table 4). This is indicative of a non-cholesterol requiring mollicutes-*Acholeplasma.* Three out of the 18 isolates hydrolysed urea in the urease test indicating that these could be ureaplasma strains (Plate 9). Therefore, 13 mycoplasmas, 3 ureaplasmas and 2 acholeplasmas have been identified from the 18 genital swab samples collected. Other microorganisms identified from the swab samples collected include *Staphylococcus spps*. *Streptococcus spps*. and *Echerichia coli* (Table 3).

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Table 3: Breakdown of Various Microorganisms isolated in this study

Organism	No isolated	Percentage
Staphylococcus spp	73	33.0
Streptococcus spp	75	33.9
Escherichia coli	91	41.2
Mycoplasma spp	13	5.9
Ureaplasma spp	3	1.4
Acholeplasma spp	2	0.9

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3.8 Discussion

The criteria for the morphological identification of animal *mycoplasmas* among others include their size range and shape of the colonies, the presence of an envelope consisting of a unit membrane, the dimensions of the ribosomes and the slender strands of DNA within the mycoplasma cell cytoplasm (Anderson, 1969). Cultures of *mycoplasma* and u*reaplasma* colonies usually have size variation possibly because of their mean generation time (Razin and Freundt, 1984). Colony sizes of isolates in this experiment were seen to vary, and when they were subcultured the mycoplasma colonies grew faster with larger and more confluent colonies than were obtained from the primary agar cultures, this observation corroborates Okwoli, (2007), who stated that this could be attributed to the inoculum size of the organism used for subcultures. Also, the presence of bigger colonies in the media could be due to inclusion of extra quantity of horse serum in the mycoplasma medium used which is known to favour mycoplasma growth. Thallium acetate a component of the mycoplasma supplements used, was diluted by the addition of exra quantity of horse serum (5ml/25ml of agar) and this diluted mycoplasma supplement was used to prepare the media used for the molecular, biochemical and serological analyses, because only pure isolates are required for use in these analyses. Penicillin was however incorporated to inhibit the growth of any contaminating bacteria. The few colonies of *ureaplasmas* that were seen on both primary isolation plates and on subsequent subcultures must have been aided by the extra quantity of horse serum added, because thallium acetate included in the mycoplasma supplement used for culture outrightly inhibits the growth of ureaplasmas, and ureaplasma specific agar was not available for use in this study. By using a dissecting microscope, an opaque central zone deep in the agar with a translucent peripheral zone which is the characteristic "fried egg" or nipple - shaped appearance was observed. These findings agree with Taylor – Robinson, *et al;* (1988) and Okwoli (2007) who observed similar colonies in their studies.

The heterogeneous nature of the morphology of *mycoplasmas* which is largely because of the lack of a rigid cell wall, has limited the use of characterizing it by morphology. As reported by Okwoli, (2007), different sizes and forms were seen within the same isolates in this study. Morphology and the size of the colony have been reported as not useful for the characterization of mycoplasma species because these properties vary within the same species and they are influenced by factors such as the degree of dehydration at the agar surface, the inoculum size, the agar

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concentration and gel strength (Razin, 1991a). Razin (1991a), also reported that under certain conditions like nutritionally poor media, inadequate pH, dry atmospheric conditions and dry medium surface, the initial central down growth might occur without formation of the peripheral surface growth. Similarly, Taylor - Robinson and Furr (1997) emphasized that colony size and appearance are hazardous criteria for identification as larger than usual *ureaplasma* colonies may form on deep agar while those of other *mycoplasma* may be small and lack peripheral zones when crowded together. However most *mycoplasmas* have a spherical, mulberry, or fried - egg appearance that differs from the spiral pattern in the centre of the pseudo colony.

Other genital mycoplasmas isolated in this study include *Ureaplasmas* and *Acholeplasmas* that were only distinguished by digitonin test that separated *Acholeplasmas* – a non cholesterol requiring mollictes, from other genital mycoplasmas and between *Mycoplasmas* and *Ureaplasmas*, urease test was employed which served as a conclusive test for *Ureaplasma* (Waites, *et al*, 2001).

From this study, 13 genital *mycoplasmas,* 3 *Ureaplasmas* and 2 *Acholeplasmas* were isolated from 221 samples taken from Kano brown goats with clinical vulvovaginitis. The frequent identification of genital mycoplasmas in most animals calls for a closer and urgent attention to the diseases caused by this zoonotic microorganism so as to forestall its epidemic.

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CHAPTER FOUR

4.0 MOLECULAR IDENTIFICATION OF MYCOPLASMA

4.1 Introduction

The genus *mycoplasma* was initially identified by it's "nipple shaped" or "fried egg" appearance on solid media (Nocard, 1954). By this period, diagnosis of infections was limited by the supply of appropriate material for culture, protein analysis or microscopy. However, as researchers developed interest in mycoplasmology, mycoplasma was identified biochemically (Ern∅ and Stipkovits, 1973) and serologically (Edward and Fitzgerald, 1954).

Molecular studies on mycoplasma began in late 20th century when Woese, Stackebrandt and Ludwig (1985) identified *Mycoplasma capricolum* subspecies *capripneumoniae* (Mccp) in goats by PCR technique. In veterinary mycoplasmology, molecular diagnosis has improved the detection and identification of mycoplasmas, specifically the polymerase chain reaction (PCR) for the *Mycoplasma "mycoides* cluster" and *Mycoplasma "mycoides* sub cluster" coupled with restriction fragment length polymorphisms (RFLP) in goats by Bashiruddin *et al*., (1994) and the PCR and denaturing gradient gel electrophoresis (DGGE) method (McAuliffe *et al*., 2005). Awan, *et al;* (2004), also recorded successfully the isolation and identification of *Mycoplasma capricolum* subspecies *capricolum (Mcc)* and *Mycoplasma putrefaciens* (*Mp)* on the basis of PCR tests from the nasal swabs and lung cultures of goats in Pishin district of Balochistan, Pakistan. Other uses of PCR in mycoplasmology include the characterization of strains within a species and for detection of a specific feature, such as the presence of an antibiotic determinant (De Barbeyrac *et al,* 1996). It is also used for detection of mycoplasmas in tissue samples when the tissues has already been processed for histologic examination or is contaminated, making culture impossible (Talkington *et al*, 1998). The PCR has also been used to study the epidemiology of genital mycoplasmas (Kong *et al*, 1997). Polymerase Chain Reaction has been employed for the laboratory diagnosis of some veterinary *mycoplasmas,* including species of the closely related *Mycoplasma mycoides* cluster (Bashiruddin*, et al*. 1994), *Mycoplasma gallisepticum* (Garcia *et al,* 1996), *Mycoplasma hyopneumonia* (Stark *et al*, 1998) and *Mycoplasma bovis* (Ayling *et al,* 1997). The PCR has the advantage of easy use, rapid availability of results, and standardization and is more suitable for processing of a large number of specimens (Foddai, *et al*; 2005).

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Previously, differentiation of *mycoplasma spps* by the PCR, based on specific primers, was limited as there is little interspecific variation in 16S ribosomal DNA (rDNA), and the identification of alternate genes suitable for PCR was hampered by the lack of sequenced animal mycoplasma genomes, but Kumar, *et al;* (2011) used species specific primers by the PCR technique to successfully isolate, identify and characterise *Mycoplasma* species from goats in india. This milestone has facilitated researches into characterizing various species of mycoplasma by the PCR.

The aim of this investigation therefore is to use the PCR technique to identify to species level the previously isolated suspected mycoplasma organisms which were identified morphologically.

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4.2 Materials and method

Method

The polymerase chain reaction was used and the following protocols were followed:

- Extraction of DNA from samples
- PCR protocol optimization
- PCR
- Running of the PCR product on the gel
- Detection from uv light

4.2.1. Extraction of DNA from samples

1. Positive cultures in liquid broth were centrifuged at 10000 x g for 5 minutes and the pellet was resuspended in 800µl of water.
2. The mixture was put in a spin column in a collecting tube, centrifuge at 10,000 x g for 1 minute and then the collection tube and the flow through were discardeded.

3. The pellets in the spin column was transfered to a new collection tube and 200µl of DNA Pre-wash Buffer was added and then centrifuged at 10,000 x g for 1 minute
4. 500ul of g-DNA wash buffer was added to the spin column and centrifuged at 10,000 x g for 1 minute

5. Spin column was transferred to a clean microcentrifuge tube, and 10µl of DNA Ellution Buffer was added to the spin column, incubated at room temperature for 5 minutes and then centrifuged at 10,000 x g for 30 seconds to elute the DNA.

The Eluted DNA is used for molecular study.

The PCR procedure applied was that the deoxyribonucleic acid (DNA) samples were tested with group – specific PCR for *Mycoplasma spp.*

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4.2.2 Protocol

The PCR were performed in a TC 512 temperature cycling system in a reaction volume of a 12.50ul, containing 1.25ul 10X PCR buffer(750Mm Tris HCL, pH 8.8, 200Mm $(NH4)_2$ SO_4, 0.1% Tween 20), 1.0ul of 50mM $MgCL_2$, 10uM of each deoxynucleotide triphosphate, 1.0U Taq DNA polymerase, 20 pmol of each primer and 25ng of template DNA. All the primer specific sequences used in this study (both for the general mycoplasma and the specie specifics except for *Mycoplasma bovis* which was as studied by Hassan, *et al*, (2011) were as developed and used by Kumar, *et al*., (2011).

Primer pairs specific to the Mycoplasma genus are:

(GPO3F 5' – TGG GGA GCA AAC AGG ATT AGA TAC C – 3' and

MGSO 5' – TGC ACC ATC TGT CAC TCT GTT ACA CTC -3').

The reaction condition for the group – specified PCR were as follows:

- one cycle of the denaturation step at 94°C for 2 minuites
- 35 cycles of denaturation at 94°C for 15 sec.
- Annealing at 53°C for 15 seconds and extension at 72°C for 15 seconds.
- One cycle of extension step at 72°C for 5 minuites

The amplification products were detected by staining with ethidium bromide (0.5 µg/ml) after electrophoresis at 80 volts for 2 h in 1.5% agarose gels. Polymerase chain reaction product with molecular size 280 base pairs (bp) was considered indicative for *Mycoplasma spps (*Plates 3 and 4 resp).

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4.2.2a Identification of *Mycoplasma* isolates up to species level

Furthermore the *Mycoplasma* species isolated were also identified by molecular methods. The process of the various DNA extraction were the same as applied to the general Mycoplasma isolates, their differences only applies to the various oligonucleotide primers that were used.

A). Oligonucleotide primers used for amplification of DNA recovered from *Mycoplasma bovis* isolates:

The PCR amplicone was a part of *M. bovis* DNA sequence, with the following primer sequences

Forward 5' - GCA ATA TCA TAG CGG CGA AT- 3' and

Reverse 5' - TCT CAA CCC CGC TAA ACA TC -3'.

The primers amplify a 227 bp fragment which is considered indicative for *Mycoplasma bovis*.

The reaction condition for *M. bovis* is as follows:

- one cycle of the denaturation step at 94^0 C for 2 minuites
- 30 cycles of denaturation at 94^0 C for 30 sec.
- Annealing at 52˚C for 1/min and extension at 72˚C for 15 seconds.
- One cycle of extension step at 72˚C for 5 minuites

The amplification products were detected by staining with ethidium bromide (0.5 µg/ml) after electrophoresis at 80 volts for 2 h in 1.5% agarose gels. Polymerase chain reaction products with molecular size 227 base pairs (bp) was considered indicative for *Mycoplasma bovis (*Plate 5).

B). PCR using the *Mycoplasma capricolum* subsp. *capricolum* specific primers

P4- 5'- ACT GAG CAA TTC CTC TT – 3' and

P8 - 5' - GTA AAC CGT GTA TAT CAA AT – 3'.

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An expected amplified product is at the 192 bp long sequence of the 16S rRNA gene of the *Mycoplasma capricolum subsp. capricolum* (Hernandez, *et al;* 2006).

The amplification was carried out using the following programme:

- One cycle of initial denaturation at 94 °C for 1 min
- 30 cycles of denaturation at 94 °C for 30 sec,
- Annealing at 56 °C for 1 min and extension at 72 °C for 1.5 min
- final extension at 72 °C for 5 min followed by 10 °C hold at infinity.

The amplification products were detected by staining with ethidium bromide (0.5 µg/ml) after electrophoresis at 80 volts for 2 h in 1.5% agarose gels. Polymerase chain reaction products with molecular size 192 base pairs (bp) was considered indicative for *Mycoplasma caprocolum subsp. capricolum.*

C). PCR using the *Mycoplasma mycoides* subsp. *capri* specific primers

P4 - 5'- ACT GAG CAA TTC CTC TT - 3' and

P6 – 5'- TTA ATA AGT CTC TAT ATG AAT – 3'.

An expected amplified product is at the 194 bp long sequence of the 16S rRNA gene of the *Mycoplasma mycoides subsp. capri* (Hernandez, *et al.,* 2006) as shown in plate 6.

The amplification was carried out using the following programme:

- initial denaturation at 94 °C for 1 min

- 30 cycles of denaturation at 94 °C for 1 min,

- annealing at , 46 °C for 1 min and extension, 72 °C for 2 min,

final extension at 72°C for 5 min followed by hold at infinity.

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To confirm the targeted PCR amplification, 5 µL of PCR product from each tube was mixed with 1 µL of 6X gel loading buffer from each tube and electrophoresed on 1.5 % agarose gel along with 1000 bp DNA Ladder (GeneRuler- Fermentas) and stained with ethidium bromide (1 % solution at the rate of 5 µL/100 mL) at a constant 80 V for 30minutes in 0.5X TBE buffer. The amplified product was visualized as a single compact band of the expected size under UV light and documented by the gel documentation system.

4.2.3 PCR protocol optimization

The PCR mix used was optimized to know which preparation will be adequate for each test. Optimum reaction for one test was then multiplied to the number of test to be done. The PCR mix is as follows:

	X1	X20
Sample DNA	5.0ul	
Master Mix	6.25ul	125ul
Primer F (20 pmole)	0.15ul	3.0ul
Primer R (20pmole)	0.15ul	3.0ul
Water	0.85µl	17.0ul

The PCR buffer, $MgCL_2$, dNTPs (deoxynucleoside triphosphate) and Taq platinum Polymerase were all produced by Fermenters (U.S.A) and packaged as a component called Master Mix. A known Mycoplasma spp. (*Mycoplasma mycoides subsp. capri* U21) was used as a positive control and sterile mycoplasma broth was used as negative control. The control organisms were processed along with the test samples in all the stages. All the materials for the PCR mix were multiplied by 20 to take care of the 18 test samples and the 2 controls.

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PCR Optimization for the Mycoplasma species.

a). *Mycoplasma bovis:*

	X1	X4
DNA	5ul	
Master Mix	6.25ul	25.0ul
Primer F (20pmole)	0.15ul	0.6ul
Primer R (20pmole)	0.15ul	0.6ul
Water	0.85ul	3.4ul
Total	7.4ul	29.6ul

b). *Mycoplasma capricolum subsp. capricolum*

	X1	X6
DNA	5ul	
Master Mix	6.25ul	37.5ul
Primer F (20pmole)	0.15ul	0.9ul
Primer R (20pmole)	0.15ul	0.9ul
Water	0.85ul	5.1ul
Total	7.4ul	44.4ul

c). *Mycoplasma mycoides subsp. capri*

	X1	X4
DNA	5ul	
Master Mix	6.25ul	25.0ul
Primer F (20pmole)	0.15ul	0.9ul
Primer R (20pmole)	0.15ul	0.9ul
Water	0.85ul	3.4ul
Total	7.4ul	29.6ul

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4.2.4. PCR

The PCR amplification of the extracted DNAs was performed using 25 µl of the PCR mix these were put in 1.5ml micro centrifuge tubes and transferred to a thermal cycler (Eppendorf, Germany). Initial denaturation was at 94°C for 2 minutes followed by:

- Denaturation at 94°C for 15 seconds
- Annealing at 53°C for 15 seconds and
- Extension (elongation) at 72°C for 15seconds.

This was done for 35 cycles. It was followed by a final extension at 72°C for 5 minutes. This is the **PCR Product** and was left at holding temperature for 20°C until ready to use.

4.2.5 Electrophoresis

Gel preparation: 1.5% of Agarose gel was prepared in 0.5M TBE buffer, melted and allowed to cool before being stained with 1.5 µl ethidium bromide. The gel was poured into a tough and comb – like structure which made holes/wells on the gel. When set, the gel block was transferred to the electrophoresis tank containing the TBE buffer.

Five (5.0) µl of the PCR product was mixed with 2 µl of the loading dye and 5 µl of each mixture was put in separate wells in the agar block already inside the tank. The loading dye enables the PCR product to sink into their respective wells. Being coloured it also enables one to observe the migration of the product during electrophoresis. The side markers (M) were put on the first and last lanes of the gel while the test samples were put on lanes 2 to 9, and the positive and negative controls were put on lanes 10 and 11 and labeled P and N respectively. The power pack of the electrophoresis tank was switched on and ran at 80mv for 2hrs. The expected band for positivity is 280bp (base pair) for all *Mycoplasma species.*

4.2.6 Detection from UV light: At the end of the electrophoresis, the gel block was taken from the tank and placed on a large camera (Kodak, U.S.A.). The amplified product was visualized and the result of the electrophoresis was photographed under UV light.

Statistical analysis

All the report values in this study were number/percent.

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4.3 Results

A total of 18 broth cultures were subjected to the PCR technique. The PCR products were visualized in an agar rose gel where 280 bp was observed as positive for *mycoplasma* isolates. Thirteen wells were created on the agar block in the electrophoresis tank. The first and the last well were used for the side markers of 1000kb, while the 2nd to the 10th wells served for the *mycoplasma* organisms, the 11th and 12th well were used for the positive and negative controls using a known mycoplasma DNA codon (*Mmc*U21) as positive and mycoplasma broth without the organism (sterile broth) as the negative, this setup was in two fold to accomondate for the total number of 18 *mycoplasma* isolates – plates 3 and 4. Digestion of the PCR product produced bands at 280bp except for the isolates on wells 3 and 5. The presence of positive band showed that *Mycoplasma* organisms are present in the sample. The uncleaved DNA fragment at 280 bp of the two samples could probably have originated from other genital but non-mycoplasmal mollicutes which by biochemical analysis were isolated as mollicutes. Furthermore, three species of the four *mycoplasma* isolates were characterzed using their specific primer pairs, they include *M. bovis*, *M. capri and M. capricolum.* These three mycoplasma species were individually analysed according to their respective thermocycler requirements of denaturation and annealing temperatures standards (Eppendorph, Germany). Three isolates were characterzed as *M. bovis* with positivity at 227bp (plate 5), four isolates were positive for *M. capricolum* with positive reaction at 192bp while three isolates were positive for *M. capri* with positive reaction at 194bp (plate 6).

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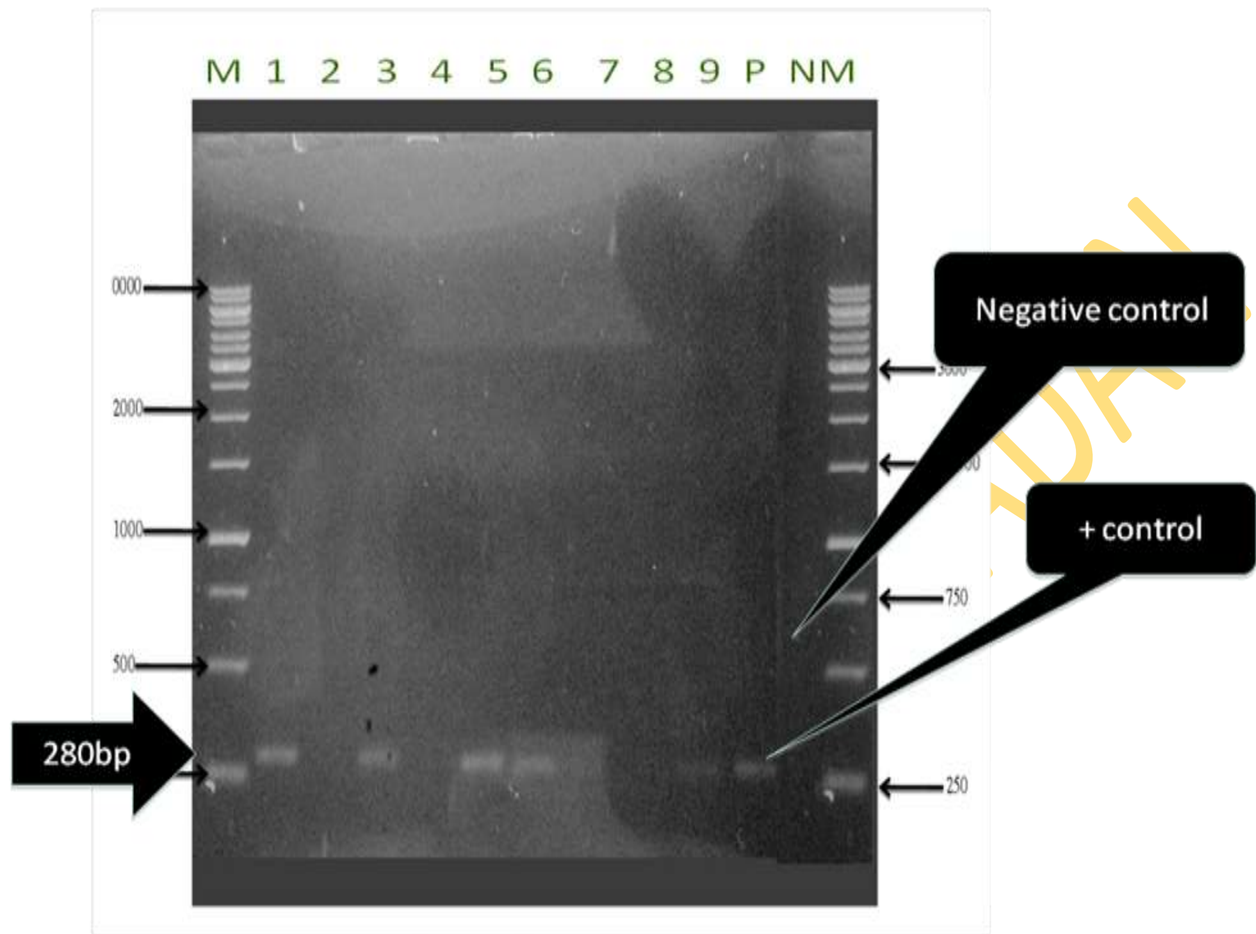

Plate 3: Result of PCR diagnostic test for Mycoplasma strain from genital swab.

Expected band for positive reaction =280bp

M = Marker lane

N = Negative control lane

P = Positive control lane

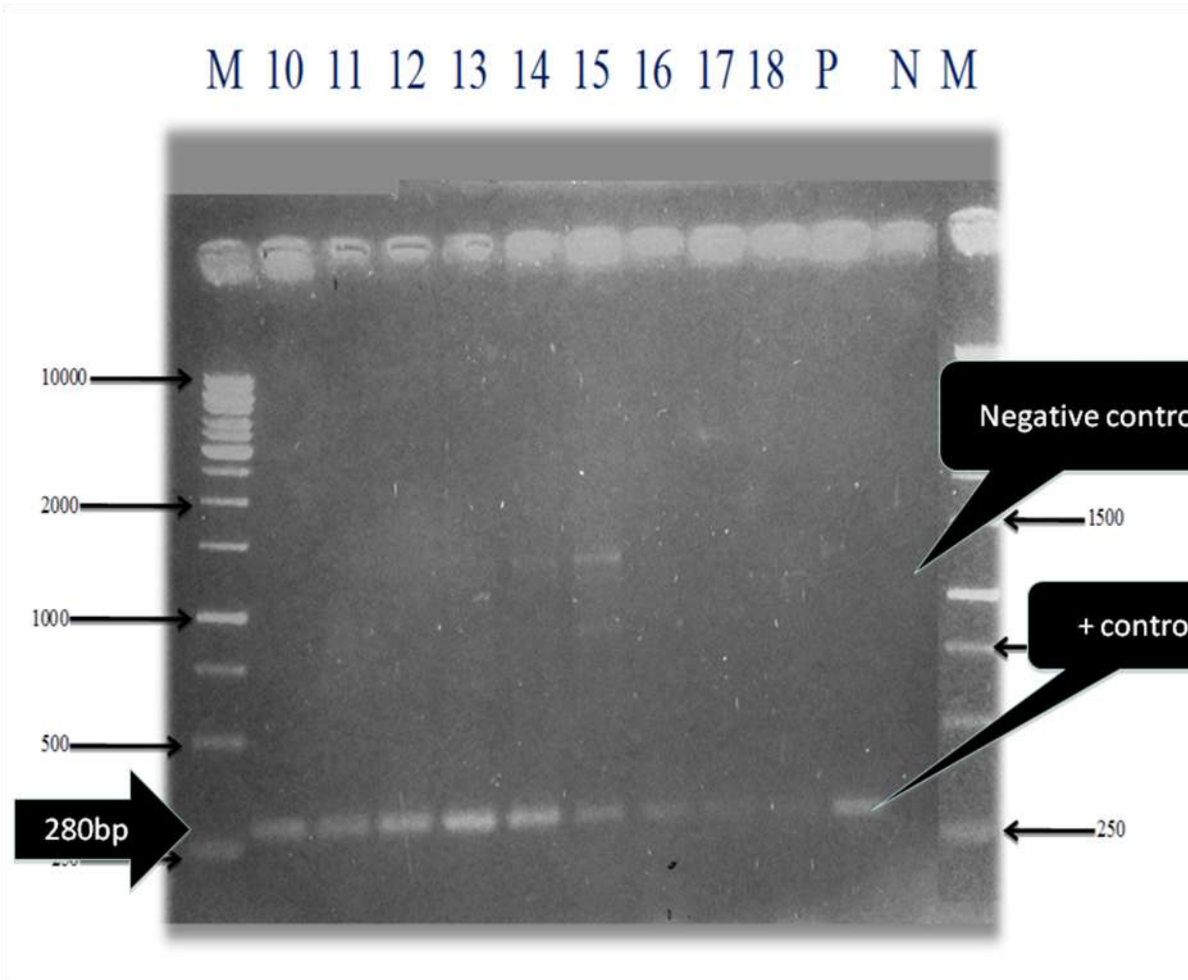

Plate 4: Result of PCR diagnostic test for Mycoplasma strain from genital swab.

Expected band for positive reaction =280bp

M = Marker lane

N = Negative control lane

P = Positive control lane

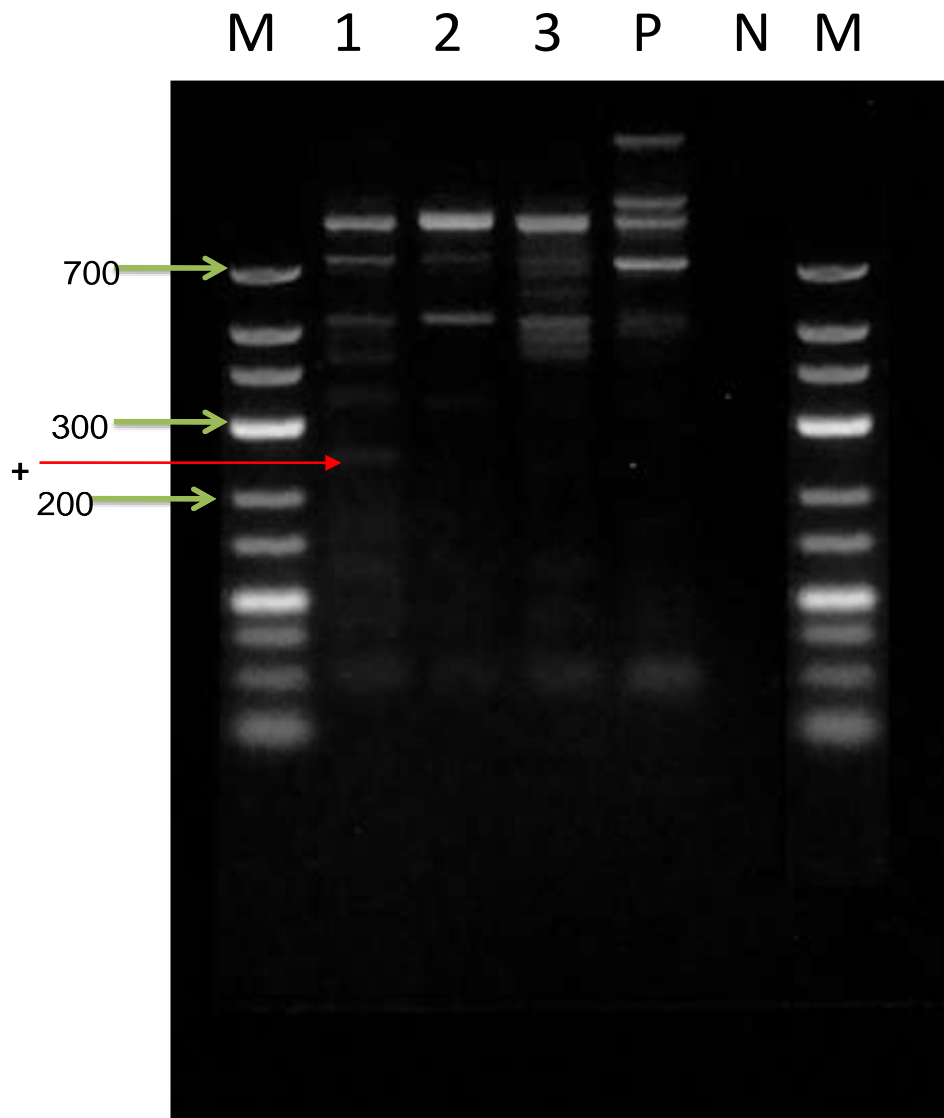

Plate 5 – *M ycoplasma bovis* specific gel amplification

Expected band for positive reaction =227bp

M = Marker lane

N = Negative control lane

P = Positive control lane

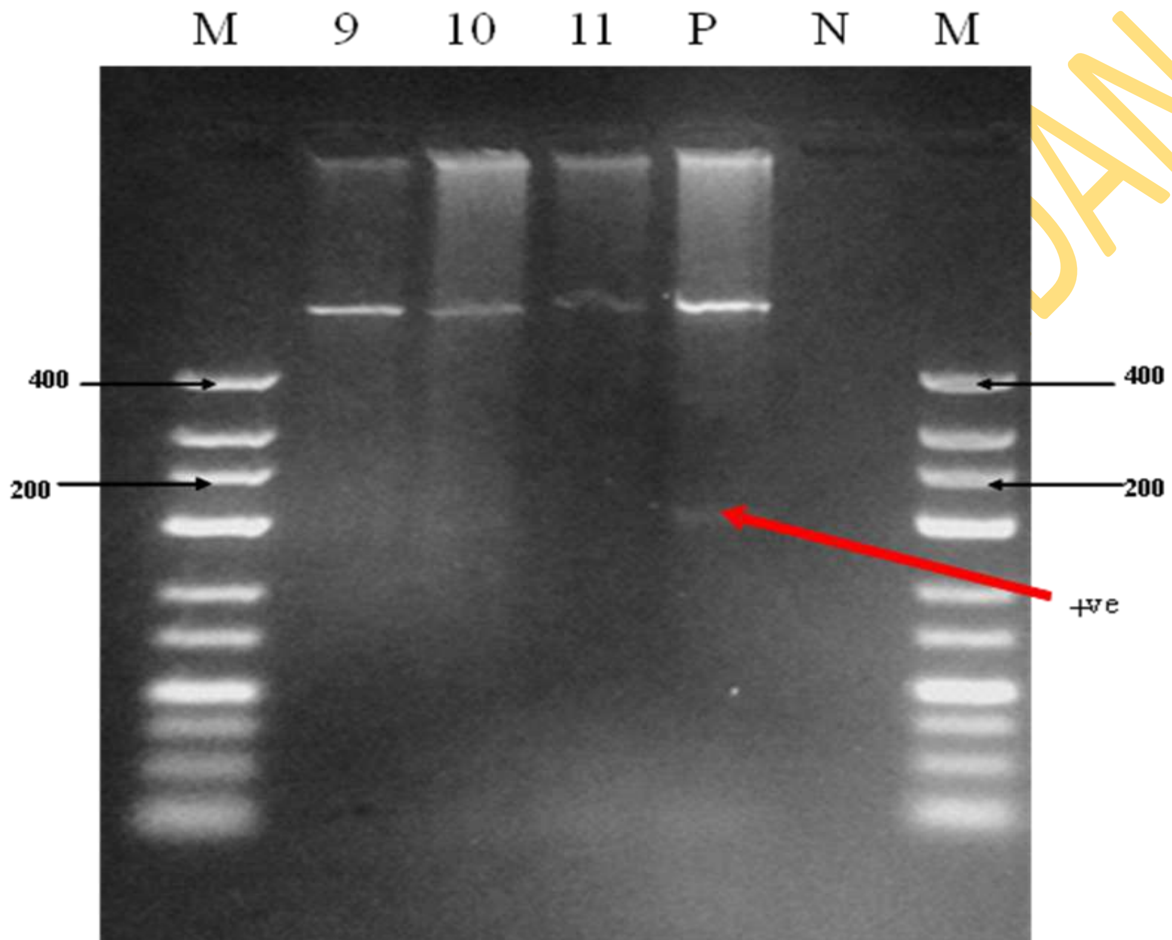

Plate 6: ***Mycoplasma capri*** - *Mycoplasma capri* specific gel amplification (194 bp)

Expected band for positive reaction =194bp

M = Marker lane

N = Negative control lane

P = Positive control lane

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4.4 Discussion

Of the 221 different samples processed, a total of 13 *Mycoplasmas*, 3 *Ureaplasmas* and 2 *Acholeplasma* isolates were obtained morphologically. PCR analysis shows that among the Mycoplasma isolates, 4 are *Mycoplasma capricolum, 3 Mycoplasma arginini*, 3 *Mycoplasma bovis*, 3 *Mycoplasma capri*. By the PCR method of classification, 2 out of the 18 isolates were found to be non-mycoplasmal isolates (possibly *Acholeplasmas* which agrees with the colonial morphological classification) due to their non-reaction to the general *Mycoplasma* primer adopted for this study, this however indicates and confirm the presence of *Mycoplasma* infection in goats in Lagos state, Nigeria.

PCRs performed were founded upon the sequences for 16S rRNA and on the genomic DNA of the 18 isolates from this study. The primer pairs specific to the *Mycoplasma genus* – GP03F and MGSO (Kumar*, et al*; 2011) were used, and they produced bands as expected at a base pair of 280bp in all positive isolates. This finding is in agreement with what Centikaya*, et al.,* (2009) observed, that designed these oligos which are complementary to each end of the sequence of the CAP-21 probe. Furthermore, molecular characterization of each *Mycoplasma species* isolated in this study was done by using a set of primers (oligos) specifically designed for each specie. The following *Mycoplasma* species were characterized in this investigation viz *M. bovis, M. capricolum* and *M. capri*.

Mycoplasma bovis - a member of the *Mycoplasma mycoides* group, has three (3) isolates identified in this study by the PCR technique and by the primer sequence employed. This primer sequence amplify the suspected isolate of *M. bovis* at approximately 227bp sequence along the sequence of the 16S rRNA gene codon of the *M. mycoides* cluster for all the isolates. This finding is in agreement with that of Hassan, *et al*., (2011) who developed a CCPP specific PCR primer that amplify at 548 bp sequence of the 16S rRNA genes from all the six members of the mycoides cluster and a digestion by the enzyme *Ehe*l resulting in the fragment 227bp, which is characteristic of *M. bovis*. Plate 5 show the gel picture of the electrophoresis of the *Mycoplasma bovis* isolates in this study. It is suggestive from this observation that most of the mycoides cluster of *Mycoplasma* may also be in the sample being analyzed but their details are not within the scope of this present study. The *Mycoplasma capricolum (Mcc)* specific primers P4 and P8 resulted in an approximately 192 bp long sequence of the amplified product of the16S rRNA gene for 4 isolates, identified as *M. capricolum* subsp. *capricolum*. None of the 3 isolates identified as *M. mycoides* subsp. c*apri* yielded

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any amplified product with this primer pairs. This finding is in agreement with that of Hotzel *et al.* (1996), Kumar (2000) and Hernandez *et al.* (2006) who used the *Mycoplasma capricolum subsp. capricolum* (Mcc) specific primer P8 in combination with another primer P4, which is common to all cluster members except MmmSC, to amplify approximately 192 bp product from only Mcc strains as positive results. PCR using the *Mycoplasma mycoides* subsp. *capri* specific primers P4 and P6 resulted in an approximately 194 bp long amplified product of the16S rRNA gene for all the 3 isolates identified as *M. mycoides* subsp. *capri,* but none of the 4 isolates identified as *M. capricolum* was amplified with this primer. This finding suggests specificity of primers which is in agreement with that of Hotzel*, et al.* (1996); Kumar, (2000) and Hernandez, *et al*. (2006), who used *Mmc* specific primers (P4 and P6) and yielded approximately 194 bp product with *Mmc* strains and not with other strains (Plate 6). Other *Mollicutes* isolated in this study are *Mycoplasma arginini, Ureaplasma spp* and *Acholeplasma spp* but they were not characterized by molecular means as their specific primer types were not available for use in this work.

In this investigation, PCR techniques with the use of specific primers were employed to identify the mycoplasma organisms and their species. Analysis of the findings from this study implies that there is indeed a confirmation of *Mycoplasma* infections in farms visited in Lagos state, Nigeria. It is however advised that more of the specific primers for typed mycoplasma genome be employed to comprehensively characterize all the mycoplasma species that may be present in clinical cases of vulvovaginitis of goats in Nigeria. Isolation and identification of *Mycoplasmas* was conducted from samples collected from goats presenting the following signs: abortion, swollen and hyperemic vulva, mastitis and loss in milk production. PCR based detection of *mycoides* cluster group and *species* is a rapid and simple method of detection and identification of the *Mycoplasma* organism and can be an effective tool for epidemiological surveys. It is however recommended that for further studies, restriction enzyme fragmentation assay can be a useful method for differentiation and identification of *Mycoplasma* isolates.

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CHAPTER FIVE

5.0: BIOCHEMICAL TESTS ON MYCOPLASMA ISOLATES

5.1 Introduction

The Order *Mycoplasmatales* consisting of the genera *mycoplasmas* and *ureaplasmas* which belong to the class *Mollicutes* have been biochemically characterized based on carbohydrate (CHO) metabolism. Some of these CHO include glucose, maltose, glycogen or arginine. Some mycoplasmas have been observed to ferment glucose with acid production, hydrolyse arginine to produce ammonia while some others do not ferment glucose nor hydrolyse arginine (Ojo, 1976). The genus ureaplasma is known to hydrolyse urea (Waites, *et al;* 2001)

When glucose is fermented, the pH of the medium falls and this is observed by a color change of the medium by a pH indicator which is most times phenol red. Other biochemical tests such as Digitonin sensitivity test (Ernø and Stipkovits, 1973), Phosphatase test, Serum digestion test, Tetrazolium and methylene blue reduction test (Aluotto, *et al*, 1970) and the 'film and spot' test (Fabricant and Freundt, 1967) have been used to classify mycoplasmas biochemically.

This investigation is carried out to biochemically identify and group *mycoplasma* and *ureaplasma species* isolated in this study.

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5.2: Materials and Method

5.2.1 Method: Processing of samples

The eighteen (18) isolates that were identified morphologically and characterized by PCR as *Mycoplasmas* (13), *Ureaplasmas* (3) *and Acholeplasmas* (2) were biochemically analysed using the following: glucose, arginine, urea, phosphate, tetrazolium chloride and serum.

5.2.1a: Glucose hydrolysis test.

Materials

The materials /reagents needed for this test include:

Mycoplasma broth with supplement	37.0ml
Horse serum	5.0ml
10% glucose	5.0ml
0.5% phenol red solution	5.0ml

Method

Into the mycoplasma broth (already sterilized by autoclaving) with mycoplasma supplement that was added on cooling, other reagents (stated above) were mixed in it and dispensed in 2.5ml amounts into rubber cork–capped tubes. Two sets of controls were used and they include:

- Media control: uninoculated media containing the appropriate test substrate.
- Substrate control: inoculated media containing sterile water in place of the test substrate.

A 0.5ml volume of 48 – hour broth culture grown in horse serum broth was used to inoculate each of the test and appropriate control tubes. These where incubated at 37°C anaerobically and read daily for 2 weeks. The day the positive reaction was observed was noted and recorded. The result

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was read by comparing the test with the two control tubes. A positive result was recorded when a colour change from pale amber to yellow was observed. No colour change by the end of incubation indicated negative result. Both control tubes remained unchanged.

5.2.1b: Arginine hydrolysis test.

Method

This is as described for glucose above however 5.0ml of 10% arginine (w/v) was used as the substrate. Incubation and inoculation of both the test and control tubes was equally as described for glucose. A positive test was recorded when a change in colour from a red clear solution to a deeper red solution was observed. Both control tubes remain unchanged. The day a positive result was observed, it was recorded. This positive result was obtained by comparing with the uninoculated control tubes.

5.2.1c: Urea hydrolysis test.

Method

The method used here is as described for glucose above. However, 5.0ml of 10% urea (w/v) was used as the substrate. Inoculation of both the test and control tubes is as described for glucose. A positive reaction was indicated by a change in colour from pale amber to intense red.

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5.2.1d: Phosphatase test.

Method

Into 36.4ml of *Mycoplasma* broth (sterilized by autoclaving), with Mycoplasma supplement added on cooling, the following were filter sterilized and aseptically incorporated:

1.0% Phenolphthalein diphosphate solution	0.5ml
Penicillin solution	0.1ml

The medium was dispensed in 1.5ml amount into 12 X 15mm tubes and covered with sterile corks. Two drops of 48 – hour broth cultures of each test isolate was incorporated into the tubes (in duplicates). These were incubated for 7 days.

On the 3rd day a drop of 5N NaOH was incorporated into each of the first set of tubes and in a control tube (without organism) and observed for an immediate change of colour to intense pink. This procedure was repeated on the 7th day with the 2nd set of tubes. A positive reaction will normally show a colour change from yellow to intense pink colour while the negative reaction will not record any colour change.

5.2.1e: Serum digestion.

Serum digestion was done using sterile photographic black and white film strips. The medium consisted of:

Sterile Mycoplasma broth	37.0 ml
Penicillin	0.1ml

Method

The method used by Okwoli, (2007) was adopted for this test. The *Mycoplasma* broth was sterilized by autoclaving and all the other components were aseptically added. The complete medium was dispensed in 1.5ml amount in sterile ½ ounce bottles. Each bottle was inoculated respectively

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with 1 drop from 48 – hour broth culture of each of the test organism. Sterile black and white photographic filmstrip – 10 X 5mm (sterilized by autoclaving) was incorporated into each bottle and incubated aerobically. A control culture containing only filmstrip without organism was set up along with the tests. A positive test was seen as the presence of dark coloured deposits at the bottom of the bottles.

5.2.1f: Tetrazolium reduction test.

Preparation of the substrate

A 2% stock solution of 2, 3, 5 – triphenyl tetrazolium chloride was prepared by dissolving 0.1gram of the substance into 5.0ml of sterile distilled water. This was filtered and sterilized.

Method

The method used by Aluotto, *et al;* (1970) and modified by Okwoli, (2007) was used and the ingredients consist of:

Sterile Mycoplasma broth with supplement	72.5ml
1.0% tetrazolium chloride	1.0ml
Penicillin	0.25ml

Apart from the *Mycoplasma* broth that was sterilized by autoclaving, other components of the medium were sterilized by filtration. The medium was well mixed and dispensed in aliquots of 3.0ml into 12 X 75mm tubes. The test was set up in duplicates and dense growth of the test organism on agar plates were cut and the agar used to inoculate the broth. Two (2) control tubes were capped with sterile Vaseline – paraffin mixture. All were incubated at 37°C for up to 7 days (Okwoli, 2007). The tubes were observed daily for the presence of a red or pink colour in the medium especially in the area of agar block, this is a positive reaction.

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5.2.1g: Methylene blue reduction test.

The method of Aluotto, *et al;* (1970) as modified by Okwoli (2007) was used for this test.

Method

Using a sterile pipette 0.05ml of 0.1% methylene blue solution (sterilized with 0.45μm Millipore filter) was added to a sterile mycoplasma broth in 12 X 75 mm tubes in duplicate. The 1.5ml of a 48 – hour broth culture of the test organism was added to each tube except the two control tubes that contains 1.45ml of uninoculated broth. The first set of tubes was covered with sterile gauze stoppers and the second sets were overlayed with 0.75ml of sterile Vaseline – paraffin mixture. These were incubated at 37°C and read at 24, 48 and 72 hours for reduction.

A positive reaction was seen as a complete dis – colourization of the broth while a blue colour shows a negative reaction. A green colour shows a weak positive reaction.

5.2.1h: Film and spot.

Method

Onto a *Mycoplasma* agar medium containing 20% (v/v) horse serum, a 48 – hour broth culture of the test organism was inoculated (by flooding and discarding excess). This was incubated for 10 days at 37°C in a candle jar (an anaerobic condition) and the plate was examined daily during the incubation for the production of film and spots.

Normally, positive reaction produces broom-like structures on *mycoplasma* agar while negative reaction would produce no morphological change. At the end of ten days there was no positive result in all samples inoculated and incubated.

Statistical analysis

All reported values in this study were as number/percent.

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5.3 Results.

Biochemical analyses were carried out on the 13*Mycoplasma,* 3 *ureaplasma* and 2 *Acholeplasma* isolates and 9 metabolic characteristics were tested for (table 5). These include Digitonin sensitivity test, glucose breakdown, Hydrolysis of arginine, Hydrolysis of urea, film and spot production test, phosphatase test, methylene blue reduction test, tetrazolium chloride reduction test and serum digestion test.

Digitonin sensitivity tests were performed on the genital mycoplasmas that showed positive feature of the typical fried egg appearance when viewed under X40 dissecting microscope. The digitonin sensitivity test is to further help in identifying specific organisms involved in this study by their reactions to the test. The result of this test is seen by the zone of inhibition or clearance that such organism displays around the digitonin sensitive disc. Such zones were measured using a straight transparent ruler and the sizes indicated (Table 4). Of the eighteen (18) isolates with positive features of the typical fried egg appearance, sixteen (16) of them show zones of inhibition with the digitonin impregnated disc. This gives us an overall *mycoplasma* and *ureaplasma* prevalence rate of 88.9% of the scope of this study.

The glucose hydrolysis tests showed that eleven isolates were positive while it was ten isolates that were positive for arginine hydrolysis in the reaction. The incubation period for both tests was for 14 days, however positive results (colour change from pale amber to yellow for glucose and from plain red to a deep red for arginine) were seen after 24hrs of incubation. Positive results were scored with a plus (+) while negative reactions were with a minus (-) as seen in table 6 and in plates 10 and 11 respectively. Seveenteen of the 18 isolates were positive for tetrazolium reduction test and three are urease producers.

Following the chat by DaMassa, *et al.*, 1987, (Table 2), the isolates were identified to be *M. bovis* (2), *M. capricolum* (4), *M. capri* (3), *M. arginini* (4), *Ureaplasma spps* (3) and *Acheleoplasma spps* (2) based on their biochemical reaction to the tests done.

The test for phosphatase activity was done on two sets and both sets exhibited positive activity for 8 isolates by colour change from light pink to intense pink colour. The second set of tubes were incubated for 7 days and 5N NaOH was added between the 3rd and the 7th day, thus producing the same positive colour change result. However, it was observed that the pink colour of test faded

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gradually on standing.There were four isolates with variable results whose colour change could not be considered as positive but were also not negative and as such were marked V. The methylene blue reduction test has 14 of the 18 isolates as positive by reducing methylene blue aerobically and anaerobically. However there are three samples that reacted weakly to the test and were marked weak (W).

All eighteen isolates were hydrolysed by proteolytic enzymes in the serum digestion test (Table 5). The positive reaction was observed as black granules / particles at the bottom of tubes. Incubation was for 5 days but the positive isolates showed up at 24hours. All isolates were positive aerobically. Positive isolates were scored plus (+) while the negative one was scored a minus (-). None of the testsed isolates was positive for the film and spot biochemical test carried out. Also according to their carbohydrate fermentation, isolates were grouped into 4 namely :(a)glucose positive/ arginine positive, (b)glucose positive/arginine negative; (c)glucose negative /arginine positive and (d)glucose negative/arginine negative. This is to aid their categorizations based on methabolic pathways employed (Okwoli, 2007). Four isolates (all 4 *M. capricolum*) were identified as group A, Seven (3 *M.mycoides subsp.capri*, 2 are *Ureaplasma spps.,* 2 *Acheleoplasma spp*) were identified as group B, Five isolates (4 *M. arginini* and 1*Ureaplasma spp*.) identified as group C while Two isolates (both *M. bovis*) were identified as group (D).

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Plate 7: Some *Mycoplasma* isolates showing varying zones of inhibition to digitonin – impregnated disks.

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Table 4: Suspected Mycoplasma Species Impregnated with Digitonin Disks and zones of inhibition.

S/N	Samples	Inhibition zone Diameter
1	1	zero
2	2	6.9mm
3	2'	7.4mm
4	2''	6.1mm
5	3	4.8mm
6	5	5.9mm
7	6	4.8mm
8	8	5.8mm
9	9	7.0mm
10	14	8.4mm
11	15	8.6mm
12	17	7.5mm
13	19	5.9mm
14	20	7.0mm
15	21	6.9mm
16	25	8.0mm
17	25'	Zero
18	28	7.9mm

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Positive

Negative

Plate 8: Hydrolysis of urea by *Ureaplasma* isolates on Urea slopes. (Urease test).

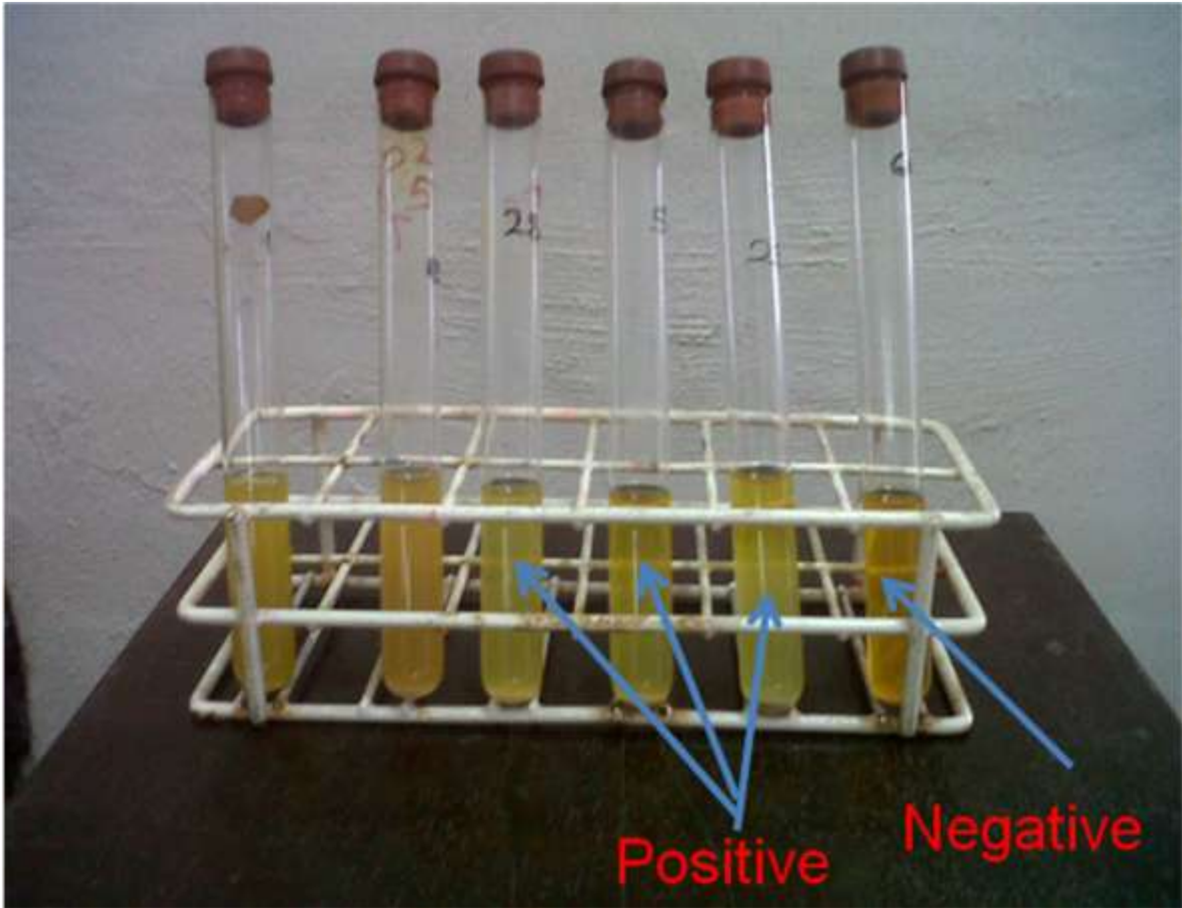

Plate 9: Some results of the Glucose hydrolysis test.

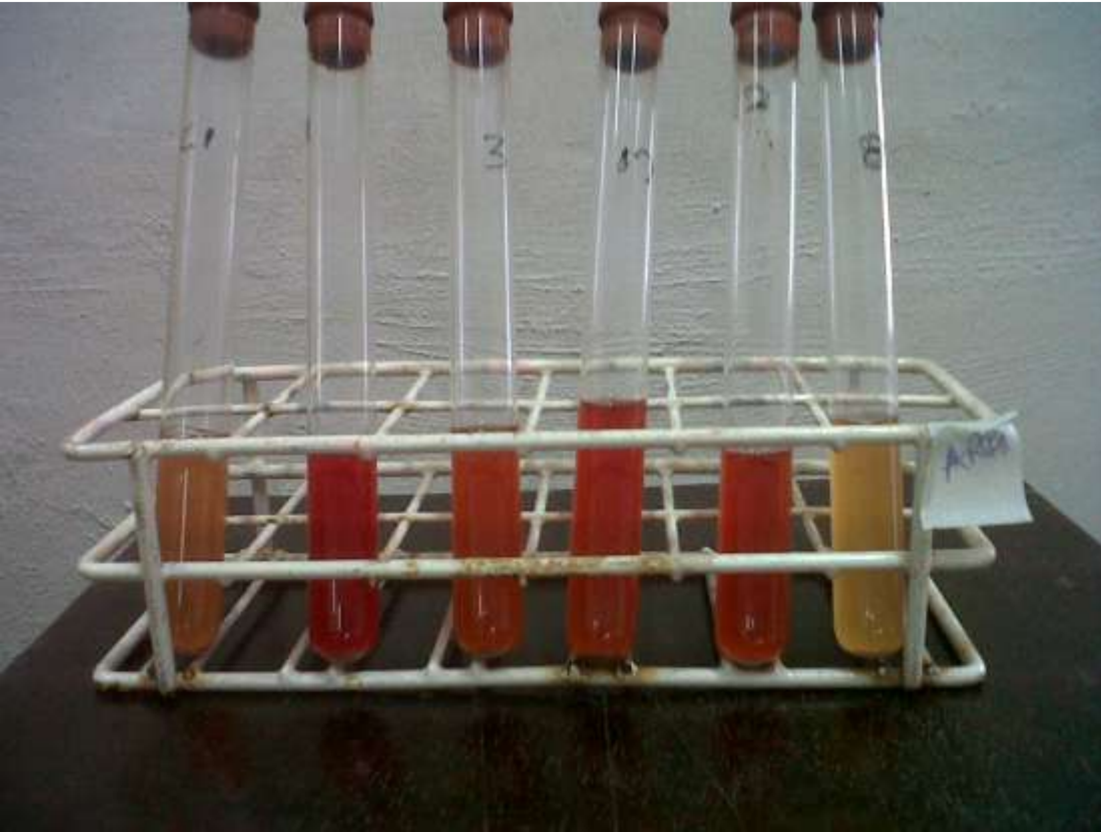

Plate 10: Some results of the Arginine hydrolysis test.

PLATE 12: Some results of the Tetrazolium reduction test.

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Table 5: RESULT OF BIOCHEMICAL TEST ON VARIOUS TEST ORGANISMS

Sample	D	G	A	U	T	C	F	Ph	Mb	Organism
1	-	+	+	-	+	+	-	+	+	*Acheloplasma spp.*
2	+	+	-	-	+	+	-	+	+	*M.capri*
2’	+	-	+	-	+	+	-	-	-	*M.arginini*
2”	+	+	-	+	-	+	-	-	+	*Ureaplasma spp*
3	+	+	-	-	+	+	-	+	W	*M.capri*
5	+	+	+	-	+	+	-	+	+	*M.capricolum*
6	+	+	-	-	+	+	-	V	+	*M.capri*
8	+	+	+	-	+	+	-	+	+	*M.capricolum*
9	+	-	+	+	+	+	-	V	W	*Ureaplasma spp*
14	+	-	+	-	+	+	-	-	+	*M.arginini*
15	+	-	+	-	+	+	-	V	+	*M.arginini*
17	+	-	+	-	+	+	-	V	+	*M.arginini*
19	+	+	+	-	+	+	-	+	+	*M.capricolum*
20	+	+	+	-	+	+	-	-	W	*M.capricolum*
21	+	-	-	-	+	+	-	-	+	*M.bovis*
25	+	+	-	+	+	+	-	-	+	*Ureaplasma spp*
25’	-	-	-	-	+	+	-	+	+	*Acheloplasma spp.*
28	+	+	-	-	+	+	-	+	+	*M.bovis*

Key

D – Sensitivity to Digitonin

G – Glucose hydrolysis.

A – Arginine hydrolysis.

U – Urea utilization.

T – Tetrazolium reduction

C – Serum digestion

F – Film and Spot.

Ph – presence of Phosphatase

Mb – Methylene blue reduction.

+ **--** Positive

V – Variable.

W – Weakly positive

-- Negative.

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Table 6: Grouping of Mycoplasma isolates according to biochemical results.

Tests	*M. bovis*	*M. capricolum*	*M. capri*	*M. arginini*	*U. spp*	*A. spp*
	(2)	(4)	(3)	(4)	(3)	(2)
Glucose	+	+	+	-	-/++	+
Arginine	-	+	-	+	-/++	-/+
Urea	-	-	-	-	+	-
Tetrazolium	+	+	+	+	-/+ +	+
MB	+	+	+	+	+	+
Phosphatase	+	+	+	-	-	+/v
FS	-	-	-	-	-	-
Serum Digest.	+	+	+	+	+	+
Digitonin	+	+	+	+	+	-

KEY.

MB - Methylene blue

FS - Film and Spot

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Table 7: Grouping the *mycoplasma* specie according to their carbohydrate fermentation

Mycoplasma spp.	Carbohydrate group			
	A (G+/A+)	**B (G+/A-)**	**C (G-/A+)**	**D(G-/A-)**
M.capricolum	4	0	0	0
M. mycoides ssp. Capri	0	3	0	0
M.arginine	0	0	4	0
M. bovis	0	0	0	2
Ureaplasma spp	0	2	1	0
Acholeplasma spp	0	2	0	0

Key:

G+/A+ Glucose positive/Arginine positive

G+/A - Glucose positive/Arginine negative

G-/A+ Glucose negative/ arginine positive

G-/A- Glucose negative/Arginine negative

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5.4 Discussion

Biochemical analyses were carried out on all the 18 genital isolates and nine metabolic characteristics were tested for, which include Digitonin sensitivity test, Glucose breakdown, Hydrolysis of arginine, Hydrolysis of urea, Film and Spot production test, Phosphatase test, Methylene Blue reduction test, Tetrazolium chloride reduction test and Serum digestion test (Table 5). Of top priority for the characterization of the mycoplasma isolates using biochemical tests are the fermentation of glucose and the test for the hydrolysis of urea and arginine (Okwoli, 2007) because they are direct reflection of the on-going metabolic processes as seen in these isolates (Table 5). *Mycoplasmas* can be classified according to whether they ferment glucose, hydrolyse arginine or hydrolyse urea, but out of all these, only the hydrolysis of urea is unique as a conclusive test for *ureaplasmas* (Waites, *et al;* 2001). Most importantly, biochemical tests are designed to detect enzyme activity, for which a suitable substrate is added to the medium and after growth, the culture is examined for evidence of substrate breakdown, as in the case of the esculin test for f-D-glucosidase (Williams and Wittler, 1971) and in the phosphatase test (Aluotto, *et al;* 1970). In all procedures it may be necessary to incubate cultures for as long as 12 days so as to allow for complete enzyme activity.

The positive result as seen for the tetrazolium reduction test for almost all isolates in this study is indicative of total substrate breakdown. The presence of these enzymes provides a rapid screening procedure for the test, as was for phosphatase activity in this study where the viable *mycoplasmas* were lysed and the enzymes react with the substrate thus making it as their own energy source in the medium. Phosphatase reactions were noticeably enhanced in the presence of Na+ ions, which presumably activated the enzyme. Phosphatase activity was also pH dependent as faster reactions were noted in the 2nd set of tubes that were incubated. It is probable that experiments performed at the acid pH (4.8) were preferred to the alkaline pH (10.5) as the reactions were detected faster in the tubes incubated upto 7 days. However a detailed investigation of the pH optimum for phosphatase would be of value.

Rice, *et al*; (2000), asserted that the ability of *Mycoplasma* isolates to utilize glucose as determined by the measurement of oxygen uptake is used to differentiate *Mycoplasma capri* and

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Mycoplasma capricolum from other members of the *Mycoplasma mycoides* cluster, which in this study was seen in their ability to ferment glucose resulting in a brightly yellow coloured product. The hydrolysis of proteolytic enzymes and methylene blue by these isolates are suggestive of high energy requirement and activity within these isolates indicating that there are high level metabolic processes going on. However, none of the test isolate is positive for the film and spot biochemical test carried out which is suggestive that the lipolytic activity of mycoplasma isolates in this study and the medium composition could not produce the desired result. In this study, it was observed that *Mycoplasma capricolum and Mycoplasma capri* both reduced methylene blue and tetrazolium chloride and in addition possessed phosphatase activity which is common amongst the *mycoplasma mycoides* group (Rice, *et al;* 2000).

The biochemical characterization of the *mycoplasmas* isolates goes further to certify the various species of *mycoplasmas* involved in this study. Biochemical tests alone may not fully identify *mycoplasmas* because some variations do occur among the isolates which may be due to factors like techniques, environmental factors, cultivation media and even the *mycoplasma* strains (Okwoli, 2007), hence there is the need for other identification methods such as serology for mycoplasma organisms isolated in this investigation.

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CHAPTER SIX

6.0 SEROLOGY TEST ON MYCOPLASMA ISOLATES

6.1 Introduction

Serology is the scientific study or diagnostic examination of blood serum especially with regard to the response of the immune system to pathogens or introduced substances (Merriam – Webster Dictionary, 2007). The blood picture in various disease conditions has been a useful tool in identifying the causative micro - organisms and this has aided recovery through therapy. Harbi *et al*; (1983) observed the following in relation to the usefulness of serologic tests in mycoplasmal disease conditions: (1) by serologic picture one can confirm whether a *mycoplasma* infection is colonized, localized or a more invasive infection and (2) by the invasiveness of the organism one can predict a better outcome either due to the activity of the infection or the presence of the organism.

Other studies that support the importance of serological tests are Kasali and Ojo, (1981) who observed a significant rise in antibodies titres to genital *mycoplasmas* from serology in low-birth weight infants, and Lin (1985) who asserted that definitive identification of genital mycoplasmas should be based on the use of one or more serological procedures because of the lack of animal models or sero-epidemic studies of substantial importance that helps to determine the role of genital *mycoplasmas* in diseases.

The growth inhibition technique (GIT) which is the inhibition of colonial development around a disc impregnated with specific antiserum has been widely used for the identification of *mycoplasmas* (Taylor-Robinson, *et al;* 1988; Taylor-Robinson, 1990). This method is with minimal sensitivity but with high specificity and has high value for the quantitative measurement of antibodies. Other techniques that enable colonies on agar to be identified directly and aids the detection of mixtures of different *mycoplasma species* or *ureaplasma* serotypes are epiimmunofluorescence and immunoperoxidase (Taylor – Robinson, 1990). Taylor – Robinson *et al;* (1988) reported that immunofluorescence and metabolic inhibition tests are serologic methods that picked out specific antigens, they showed little cross-reaction between species and were both sensitive and specific. Other such tests include gel-diffusion and immunoelectrophoresis with specific antisera and they have been reported as powerful means of studying the antigenic structure of *mycoplasmas* and the relationships between them (Taylor-Robinson, *et al;* 1988). They also reported

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that the western blot technique is a new approach to assessing the importance of particular antigens. It is reported that genital *mycoplasmas* infections stimulate antibody responses that may be used for diagnostic purposes, although their ubiquitous nature in animals makes interpretation of antibody titres difficult (Waites *et al,* 2001).

Some of the serologic methods that have been described for *Mycoplasma capricolum*, *Mycoplasma mycoides subspecie mycoides* and *Mycoplasma bovis* include indirect haemagglutination test, immunofluorescence test, enzyme immunoassays and growth inhibition test (Chima, *et al*., 1986; Ajuwape, *et al,* 2003). The heterogenecity of strains of *Mycoplasma capricolum* was reported in earlier studies by DaMassa *et al*, (1992) stating that antisera to several isolates may be necessary to identify all strains. These reports, coupled with the inconsistent biochemical analyses, made the serological identification of *mycoplasma* isolates necessary.

Serological characterization of all the isolates in this study was carried out using the growth inhibition technique as previously described by Clyde, (1964) and ErnØ and Stipkovits (1973).

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6.2 Materials and Method

6.2.1 Materials:

Ten New Zealand white breeds of female rabbits aged between 6 – 8 months were used. They were obtained from the Department of Veterinary Physiology, Pharmacology and Biochemistry, University of Ibadan, and were kept in cages in the experimental animal unit belonging to the same Department.

Five well spaced cages were washed, disinfected and allowed for proper ventilation.They were labeled A, B, C, D and E to allow for two rabbits per cage. The rabbits were allowed to stay for one (1) week to acclamatize. They were thereafter screened for the presence of *Mycoplasma / Ureaplasma* and other bacteria. The method for the isolation of *Mycoplasma, Ureaplasma* (Ern∅ and Stipkovits, 1973) and those for other bacteria (Barrow and Feltham, 1995) were used.

6.2.2 Method:

6.2.2a Preparation of Antigen of *Mycoplasma.* This was done according to the method of Edward and Fitzgerald (1954).

Mycoplasma species that were molecularly and biochemically identified was used for antigen production. Single colony each of *M. bovis*, *M. arginini, M.capri* and *M. capricolum* respectively were randomly selected and inoculated unto *Mycoplasma* broth and incubated at 37°C for 72hrs, these were used as antigens. The following steps were taken in preparing the antigen:

1. The *mycoplasma* broth medium of the antigen was grown in 500 – 1000 ml of *mycoplasma* broth. The culture was allowed to become slightly acidic by allowing it to stand on the bench for 24hrs and observing with a litmus paper before harvesting as in many cases this would protect the immunogenicity of the preparation.
2. Centrifuge broth culture at 25000 to 30000 rpm for 40 – 45 minutes, discard the supernatant fluid and wash the sedimented pellet twice in 100 – 200 ml of phosphate buffered saline (PBS) pH 7.5.
3. Step 2 above was repeated twice by re-suspending the pellet each time in the wash fluid to ensure adequate washing.

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4. Re-suspend the final pellet in 4 – 8 ml PBS. The solution was mixed carefully in a vortex mixer to ensure complete suspension of the *mycoplasma* cells.
5. Four (4) ml of the antigen suspension was emulsified with 4ml of Freund's complete adjuvant.
6. The emulsified antigen was used for immediate injection of animals.
7. The remaining antigen was stored in the aqueous phase at -70°C for use in the booster injections.

6.2.2b Immunization Procedures

1. Each rabbit was pre – bled by taking 10 ml of blood, this is to get the pre-immunization serum.
2. The rabbits were Immunized accordingly:

1. Day 1: 0.5ml vol. of the emulsified antigen was injected into each of four intramuscular sites on each rabbit (2 ml total/rabbit).
2. A 0.2 ml vol. of emulsified antigen was injected intradermally into each of four sites on each rabbit (0.8 ml/rabbit)
3. On day 21 a 10ml vol. blood sample was taken from each rabbit as a test bleeding.
4. On same day 21, a booster injection of 0.5ml vol. of the emulsified antigen was given into each of the four intramuscular sites of each rabbit.
5. Each animal was bled on days 28, 35 and 42 respectively.
6. The blood samples were centrifuged at 3000rpm for 20mins to separate the cells from the serum.
7. The serum was decanted and is now used as the Antisera.

The determination of titre of the serum from each rabbit was done according to the method described by Nicole and Edwards, (1953), Edward and Fitzgerald, (1954). The antisera produced from the rabbits as described above were used to serogroup the mycoplasma strains which were identified biochemically as belonging to groups A, B, C and D respectively using growth inhibition test as described by Erno and Stipkovits (1973).

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6.2.2c Media for the Isolation of *Mycoplasma* of Goats.

Content :- Mycoplasma agar 35gm

Distilled water 65ml

Mycoplasma supplement G contains the following:

a). Horse serum 20ml

b). Yeast extract (25% w/v) 10.0ml

c). Thallous acetate 25.0mg

d). Penicillin 20,000IU

The agar is initially boiled to dissolve and then cooled to room temperature; add the constituted mycoplasma supplement G, mix properly and then 15ml vol. was delivered each into the glass petri dishes and then spread evenly.

6.3 Agglutination test.

Mycoplasma isolates that were biochemically identified as *M. capri*, *M. arginini, M. bovis* and *M. capricolum* were used for the agglutination test to ascertain the antibody potency in the serum collected. A pure colony of each organism was placed on clean glass slides with a drop of sterile distilled water to form a solution and a drop of the corresponding test serum was added. This solution is expected to form agglutination for a positive result and if no agglutination is seen, it is taken as a negative result. The time for agglutination to form for each antigen antisera solution was noted and recorded as shown in table 11.

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6.4 Growth inhibition test

It has been established by Nicole and Edwards (1953) and Edward and Fitzgerald (1954), that antiserum inhibit the growth of *mycoplasmas.* This was demonstrated in this study as follows:

1. Mycoplasma agar with supplement was prepared and poured into Petri-dishes and was allowed to set and dry by placing them in the incubator at 37°C.
2. A dilution of a late log – phase culture containing approximately 10^4, 10^5 and 10^6 CFU/ml was made.
3. A drop of 0.1ml vol. of the culture dilution was placed on the pre - dried agar surface and spread evenly along a broad line using a bent glass rod that has been dipped in alcohol, flamed and cooled. The inoculated plate was left at room temperature, agar side down until entire inoculum has been absorbed unto the medium.
4. Appropriate antiserum was placed carefully at the middle of the line on the inoculated agar surface using alcohol flamed wire-loop. Each antiserum was identified with a code number on the outside of the glass petri dish.
5. The plates were incubated aerobically at 37°C under increased carbondioxide atmospheric conditions and placed in the incubator for 5 days.
6. The surfaces of the plates were examined daily for zones of inhibition around the antisera spot.
7. Results were recorded by measuring the zone of inhibition (in millimeters) from one edge to the other and this represents positive results. Absence of inhibition zone was recorded as negative result (Table 10).

Antisera were raised against each of the *Mycoplasma* organism – *M. bovis, M. arginini, M. capri* and *M. capricolum subsp. capricolum*. This experiment lasted 49 days.

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6.5 Results

Large quantity of serum was obtained from the rabbits used in this study for the various tests performed. The agglutination test show that the clumping-time (agglutination) for the antigen and the serum for each specie was quite fast and that only antigen - antisera of the same specie can agglutinate while antisera from different species did not form agglutination. Also within same species it was observed that the time for the agglutination to form was relatively very close (Table 8) thus suggesting that there is an attraction to similar sites for binding. The Growth inhibition technique showed clearly marked inhibition zones after 5 days of incubation, indicating that sufficient immunoglobulins in the antibody was developed to protect the body against invading antigen. The plates were observed even after 10 days of incubation to know whether there will be a decline in the inhibition zones seen at day 5, the result was as good as seen previously at day 5 (plates 12 a&b). The observed inhibition zone measures in diameter of between 3.5 to 4.0mm for *M. capricolum*, between 5.6 to 6.0mm for *M. arginini*, between 5.8 to 6.0mm for *M bovis* and between 3.7 to 4.0mm for *M. capri* (table 9). However, plate 13b shows a situation were the antibody could not inhibit the growth of the invading antigen; this could be due to insufficient immunoglobulin in the antibody.

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Table 8: Agglutination time observed in the antigen – antibody reaction.

Micro-organism :					
	M. capricolum	5.	8.	19.	20.
	Time interval:	1.45s	1.45s	2min	2min
	M. capri.	2.	3.	6.	
	Time interval.	1.30s	1.30s	1.30s	
	M. arginini.	2’	14.	15.	17.
	Time interval:	1.20s	1.20s	1.20s	1.22s
	M. bovis.	21.	28.		
	Time interval:	2m	2min		

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Table 9: Diameter of inhibition zones observed in the GIT method.

Micro-organism	Diameter of size
Mycoplasma capricolum (4 in number)	1). 4.0mm
	2). 3.5mm
	3). 4.0mm
	4). 3.8mm
Mycoplasma arginini (4 in number)	1). 6.0mm
	2). 5.6mm
	3). 6.0mm
	4). 5.6mm
Mycoplasma bovis (2 in number)	1). 6.0mm
	2). 5.8mm
Mycoplasma capri (3 in number)	1). 4.0mm
	2). 4.0mm
	3). 3.7mm

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Plate 12(a): Growth Inhibition Test of *M. arginini* after 5 days incubation.

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Plate 12 (b): Growth Inhibition Test of *M. arginini* after 10 days incubation

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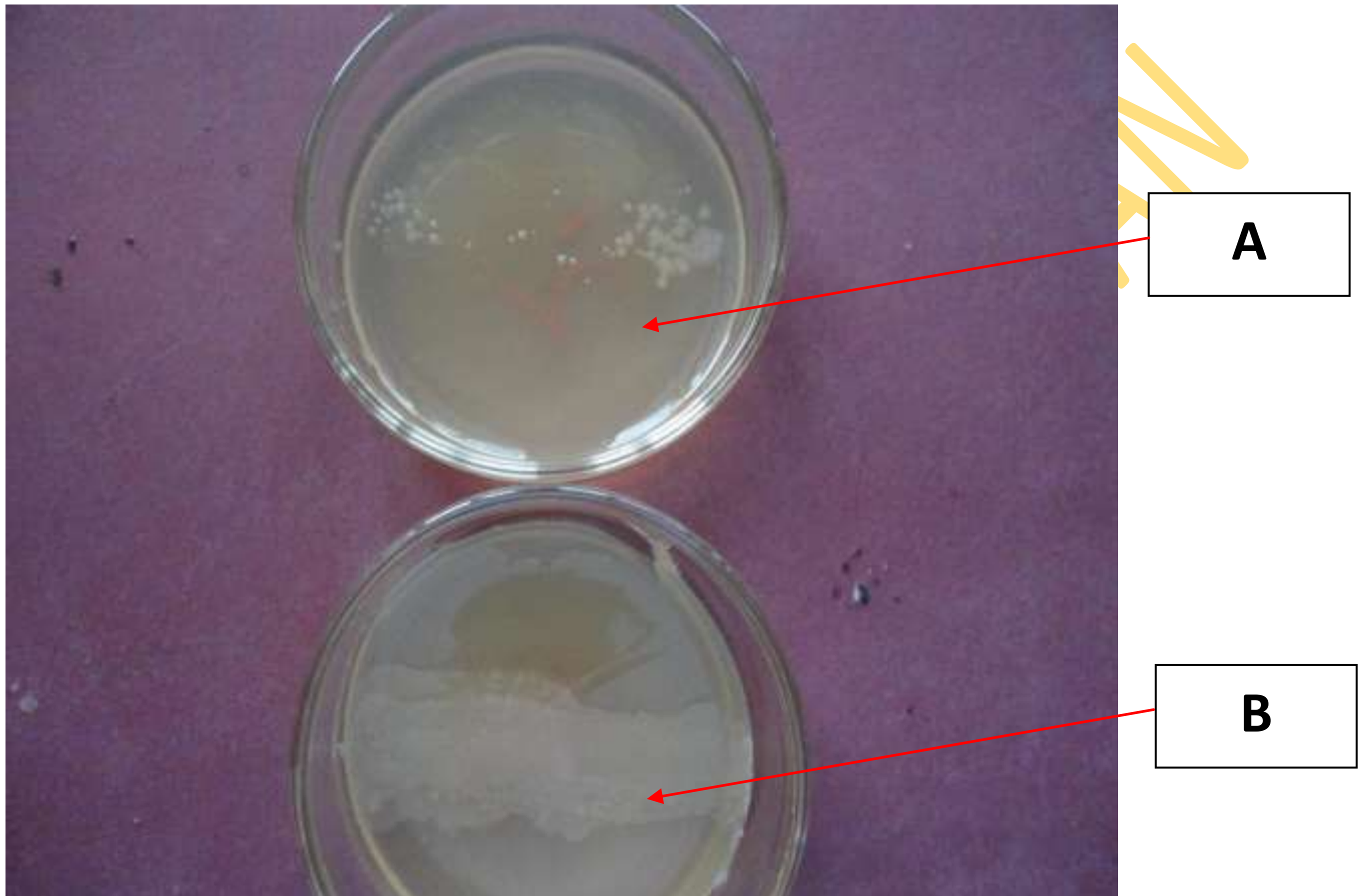

Plate 13: Growth Inhibition Test A—positive for *M. arginini,*
B—negative for *M. arginini* abtibody on *M. bovis* antigen

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6.6 Discussion

Different serological test methods are available for the identification of *Mycoplasma species* and they include: Growth Inhibition test (Edward and Fitzgerald, 1954; Chima, *et al;* 1986, Taylor-Robinson, *et al;* 1988), Immuno-diffussion test (Taylor-Robinson, 1988), Complement Fixation Test (CFT), Immunofluorescence Test (IFT) (Taylor-Robinson, 1990), Indirect haemagglutination test (Chima, *et al*; 1986), Enzyme Linked Immunoassays (ELISA) (Chima, *et al*; 1986, Ajuwape, *et al*; 2003) and Western blot technique (Waites, *et al*; 2001).

In this study however, the growth inhibition technique was adopted for the identification of the *Mycoplasma species* and *Ureaplasma species* because of its high specificity (Taylor – Robinson, 1988) and also it is a universally accepted technique. According to Edward and Fitzgerald (1954) the inhibition effect of antisera in GIT test are mediated by IgG and to a lesser extent by IgM and IgA, hence the importance of screening the rabbits to show that they have negative sera before the experiment. From this study, it was observed that 16 out of the 18 genital mycoplasma isolates subjected to the growth inhibition test showed zones of inhibition to the autoimmune antisera raised against the antigens - *M. capri, M. arginini M. capricolum, M. bovis* and *Ureaplasma spp* which corroborates an earlier work by Okwoli, (2007).

It is interesting to note that the two isolates which did not show any reaction to digitonin test and to the PCR procedure also did not show any inhibition to other *mycoplasma* isolates that were biochemically characterized except with themselves when they were reacted. The implication of this is that serological tests are thus necessary not only in identifying strains of *mycoplasmas* but also in confirming the identity of the strains biochemically characterized.

It is observed from this study that the sizes of the zones of inhibition formed by each antibody to the autoimmune antigen raised against each of them vary slightly, which could be traceable to varying concentration of the antibody and or the titre of the autoimmune antisera present in the solution which is in line with what Edward and Fitzgerald (1954) stated "the size of the zone of inhibition is directly proportional to the antibody titre of the serum as a result of the relative sensitivity of the method" (Table 9). It is therefore not uncommon to find instances of circular precipitin band where diffusing antiserum and soluble *mycoplasma* antigen have come together in a proper concentration, this is likely possible where multiple inoculum of the antisera is present on the antibody on the agar plate as observed in this study (Plate 13 a&b). By this investigation, serogrouping of the mycoplasma

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isolates as compared with the biochemical characterization on metabolic pathway utilization reveals that group A reacted with antisera of *M. capricolum*, group B with antisera of *M. capri*, group C with antisera of *M. arginine* while group D reacted with antisera of *M. bovis*

Serologically, the autoimmune antisera developed naturally within the rabbit host in this study, was able to generate enough titre against the antigen from which they were raised indicating that potent and viable antisera can be raised from animal host against the invading antigen as long as the test organism is a pure culture.

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CHAPTER SEVEN

7.0 ANTIBIOTIC SENSITIVITY TESTING

7.1 Introduction

The search for antibiotics began in the late 1800s, with the growing acceptance of the germ theory of disease, a theory which linked bacteria and other microbes as causative agent of a variety of ailments. As a result, scientists began to devote time to search for antibiotics that would kill these disease-causing bacteria. The goal of such research was to find the so-called "magic bullets" that would destroy microbes without toxicity to the person and animal taking the medication (Levy, 1998).

Antibiotics have long been hailed as wonder drugs, which are capable of killing disease-causing bacteria with little or no harm to the infected person or animal. The use of antibiotics as feed additive, chemotherapeutic and prophylactic agents, in Agriculture, Medicine and Veterinary Medicine has brought about great relief to the infected human and animals and has brought increase in food production (Freischem, 2012). However indiscriminate use and abuse of antibiotics has led to emergence of antibiotic drug resistance. The rise of antibiotic resistant bacteria is a global health concern. However, strains of pathogenic bacteria carrying resistance genes to one or more antibiotics has prevented modern medicine from treating common infectious diseases with a single antibiotic agent (Todar, 1996).

The increase in antibiotic resistant bacteria is largely due to the widespread indiscriminate use of antibiotics—in medicine, in animal health, and in agriculture. Two million pounds of antibiotics were produced in the United States in 1954; by 1996 this figure had skyrocketed to 50 million pounds (Todar, 1996). Antimicrobials, and especially antibiotics are indispensable tools in the control of animal disease through appropriate prevention and treatment; their contribution to animal health and basic animal welfare, as well as to human health through treatment of potentially contagious diseases is crucial (Freishem, 2012).

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7.2 Materials and method

Materials:

- Mycoplasma Agar with Supplements (Oxoid LTD, Basingstoke, Hampshire, England).

- 17 Antibiotic discs (Abtek Biologicals Ltd, Liverpool): gentamicin GN,(10µg), cotrimaxazole CO,(50µg), chloramphenicol C,(10µg), ampicillin AM,(30µg), cefuroxime CF,(30 µg), Tylosin TL,(30 µg), nitrofurantoin N,(100µg), erythromycin E,(10µg), tetracycline TE(50µg), norfloxacin NB,(10µg), ciprofloxacin CIP,(5g), augumetin AU,(30µg), ofloxacin OF,(5µg), ampicillin /cloxacillin AP,(30µg), ceftraxone FX,(30µg), clindamycin CD,(10µg) and amoxicillin AX,(30µg).

- Test Organisms in broth: These consists of the *Mycoplasma* isolates (13), *Ureaplasma* isolates (3) as well as *Acholeplasma* isolates (2) which were biochemically, serologically and molecularly (by PCR) identified.

7.3 Method

Antibiotic sensitivity testing:

Disc diffusion technique was employed for *Mycoplasma* species. The test organisms were respectively inoculated aseptically into sterile mycoplasma broth and incubated for 48 hours. The culture was used to flood the surface of antibiotic sensitivity test agar as described by Ericson and Sherris (1971). Excess culture medium was discarded into a disfectant container and the surface of the inoculated agar was allowed to dry for 15 – 20 minutes.

After the surface had dried, the above mentioned antibiotics were placed on the inoculated sensitivity test agar plates and pressed gently to make good contact using a sterile forcep (Mackie and MacCartney, 1996), (Abtek Biologicals Ltd, Liverpool, UK). The antibiotics were allowed to diffuse into the medium for 30 minutes and then incubated at 37°C for 24 hours. The plates were examined for areas of no microbial growth around the disc and zones of inhibition were measured with transparent ruler. Microbial organisms sensitive to the antibiotics were inhibited at a distance from the disc whereas resistant strain grew up to the edge of the discs.

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The results were interpreted according to the recommendation of National Committee for Clinical Laboratory Standards, sub-committee on antimicrobial susceptibility testing (1971).

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7.4: Results

All the *Mycoplasma* isolates in this study were clearly sensitive to the Nitrofurantoin and Ciprofloxacn. On the agar plates it was observed that zones of inhibition created by these antibiotics to the mycoplasmal isolates were upto 6.00mm in diameter. There were no traces of any microbial growth within the radius of the antibiotic influence. Resistances to all other antibiotics were observed including tylosin which is the clinical drug of choice to *mycoplasma* infections. However, there were selective sensitivity of these *mycoplasma* isolates to Erythromycin, Ofloxacine, Ampicillin / Cloxacillin combination, Augumetin, Cotrimaxazole and Chloramphenicol, which was lost after further incubationfor upto 5 days. As observed total resistance was displayed by the *Mycoplasma* isolates in this study to other antibiotics used which include the aminoglycosides, macrolides, lincosamides and the cephalosporin of the 1st and 2nd generation. This alarming resistance pattern observed with the mycoplasma isolates could not be unconnected with previous exposure to the antibiotics possibly at subnormal doses orght abuse of the medicines. From table 11 below, the resistance pattern shows that all the organisms were resistant to tylosin. Sensitivity was mostly toward the Nitrofurans and the Quinolones where upto 95% efficacy was obtained.

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Table 10: ***Mycoplasma spp* RESISTANCE PATTERN TO ANTIBIOTICS.**

Org	GN	CO	C	AM	CF	TL	N	E	TE	NB	CIP	AU	OF	AP	FX	CD	AX
1	--	+	--	--	+	--	+	+	--	--	+	+	--	+	---	---	--
2	+	+	+	--	+	--	+	--	+	--	+	--	--	+	--	--	--
2’	+	--	+	---	---	--	+	+	+	--	+	--	+	--	---	--	--
2”	--	--	+	--	--	+	+	--	+	--	+	--	+	+	--	--	--
3	+	--	--	--	+	--	+	--	--	--	+	+	--	+	+	--	--
5	+	+	+	--	--	--	+	+	--	+	--	--	--	+	--	--	--
6	--	+	+	---	--	+	+	+	+	--	--	+	---	--	---	--	--
8	+	--	--	--	+	+	+	--	+	--	--	--	--	--	+	--	--
9	+	+	--	--	--	--	+	--	+	---	---	--	+	+	--	--	--
14	+	--	+	--	--	--	+	--	+	--	+	--	--	+	--	--	--
15	+	--	--	--	+	--	+	+	+	--	+	--	--	+	+	--	--
17	--	--	--	--	--	--	+	+	--	--	+	--	--	--	--	--	--
19	--	--	--	--	+	--	+	+	--	---	--	---	--	---	--	---	--
20	+	--	--	--	--	--	+	--	+	--	--	--	--	+	--	--	--
21	--	+	--	--	--	--	+	--	+	--	--	--	--	+	--	--	--
25	+	--	--	--	--	--	+	+	+	--	+	--	--	--	+	--	--
25’	+	--	--	--	--	--	+	--	+	--	+	--	--	--	+	--	--
28	--	--	--	--	--	--	+	--	--	--	+	--	--	--	+	--	--

KEY

+ SUSCEPTIBLE

__ RESISTANT

Table 11: SUMMARY OF THE RESISTANT PATTERN OBSERVED IN MYCOPLASMA

S/N	Mycoplasma organism	Mycoplasma Resistant to
1	*Mycoplasma arginini*	Tylosin 30µg, Gentamycin 10µg, Ampicillin 30µg, Ofloxacin 5µg, Erythromycin 10µg, Norfloxacin 10µg, Tetracycline 50µg, Ampicillin/Cloxacillin 30µg.
2	*Mycoplasma bovis*	Tylosin 30µg, Erythromycin 10µg, Gentamycin 10µg, Ampicillin/Cloxacillin 30µg, Ceftraxone 30µg, Ofloxacin 5µg Tetracycline 50µg, Amoxicillin 30µg, Augumetin 30µg.
3	*Mycoplasma capri*	Cefuroxime 30µg, Cotrimaxazole 50µg, Tylosin 30µg, Erythromycin 10µg, Gentamycin 10µg, Augumetin 30µg Ceftraxone 30µg, Tetracycline 50µg, Ofloxacin 5µg.
4	*Mycoplasma capricolum*	Cotrimaxazole 50µg, Erythromycin 10µg, Tylosin 30µg, Ceftraxone 30µg, Ampicillin/Cloxacillin 30µg, Ofloxacin 5µg, Norfloxacin 10µg, Chloramphenicol 10µg, Amoxicillin 30µg.
5	*Ureaplasma spp*	Norfloxacin 10µg, Ofloxacin 5µg, Tetracycline 50µg, Tylosin 30µg, Ampicillin/Cloxacillin 30µg, Tetracycline 50µg.
6	*Acholeplasma spp*	Augumetin 30µg, Chloramphenicol 10µg, Cotrimaxazole 50µg Tylosin 30µg, Erythromycin 10µg, Amoxicillin 30µg.

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7.5 Discussion

Mycoplamas are bounded by a triple-layered peptidoglycan membrane and do not have a rigid cell wall, hence they are resistant to penicillins and other antibiotics that act on this cell-wall. They are, however, susceptible to a variety of other broad spectrum antibiotics, most of which only inhibit their multiplication and do not kill them (bacteriostatic antibiotics) (Taylor-Robinson & Bebear, 1997). A bacterial organism is resistant if it can tolerate concentrations of an antibiotic much higher than the concentration that inhibits development of most strains of the same species. Resistance of bacteria is governed by two types of genetic control – chromosomal (where there are resistant genes in the chromosome) and plasmid (which contains resistant factor that produces substances that decrease the uptake of antibiotics or modify them by enzymatic processes such as acetylation, phosphorylation or adenylation) (Ferrando, 1975). Resistance of micro organisms to antibiotic effect has been a most disturbing phenomenon in microbial infections. Over the years the pattern of resistances observed has resulted in promulgation of laws and scientific researches.

The resistance patterns displayed by the micro-organisms to the antibiotics used in this study depicts the frightening fact that most of our commonly used antibiotics are fast loosing their bacteriostatic and bacteriocidal effects on the most commonly encountered micro-organisms. The indication of this is that resistances are built up in the plasmids harbored in the micro-organisms which can then evade antibiotic actions with ease thereby causing prolonged diseases in animals and humans. The tetracyclines, which inhibit protein synthesis, have always been in the forefront of antibiotic usage, particularly for genital infections (Hannan and Woodnutt, 2000) and macrolides which are widely used for respiratory tract infections (Ferrando, 1975) have by our observation in this study seen to be loosing their potency. For example, the tetracyclines resistant plasmids seems to have a strong effect which has resulted in most micro organisms exhibiting a profound resistant pattern to the tetracycline antibiotic as observed with the *Mycoplasma* isolates in this study, this finding corroborates the acquired resistance to tetracyclines reported by Degrange *et al*., (2006) for *Mycoplasma hominis* and *Ureaplasma spp*. by the acquisition of the tet (M) gene. Several authors have also reported the sensitivity of *U. urealyticum* to a wide range of broad spectrum antibiotics like gentamycin, azithromycin, oflaxacin, streptomycin, minocycline and erythromycin (Shepard *et al.*, 1966; Braun *et al.*, 1970; Razin, *et al.,* 1991; Waites *et al.*, 1993; Matlow *et al.*, 1998 and Ogasarawa and Goodwin, 1999). Taylor-Robinson, 1990 also stated that *Mycoplasmas* have been found to be

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resistant to erythromycin, trimethoprim, sulphonamides and rifampicin. It is observed from this study that the *Mycoplasmas and Ureaplasma species* are resistant to gentamycin (10µg), ampicillin (30µg) and tetracycline (50µg) but were sensitive to nitrofurantoin (100µg) and ciprofloxacin (5g). It is however possible that at a higher concentration of these antibiotics sensitivity is realizable.

A new quinolone, gemifloxacin was found to be a more effective agent against different species of *Mycoplasmas* and *Ureaplasmas* than tetracycline, clindamycin and other quinolones (Duffy *et al.,* 2000). This view is shared by Hannan and Woodnutt (2000) who reported that the antibiotic gemifloxacin was effective against all the human respiratory and urogenital *mycoplasmas* and *ureaplasmas* tested, and they also found it to be 5 to 100 fold more active than ciprofloxacin. In their own study, Bebear *et al.,* (2000a) reported that trovafloxacin exhibited greater efficacy than other fluoroquinolones tested against fluoroquionolone – resistant *Mycoplasma hominis* and *Ureaplasma* isolates. Ogasarawa and Goodwin (1999) did a study of the efficacy of azithromycin in reducing lower genital *U. urealyticum* colonization in women at risk for preterm delivery and suggested that a single dose of azithromycin is ineffective. The resistant pattern observed in this study differs slightly from Braun *et al*, (1970) and Ogasarawa and Goodwin, (1999), in that the macrolide antibiotics could not selectively inhibit the growth of the *mycoplasmas.* Tylosin which clinically is the choice drug for *mycoplasma* infections could also not selectively inhibit the isolates from this study.

From this study, it observed that two organisms – *Mycoplasma bovis* and *Mycoplasma capri* were resistant to seventeen antibiotics but completely sensitive to two antibiotics. Three of the eighteen *mycoplasma* isolates tested (two *M. capricolum* and one *M. arginini*) were strongly resistant to fifteen of the antibiotics but weakly to two which later show resistance after two days of incubation. This observation therefore suggests that the *Mycoplasma* isolates are likely to have resistances within their plasmids which resulted from previous exposure to various antibiotics, some of which are included in this study. This exposure could however be in feed or drinking water at subnormal doses resulting in the resistant pattern displayed. Also contact with other *mycoplasma* resistant plasmid carrier animals could result in the spread of these antibiotic resistant organisms as a result of plasmid transfer. Should these infected animals contaminate food, water, milk and other consumables with their droppings or vaginal discharge that is consumed by man and animals, this would ultimately result into transfer of organism's inhibitory resistant genes and the cycle of infection continues.

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Another important but often neglected reason for this resistance, is the fact that antibiotic abuse is very rampant amongst farmers and farm attendants and the lack of adequate legislation against the prescription of antibiotic by qualified personnel has made control difficult. Laws are made but they are not enforced, so quarks, farmers and farm attendants use antibiotics indiscriminately without carrying out antibiotic- sensitivity tests, no clinical investigations and as a result antibiotics are not selective which finally results in loss of antibiotic selective pressure. Emergence of resistance might lead to ineffective treatment of the disease causing pathogen; this might lead to loss of animals and economic loss of valuable proteins needed to feed the ever growing population of Nigeria.

It is however advised that self medication of all forms (antibiotics in particular) on farms be completely avoided and adequate consultation of a qualified veterinarian be sought before any medication (vaccination inclusive) is carried out. Antibiotics need to be used responsibly, with care and discrimination (as little as possible, as much as necessary) to help maintain their effectiveness and limit resistance development.

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CHAPTER EIGHT

IDENTIFICATION OF OTHER BACTERIA

8.1 Introduction

The bacteria associated with vulvovaginitis may be normal bacteria resident in the vagina and vulva that could become pathogenic as a result of a change in the vaginal and vulvular micro-environment. The healthy vagina contains normal bacterial flora therefore, culture results of samples should be interpreted cautiously as it pertains to disease condition because a heavy growth, especially of one organism, is probably more significant than a light growth of several organisms (Aiello, 1998). Ajala*, et al*; (2011) reported that in clinical cases of vulvovaginitis of does in Ibadan, there have been no infectious agents consistently isolated, but there are some evidences that bacteria (Bamgboye, 2006), *Ureaplasma spps* (McCaughey and Ball, 1983) and *Mycoplasma spps* (Trichard, *et al;*1993) may have been involved and have been recovered. They, however reported the following as bacteria isolated from does in Ibadan, Nigeria, with clinical signs of vulvovaginitis in their study: *Gemella, Staphylococcus, Streptococcus species, E. coli* and *Proteus mirabilis*.

In view of the above reasons it was necessary to identify the presence of other likely pathogens besides *mycoplasmas* and *ureaplasmas* that may be present in the vaginal swabs of goats examined in this study

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8.2 **Materials** and **Methods**

The method described by Okwoli, (2007) was adopted for this test.

8.2.1 **Samples**: These are the same vaginal swabs collected from 221 goats using commercially prepared sterile cotton swabs.

8.2.2**: Media**: These are routine media comprising of Tryptose soy broth (TSB), Blood agar (BA) and MacConkey agar. They were used for the isolation of pathogens that are non-mycoplasma and ureaplasma pathogens.

8.2.3: **Atmosphere of cultivation**: All media used were incubated in air at 37°C.

8.2.4: **Temperature of growth:** All cultures including those on solid media and broth were incubated at 37^{0} C.

8.2.5 **Inoculation and Incubation of routine media**: Each of the swabs was inoculated into tryptose soy broth and incubated in air at 37°C. After 24hrs of incubation subcultures were made respectively from each tryptose soy broth cultures onto the Blood agar as well as MacConkey agar. All plates were examined after 24 and 48 hours of incubation. Suspected colonies were identified according to standard methods (Cheesbrough, 2000).

8.2.**6 Smears**

The swab was also used to make smears on clean glass slides and stained by the Gram techniques. The Gram stained smears were examined for the presence of leucocytes and bacteria.

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8.3 Biochemical identification of other bacteria

Samples were inoculated onto tryptose soy broth (TSB) and incubated at 37^0C over night under aerobic conditions.

They were then subcultured on to Blood agar and MacConkey agar and were incubated overnight at 37^0C under aerobic conditions. They were studied morphologically and Gram stained.

8.3.1 ***Streptococcus faecalis***

On blood agar hemolysis was observed. Beta hemolysis was observed on these cultures.

8.3.1.1**: Gram Stain**. – On Gram staining of the vulva swab samples collected from the swollen and hemorrhagic vulva of goats, beta – hemolytic streptococci appear as Gram-positive cocci. Some are single, in pairs and others in short chains

8.3.1.2**: Culture**: On blood agar they appear as small grayish-white, shining colonies with irregular outline and are beta-hemolytic. There was no growth on MacConkey agar.

8.3.1.3**: Catalase test:** The method of Cheesbrough (2000) was employed.

Colonies of the organism were removed with a sterile straight wire and immersed in a test tube containing 10ml of 3% hydrogen peroxide solution. The absence of immediate bubbling (release of oxygen) indicated a negative test. Thus beta – haemolytic *streptococci* are catalase negative.

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8.3.2: ***Staphylococcus aureus***.

These micro organisms are Gram positive cocci in clusters, non-motile, non-sporing, aerobic and attacks sugars by fermentation.

8.3.2.1: **Culture**:

- On blood agar plates, the organism appeared as raised, smooth, cream-coloured colonies with entire edges.
- On MacConkey agar, the colonies appeared pinkish (lactose – fermenters) and small in size.

8.3.2.2: **Gram stain**: On staining a colony from the blood agar plate, Gram-positive cocci in clusters were seen.

8.3.2.3**: Coagulase test**: This was done as described by Cheesbrough, (2000).

8.3.2.3a.: Slide coagulase test (this test detects the bound coagulase i.e clumping factor): Two thick suspensions of the test organism were made in sterile distilled water on two ends of a clean glass slide. A drop of rabbit plasma was put in one of the suspension and mixed properly. Plasma was not placed on the second suspension that acted as negative control. The presence of clumping within 10 seconds in the first suspension indicated coagulase positive reaction while absence of bubbles within 10 seconds indicates a negative reaction. Thus *Staphylococcus* is coagulase positive.

8.3.2.4: **Catalase test**: The method of Cheesbrough (2000) was employed.

Colonies of the organism were removed with a sterile straight wire and immersed in a test tube containing 10ml of 3% hydrogen peroxide. The presence of immediate bubbling (release of oxygen) indicated a positive reaction while no bubbles indicates a negative reaction. Thus *Staphylococcus aureus* is catalase positve.

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8.3.3: ***Escherichia coli***.

E. coli are Gram negative rods, often motile, aerobic and facultatively anaerobic, coagulase positive, Oxidase negative, citrate negative, attacks sugars fermentatively and gas is normally produced.

8.3.3.1: **Culture:**

- Blood agar colonies of *E.coli* appear as smooth, large, sometimes mucoid and sometimes haemolytic.
- MacConkey agar: *E. coli* ferment lactose producing smooth pinkish colonies.

8.3.3.2**: Biochemical tests**; Several biochemical tests as described by Cheesbrough (2000) were done to identify *E. coli.* (Table 13) and some are stated below:

8.3.3.3**. Motility test**: A drop of an overnight broth culture of *E. coli* in peptone water was placed on a clean glass slide, covered with cover slip and examined microscopically with X40 objective. All the *E. coli* isolates were found motile.

8.3.3.4**. Indole test**: The test organism was grown in peptone water overnight and a drop of Kovac's reagent was put into it. The bottle was gently shaken and allowed to stand. A red colour was seen in the surface layer within 10 minutes and this indicated that organism was indole positive.

8.3.3.5. **Carbohydrate fermentation test**: Three bottles of peptone water each containing a separate sugar – glucose, lactose and mannitol, and also a Durham tube were inoculated with the test organism. After an overnight incubation at 37^0C the organism was found to have fermented all carbohydrate with gas production.

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8.4 **Antibiotic sensitivity tests**

Disc diffusion technique was employed for *E. coli, Staphylococci* and *Streptococci species*. Each of the isolate were respectively inoculated into 5ml TSB, and incubated for 8 hours then 0.1ml of each culture was inoculated into 4ml of sterile TSB and shaken properly to make a dilution of 1:2000 as described by Adetosoye (1984). The diluted culture was used to flood the isosensitive agar plate, the excess broth was discarded into a container of disinfectant. After the surface had dried, antibiotic discs including: gentamicin GN,(10 µg), cotrimaxazole CO,(50µg), chloramphenicol C,(10 µg), ampicillin AM,(30µg), tylosin TL,(30 µg), cephalexin CX,(30 µg), nitrofurantoin N,(100µg), erythromycin E,(10µg), tetracycline TE(50µg), norfloxacin NB,(10µg), ciprofloxacin CIP,(5g), augumetin AU,(30µg), ofloxacin OF,(5µg), ampicillin /cloxacillin AP,(30µg), ceftraxone FX,(30µg), clindamycin CD,(10µg) and amoxicillin AX,(30µg), (Abtek Biologicals Ltd, Liverpool, England) were carefully placed on the inoculated sensitivity test agar plates and pressed gently to make good contact with the antibiotics sensitivity test agar using a sterile forcep. The antibiotics were allowed to diffuse into the medium and then incubated at 37°C for 18 hours. The plates were examined for areas of no microbial growth around the disc and zones of inhibition were measured with transparent ruler. Microbial organisms sensitive to the antibiotics were inhibited at a distance from the disc whereas resistant strain grew up to the edge of the discs. The results were interpreted according to the recommendation of National Committee for Clinical Laboratory Standards, sub-committee on antimicrobial susceptibility testing (1971).

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8.5 Results

The isolation of other micro organisms in this study was done using standard methods (Barrow and Feltham, 1995). It was observed that most of the does with signs of vulvovaginitis had *Staphylococci* and *Streptococci* species isolated from them. This agrees with Aiello, (1998) that the vagina microflora contains some microorganisms that may be pathogenic when there is change in the vaginal and vulva micro-environment. In this experiment the bacteria pathogens isolated include *Staphylococcus species*, *Streptococcus species* and *Escherichia coli.* The Mixed population of 3 species of bacteria isolated from the vulvovaginitis cases in this study is in accordance with the findings of Webb and Chick, (1976, in sheep vulvovaginitis) and Ajala, *et al;* (2011, in goat vulvovaginitis) who stated that vulvovaginitis in sheep and goats was associated with mixed bacteria populations.

Biochemical results obtained were in accordance to the findings by standard methods of isolation (Cheesbrough, 2000). *Staphylococcus species* isolated were coagulase and catalase positive, while the *Streptococcus faecalis* is non-motile, non-sporing and catalase negative.The *Escherichia coli* isolates were characteristically indole producers, catalase and lactose positive(Table 13).

From the antibiotic resistance tests (Table 14) it was discovered that all of the bacteria were sensitive to Ciprofloxacin and Nitrifuratoin while some were partially sensitive to Cotrimazole, Ofloxacin, Norfloxacin and Erythromycin, as their growth though were not to the antibiotic disc but zones of inhibition were not distinctly marked while they were resistant to all other antibiotic agents that were used.

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Table 12: Breakdown of Other bacteria isolated in this study

Organism	**No isolated**	**%ages**
Staphylococcus spp	73	33.0
Streptococcus faecalis	75	33.9
Escherichia coli	91	41.2

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Table 13: Biochemical Test results of other Bacteria isolates

TEST	MICRO ORGANISM		
	Staph.aureus	*Streptococci faecalis*	*E. coli*
Coagulase	+	-	-
Urease	-	-	-
Lactose	-	-	+
Pyruvate	+	+	-
Ribose	+	+	-
Sucrose	+	-	+
Xylose	-	-	+
Catalase	+	+	+
Indole Production	-	-	+
Heamolysis	+	+	-
Oxidase	-	-	-
Nitrate Reduction	+	-	-
Methyl red	+	-	-
Voges Proskeur	+	-	-

KEY: + **P**ositive

\- Negative

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Antibiotic resistance observed.

Antibiotics tests were also carried out on other pathogens isolated in this study. The results observed showed a varying degree of resistance pattern exhibited by the organisms. There was a general trend of high resistance exhibited to other antibiotics. It was observed that susceptibility was majorly to the quinolones (third generation antibiotics) used. The results obtained are as shown in table 14 below:

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Table 14:

(a) Antibiotic Resistance Pattern for Other bacterial isolates.

S/NO	Bacterial Isolates	Bacteria Resistant to
1.	*Streptococcus faecalis*	Amoxicillin 30µg, Gentamycin 10µg, Tetracycline 50µg, Cotrimaxazole 50µg, Erythromycin 10µg, Ampicillin 30µg.
2	*Staphylococcus species*	Gentamycin 10µg, Cotrimaxazole 50µg Ampicillin/Cloxacillin 30µg, Ofloxacin 5µg, Amoxicillin 30µg, Erythromycin 10µg, Chloramphenicol 10µg, Augumetin 30µg.
3	*Escherichia coli*	Chloramphenicol 10µg, Erythromycin 10µg Cotrimaxazole 50µg, Norfloxacin 10µg Gentamycin 10µg, Amoxicillin 30µg, Augumetin 30µg, Ampicillin 30µg.

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8.6 Discussion

The list of other bacteria isolated from does with clinical signs of vulvovaginitis in this study include, (a) *Escherichia coli* which were isolated at a higher frequency (41.2%) from both the mucus and bloody samples collected, (b) *Staphylococcus species* (33.0%) isolated also from mucopurulent and bloody samples collected and (c) *Streptococci faecalis* (33.9%) which were also isolated from the mucopurulent and bloody discharges.

This high prevalence rate of microorganisms in goats with obvious vulvovaginitis could be due to immunocompromised state which increases their susceptibility to microorganisms and eventually diseases.

From the biochemical tests carried out on these bacteria isolates (Table 13), it was observed that the bacteria reactions were in accordance to their various metabolic requirements, which indicates that is these bacteria were actively alive and replicating. The various results suggest that the vaginal environment was supportive of such growth which may be traceable to compromise of the host immune system. Although the nomal vagina contains bacteria but a heavy growth of one bacteria is significant (Aiello, 1998) when daeling with vaginal infections than a light growth of several bacteria. The mixed population of 3 species of bacteria isolated from does with vulvovaginitis in this study is in accordance with the report of Webb and Chick, (1976) and Ajala, et al; (2011), who stated that vulvovaginitis in sheep and goats was associated with mixed bacteria populations.

The antibiotic tests shows that all bacteria were sensitive to Ciprofloxacin and Nitrofurantoin while a few were sensitive to Cotrimaxazole, Norfloxazone, Amoxicillin and Erythromycin while all the bacteria isolates were resistant to other eleven (11) antibiotics that were used in this study. This resistance shown by these bacteria to antibiotics used suggests that for the effective treatment of vulvovaginitis in the field antibiotic sensitivity test be carried out to avoid treatment failure. The resistance pattern shown to other antibiotics is not unconnected to resistances developed as a result of previous exposure to those antibiotics at subnormal doses in the name of prevention of diseases as they speculate in the farms. Chima, *et al*;1986 reported that other isolated bacteria from cases of caprine vulvovaginitis should be considered equally important as most times they accelerate the pathology seen in the disease condition. Ajala, et al; (2011) reported *Staphylococcus spps* as an opportunistic pathogen likely to be more virulent in pathology seen in vulvovaginitis of does, and in

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this study 33.0% of *Staphylococcus species* was isolated, this high rate of occurence is likely also to contribute to the pathology observed thus agreeing with Ajala, *et al;* (2011). All isolates from these goats may play various roles in the pathogenecity of vulvovaginitis and urogenital tract infections, though they could serve as opportunistic agents which could lead to adverse conditions on the long run.

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CHAPTER NINE

ANIMAL PATHOGENICITY TEST

9.1: Introduction

Mycoplasma has been known to cause infections in animals resulting in the death of the animal and economic losses to farmers and the livestock industry. To evaluate the pathogenicity of this infection, reseachers have used various animal models (Agnello, *et al*; 2012). For those involved in the respiratory problems, researchers have used endobronchial inoculation to demonstrate the pathogenicity of these organisms by using calves (Ajuwape, 2004), goats (DaMassa, *et al;* 1986). Other animal models that have been used to reproduce mycoplasmal infections include sheep by Agnello, et al; (2012) in cases of arthritis of sheep, Ajuwape, et al; (2005) in cases of contagious bovine pleuropneumonia (CBPP) in sheep and DaMassa, *et al;* (1991) in a case of caprine mastitis in sheep. Also Taylor-Robinson and Furr, (1997) inoculated mice intravaginally with *Ureaplasma urealyticum* serotype 8 and established susceptibility as the mice came down with the infection.

Species of *Mycoplasmas* and *Ureaplasmas* have been reproduced experimentally from various animals' anatomical sites like genital tract of sheep (Le Grand *et al*, 1995; chima *et al,* 1995), the lungs of cattle, sheep and goats (Howard & Gourlay, 1982), the mammary glands of goats (Gourlay *et al*, 1973) and at the ankle joints of goats (Agnello *et al*, 2012).

The aim of this experiment is to reproduce vulvovaginitis in Kano brown goats by scarifying the vulva of these goats with mycoplasmas isolated in this investigation.

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9.2: Materials and methods

Experiment using goats

This study was performed on four (4) groups of 4 experimental animals each with 4 control animals. None of these animals had been vaccinated against any *Mycoplasma spps.* They are Kano brown female goat breeds of ages between 8 and 12 months old with mean body weight of 20 – 25kg purchased from the goat market at Bodija in Ibadan, Oyo state. All animals were under the same care and feeding conditions. They were kept at the goat section of the Veterinary Research farm unit for acclimatization and screening for ten days after washing and disinfection of the pen.

Screening:

The animals in this study were screened for *Mycoplasmas*, *Ureaplasma* and other bacteria prior to use, this was to rule out the presence of existing *Mycoplasma / Ureaplasma* species and any other bacteria that may interfere with the results. The screening for the presence of *Mycoplasma/Ureaplasma* and other bacteria was done by taking swabs from all orifices and inoculated unto *Mycoplasma* broth and subsequently unto *Mycoplasma* agar for the mollicutes, and also unto Tryptose soy broth and later unto blood and McConkey agar (for other bacteria) to observe for any growth before the experiment could commence. The goats were kept in an intensive care unit and were fed with rice bran and wheat ofal and water was given *ad lib* for the period of the experiment.

Four *Mycoplasma* isolates are being tested for in this experiment viz: *Mycoplasma arginine, Mycoplasma bovis, Mycoplasma capri and Mycoplasma capricolum*. For each *mycoplasma* isolate being tested for, 5 goats were assigned, 4 for the experiment and 1 as control.

Method:

The vaginal canal of the experimental animals were gently scarified using a sterilized dental brush and 2ml of 4.0 X10^7 CFU/ml of the culture of each *mycoplasma* isolate was introduced into the vaginal canal while 2ml of sterilized mycoplasma broth was introduced into the vaginal canal of the control does. Daily rectal temperatures were taken and observation for any gross change in vulva appearance and abnormal discharges if any were noted.

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9.3. Results.

Clinically, the appetite of all the goats used in this experiment remained unaffected during the experiment. However, their body temperature showed a mild rise of 38.9^0C to 39.6^0C for up to 3-4 days post innoculation. From the 3rd day post inoculation, the vulvar lips of all the infected goats were swollen and hyperemic up to 10-12 day post innoculation. Mucus discharges from the vulva of all test animals were observed from day 13 post inoculation but with varying severity. Plate 14 shows one of the control animals with absolutely normal vulva size and shape and no form of abnormality observed after 42 days of the experiment. In plate 15 we see the animal infected with *M. capri* isolate showing swollen, hyperemic and vulva with mucus surfaces. This was observed 28 days post inoculation. The gait of the animal is affected at this point. Plate 16 in like manner is showing the infection caused by *M. capricolum* isolate 28 days post inoculation, there is marked distention of the vulva lips due to massive swelling, hyperemia and scabs on the vulva suface. *M. arginini* infection is seen on plate 17 also with swelling, hyperemia 28 days post inoculation. Although, the pathology seen is not as extensive as in *M. capri*, the animal was still very restless. *M. bovis* infection is seen on plate18, the pathology observed seems to be more because as at 28 days post inoculation mucus discharges were still seen. There is sloughing of the vulva surfaces and swellings.

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Plate 14: Control animal at day 42 post innoculation

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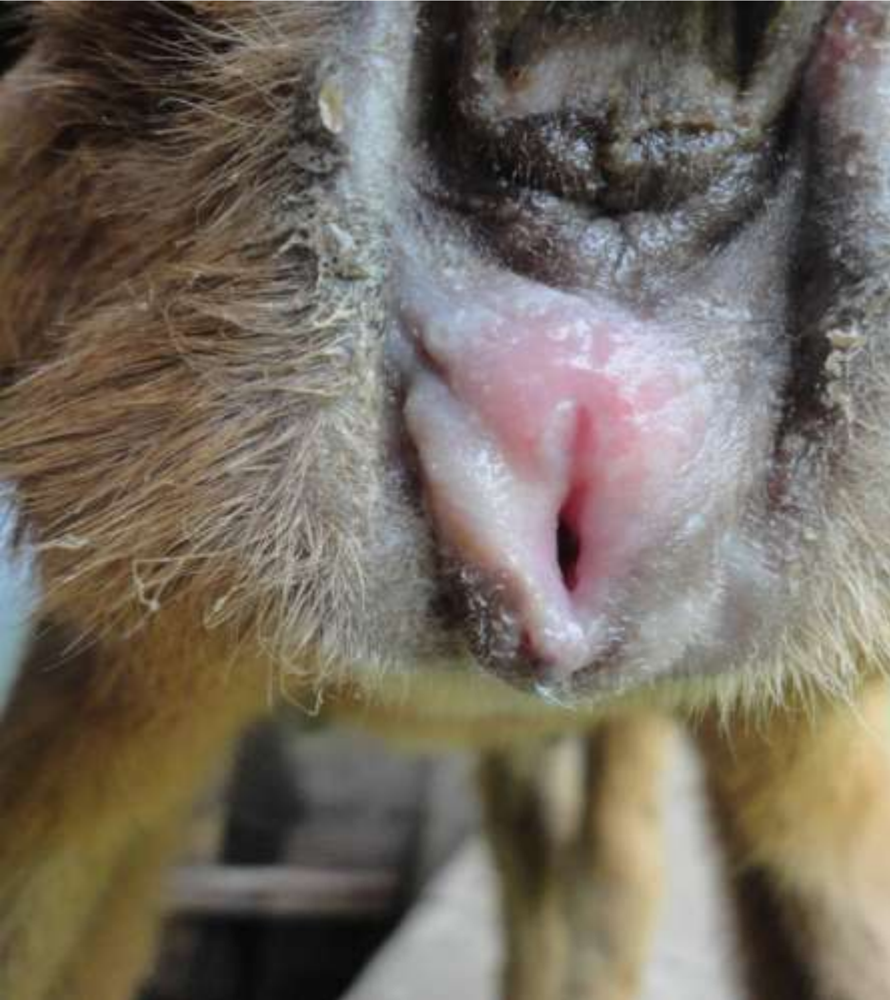

Plate 15: *Mycoplasma capri* infection – Day 28 post inoculation

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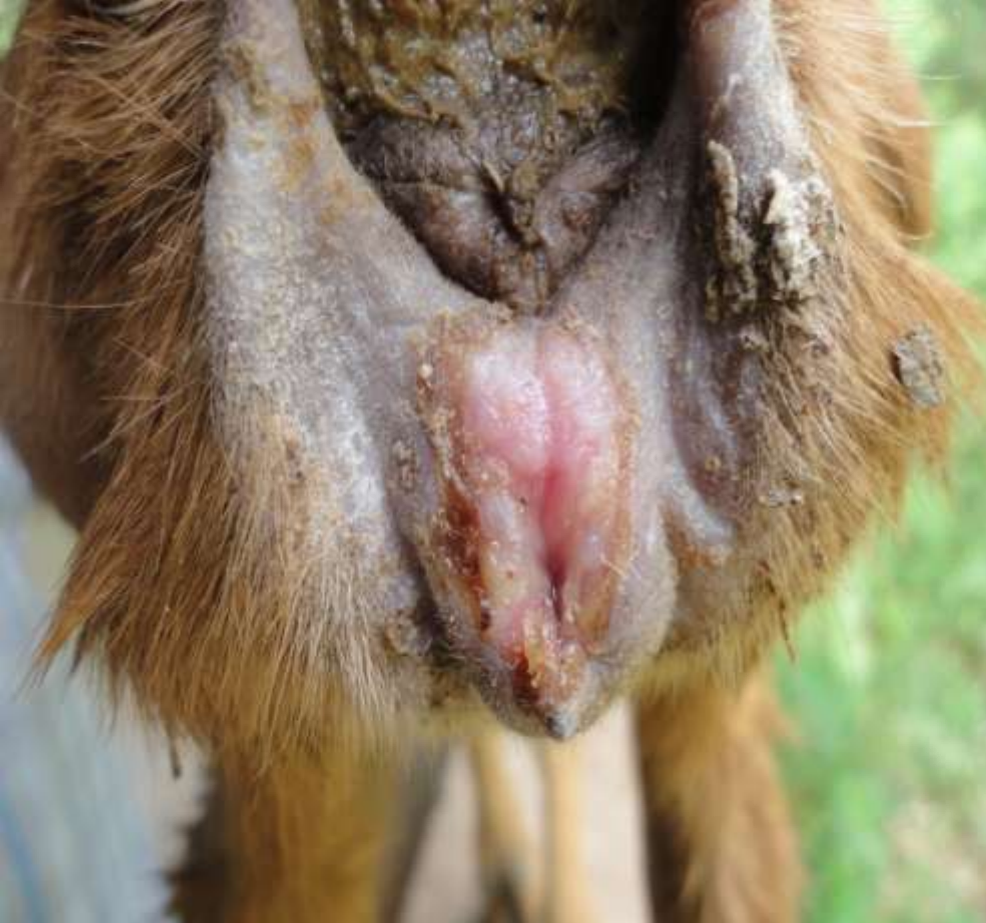

Plate 16: *Mycoplasma capricolum* infection – Day 28 post inoculation

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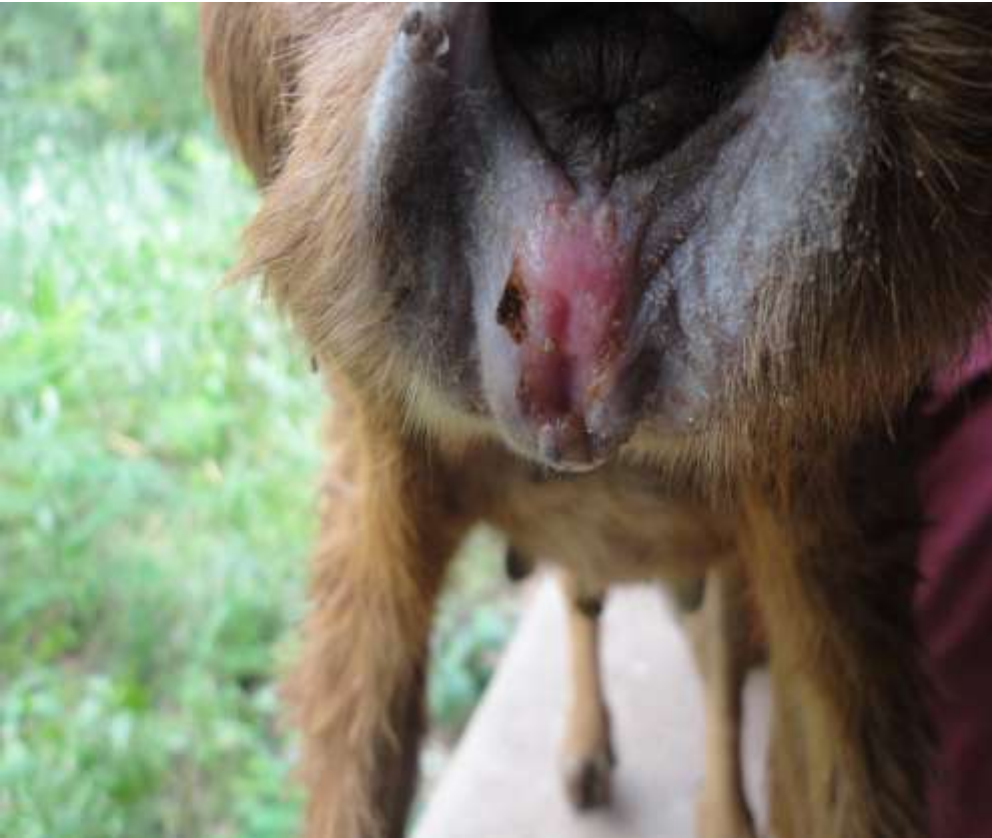

Plate 17; *Mycoplasma arginini* infection – Day 28 post inoculation

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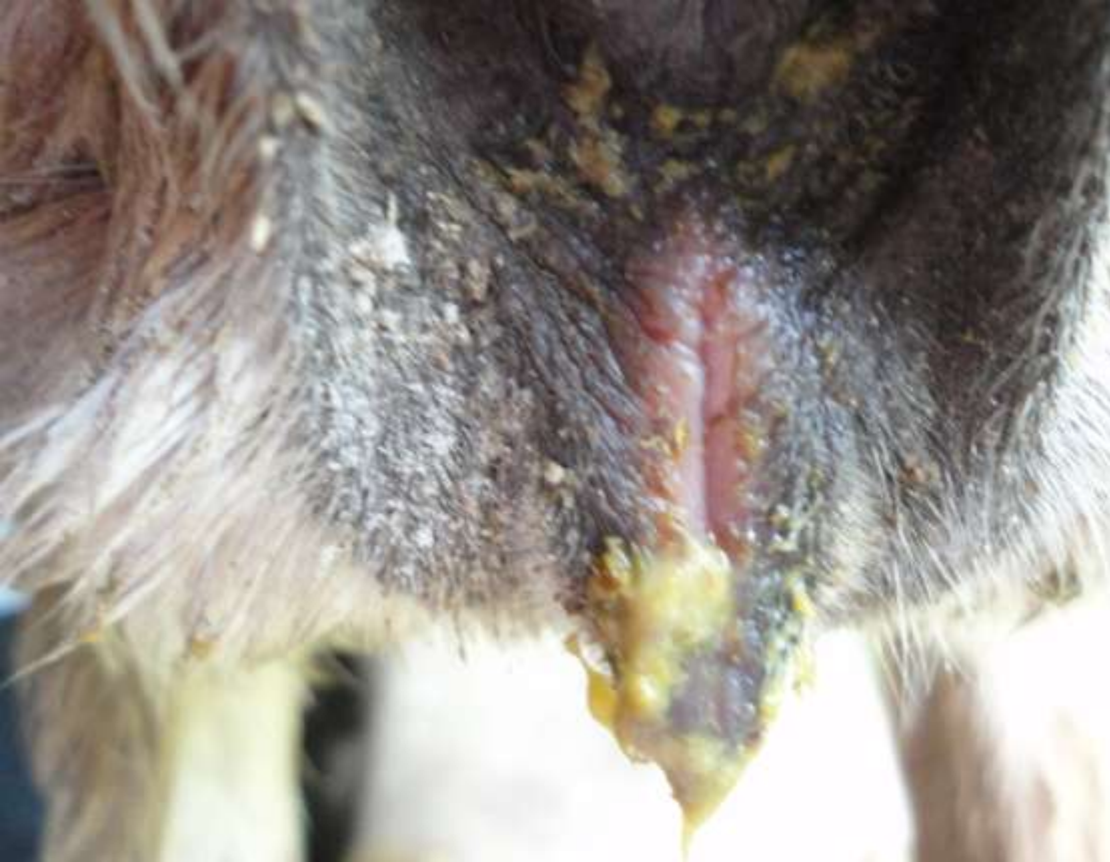

Plate 18: *Mycoplasma bovis* infection – Day 28 post inoculation

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As the experiment progressed all infected animals died at one point and the other. *Mycoplasma bovis* infected goats died on day 30 post inoculation, *Mycoplasma capri* infected goats died day 33 post inoculation, *Mycoplasma capricolum* infected goats died day 38 post inoculation while the *Mycoplasma arginini* infected goats died day 41 post inoculation.

Post Mortem examinations were carried out and congestion and edema of the lungs was a common but pronounced lesion on all animals posted, Plate 19 is the necropsy result of one of the *M. bovis* infected goats showing generalized hyperemia of organs in situ. The necropsy result of one of the *M. capri* infected goats shows very friable liver and pale lungs as seen in plate 20.

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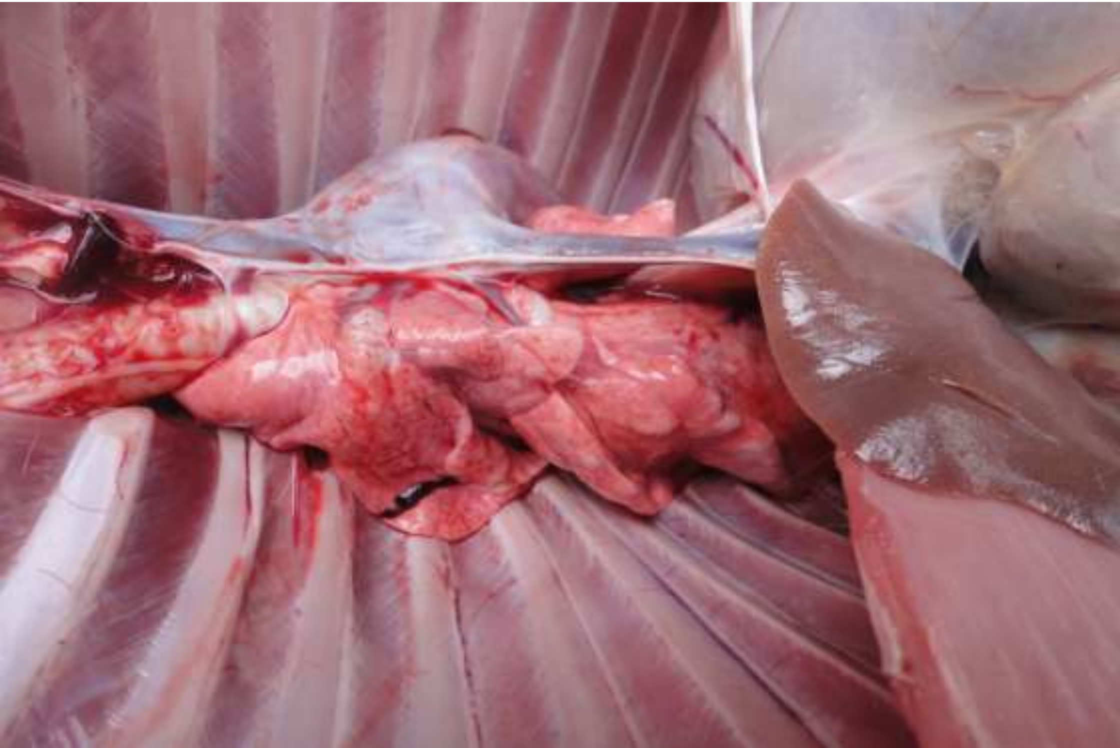

Plate 19: Hyperemia of the Lungs: *M.bovis* infection

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Plate 20: in situ viscera of *Mycoplasma capri* infected goat showing friable liver.

9.4 Histopathology.

This is the histological study of the pathology created by the micro- organisms invading cells, tissues and or organs. It deals with the composition of the cell constituents and the pathology caused to any of the constituent.

Method:

The procedure followed is from "The basic labouratory Histopathology techniques" and they include the following:

a**). Fixation**: This involves the physical and chemical treatment with various reagents of the cell and or tissues. By this process the cell constituents are free from distortion and decomposition from endogenous and exogenous factors. The fixative agent used for this experiment is Formaldehyde (formalin) at a concentration of 40% gas in water.

b). **Dehydration:** This is the process of removing the inherent water content of a given specimen of a cell or tissue in a gradual way considering osmotic dynamics. 70% of ethanol for 2hrs was used for this experiment.

c). **Clearing:** This involves the removal of the alcohol (ethanol) that the cell or tissues had bathed in and to initiate and complete a process that will make cells transparent at microscopic view.

d**). Infiltration**: This is subjecting the cells and tissues into a molten paraffin wax which serves as support to the cells and tissues for subsequent stage of sectioning. Paraffin wax permeates into the tissues to fill up vacuoles that have been left by dehydration. Three rounds of treatment at 2hrs each was used for this experiment.

e). **Embedding**: This is the positioning of the processed infiltrated cells or tissues in molten paraffin wax in an enclosure called a Mould. The embedded tissues are left until the wax solidifies.

f**). Blocking**: Here the tissues are cut into little chunks or blocks. These blocks are placed on hot spatula and attached to pieces of wood that serves as clamping and positioning for sectioning.

g**). Sectioning**: This is done in a microtone and it cuts only a thin slice of the original tissue at a preset thickness i.e 4μm. This section was taken up from a waterbath by a clean glass slide coated on

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one side with glycerin-egg albumin. They are then arranged in a slide carrier and air dried for 30mins and allowed to fix in preparation for staining.

h). **Staining**: The routine Haematoxyline and Eosin (H & E) staining method was used for the tissues in this experiment.

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Result of the histopatholgy experiment

1. **The Lungs** – There is infiltration of many alveoli and interalveolar septae by moderate numbers of Neutrophils, Lymphocytes and a few Plasma cells. There is mild Harmorrhage into the alveoli. Most of the alveoli capillaries are engorged with red blood cells (RBC). There is congestion and edema of the lungs.

2. **The Liver**: – There is infiltration of many alveoli and interalveolar septae by moderate numbers of Neutrophils, Lymphocytes and a few Plasma cells. There is mild Harmorrhage into the alveoli. Most of the alveoli capillaries are engorged with red blood cells (RBC).

3. **Lymph node**: Scattered tissue; absence of regular lymphoid follicles in cortex. Many plasma cells seen in section and massive depletion of lymphocytes.

4. **Vulva**: Severe infiltration of the epidermis and dermis by neutrophils.

The plates below show the various histopathological lesions observed

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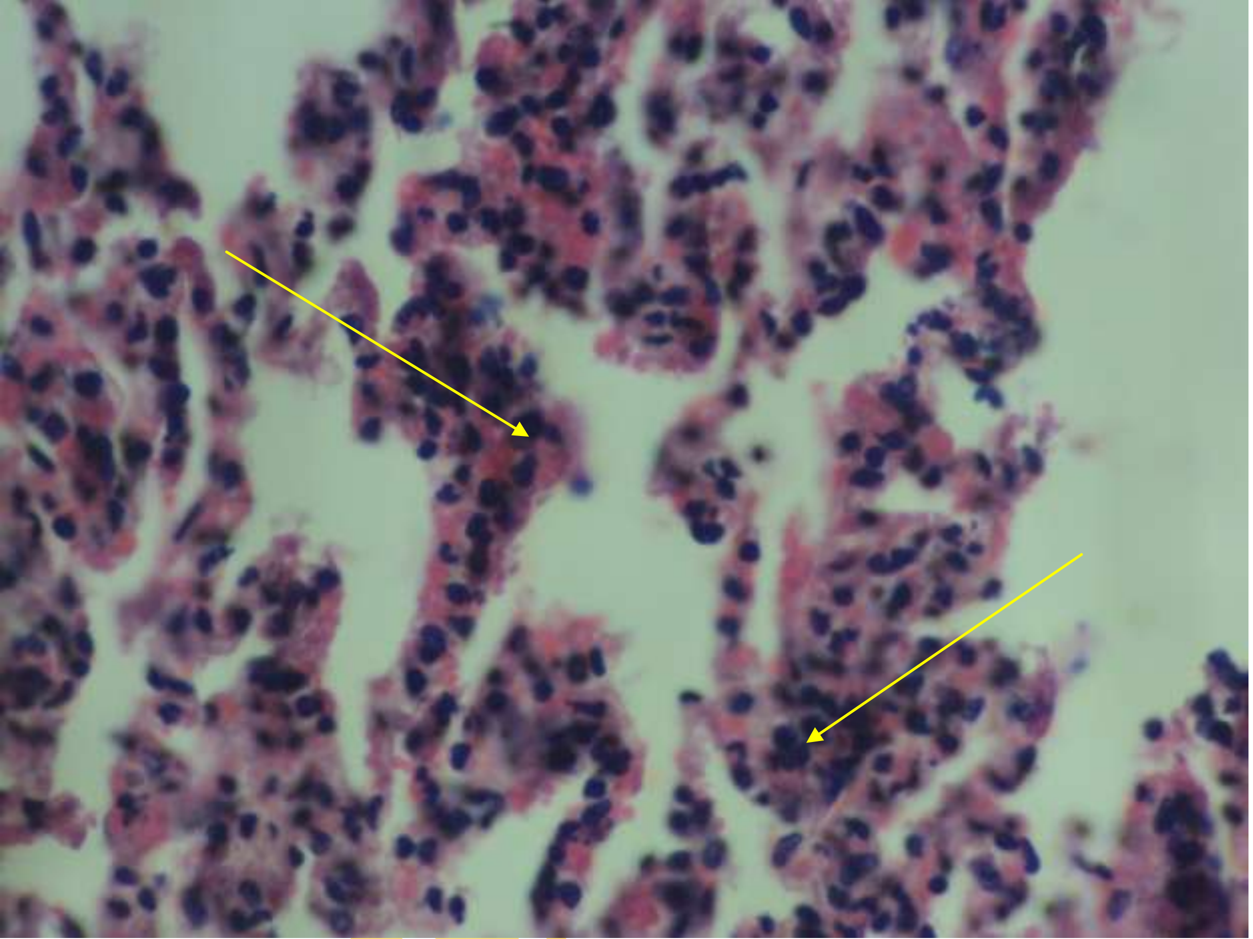

Plate 21: Lungs section in *M. bovis* infection

Cellular infiltrations and diffuse hyperemia in the Lungs. H & E X 400.

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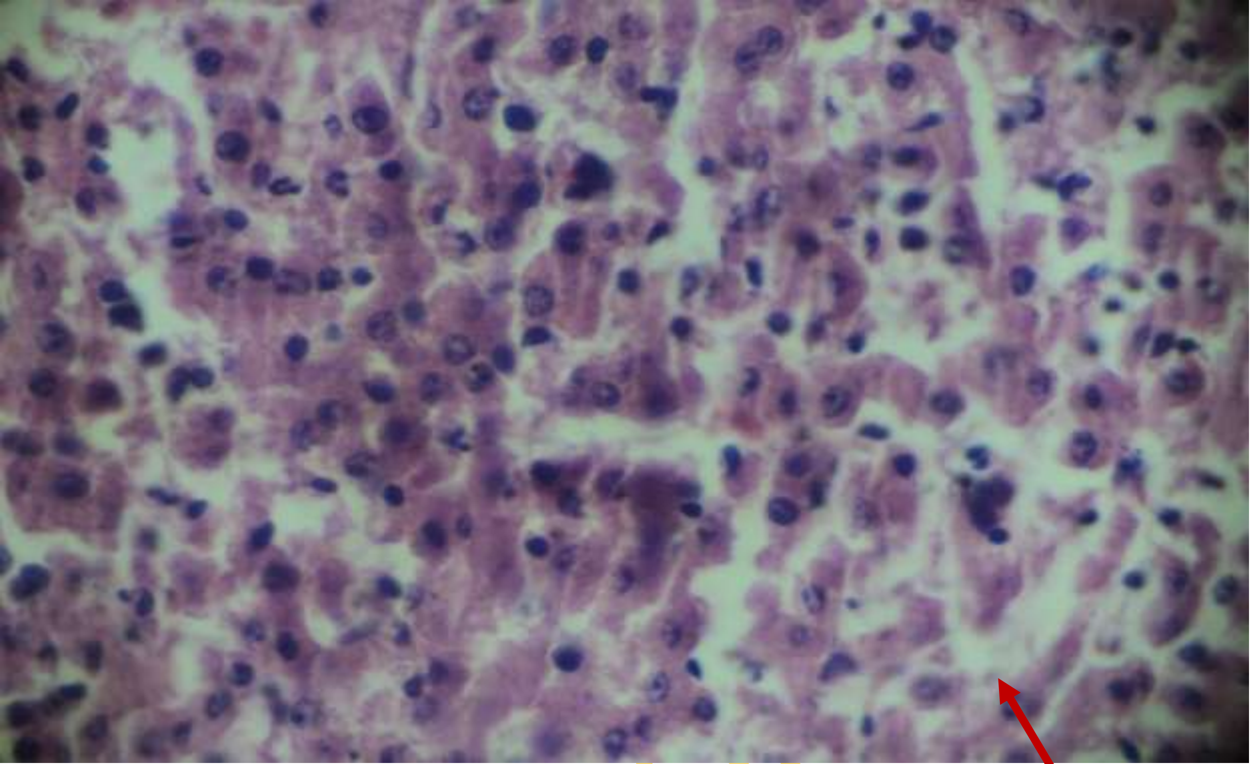

Plate 22: Lymphnode section with lymphocytes depletion in *M. bovis* infection

Extensive lymphoid necrosis in lymphoid follicles. H & E. X400.

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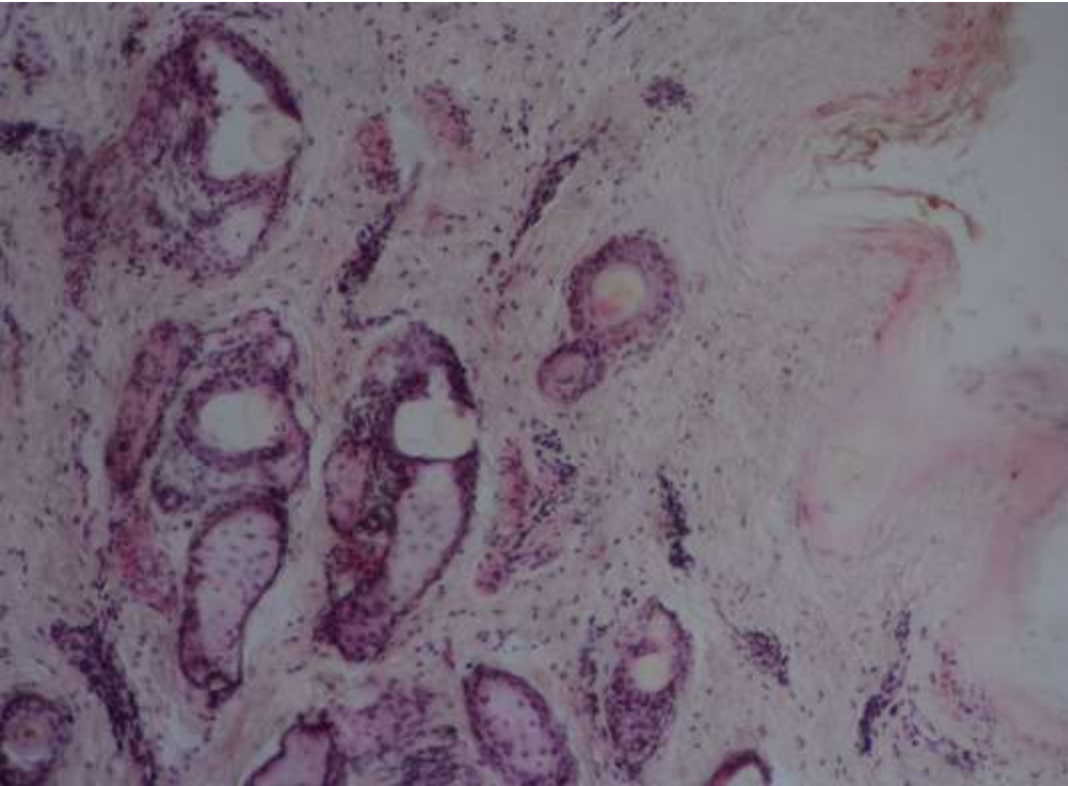

Plate 23: Skin section of Vulva – control animal X40

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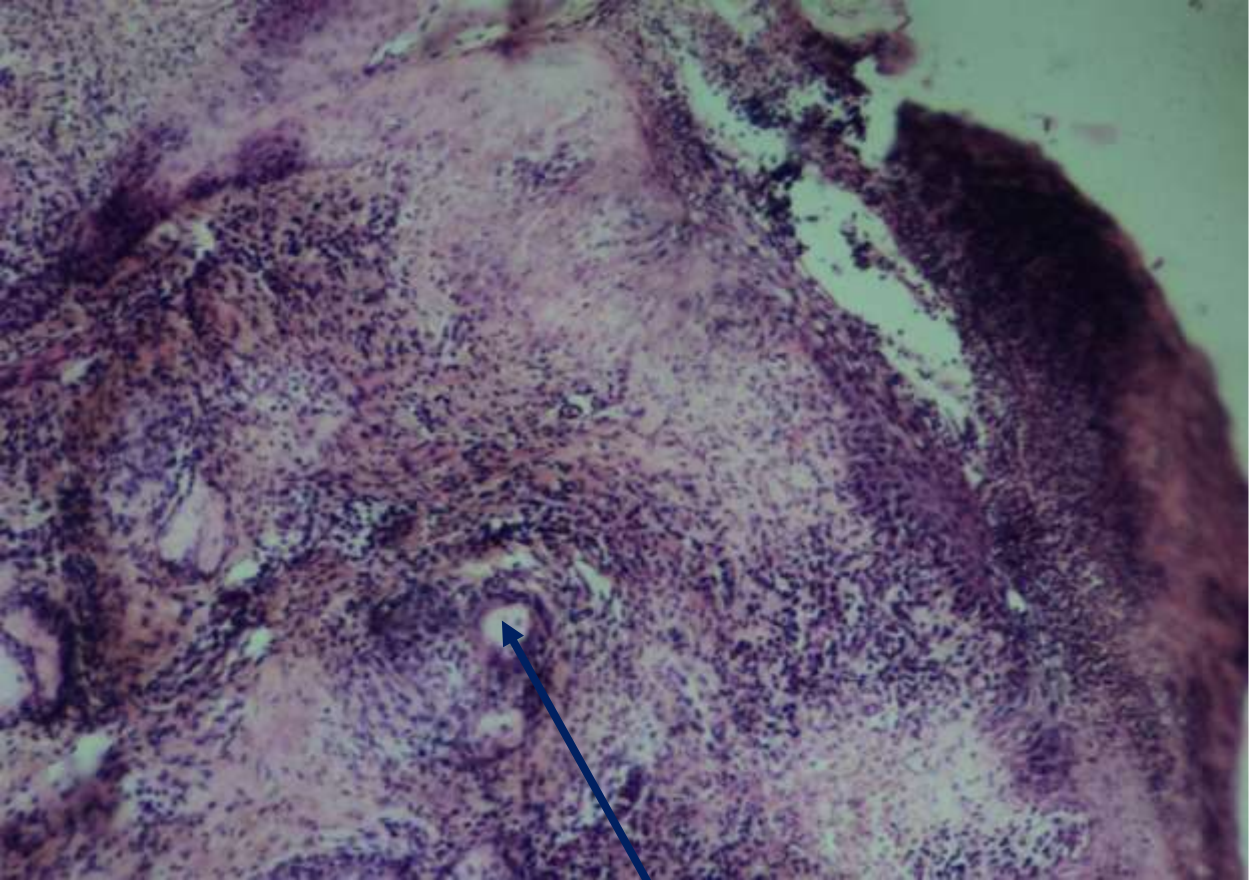

Plate 24: Skin section of Vulva – *M.bovis* infection

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9.5 Discussion

Among the important goat diseases, mycoplasmal infections result in significant losses on the African continent and in countries such as Greece, France, India, Israel, Italy, Portugal, Spain, and the United States. Morbidity and mortality can be upto 100% and important outbreaks have been described, that required the destruction of an entire herd of 700 goats in the United States (DaMassa, *et al;* 1992). The economic impact of the disease lies in its high morbidity and resultant loss of milk and meat production rather than in its mortality (DaMassa, *et al;* 1987c).

The clinical observations of pyrexia and mucus discharges from the vulva in this study are in accordance with those reported by other workers in cases of spontaneous Granular Vulvovaginitis in sheep due to *ureaplasmas*, *Mycoplasma spp*. and *Acholeplasma laidlawii* (Doig and Ruhnke 1977) and in goats due to *Mycoplasma bovis* and *Mycoplasma capri* of the caprine serogroup 11 and *Acholeplasma oculi* (Tiwana and Singh 1982). There was a significant increase relative to body temperature (pyrexia) in the goats with mycoplasma infections (39.6^{0}C) compared to control animals (38.5^{0}C). It was also observed that there was no decrease in the appetite of the goats used in this experiment all through the study period contrary to the report by other authors (Misri, *et al;* 1998, Tracee, 2010). Based on the results obtained in our study we can conclude that the *Mycoplasma arginine, Mycoplasma bovis Mycoplasma capri and Mycoplasma capricolum* infection in goats was not anaemic but septicaemic in nature, although the goats were affected clinically and metabolically.

The gross and microscopic lesions in the vulva and vagina were almost similar but different in severity to those observed by other workers in-experimentally induced Granular Vulvovaginitis in sheep due to *ureaplasmas* (Doig and Ruhnke 1977), and in goats due to *Mycoplasma agalactiae* (Singh *et al*. 1975), *A. laidlawii* and *A. oculi* (Gupta *et al*. 2006).

The infiltration of large number of lymphocytes and plasma cells in the epithelial, sub-epithelial, muscular and serosal layers and also around the blood vessels in the genital tract of the infected goats indicates that strong cell-mediated responses are directed against the invading *Mycoplasma* organisms. *Mycoplasma capri* was re-isolated from the vaginal swabs of the infected goats, indicating that local infection with this organism remains confined to the genital tract and this findings agrees with Barile (1973) who observed that most *Mycoplasma* pathogens are not highly invasive but confine themselves to the epithelial surfaces to produce localized infections. However, also in this study, the congestion and hemorrhages observed in the lungs of the *M. bovis* infected

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goats is a divergent finding from Barile (1973) but goes to support Ajuwape, *et al*; (2005) that the Mycoides group of mycoplasmas to which *M.bovis* is a member are very invasive since they are found in important body organs during infections. It is also very possible to infer from this study that the invasiveness of members of this mycoides group may vary since *M. capri* is also a member of the group whose re-isolation was from the vagina swab.

M. capricolum is an important disease agent in goats and the rearing of goats together with cattle has made this organism to produce lesions in the kidney, marbling in the lungs and depletion of lymphocytes in cattle that clearly resembles contagious bovine pleuropneumonia (CBPP).(Ajuwape *et al;* 2005).

Observations made in this present study compel us to conclude that the Mycoplasmal isolates of *M bovis, M.capri, M. capricolum* and *M.arginini* are pathogenic for female genital tract of goats with decreasing order of pathogenicity as listed above (*M. bovis* is most pathogenic while *M. arginini* is least) and they cause vulvovaginitis. Moreover, *M.bovis* has been implicated in cases of pneumonia (Ikheloa*, et al*., 2004), Mastitis (Amosun, 2011), necrotic dermatitis in calf and cattle and also dermatitis in man (Senturk, *et al*., 2012). Attention should be placed on this organism and vaccines should be prepared against it to prevent infections in livestock which can easily transmit the disease to man so as to prevent public health harzards. It is important to remember that all of the *Mycoplasma* species known to affect goats may be carried by clinically healthy animals, and that also carrier animals may be seronegative. Thus, continued surveillance by the national agricultural institutes on flocks for diseases attributable to *Mycoplasma* species should be ongoing. *Mycoplasmas* should be considered in the differential diagnosis of all outbreaks of mastitis/agalactiae, pleuropneumonia, arthritis, keratoconjunctivitis, and vulvovaginitis in goats.

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CHAPTER TEN

CONCLUSION AND RECOMMENDATIONS.

10.1 Conclusion.

In this study *Mycoplasma arginine, Mycoplasma bovis, Mycoplasma capri, Mycoplasma capricolum, Ureaplasma spps* and *Acholeplasma spps* were isolated from the vulva and vagina swabs of vulvovaginitis cases of goats in Lagos state, Nigeria. The isolation of *mycoplasmas* is considered to be one of the most difficult tasks for diagnostic laboratories due to their inability to grow easily in laboratory media in spite of the great improvement in media formulations. In this study, samples of vaginal swabs were taken and *Mycoplasma*s isolates were grown on a special mycoplasma broth and mycoplasma agar with supplements (Oxoid, USA) enhancing the growth and thus 13 *mycoplasma*, 3 *Ureaplasma* and 2 *Acholeplasma* isolates were identified. The *Mycoplasma* isolates from this study were isolated by culture and identified and characterized by biochemical, serology and molecular (PCR) methods.

Predominant among the biochemical tests needed for the characterization of the mycoplasma isolates were the glucose fermentation test and the test for the hydrolysis of urea and arginine. In this study, nine (9) metabolic characteristics were tested for and they include Digitonin sensitivity test, Glucose breakdown, Hydrolysis of arginine, Hydrolysis of urea, Film and Spot production test, Phosphatase test, Methylene Blue reduction test, Tetrazolium Chloride reduction test and Serum Digestion test. The results obtained on the biochemical characteristics of the mycoplasmas show some variation among the *mycoplasma* isolates. This could be adduced to factors like techniques, the environment, cultivation media and even the mycoplasma strains involved (Okwoli, 2007). The operational schedule of dividing the genus *mycoplasma* into groups on the basis of the results of the glucose and arginine tests, leaves room for serological or biochemical variants within a given species (Chima, *et al,* 1986). This further goes to confirm that biochemical tests alone may not be enough to fully identify *mycoplasmas* hence the need of other identification methods like serology tests and molecular techniques.

Although serological methods are easier to perform and less costly, however, they are pointers to detection of homologous species in test isolates as seen in the agglutination test. Heterologous reactions are generally not favoured by serology as cross reactions of organisms are not possible. Serological tests are also helpful in identifying specific specie but have low sensitivity when

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compared to the PCR technique. The use of Serology test in this study has enhanced the quality of decision taken as to the species we are working with and their inter-relations.

PCR-based technology for *Mycoplasma* yields the highest level of sensitivity and specificity. The detection of *Mycoplasma spp.* in cattle, buffaloes, sheep and goats by polymerase chain reaction (PCR) was based on the *in vitro* amplification of the highly-conserved 16S rRNA gene, so using PCR technique to differentiate between *M. bovis* and *M. capri* because of the close relation between both species reflects the specificity in the nature of this technique which is also rapid and sensitive. The evolution within *mycoplasmas* has been reported to be unusually rapid (Woese, *et al*., 1985). Petersson, *et al.,* (1996), suggested that members of the *M. mycoides* cluster could be used as a model system for molecular evolution because of their very close and similar sequences. As reported by Weisburg, et al; (1989), two kinds of phylogenic trees were constructed from strains of *M. capricolum*, one was based on mutational events from consensus sequences, and the other one on individual operons. Therefore, strains of *M. capricolum* constitute a very useful model for studies of molecular evolution. *M. capricolum* and *M. bovis* may also be suitable subspecies for comparing the evolution of other genes with the evolution observed for the 16S rRNA genes.

Mycoplamas are bounded by a triple-layered peptidoglycan membrane and do not have a rigid cell wall, hence they are not susceptible to penicillins and other antibiotics that act on the cell wall. They are, however, susceptible to a variety of other broad spectrum antibiotics, most of which only inhibit their multiplication and do not kill them (Taylor-Robinson & Bebear, 1997). The resistance patterns to antibiotics obtained for *Mycoplasma species* in this study depicts clearly that the commonly used antibiotics have been grossly abused in the field. Of the seventeen antibiotics employed in this study, the *Mycoplasmas* were susceptible to just two of them - Nitrofurantoin and Ciprofloxacin while to other antibiotics the *mycoplasma*s showed resistance.

The Morbidity and Mortality caused by the *Mycoplasma* isolates in the experimental animals in this study is consistent with the pathogenecity picture produced in caprine as reported by Kumar*, et al;* 2011. There was pyrexia in the goats used in this study but their appetite was not affected. The vulva lesions of mucus discharges, swellings and hyperemia were present. There was evidences of discomfort as the goats were mostly restless, walking around looking for places of comfort within the pens. Post mortem findings of hyperemia of the lungs and infiltration of cells (neutrophils – as

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observed histologically) into the tissues affected were classical for *Mycoplasma* infections of goats and this is in agreement with the findings of DaMassa, *et al;* (1987b).

10.2 Recmmendations

This study recommends that further work be done on the following aspects as they relate to *Mycoplasma* of goat characterization in Nigeria

- A Phylogenetic study of the *Mycoplasma* isolates from this work which will place the species of *Mycoplasma* isolates of goats in Lagos state, Nigeria on the gene bank library.
- The Development of Heterogenous Vaccine for *Mycoplasma* organisms using autoimmune antisera that will prevent pneumonia and vulvovaginitis.
- A Research on the haematological and biochemical profile of these *Mycoplasma* infections in goats in Nigeria.

The following are suggested remedy to cater immediately for the present challenges:

(ii) Hygienic management practices- this includes:

- The provision of clean and ventilated pens, clean feeding troughs and controlled grazing habits.
- Avoid any form of injury to the animals.
- Clean and portable drinking water should be supplied.
- Adequate and regular disinfection of the herd.

(ii) Accurate and appropriate medication to animals when needed.

(iii) Accurate and complete diagnosis of the disease condition to ensure appropriate antibiotic medications at all times.

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(iv) Adequate training and re-training of farm and herd personnel and attendants including workshops, to be on a quarterly basis on farm management practices and the judicious use of antibiotics where and when neccesary.

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APPENDIX 1.

SAMPLE	Gram staining	Antibiotics bacteria are sensitive to.
3	G+	-
	G-	-
4	G+	OF ,CIP ,FX ,
	G-	CIP ,OF ,GN.
6	G+	OF ,CIP ,CO ,FX ,AU.
	G-	TE ,OF .
8	G+	CIP ,FX .
	G-	CIP.
11	G+	CIP ,CO ,FX .
	G-	TE ,OF .
14	G+	OF ,CIP ,CO ,FX .
	G-	CIP ,OF .
16	G+	OF ,CIP ,AP ,FX ,AU .
	G-	CIP ,CF .
17	G+	OF ,E ,CIP ,FX .
	G-	-
19	G+	OF ,CIP ,FX ,
	G-	CIP ,TE ,OF ,C .
25	G+	OF ,E ,GN ,FX .
	G-	-
28	G+	OF ,CIP ,CO .
	G-	NB ,OF .
29[1]	G+	OF ,CIP .

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	G-	TE ,OF ,CF .
29"	G+	OF ,CIP ,CO ,FX .
	G-	CIP ,TE ,OF .
30	G+	GN ,FX .
	G-	-
31	G+	OF ,E ,GN ,FX .
	G-	CIP ,CF .
43'	G+	OF ,CIP ,FX ,AU .
	G-	TE ,OF ,CF ,AM ,GN .
43"	G+	OF ,CIP ,CO ,FX ,AU.
	G-	CIP ,OF ,AM .
45'	G+	AU .
	G-	TE .
45"	G+	AU .
	G-	CIP ,TE .
64	G+	OF ,E ,AU .
	G-	CIP ,OF .
65	G+	-
	G-	OF ,GN .
73	G+	OF ,E ,CIP ,CD ,CO

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		,FX ,AU .
	G-	CIP ,TE .
75	G+	OF .
	G-	NB ,OF .
77	G+	-
	G-	-
78	G+	OF ,CIP .
	G-	OF ,CF .
80	G+	OF ,CIP ,FX .
	G-	CIP ,AM .
87	G+	CO .
	G-	CIP ,OF .
89	G+	OF ,CIP ,CD .
	G-	OF.
92	G+	E ,CIP ,CD ,FX .
	G-	TE ,C .
99	G+	CIP ,FX .
	G-	TE ,OF ,C .
103	G+	CIP .
	G-	OF ,CF .
107	G+	E ,CIP ,FX .
	G-	CIP ,OF .

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132	G+	OF ,CIP .
	G-	CIP ,OF ,CF .
133	G+	OF ,CIP .
	G-	CIP ,NB ,OF ,
134	G+	CIP .
	G-	C , CF .
135	G+	CIP .
	G-	-
136	G+	-
	G-	-
137	G+	CIP ,CO .
	G-	NB ,CF ,OF .
138	G+	CIP ,FX .
	G-	-
139	G+	OF ,GN ,AP .
	G-	CIP .

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G-	OF ,GN .
G+	OF ,E ,CIP ,CD ,CO ,FX ,AU .
G-	CIP ,TE .
G+	OF .
G-	NB ,OF .
G+	-
G-	-
G+	OF ,CIP .
G-	OF ,CF .
G+	OF ,CIP ,FX .
G-	CIP ,AM .
G+	CO .
G-	CIP ,OF .
G+	OF ,CIP ,CD .
G-	OF.
G+	E ,CIP ,CD ,FX .
G-	TE ,C .
G+	CIP ,FX .
G-	TE ,OF ,C .
G+	CIP .
G-	OF ,CF .

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G+	E ,CIP ,FX .
G-	CIP ,OF .
G+	OF ,CIP .
G-	CIP ,OF ,CF .
G+	OF ,CIP .
G-	CIP ,NB ,OF ,
G+	CIP .
G-	C , CF .
G+	CIP .
G-	-
G+	-
G-	-
G+	CIP ,CO .
G-	NB ,CF ,OF .
G+	CIP ,FX .
G-	-
G+	OF ,GN ,AP .
G-	CIP .

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Media used in this work

Mycoplasma broth medium: consists of the following

Mycoplasma broth base	25g
Distilled water	75ml

Mycoplasma Agar medium: consists of the following

Mycoplasma agar base	35gm
Distilled water	65ml

Mycoplasma supplement G contains the following:

a). Horse serum	20ml
b). Yeast extract (25% w/v)	10.0ml
c). Thallous acetate	25.0mg
d). Penicillin	20,000IU

Both mycoplasma broth and agar plus the supplements used were supplied by Oxoid USA.

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APPENDIX 11

***MYCOPLASMA* RESISTANCE PATTERN TO ANTIBIOTICS.**

Org	GN	CO	C	AM	CF	CX	N	E	TE	NB	CIP	AU	OF	AP	FX	CD	AX
1	--	+	-	--	+	--	+	+	--	--	+	+	--	+	---	---	--
2	+	+	+	--	+	--	+	-	+	--	+	--	--	+	--	--	--
2’	+	--	+	---	---	--	+	+	+	--	+	--	+	--	---	--	--
2”	--	--	+	--	--	+	+	-	+	--	+	--	+	+	--	--	--
3	+	--	-	--	+	--	+	-	--	--	+	+	--	+	+	--	--
5	+	+	+	--	--	--	+	+	--	+	--	--	--	+	--	--	--
6	--	+	+	---	--	+	+	+	+	--	--	+	---	--	---	--	--
8	+	--	-	--	+	+	+	-	+	--	--	--	--	--	+	--	--
9	+	+	-	--	--	--	+	-	+	---	---	--	+	+	--	--	--
14	+	--	+	--	--	--	+	-	+	--	+	--	--	+	--	--	--
15	+	--	-	--	+	--	+	+	+	--	+	--	--	+	+	--	--
17	--	--	-	--	--	--	+	+	--	--	+	--	--	--	--	--	--
19	--	--	-	--	+	--	+	+	--	---	--	---	--	---	--	---	--
20	+	--	-	--	--	--	+	-	+	--	--	--	--	+	--	--	--
21	--	+	-	--	--	--	+	-	+	--	--	--	--	+	--	--	--
25	+	--	-	--	--	--	+	+	+	--	+	--	--	--	+	--	--
25’	+	-	-	--	--	--	+	-	+	--	+	--	--	--	+	--	--
28	-	-	-	-	-	-	+	-	-	-	+	-	-	-	+	-	-

KEY

+ SUSCEPTIBLE

__ RESISTANT

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***Staphylococcus* RESISTANCE TO ANTIBIOTICS USED**

Org	GN	CO	C	AM	CF	CX	N	E	TE	NB	CIP	AU	OF	AP	FX	CD	AX
1	–	–	–	–	–	–	–	–	+	–	+	–	+	+	–	+	–
2	–	–	–	+	–	–	–	+	–	–	+	–	+	–	–	+	–
3	+	-	–	–	–	–	+	–	–	–	+	–	+	–	–	–	–
4	–	–	+	–	–	–	+	–	–	–	+	–	+	–	+	–	–
5	–	–	+	–	+	–	–	–	–	–	+	–	+	–	+	–	–
6	+	–	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–
7	+	–	–	-	–	–	–	–	–	–	+	–	+	–	+	–	–
8	+	–	–	–	–	–	–	–	–	–	+	–	+	–	+	–	–
9	–	–	–	–	–	+	–	–	–	–	–	–	+	–	–	–	–
10	–	+	–	–	–	–	+	–	–	–	–	–	+	–	–	–	–
11	–	+	–	–	–	+	–	–	–	–	+	–	+	–	–	–	+
12	–	–	–	–	–	–	–	–	+	+	+	–	+	–	–	–	+
13	–	–	–	+	–	–	–	–	–	–	+	–	+	–	–	+	–
14	–	–	–	+	–	–	–	–	–	–	–	–	–	–	+	–	–
15	–	–	–	–	–	–	–	–	–	–	+	–	–	–	+	–	–
16	+	-	–	-	–	–	+	–	–	–	+	–	–	–	–	–	–
17	–	–	–	–	–	–	–	–	+	–	–	–	+	+	–	–	–
18	–	–	+	–	–	–	–	–	–	–	+	+	+	+	–	–	–
19	+	–	–	–	–	–	–	–	–	–	+	–	+	–	-	–	–
20	–	–	–	–	+	–	–	–	–	–	+	–	+	–	-	–	–
21	+	-	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–
22	+	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–
23	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–	–
24	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–	–
25	–	-	–	–	–	–	–	–	–	–	+	–	+	+	–	–	–
26	–	+	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–

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27	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
28	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–
29	–	–	–	–	+	–	–	–	–	–	+	–	+	–	–	–	–
30	–	–	–	-	–	–	–	-	–	–	+	–	+	–	–	–	–
31	–	+	–	–	–	+	–	+	–	–	+	–	+	–	–	–	–
32	–	–	–	–	–	–	–	–	–	+	+	–	+	–	–	–	–
33	–	–	–	–	–	–	–	–	–	+	+	–	+	–	–	–	–
34	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–
35	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–
36	–	–	+	–	-	–	–	–	–	–	+	+	+	–	–	–	–
37	–	–	+	–	–	–	–	–	–	–	+	+	+	–	–	–	–
38	+	–	–	–	–	–	+	–	–	–	+	–	+	–	–	–	–
39	+	–	–	+	–	–	–	–	–	–	+	–	+	-	–	–	–
40	–	–	–	+	–	–	–	–	–	–	+	–	–	–	–	–	–
41	–	+	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
42	–	–	–	-	–	+	–	–	+	–	+	–	–	–	–	–	–
43	–	-	-	-	–	–	–	–	–	–	+	–	+	–	–	–	–
44	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–
45	-	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–
46	-	–	–	–	–	–	–	–	–	+	+	–	+	–	–	–	–
47	–	–	–	+	–	–	–	–	–	+	+	+	–	–	–	–	–
48	-	–	–	–	–	–	–	–	–	+	+	+	–	–	–	–	–
49	–	–	–	–	–	–	–	–	–	–	+	+	-	–	–	–	–
50	-	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–
51	-	–	-	–	–	–	+	–	–	–	+	–	+	–	–	–	–
52	-	–	–	–	–	–	–	–	–	–	+	–	+	–	-	–	–
53	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–
54	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–
55	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–

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56	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
57	–	–	–	–	+	–	–	–	–	–	+	–	–	–	–	–	–
58	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
59	-	–	–	–	–	–	–	–	–	–	+	–	+	–	–	+	+
60	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–
61	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–
62	–	–	-	–	–	–	–	–	–	–	+	–	+	–	–	–	–
63	–	–	-	–	–	–	–	+	–	–	+	–	+	–	–	–	–
64	–	–	–	–	+	–	–	+	–	–	+	–	+	–	-	–	–
65	–	-	+	–	–	–	–	–	–	–	+	–	+	–	–	–	–
66	+	–	+	-	-	–	+	–	–	–	+	–	+	–	–	–	–
67	–	–	–	–	–	–	–	–	–	+	+	–	+	–	–	–	–
68	–	+	–	+	–	+	–	+	–	+	+	–	+	–	–	–	–
69	–	–	–	–	–	–	–	–	+	–	+	–	+	–	+	–	+
70	–	+	–	–	–	+	–	–	+	–	+	–	+	–	+	–	+
71	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–
72	–	–	–	–	+	–	–	–	–	–	+	–	+	–	–	–	–
73	–	–	–	–	+	–	–	+	–	–	+	–	+	–	–	–	–

KEY

+ SUSCEPTIBLE

__ RESISTANT

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Streptococcus RESISTANCE TO ANTIBIOTICS USED

Org	GN	CO	C	AM	CF	CX	N	E	TE	NB	CIP	AU	OF	AP	FX	CD	AX
1	–	–	–	–	–	–	–	–	+	–	+	–	+	+	–	+	–
2	–	–	–	+	–	–	–	+	–	–	+	–	+	–	–	+	–
3	+	-	–	–	–	–	+	–	–	–	+	–	+	–	–	–	–
4	–	–	+	–	–	–	+	–	–	–	+	–	+	–	+	–	–
5	–	–	+	–	+	–	–	–	–	–	+	–	+	–	+	–	–
6	+	–	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–
7	+	–	–	-	–	–	–	–	–	–	+	–	+	–	+	–	–
8	+	–	–	–	–	–	–	–	–	–	+	–	+	–	+	–	–
9	–	–	–	–	–	+	–	–	–	–	–	–	+	–	–	–	–
10	–	+	–	–	–	–	+	–	–	–	–	–	+	–	–	–	–
11	–	+	–	–	–	+	–	–	–	–	+	–	+	–	–	–	+
12	–	–	–	–	–	–	–	–	+	+	+	–	+	–	–	–	+
13	–	–	–	+	–	–	–	–	–	–	+	–	+	–	–	+	–
14	–	–	–	+	–	–	–	–	–	–	–	–	–	–	+	–	–
15	–	–	–	–	–	–	–	–	–	–	+	–	–	–	+	–	–
16	+	-	–	-	–	–	+	–	–	–	+	–	–	–	–	–	–
17	–	–	–	–	–	–	–	–	+	–	–	–	+	+	–	–	–
18	–	–	+	–	–	–	–	–	–	–	+	+	+	+	–	–	–
19	+	–	–	–	–	–	–	–	–	–	+	–	+	–	-	–	–
20	–	–	–	–	+	–	–	–	–	–	+	–	+	–	-	–	–
21	+	-	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–
22	+	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–
23	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–	–
24	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–	–
25	–	-	–	–	–	–	–	–	–	–	+	–	+	+	–	–	–
26	–	+	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–

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27	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
28	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–
29	–	–	–	–	+	–	–	–	–	–	+	–	+	–	–	–	–
30	–	–	–	-	–	–	–	-	–	–	+	–	+	–	–	–	–
31	–	+	–	–	–	+	–	+	–	–	+	–	+	–	–	–	–
32	–	–	–	–	–	–	–	–	–	+	+	–	+	–	–	–	–
33	–	–	–	–	–	–	–	–	–	+	+	–	+	–	–	–	–
34	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–
35	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–
36	–	–	+	–	-	–	–	–	–	–	+	+	+	–	–	–	–
37	–	–	+	–	–	–	–	–	–	–	+	+	+	–	–	–	–
38	+	–	–	–	–	–	+	–	–	–	+	–	+	–	–	–	–
39	+	–	–	+	–	–	–	–	–	–	+	–	+	-	–	–	–
40	–	–	–	+	–	–	–	–	–	–	+	–	–	–	–	–	–
41	–	+	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
42	–	–	–	-	–	+	–	–	+	–	+	–	–	–	–	–	–
43	–	-	-	-	–	–	–	–	–	–	+	–	+	–	–	–	–
44	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–
45	-	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–
46	-	–	–	–	–	–	–	–	–	+	+	–	+	–	–	–	–
47	–	–	–	+	–	–	–	–	–	+	+	+	–	–	–	–	–
48	-	–	–	–	–	–	–	–	–	+	+	+	–	–	–	–	–
49	–	–	–	–	–	–	–	–	–	–	+	+	-	–	–	–	–
50	-	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–
51	-	–	-	–	–	–	+	–	–	–	+	–	+	–	–	–	–
52	-	–	–	–	–	–	–	–	–	–	+	–	+	–	-	–	–
53	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–
54	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–
55	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–

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56	_	_	_	_	_	_	_	_	_	_	+	_	_	_	_	_	_
57	_	_	_	_	+	_	_	_	_	_	+	_	_	_	_	_	_
58	_	_	_	_	_	_	_	_	_	_	+	_	_	_	_	_	_
59	-	_	_	_	_	_	_	_	_	_	+	_	+	_	_	+	+
60	_	_	_	_	_	_	_	_	_	_	_	_	+	_	_	+	_
61	_	_	_	_	_	_	_	_	_	_	+	_	+	_	_	_	_
62	_	_	-	_	_	_	_	_	_	_	+	_	+	_	_	_	_
63	_	_	-	_	_	_	_	+	_	_	+	_	+	_	_	_	_
64	_	_	_	_	+	_	_	+	_	_	+	_	+	_	-	_	_
65	_	-	+	_	_	_	_	_	_	_	+	_	+	_	_	_	_
66	+	_	+	-	-	_	+	_	_	_	+	_	+	_	_	_	_
67	_	_	_	_	_	_	_	_	_	+	+	_	+	_	_	_	_
68	_	+	_	+	_	+	_	+	_	+	+	_	+	_	_	_	_
69	_	_	_	_	_	_	_	_	+	_	+	_	+	_	+	_	+
70	_	+	_	_	_	+	_	_	+	_	+	_	+	_	+	_	+
71	_	_	_	_	_	_	_	_	_	_	+	_	+	_	_	_	_
72	_	_	_	_	+	_	_	_	_	_	+	_	+	_	_	_	_
73	_	_	_	_	+	_	_	+	_	_	+	_	+	_	_	_	_
74	_	_	_	_	+	_	_	_	_	_	+	_	+	_	_	_	_
75	_	_	_	_	+	_	_	+	_	_	+	_	+	_	_	_	_

KEY

+ SUSCEPTIBLE

__ RESISTANT

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Esherichia coli **RESISTANCE TO ANTIBIOTICS USED**

Org	GN	CO	C	AM	CF	CX	N	E	TE	NB	CIP	AU	OF	AP	FX	CD	AX
1	–	–	–	–	–	–	–	–	+	–	+	–	+	+	–	+	–
2	–	–	–	+	–	–	–	+	–	–	+	–	+	–	–	+	–
3	+	-	–	–	–	–	+	–	–	–	+	–	+	–	–	–	–
4	–	–	+	–	–	–	+	–	–	–	+	–	+	–	+	–	–
5	–	–	+	–	+	–	–	–	–	–	+	–	+	–	+	–	–
6	+	–	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–
7	+	–	–	-	–	–	–	–	–	–	+	–	+	–	+	–	–
8	+	–	–	–	–	–	–	–	–	–	+	–	+	–	+	–	–
9	–	–	–	–	–	+	–	–	–	–	–	–	+	–	–	–	–
10	–	+	–	–	–	–	+	–	–	–	–	–	+	–	–	–	–
11	–	+	–	–	–	+	–	–	–	–	+	–	+	–	–	–	+
12	–	–	–	–	–	–	–	–	+	+	+	–	+	–	–	–	+
13	–	–	–	+	–	–	–	–	–	–	+	–	+	–	–	+	–
14	–	–	–	+	–	–	–	–	–	–	–	–	–	–	+	–	–
15	–	–	–	–	–	–	–	–	–	–	+	–	–	–	+	–	–
16	+	-	–	-	–	–	+	–	–	–	+	–	–	–	–	–	–
17	–	–	–	–	–	–	–	–	+	–	–	–	+	+	–	–	–
18	–	–	+	–	–	–	–	–	–	–	+	+	+	+	–	–	–
19	+	–	–	–	–	–	–	–	–	–	+	–	+	–	-	–	–
20	–	–	–	–	+	–	–	–	–	–	+	–	+	–	-	–	–
21	+	-	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–
22	+	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–
23	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–	–
24	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–	–
25	–	-	–	–	–	–	–	–	–	–	+	–	+	+	–	–	–
26	–	+	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–

27	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
28	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–
29	–	–	–	–	+	–	–	–	–	–	+	–	+	–	–	–	–
30	–	–	–	-	–	–	–	-	–	–	+	–	+	–	–	–	–
31	–	+	–	–	–	+	–	+	–	–	+	–	+	–	–	–	–
32	–	–	–	–	–	–	–	–	–	+	+	–	+	–	–	–	–
33	–	–	–	–	–	–	–	–	–	+	+	–	+	–	–	–	–
34	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–
35	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–
36	–	–	+	–	-	–	–	–	–	–	+	+	+	–	–	–	–
37	–	–	+	–	–	–	–	–	–	–	+	+	+	–	–	–	–
38	+	–	–	–	–	–	+	–	–	–	+	–	+	–	–	–	–
39	+	–	–	+	–	–	–	–	–	–	+	–	+	-	–	–	–
40	–	–	–	+	–	–	–	–	–	–	+	–	–	–	–	–	–
41	–	+	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
42	–	–	–	-	–	+	–	–	+	–	+	–	–	–	–	–	–
43	–	-	-	-	–	–	–	–	–	–	+	–	+	–	–	–	–
44	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–
45	-	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–
46	-	–	–	–	–	–	–	–	–	+	+	–	+	–	–	–	–
47	–	–	–	+	–	–	–	–	–	+	+	+	–	–	–	–	–
48	-	–	–	–	–	–	–	–	–	+	+	+	–	–	–	–	–
49	–	–	–	–	–	–	–	–	–	–	+	+	-	–	–	–	–
50	-	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–
51	-	–	-	–	–	–	+	–	–	–	+	–	+	–	–	–	–
52	-	–	–	–	–	–	–	–	–	–	+	–	+	–	-	–	–
53	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–
54	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–
55	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–

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57	_	_	_	_	+	_	_	_	_	_	+	_	_	_	_	_	_
58	_	_	_	_	_	_	_	_	_	_	+	_	_	_	_	_	_
59	-	_	_	_	_	_	_	_	_	_	+	_	+	_	_	+	+
60	_	_	_	_	_	_	_	_	_	_	_	_	+	_	_	+	_
61	_	_	_	_	_	_	_	_	_	_	+	_	+	_	_	_	_
62	_	_	-	_	_	_	_	_	_	_	+	_	+	_	_	_	_
63	_	_	-	_	_	_	_	+	_	_	+	_	+	_	_	_	_
64	_	_	_	_	+	_	_	+	_	_	+	_	+	_	-	_	_
65	_	-	+	_	_	_	_	_	_	_	+	_	+	_	_	_	_
66	+	_	+	-	-	_	+	_	_	_	+	_	+	_	_	_	_
67	_	_	_	_	_	_	_	_	_	+	+	_	+	_	_	_	_
68	_	+	_	+	_	+	_	+	_	+	+	_	+	_	_	_	_
69	_	_	_	_	_	_	_	_	+	_	+	_	+	_	+	_	+
70	_	+	_	_	_	+	_	_	+	_	+	_	+	_	+	_	+
71	_	_	_	_	_	_	_	_	_	_	+	_	+	_	_	_	_
72	_	_	_	_	+	_	_	_	_	_	+	_	+	_	_	_	_
73	_	_	_	_	+	_	_	+	_	_	+	_	+	_	_	_	_
74	_	_	_	_	+	_	_	_	_	_	+	_	+	_	_	_	_
75	_	_	_	_	+	_	_	+	_	_	+	_	+	_	_	_	_
76	_	_	_	_	_	_	_	_	_	_	+	_	+	_	_	_	_
77	_	_	_	_	_	_	_	_	_	_	+	_	_	_	_	_	_
78	_	_	_	_	_	_	_	_	_	_	+	_	_	_	_	_	_
79	_	_	_	_	+	_	_	_	_	_	+	_	_	_	_	_	_
80	_	_	_	_	_	_	_	_	_	_	+	_	_	_	_	_	_
81	-	_	_	_	_	_	_	_	_	_	+	_	+	_	_	+	+
82	_	_	_	_	_	_	_	_	_	_	_	_	+	_	_	+	_
83	_	_	_	_	_	_	_	_	_	_	+	_	+	_	_	_	_
84	_	_	-	_	_	_	_	_	_	_	+	_	+	_	_	_	_

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85	_	_	-	_	_	_	_	+	_	_	+	_	+	_	_	_	_
86	_	_	_	_	+	_	_	+	_	_	+	_	+	_	-	_	_
87	_	-	+	_	_	_	_	_	_	_	+	_	+	_	_	_	_
88	+	_	+	-	-	_	+	_	_	_	+	_	+	_	_	_	_
89	_	_	_	_	_	_	_	_	_	+	+	_	+	_	_	_	_
90	_	+	_	+	_	+	_	+	_	+	+	_	+	_	_	_	_
91	_	_	_	_	_	_	_	_	+	_	+	_	+	_	+	_	+

KEY

+ SUSCEPTIBLE

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APPENDIX 111

Average Daily rectal temperature of experimental goats

DAY	INFECTED GOATS TEMP				CONTROL GOATS TEMP			
	Ma.	Mb.	Mc	Mcc	Ma.	Mb.	Mc	Mcc
1	38.5	38.5	38.6	38.5	38.6	38.5	38.6	38.6
2	38.6	38.5	38.5	38.6	38.5	38.6	38.6	38.7
3	38.5	38.5	38.6	38.6	38.5	38.6	38.6	38.6
4	38.6	38.6	38.7	38.6	38.6	38.6	38.7	38.7
5	38.7	38.6	38.7	38.7	38.6	38.6	38.6	38.8
6	38.8	38.8	38.9	38.7	38.7	38.7	38.7	38.7
7	38.6	38.7	38.9	38.7	38.6	38.6	38.7	38.7
8	38.8	38.8	38.9	38.8	38.6	38.6	38.7	38.8
9	38.9	38.8	38.9	38.8	38.7	38 7	38.8	38.8
10	38.8	38.8	38.9	38.8	38.6	38.7	38.7	38.7
11	38.6	38.9	38.9	38.9	38.6	38.7	38.7	38.7
12	38.9	38.9	39.0	38.9	38.7	38.7	38.6	38.6
13	39.0	39.0	39.1	39.1	38.7	38.7	38.6	38.6
14	38.9	38.9	39.0	39.0	38.7	38.7	38.6	38.6
15	38.9	38.9	38.9	39.0	38.7	38.7	38.7	38.7
16	38.9	38.9	39.0	39.0	38.7	38.7	38.6	38. 7
17	38.9	39.0	39.0	39.1	38.7	38.7	38.6	38.7
18	38.9	38.9	39.0	39.0	38.6	38.7	38.6	38.7
19	38.9	39.0	39.1	39.1	38.6	38.7	38.6	38.8
20	39.0	39.0	39.1	39.0	38.6	38.7	38.6	38.8

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21	39.1	39.0	39.2	39.1	38.7	38.7	38.7	38.8
22	38.9	39.0	39.1	39.1	38.7	38.8	38.7	38.8
23	38.9	39.0	39.0	39.0	38.7	38.8	38.7	38.8
24	39.0	39.1	39.1	39.2	38.7	38.8	38.6	38.7
25	39.2	39.3	39.3	39.3	38.7	38.7	38.6	38.7
26	39.1	39.3	39.4	39.3	38.7	38.6	38.6	38.7
27	39.1	39.2	39.4	39.2	38.7	38.6	38.7	38.6
28	39.1	39.2	39.3	39.2	38.6	38.6	38.7	38.6
29	38.9	38.9	39.0	39.3	38.6	38.6	-	38.6
30	38.9	39.0	-	39.4	38.6	38.6	-	38.6
31	38.9	39.4	-	39.6	38.6	38.6	-	38.6
32	39.0	39.2	-	39.4	38.6	38.7	-	38.6
33	38.9	39.6	-	-	38.6	38.7	-	38.6
34	39.6	39.6	-	-	38.6	38.6	-	38.6
35	39.4	39.6	-	-	38.6	38.6	-	38.7
36	39.4	39.5	-	-	38.5	38.6	-	38.7
37	39.4	39.5	-	-	38.5	38.6	-	38.5
38	-	39.5	-	-	38.5	38.6	-	38.5
39	-	39.5	-	-	38.5	38.6	-	38.5
40	-	39.4	-	-	38.5	38.6	-	38.5
41	-	39.4	-	-	38.4	38.5	-	38.5
42	-	-	-	-	38.4	38.5	-	38.5

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