MOLECULAR STUDIES ON HUMAN UROGENITAL MYCOPLASMOSIS IN LAGOS AND JOS, NIGERIA.

A THESIS SUBMITTED TO THE SCHOOL OF POST GRADUATE STUDIES, UNIVERSITY OF LAGOS, IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD) IN THE DEPARTMENT OF MEDICAL MICROBIOLOGY AND PARASITOLOGY, COLLEGE OF MEDICINE UNIVERSITY OF LAGOS, NIGERIA.

BY

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NOVEMBER, 2008

CERTIFICATION

This is to certify that the Thesis:

MOLECULAR STUDIES ON HUMAN UROGENITAL MYCOPLASMOSIS IN LAGOS AND JOS, NIGERIA.

Submitted to the
School of Postgraduate Studies
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For the award of the degree of
DOCTOR OF PHILOSOPHY (Ph.D)
is a record of an original research carried out

By
OLORUNSHOLA, ISAAC DAYO
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DECLARATION

We thereby declare that the thesis titled "**MOLECULAR STUDIES ON HUMAN UROGENITAL MYCOPLASMOSIS IN LAGOS AND JOS, NIGERIA**" is a record of original research work carried out by **OLORUNSHOLA, Isaac Dayo,** in the Department of Medical Microbiology and Parasitology, College of Medicine of the University of Lagos, Nigeria.

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DEDICATION

THIS THESIS IS DEDICATED FIRST TO:

GOD ALMIGHTY, From Whom All Blessings Flow, The I Am That I Am, The Only One With The Throne Of Mercy, "for every good gift and every perfect gift is from above and cometh down from the father of lights with whom is no variableness, neither a shadow of turning" (James 1:17).

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF PLATES

LIST OF FIGURES

ABSTRACT

Information on the involvement of genital mycoplasmas in human diseases in several African countries is either lacking or is limited to a few colonization reports. In Nigeria, *Ureaplasma spp,* or *Mycoplasma genitalium*, has been implicated in male and female infertility. This work presents the current distribution pattern of these pathogens in sexually transmitted disease (STD) patients in Nigeria, and provides evidence for the occurrence of *Mycoplasma genitalium* in a Nigerian study population. The study population is more diverse than earlier investigations, utilizing patients from two Federal Government Reference Centers for HIV/AIDS/STI located at the Jos University Teaching Hospital (JUTH) in the north and the Lagos University Teaching Hospital (LUTH) in the south. Urethral swabs and high vaginal or endocervical swabs were taken from adult male and female patients and controls, respectively. Participants' consent was sought as demanded by ethical committees. Swabs were snapped off into complete ureaplasmal broth and transported in ice coolers to the laboratory. Altogether, 824 swab specimens from patients and controls were cultured routinely in standard ureaplasmal broth, and *Ureaplasma spp* was identified by the usual test for urease production on agar plate. *Ureaplasma spp* was isolated from 22 of 70 males (31.4%) at the JUTH location, and from 19 of 70 males (27.1%) at the LUTH location. In females, 96 of 180 (53.3%) swabs were positive at JUTH, and 86 of 180 (47.8%) at LUTH. This gave an overall prevalence of 44.6% (223 of 500) in the male and female patients. At the two locations, a total of 324 asymptomatic participants were sampled, with a total of 140 (10.7%) males and 184 (7.1%) females. The breakdown comprises of 5 of 70 males (7.1%) at the JUTH location, and from 10 of 70 males (14.3%) at the LUTH location. In females, 6 of 92 (6.5%) swabs were positive at JUTH, and 7 of 92 (7.6%) at LUTH. This gave an overall prevalence of 8.6% (28 of 324) in the male and female controls. Thus, the rate of isolation of *U urealyticum* was higher in patients (44.6%) with clinical evidence of sexually transmitted infection (STI) than in asymptomatic (control) groups (8.6%), supporting previous studies in Nigeria and various other parts of the world.

The isolates in broth were lyophilized and transported abroad to a host laboratory in the United States of America for Polymerase Chain Reaction (PCR) technology. Two hundred and thirty six (236) lyophilized samples made up of 119 cases and 117 controls were subjected to Polymerase Chain Reaction (PCR) assays for Uu and Mg. In PCR assays, two primers UU8 and UPA were used to bind with either the target gene located at a 650-bp

conserved region of *Ureaplasma urealyticum* (Uu) 16S rRNA gene for the identification of *U. urealyticum*, or the target gene at a 400-bp conserved region of *Ureaplasma parvum* (Up) urease gene for the identification of *U. parvum* biovar. Uu was detected in 26 of 119 patients (21.85 %), while 49 (41.18%) were positive for Up. Of the 117 controls examined, 15 (12.82%) and 20 (17.09%) were positive for Uu and Up respectively. Thus, *Ureaplasma parvum* biovar was detected at a higher rate from both patient and control groups. Real-time PCR technology was specifically employed for the rapid detection of *Mycoplasma genitalium*: 17 of the 119 patient specimens (14.29%) and 7 of the 117 of control specimens (6.0%) were positive. Overall, 24 of 236 specimens examined or 10.17% were positive for Mg. The association of *Mycoplasmas genitalium* with Nongonococcal urethritis (NGU) was strongest in males <35 ($P \leq 0.050$) years of age. In conclusion, genital mycoplasma including Uu, Up and Mg may play some role in cases of non gonococcal urethritis and infertility in males and females attending STD clinics in LUTH and JUTH. Out of the 119 infertile patients selected for PCR analysis, 44 (36.97%) patients were positive for *Ureaplasma urealyticum* by culture. The sensitivity and specificity of all the techniques employed in this study were compared, and it was discovered that RT-PCR was the most sensitive (100%), specific (98%), fastest and most accurate. It was also the only technique that could be employed to identify *Mycoplasma genitalium* from lyophilized broth culture in this study. PCR technique was faster and more specific than cultural technique. It has also enabled the speciation of Nigerian Uu isolates into Uu and Up serovars for the first time. Culture and identification remain the fundamental procedure in clinical diagnosis, whereby an organism could be morphologically studied and confirmed. In modern diagnostic work, however, culture has great limitations as far as speed, specificity, and scope of capability are concerned. The trend now is molecular diagnostic approach which is faster, more specific, permits serovar determination of Uu, and affords detection of highly fastidious mycoplasmas such as Mg. Participants drawn from 30 of the 37 states of Nigeria unanimously completed the questionnaire covering demographic information, health status; number of sexual partners, history of STI, use of contraceptives, primary and secondary STD clinical conditions The data generated against the risk factors examined in this study was tested statistically and it was discovered that the following were significant, since their P values were less or equal to 0.05. This includes history of STI, number of sexual partners, use of condon, age, occupation and level of education. The association of genital mycoplasmas infections was strongest in participants <40 years of age ($P = 0.059$), participants with history

of STI (0.030), those with multiple number of sexual partners (0.040), lack of the use of condon (0.014), age of debut <18 years of age (0.023) and those categories of low socioeconomic status in occupation (0.020), and level of education (0.025). This study has paved the way for future studies that could focus on determining *Ureaplasma* serovars in human subjects and the development of Polymerase Chain Reaction diagnostic facilities for *Ureaplasma spp*, *Mycoplasma genitalium*, and related microorganisms in Nigeria.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 GENERAL INTRODUCTION

Mycoplasmosis is the terminology used to describe infections/diseases caused by a group of microorganisms belonging to the order Mycoplasmatales, class Mollicutes, family Mycoplasmataceae and genus *Mycoplasma*. A closely related organism, formerly known as T-mycoplasma but now grouped under *Ureaplasma spp.* is also often discussed under mycoplasmosis. This Ph.D project focuses on urogenital infections associated with these groups of organisms. Only two species of ureaplasmas are currently recognized but several species of mycoplasmas have been isolated or confirmed from the urogenital system.

1.2 SEXUALLY TRANSMITTED DISEASES/INFECTIONS

Based on the pathogenic potentials of these organisms in humans, this Ph.D project has concentrated on *Ureaplasma spp* and *Mycoplasma genitalium*. The study has applied molecular techniques to their investigation for the first time in Nigeria. In this introductory chapter, an overview of sexually transmitted diseases (STD) or sexually transmitted infections (STI) will first be presented in as much as the subject matter for the project is related to STI. Genitourinary infections are caused by an assembly of diverse microbial agents that cause considerable morbidity and mortality in humans worldwide (Schlicht *et al.*, 2004). Classical examples of such pathogens include *Neisseria gonorrhoea, Treponema pallidium, chlamydia trachomatis, Haemophilus ducreyi*, etc. The World Health Organization (WHO) in 1995 ranks these four bacterial pathogens and *Trichomonas vaginalis* among the most common curable infections in the world (WHO, 1995). In year 2001, WHO also estimated that 340 million fresh cases of gonorrhea, syphilis, *Chlamydia* infections, chancroid and trichomoniasis occurred in men and women within the age range of 15 to 49 years. In the same publication, WHO expanded the list of curable STI pathogens to cover several groups of bacteria, viruses, fungi, Chlamydia, protozoa and ectoparasites. (WHO, 2001). Table 1.1, presents a summary of all the major known STD/STI.

Table 1: Sexually Transmitted Infections.

Diseases	Etiology	Risk Factors	Clinical features	Diagnosis	Management	Prevention/ Control
Acquired Immunodeficiency Syndrome (AIDS)	*Human immunodeficiency virus (HIV-1 and HIV-2*	Unprotected sexual activities Blood transfusion Injury Concurrent STIs	Swollen lymph nodes, night sweats, diarrhea, weight loss, fatigue, susceptibility to opportunistic infections	Serology, ELISA and PCR	Antiretroviral drugs	Complete abstinence, Or protected sex, blood screening prior to transfusion
Bacterial vaginosis	*Bacteriodes spp. Gardnerella and Mollusca spp*	Sexual contact.	Systemic disorders, characterized by fever, chills, malaise and vaginal discharge. with characteristic odor	Culture, wet mounts and PCR	Erythromycin and Tetracycline, Trimethroprim	Good hygiene and protected sex
Chancroid	*Haemophilus ducreyi*	Sexual contact	Chancre-like ulcers in the genital region, known as genital ulcer	Culture, and PCR	Erythromycin	Good hygiene and protected sex
Chlamydia infections Pelvic Inflammatory Disease NGU and LGV	*Chlamydia trachomatis*	Sexual contact, intrapartum, perinatal	A burning sensation during urination, penile discharge and Epididymitis: NGU	Culture, ELISA and PCR	Doxycycline	Good hygiene and protected sex
Cytomegalovirus Infections	*Cytomegalovirus*	Sexual contact	Urethral and cervical Discharges and Epididymitis: NGU inflammation, and/or small hard swelling at the bottom of the testicle.*	Serology, ELISA and PCR	Chemotherapy	Complete abstinence, Or protected sex, blood screening prior to transfusion
Enteric infections	*CampylobShigellasp.EscherichiSalmonell*	Faecal oral	Diarrhea	Culture and PCR	Trimethroprim	Good hygiene and protected sex
Amebiasis	*Entamoeba histolytica (protozoan*	Faecal oral	Diarrhea	Culture and PCR	Trimethroprim	Good hygiene and protected sex

Giardiasis	*Giardia lamblia*	Faecal oral	Diarrhea	Culture and PCR	Trimethroprim	Good hygiene and protected sex
Genital herpes	*Herpes simplex virus*	Sexual	Periodic outbreaks of small blisters, usually on vaginal lips, fever, painful urination, and headaches.	Serology, ELISA and PCR	Chemotherapy	Complete abstinence, Or protected sex, blood screening prior to transfusion
Genital (venereal) warts	*Human papillomavirus*	Sexual	Cauliflower-like warts on the vulva, vaginal walls, or cervix.	Serology, ELISA and PCR	Chemotherapy	Complete abstinence, Or protected sex, blood screening prior to transfusion
Gonorrhea	*Neisseria gonorrhea*	Sexual contact	Discharge from the cervix that is yellow-green in color and irritates the vulva;	Culture and PCR	Diprofloxacin	Good hygiene and protected sex
Granuloma inguinale (donovanosis)	*Campylobacter spp* *Shigella spp*	sexual	Cauliflower-like warts around the head or shaft of the penis or the scrotum.	Culture and PCR	Trimethroprim	Complete abstinence, Or protected sex, blood screening prior to transfusion
Group B streptococcal infections	*Streptococcus agalactiae*	Sexual	Bacterial infections such as Pneumonia,septicemia, meningitis	Culture and PCR	Erythromycin	Good hygiene and protected sex

Adapted, with modifications, from The Sexpert Department of Sociology, University of California, Santa Barbara' s Sexfo. (Chamberlain, 2007).

STI remain a public health problem of major significance in most parts of the world. The incidence of acute STI is believed to be high in many countries. Failure to diagnose and treat STI at an early stage may result in serious complications and sequalae, including infertility, fetal wastage, ectopic pregnancy, anogenital cancer, premature death, and neonatal and infant infections. The appearance of HIV/AIDS created more awareness about the increasing rate of STI transmission and complications associated with management and control due to antimicrobial resistance. STI are passed from one person to another, through various types of sexual acts and risk factor practices. Although more commonly referred to as sexually transmitted diseases STD, many infections are asymptomatic, meaning that an individual may not display any symptom or the symptoms go unnoticed. Subsequently, the terminology for STD has been changing more towards the new term of STI, as defined by WHO, 2002. In developing countries, STD and their complications rank in the top five disease categories for which adults seek health care. The scale of the STD problem is too great to be dealt with in specialized STD centers alone, and steps must be taken to expand and integrate STD management in primary health and other health centers as recommended by the World Health Organization (WHO, 1995).

1. 3 RATIONALE FOR THE STUDY

Infertility or the inability of a married couple to have children is a stigma in many African countries. Invariably, the wife is blamed for the failure, but modern medical practice, which is available in urban cities, is enabling male partners to be investigated as well as the females. In the African culture, for an infertile couple, the society views infertility as a social stigma with considerable emotional stress (Ladipo, 1986). Family and societal pressure on the infertile couple often predispose to marital stress and inability, polygamy, divorce, prostitution and extreme cases, suicidal tendencies (Ladipo, 1986, Jimoh, 2004).

Microbial causes of infertility have not been exhaustibly investigated in Nigeria. Moreover there are no available data in this environment regarding the role of mycoplasmas in STD maladies. The role of microorganisms of the class mollicutes including *Mycoplasma spp* and *Ureaplasma spp* has not been well studied in Nigeria because of the special skills required for culturing and identifying these agents. From the wealth of literature, molecular tools such as PCR have not been applied to investigating the role of these agents in female and male infertility in Nigeria.

1.4 Previous Studies on Human *Ureaplasma urealyticum** in Nigeria

Table 2. Previous Studies on Human *Ureaplasma urealyticum in Nigeria, by cultural isolation.**

Authors	Site	Study Population	Prevalence
Osoba (1972)	South (Ibadan)	Female patients & cohorts Overall	60%
Ladipo & Osoba (1978)	South (Ibadan)	100 semen samples - low count males - normal count males - overall	 57.1% 15.0% 39%
Adegboye *et al.*, (1979)	North (Zaria)	Females - infertility problems - apparently healthy - Overall	 84.6% 55.6% 65%
Bakare *et al.*,(2002)	South (Ibadan)	Men with NGU (154)	14.3% (Rate higher than in controls $P < 0.001$)
Olumide and Mohammed (2004).	Early diagnosis and management of sexually transmitted infections in the prevention of HIV/AIDS, in Nigeria.(references to other STD pathogens)		
Ogunsola (2005)	Reviewed the roles of STD pathogens in HIV transmission in Nigeria and concluded the need for more studies in human mycoplasmology		
Olorunshola *et al.*, (2006). Paper presented at an international conference at Ferarra, Italy (being the preliminary epidemiological data aspect of this Ph.D project)	South (Lagos) and North (Jos)	Male and female patients attending STD clinics in Nigeria Males with NGU and infertility (semen) Female patients with history of infertility	60% 44.6%

Legend= ***Some isolates might actually have been *U. parvum***

There are previous reports of isolation of *Mycoplasma hominis* (Mh) and *Ureaplasma urealyticum* (Uu) (Osoba 1972) and Adegboye *et al.*, (1979), showed higher prevalent rates for both organisms in females with infertility. Ladipo and Osoba (1978) specifically associated Uu with sperm abnormalities and male infertility. Bakare *et al.*, (2002) associated Uu with non-gonococcal urethritis in males in Ibadan. Thus far, no studies have

been performed that have specifically evaluated the role of *genital* mycoplasmas in postpartum or post abortion fever and endometritis in Nigeria. Moreover, the role of microorganisms of class mollicutes including *Mycoplasmas spp* and *Ureaplasma spp* has not been extensively studied in Nigeria because of the non availability of modern technology, special skills required for culturing and identifying these agents (Ogunsola, 2005) (Table 2). The physician does not pay routine attention like other STD pathogens during the course of managements. Hence, more knowledge is required about human mycoplasmology in Nigeria.

The modern techniques have made available the complete genome sequence of *Ureaplasma urealyticum* (Uu), *Ureaplasma parvum* (Up) and *Mycoplasma. genitalium* (Mg). This has highly improved understanding of their basic biology and pathogenic properties. Recently, the use of vero cell line in tissue culture has also enhanced the isolation of *Mycoplasma genitalium*, which is much cheaper in our settings than PCR or RT-PCR. (Jensen *et al.*, 1994). Serological tests such as ELISA are also very useful as reported by Moller *et al.*, (1985). Several studies have reported that these microorganisms are capable of causing non-gonococcal urethritis (NGU) and pregnancy complications in males and female patients. Until recently, not many laboratories in the world handle ureaplasmas or mycoplasmas. Their requirement for highly complex growth media to which serum, yeast extract, etc, must be added (Tables 3.1 and 3.2), the fact that they produce tiny, microscopic colonies (Plates 1 and 2), and their notoriety as contaminants of tissue cultures used for growing viruses, make them unappealing to most laboratories. Hence more knowledge is required in this field- human mycoplasmology.

In Nigeria there are no work done on *Mycoplasma genitalium* in both males and females, except the current report (Table 2). The aim of this study is to test the hypothesis of the association of mycoplasmas with non gonococcal urethritis (NGU) and infertility among sexually transmitted disease (STD) patients in Nigeria, through the prevalent determination of genital mycoplasmas among the patients attending STD clinics in Lagos and Jos, Nigeria. Preliminary data emanating from this study are expected to provide a strong basis for a comprehensive investigation of the role of ureaplasmas and its related microorganism, Mg, in the overall picture of STD/STI/HIV/AIDS in the states of Nigeria. Physicians and epidemiologists need such data in their efforts to control STD in national health programmes.

1.5 MAIN OBJECTIVE OF THS STUDY:

The overall objective of this study is to: determine the prevalence of genital mycoplasmas, among STD patients, evaluate the association of *Mycoplasma genitalium* and *Ureaplasma spp* with urethritis and infertility using high technology, and speciate *Ureaplasma spp* that will be recovered in the course of this study.

1.6 SPECIFIC OBJECTIVES ARE TO:

1. Isolate and compare the prevalence of genital *Ureaplasma spp* in patients attending STD clinics in LUTH and JUTH by culture.

2. Speciate *Ureaplasma spp* by PCR technology and show their distribution pattern among patients attending STD clinics in Nigeria.

3. Investigate the prevalent of *Mycoplasma genitalium* (an emerging pathogen) in Nigerian male and female subjects.

4. Show the sensitivity and specificity of improved techniques used, over cultural isolation methods employed, in previous studies.

5. Evaluate the statistical correlation of some STD high risk factors in epidemiology of genital mycoplasmas in Nigeria by culture and molecular technology.

CHAPTER TWO

LITERATURE REVIEW

2.1 INTRODUCTION

The first report of a *Mycoplasma* in association with human pathological condition was by Dienes and Edsall (1937) who isolated an organism which was probably the one known now as *Mycoplasma hominis* from a Bartholin's gland abscess. At that time, mycoplasmas were called pleuropneumonia-like organisms because the microbe now known as *Mycoplasma mycoides* had been shown to cause bovine pleuropneumonia. The term mycoplasma (Greek: mykes = fungus and plasma = formed) was first used to describe the pleuropneumonia-like organisms in the 1950s. This designation was initially intended to describe the growth form of *M. mycoides*, but the term soon gained widespread usage and was applied to all pleuropneumonia-like organisms of humans and animals, identified at that time.

In 1954 Shepard provided the first description of T-strain *Mycoplasmas,* when he was able to cultivate them *in vitro* from the urethras of men with nongonococcal urethritis (Shepard, 1954). The possible role of genital mycoplasmas in diseases of the female reproductive tract that affect pregnancy outcome or lead to infertility has been debated since the 1970s, and there are still no clear answers to the many questions that remain. The initial associations with infertility came following reports that ureaplasmas could be isolated from the lower genital tract more commonly in infertile couples than in fertile couples, but this was not found consistently in subsequent investigations (Gnarpe and Friberg, 1972, Mathews *et al.*, 1972, Upadhyaya, 1983). Tissue obtained at laparoscopy has also shown that ureaplasmas can be recovered more commonly from infertile women than fertile women, even when cervicovaginal isolation rates from the two groups are similar (Stray-Pedersen *et al.,* 1978 and Stray-Pedersen *et al.* 1982). Ureaplasmas are known to attach to sperm and decrease motility, explaining the association with male factor infertility seen in some studies (Taylor-Robinson *et al.,* 1999). Elimination of ureaplasmas by antimicrobial treatment has been correlated with improvement in sperm motility, quantity, and appearance by some investigators (Swenson *et al.*, 1979 and Tooth and Lesser 1982).

Ogunsola in 2005 pointed out various factors militating against the lack of national record in some of the STI data in Nigeria. This include poor diagnostic system, failure of some patients to seek proper medical care, inadequate financial support for hospital diagnostic units, and non-availability of modern technology such as PCR. The role of microorganisms of the class mollicutes including *Mycoplasma spp* and *Ureaplasma spp*

has not been well studied in Nigeria because of the special skills required for culturing and identifying these agents. To the best of our knowledge molecular tools such as PCR has not been applied to investigating the role of these agents in female and male infertility in Nigeria. There are previous reports of isolation of *Mycoplasma hominis* and *Ureaplasma urealyticum* (Osoba 1972) and Adegboye, Briggs and Lister (1979). The latter authors showed higher prevalent rates for both organisms in females with infertility. Ladipo and Osoba (1978) specifically associated *U urealyticum* with sperm abnormalities and male infertility. Bakare *et al* (2002) associated *Ureaplasma urealyticum* with non-gonococcal urethritis in males. Mycoplasmas have long been recognized as pathogens of respiratory, urogenital tract, and joints in a variety of animal species. And with rare exceptions, they produce diseases that are chronic and multifactorial origin the syndrome produced depending on environmental conditions., the genetic predisposition of the host and to a lesser extent the nature of the infecting microbe. Thus the diffulty in establishing the aetiological importance of mycoplasmas. In the past decade, these organisms have received increasing attention as agents of human disease. Ureaplasma urealyticum [formerly known as T-strains Mycoplasmas] has been proved to be the cause of non-gonococcal urethritis in men and continue to be implicated in in infertility stillbirth and spontaneous abortion. The purpose of this review chapter is to present a comprehensive account of the field of mycoplasmology and show areas where knowledge is lacking to justify the objective of this project. Thus mycoplasmas and ureaplasmas associated with urogenital infections receive special attention in the discussions.

2.2 Historical Perspectives

In 1898 Nocard and Roux reported the cultivation of the causative agent of contagious bovine pleuropneumonia (CBPP); at the time a grave disease in agriculture and today a concern of cattle ranchers particularly in Africa and Southern Europe, and of customs officials elsewhere. The disease is caused by *M mycoides subsp. mycoides* SC (small-colony type), and the work of Nocard and Roux represented the first isolation of a *Mycoplasma.* Its culture was difficult because of the complex growth requirements (Nocard and Roux, 1898).

These researchers succeeded by inoculating a semi-permeable pouch of sterile medium with pulmonary fluid from an infected animal and depositing this pouch intraperitoneally into a live rabbit. After fifteen to twenty days, the recovered pouch had an opacity that an

uninoculated control lacked. This turbid broth could then be used to inoculate a second and third round and subsequently introduced into a healthy animal, causing disease. However this did not work if the material was heated, indicating a biological agent at work. Uninoculated media in the pouch, after removal from the rabbit, could be used to grow the organism *in vitro*, demonstrating cell-free culture and ruling out viral causes, although this was not fully appreciated at the time (Nocard and Roux, 1898).

The earliest reports of mycoplasmas as infectious agents in humans appeared in the 1930s and 1940s (Dienes and Edsall 1937). At that time, primary atypical pneumonia was associated with an infectious agent, because of its minute size and innate biological properties unknown at that time, passed through bacteria-retaining filters, resisted penicillin and sulfonamide therapies, and adapted to growth in embryonated eggs and tissue culture cells (Edward and Freundt, 1956).

Correlations between the etiologic agent of "walking pneumonia" with viruses, L-forms, and pleuropneumonia-like agents (referred to as PPLOs in publications and textbooks of that era) were frequent and often misleading. In the early 1960's, there were reports linking Eaton's Agent to the PPLOs or mycoplasmas, well known then as parasites of cattle and rodents, using sensitivity to antimicrobial compounds (i.e. organic gold salt) (Marmion and Goodburn, 1961). The ability to grow Eaton's Agent, now known as *Mycoplasma pneumoniae*, in cell free media allowed an explosion of research into what had become the most medically important and the most studied mycoplasma (Marmion and Goodburn, 1961).

The name *Mycoplasma*, from the Greek mykes (fungus) and plasma (formed), was proposed in the 1950's, replacing the term pleuropneumonia-like organisms (PPLO) referring to organisms similar to the causative agent of CBPP (Edward and Freundt, 1956). It was later found that the fungus-like growth pattern of *M. mycoides* is unique to that species. The confusion about mycoplasmas and virus surfaced again 50 years later when Eaton and colleagues cultured the causative agent of human primary atypical pneumonia (PAP) or 'walking pneumonia.' This agent could be grown in chicken embryos and passed through a filter that excluded normal bacteria, but could not be observed by the high magnification light microscopy of the day, and caused disease that could not be treated with the popular antimicrobials sulphonamides and penicillin (Eaton *et al.*, 1945).

Eaton did consider the possibility that disease was caused by a *Mycoplasma,* but the agent did not grow on the standard PPLO media of that time. These observations led to the conclusion that PAP had a viral etiology. Research at that time showed the cultured agent could induce disease in experimentally infected cotton rats and hamsters. In spite of controversy then about whether the researchers had truly isolated the causative agent of PAP (based largely on the unusual immunological response of patients with PAP), in retrospect their evidence along with that of colleagues and competitors appears to have been quite conclusive (Marmion, 1990).

In 1954 Shepard provided the first description of T-strain *Mycoplasmas,* later known as *Ureaplasmas*, when he was able to cultivate them *in vitro* from the urethras of men with nongonococcal urethritis (Shepard, 1954). There has been considerable controversy as to the role of *Ureaplasma* in cases of NGU, since Ureaplasmas were first isolated. For some twenty years after the initial isolation of *U urealyticum*, scientists all over the world could not agree that it could cause NGU, despite encouraging transmissions studies in animals.

The challenge to conclusively prove a causal relationship was taken up by Dr David Taylor-Robison, a consultant venerologist and the then head of the Medical Research Center, Sexually Transmitted Disease Clinic at Watford, Harrow, and a famous research institute under the University of London. He and a physician colleague infected themselves with a pure culture of *U urealyticum* previously isolated from a patient, and they developed NGU (Taylor-Robinson *et al.,* 1977). That work established *U urealyticum* as a very important cause of NGU.Two mycoplasmas commonly found in the urogenital tracts of healthy persons are *Mycoplasma hominis* and *Ureaplasma urealyticum.* Over the years, the pathogenic roles of these mycoplasmas have been proven in adult urogenital tract diseases, neonatal respiratory infections, and a range of other diseases usually in immunocompromised patients (Abele-Horn *et al.,* 1998).

Several recent reports (Andrews *et al.*, 2003 and Arya *et. al.*, 2001); illustrate the increasing impact of *Mycoplasma spp* on emerging diseases. *Mycoplasma fermentans* was first isolated from the lower genital tract of both adult men and women in the early 1950s, but their role in classic lower genital tract disease has not been established (Andrews *et al.,* 2003). Reports in the 1970s of *M. fermentans* in the joints of rheumatoid arthritis patients

and in the bone marrow of children with leukemia raised expectations for its pathogenic potential (Andrews *et al.*, 2003 and Arya *et. al.*, 2001); these findings have not been adequately confirmed. Sufficient evidence, however, has accumulated recently to establish an important and emerging role for *M. fermentans* in human respiratory and joint diseases.

For example, *M. fermentans* has been detected by specific gene amplification techniques such as polymerase chain reaction (PCR) in the synovial fluid of patients with inflammatory arthritis, but not in the joints of patients with juvenile or reactive arthritis (Baier *et al., 2003*). In two other studies using PCR, *M. fermentans* was identified in the upper respiratory tract of 20% to 44% of both healthy and HIV-infected patients (Bancalari *et al.*, 2003 and Baseman *et al.*, 2004) and was associated with acute respiratory distress syndrome in nonimmunocompromised persons (Baseman *et al.*, 1988).

Mycoplasma genitalium was detected in the urogenital tract of two patients with nongonococcal urethritis (Taylor-Robinson *et al.*, 1995), but for more than a decade, very little was known about its host distribution and pathogenicity. Early experimental studies established that the organism caused lower genital tract infections in both male and female chimpanzees, with extensive urethral colonization in males and apparent tissue invasion, eventually leading to overt bacteremia (Bebear *et al.*, 1999).

However, the fastidious growth requirements of *M genitalium* from human hosts severely limited further study until the advent of molecular detection techniques. Specific sequences in the 140 kDa adhesin protein gene of *M genitalium* were selected as targets in a PCR-based detection assay (Benn *et al.*, 2002 and Benstein *et al.*, 2003). Subsequent application of these techniques in cases of acute NGU, not including those of patients colonized or infected with *Chlamydia trachomatis,* has provided mounting evidence for the involvement of *M genitalium* as an etiologic agent of this disease (Benstein *et al.*, 2003; Blanchard and Bebear 2002, and Blanchard, 1993). Also, *M genitalium* has been suspected in chronic NGU and PID (Blanchard, 1993).

The discovery in 1988 of *M genitalium* strains in human nasopharyngeal throat specimens, where they were frequently mixed with strains of *M pneumoniae*, not only changed dramatically the concept of host distribution of *M genitalium* but also prompted critical questions about the role of this *Mycoplasma* in human respiratory disease (Boesen

et al, 2001). However, the immunologic cross-reactivity between *M genitalium* and *M pneumoniae* and the inability of most conventional diagnostic serologic tests to conclusively identify *M. genitalium* have complicated its delineation in acute human respiratory disease.

PCR assays specific for the organism have detected *M genitalium* in throat specimens of patients infected with HIV-1 (Boesen *et al,* 2001). However, these probes have not been applied to control groups and patients in outbreaks of acute respiratory disease and/or pneumonia to determine whether *M genitalium* alone is an etiologic agent in respiratory infections.

M. genitalium has been implicated as an etiologic agent in certain human joint diseases. This clinical correlation began with the observation of a mixed infection of *M pneumoniae* and *M genitalium* in synovial fluid specimens of a nonimmunocompromised patient after an acute respiratory infection (Bonnin *et al.,* 1995). A predominant role was not established for either *Mycoplasma spp* in the initial respiratory disease or in the joint manifestations, although evidence to implicate postinfectious autoimmunity to both organisms was described. These findings prompted a PCR assay on synovial fluids from patients with various arthritic syndromes, which presented case reports on two of 13 patients with *M genitalium* detected in joint fluids (Bowman *et al.,* 1998).

Another area of emerging mycoplasmal infections concerns immunodeficiency. Although patients with congenital or acquired disorders of antibody production are susceptible to a wide variety of microbial infections, the unique susceptibility of such patients to mycoplasmal infections is a growing concern, especially considering the number of occurrences, the types of mycoplasmas involved, and the difficulties posed in the therapeutic management of such infections. In addition, the increased use of prolonged or permanent immunosuppressive chemotherapy required for patients undergoing tissue or organ transplantation or treatment of various malignant diseases has also increased the risk for mycoplasmal infections from mycoplasmas that are part of the normal human mollicute flora to those acquired through animal contact (Bowman *et al.,* 1998).

The association between immunodeficiency and mycoplasmal infections was first reported in the mid 1970s in patients with primary hypogammaglobulinemia and infection with *U*

urealyticum, *M pneumoniae*, *M salivarium*, and *M hominis* that localized in joint tissue, frequently with destructive arthritis. Similar joint infections in hypogammaglobulinemic patients with these mycoplasmal species continue to be reported (Braun *et al.*, 1971). Since most of these mollicutes, with the possible exception of *M pneumoniae*, occur as part of the normal human flora, the origin of such joint infections is considered endogenous. Patients with hypogammaglobulinemia and other antibody deficiencies are also especially susceptible to mycoplasmal infections of the upper respiratory and urinary tracts caused most frequently by *M pneumoniae* or *U urealyticum*, respectively (Brown *et al.*, 1987).

Mycoplasmal infections following organ transplantation and immunosuppressive chemotherapy were observed in the early 1980s, with both *M hominis* and *U urealyticum* reported most often (Brown *et al.*, 1987, Brus *et al.*, 1991 and Buckingham *et al.*, 2003).

While patients with antibody defects or those receiving immunosuppressive drugs appear to be the most susceptible to infections with mycoplasmas present in healthy tissues, emerging evidence indicates that contact with other mycoplasmas in the environment is an important hazard. For example, the direct isolation of a feline mycoplasma (*M felis*) from the joint of a hypogammaglobulinemic patient with septic arthritis was recently reported (Busolo *et al.*, 1984), with suspected transmission occurring through a cat bite 6 months before the onset of arthritis. Other examples include fatal septicemia caused by *M arginini*, a common animal mycoplasma, from blood and multiple tissue sites in a slaughter house employee who had advanced non-Hodgkin's lymphoma and hypogammaglobulinemia (Carrey *et al.*, 1991). One of the most critical aspects of mycoplasmal infections in immunodeficient patients is the frequent inability to control such infections with appropriate broad spectrum antibiotics (Carrey *et al.*, 1991). Although the tetracyclines and erythromycins are effective chemotherapeutic agents for many mycoplasmal infections, *M fermentans* and *M hominis* strains are usually resistant to erythromycin, and tetracycline-resistant strains of *M hominis* and *U urealyticum* have been reported from the lower urogenital tract of patients (Carrey *et al.*, 1991). However, these antibiotics and most other broad spectrum agents have limited mycoplasmacidal activity *in vivo*, and their efficacy eventually depends on an intact host immune system to eliminate the mycoplasmas (Braun *et al.*, 1971 and Brown *et al.*, 1987).

2.3 Taxonomy and phylogeny

The medical and agricultural importance of members of the genus *Mycoplasma* and related genera has led to the extensive catalogue of many of these organisms by culture, serology, and small subunit rRNA gene and whole genome sequencing. A recent focus in the sub-discipline of molecular phylogenetics has both clarified and confused certain aspects of the organization of the class Mollicutes, and while a truce of sorts has been reached, the area is still somewhat of a moving target (Johansson and Pettersson, 2002). The name mollicutes is derived from the Latin *mollis* (soft) and *cutes* (skin), describing the absence of a cell wall and the genetic capability to synthesize peptidoglycan.

Despite the lack of a cell wall, *Mycoplasma* and relatives have been classified in the phylum Firmicutes consisting of low G+C Gram-positive bacteria such as *Clostridium, Lactobacillus*, and *Streptococcus,* based on 16S rRNA gene analysis (Hammerschlag *et al.,* 1987) The cultured members of Mollicutes are currently arranged into four orders: Mycoplasmatales Acholeplasmatales, Anaeroplasmatales and Entomoplasmatales. The order Mycoplasmatales contains a single family, Mycoplasmataceae and two genera: *Mycoplasma* and *Ureaplasma* (Table 2.1). The remaining species in the genus *Mycoplasma* are divided into two non-taxonomic groups, *M hominis* and *M pneumoniae,* based on 16S rRNA gene sequences. The *hominis* group contains the phylogenetic clusters of *M bovis, M pulmonis*, and *M hominis*, among others. The *pneumoniae* group contains the clusters of *M muris*, *M fastidiosum*, *U urealyticum*, the currently unculturable haemotrophic mollicutes, informally referred to as haemoplasmas (recently transferred from the genera Haemobartonella and Eperythrozoon), and the *M pneumoniae* cluster (Maniloff, 2002). The *M pneumoniae* cluster contains the species (and the usual or likely host) *M alvi* (bovine), *M amphoriforme* (human), *M gallisepticum* (avian), *M genitalium* (human), *M imitans* (avian), *M pirum* (uncertain/human), *M testudinis* (tortoises), and *M pneumoniae* (human) (Maniloff, 2002).

Then the evolution rate of these bacteria appears to have also increased significantly. This is an attractive hypothesis, but while it tracks the emergence of several of the unusual characteristics of *Mycoplasma* and related organisms, it does not address the selective pressures driving their evolution, except perhaps the widespread closes association of a parasite with a specific host. The advantages of a reduced genome, cell wall-less structure,

and alternate genetic code remain murky (Maniloff, 2002). Table 2.1 below summarizes the Major characteristics and taxonomy of the class mollicutes as approved by International Committee on Systematic Bacteriology - Subcommittee on the Taxonomy of Mollicutes and published by Razin, (1992).

TABLE 2.1 MAJOR CHARACTERISTICS AND TAXONOMY OF THE CLASS MOLLICUTES

Taxonomy	Classification	No of Spp	Genome Size	G+C of genome	Cholesterol Requirements	Distinctive properties	Habitat
Order I	Mycoplasmatales						
Family	Mycoplasmataceae						
Genus	Mycoplasma	102	580-1350	23-40	Yes	Optimun growth (37^0_C)	Humans and Animals
Genus	Ureaplasma	6	760-1170	27-30	Yes	Urea hydrolysis	Humans and Animals
Order II	Entomoplasmatales						
Family	Entomoplasmataceae						
Genus	Entomoplasma	5	790-1140	27-29	Yes	Optimun growth (37^0_C)	Insects and Plants
Genus	Mesoplasma	12	870-1100	27-30	No	Tween 80 required in serum free medium	Insects and Plants
Family	Spiroplasmataceae					Helical,motie filaments	
Genus	Spiroplasma	33	780-2220	24-31	Yes	Optimun growth (37^0_C)	Insects and plants
Order III	Acholeplasmatales						
Family	Acholeplasmataceae						
Genus	Acholeplasma	13	1500-1650	26-36	No	Optimun growth (37^0_C)	Animals and some Plants and insects
Order IV	Anaeroplasmatales						
Family	Anaeroplasmataceae						
Genus	Aaeroplasma	4	1500-1600	29-34	Yes	Oxygen-sensitive anaerobes	Bovine/Ovine rumen
Genus	Asteroleplasma	1	1500	40	No	Oxygen-sensitive anaerobes	Bovine/Ovine rumen
Undefined Taxa							
Genus	Phytoplasma	ND	640-1185	23-29	Unknown	Uncultured invitro	Insects and plants

Legend= Order 1 contains mycoplasmas of medical importance. Adapted with modification from Razin, (1992).

2.4 The Human Mycoplasmas

Several species of mollicutes of medical importance are considered commensals, but three in the genus *Mycoplasma* are proven pathogens: *M pneumoniae*, *M genitalium,* and *M hominis. M fermentans* is an organism which may play a role in human disease in some circumstances. Considerable evidence has accumulated in recent years to suggest its etiologic role as an opportunist in persons with human immunodeficiency virus infection and AIDS (Ainsworth *et al.,* 2000) and a possible association with chronic arthritic conditions (Horowitz *et al.,* 2000, and Johnson *et al.,* 2000).

Other organisms such as *M penetrans* appear to have the potential for being human pathogens (Blanchard and Bebear, 2002), but no conclusive proof demonstrating this has been offered to date. The most recent human mycoplasmal species to be recognized is *Mycoplasma amphoriforme*, an organism that has been detected in the lower respiratory tract of several immunocompromised persons in association with chronic bronchitis, and investigations are now under way to determine whether a role in human disease can be established with certainty (Webster *et al.,* 2003).

Table 2.2 The diseases caused by Mycoplasmas of Medical importance

Organism	**Disease**
M. pneumoniae	Upper respiratory tract disease, tracheobronchitis, atypical pneumonia
M. hominis	Pyelonephritis, pelvic inflammatory disease, postpartum fever
M. genitalium	Nongonococcal urethritis
U. urealyticum	Nongonococcal urethritis

Table 2 (Adapted from: Murray *et al., 1994*).

2.4.1 Human Ureaplasma species, biovars, and serovars (As displayed in Table 2.3)

Table 2.3 *Ureaplasma urealyticum* (UU) and urogenital tract infections in humans and recent advancement in mycoplasmology.

Criterium	*Ureaplasma spp*		Authors
Classification	*Ureaplasma parvum*	*Ureaplasma urealyticum*	(Harasawa, 1999, Kong *et al.*, 1999 and 2000, Robertson *et al.*,1986, 1993 and 2004).
Biovars	Biovar 1 or parvo biovar	Biovar 2 or T960	(Harasawa, 1999, Kong *et al.*, 1999 and 2000, Robertson *et al.*, 1986, 1993 and 2004).
Serotypes/serovars	1, 3, 6 and 14	2, 4, 5, 7, 8, 9, 10, 11, 12, 13	(Robertson and Stemke, 1982).
Genes targets for PCR assays used to detect Ureaplasmas	Urease gene cluster (Robertson *et al.*, 1993)	The rRNA gene clusters, (Blanchard *et al.*, 1993)	(Robertson *et al.*, 1993) and Blanchard *et al.*, 1993) respectively
U. parvum is more common than *U urealyticum*, but co-infections may occur.			(Harasawa, 1999, Kong *et al.*, 1999 and 2000, Robertson *et al.*, 1986, 1993 and 2004).
Several studies have found serotype 4 to occur more commonly in men with NGU.			Shepard *et al.*, 1978
Lin, 1977 found no differences in the serotype distribution in normal college women, women with salpingitis, and pregnant women who delivered normal, low-birth-weight, or stillborn infants.			Lin, 1977
Naessens *et al.*, 1988 typed 240 *Ureaplasma* strains isolated from cervices, placentas, or fetal tissues in women with a history of recurrent abortion.			Naessens *et al.*, 1988
Serotype 6 was the predominant type detected in urine samples according to one study.			Hewish *et al.*, 1986).
Serotype 4 was significantly more common in the cervices of women with recurrent abortions (20.8%) than in those of control patients (5.1%).			Quinn *et al.*, 1983
The fact that serotype 8 produces more phospholipase A2 than serotypes 3 and 4 may explain involvement of this serotype in premature birth			Quinn *et al.*, 1983
Studies have also linked serotype 4 to urethritis and spontaneous abortion.			Robertson *et al.*, 1985).
Zheng *et al.*, 1995 evaluated 10 ureaplasmal isolates from neonatal cerebrospinal fluid and three blood stream isolates using serotype-specific reagents and monoclonal antibodies.			Zheng *et al.*, 1995
Of the two ureaplasma species, there are differences between *U. parvum* and *U. urealyticum* in distribution and even some possible pathogenicity in different populations			(Deguchi *et al.*, 2004; Domingues *et al.*, 2002; Knox *et al.*, 2003).
U. urealyticum is more common than *U. parvum* in young males with NGU.			Povlsen *et al.*, 2002; Deguchi *et al.*, 2004).
Recently Deguchi and coworkers in 2004 reexamined the presence of *U. parvum* and *U. urealyticum* in NGU and found a significantly higher prevalence of *U. urealyticum* compared to *U. parvum*.			Deguchi *et al.*, 2004
U. urealyticum has been found to be dominant in patients with pelvic inflammatory disease, miscarriage, birth weight, gestational age, and preterm delivery, than *U. parvum*			Abele-Horn *et al.*, 1997
Heggie *et al.*, 2001, found no differences between infants harboring *U. parvum* compared to those with *U. urealyticum* in. broncho pneumonia dysplacia (BPD).			Heggie *et al.*, 2001,

However, Abele-Horn, 1997, reported that 10 of 18 (56%) of infants colonized by *U. urealyticum* developed BPD versus 12 of 48 (25%) infants with *U. parvum* ($P < 0.05$).	Abele-Horn, 1997
Katz, 2005 in his own study, compared the occurrence of *U. urealyticum* and *U. parvum* in 181 infants with birth weights of <1,500 g whose endotracheal aspirates were culture positive for *Ureaplasma spp.* over a 10-year period and found no significant difference or trend in prevalence of either species in infants with or without BPD.	Katz, *et al.*,2005
When PCR was used to compare the distribution of species and prevalence of tetM in 63 isolates of *Ureaplasma spp.* no differences were seen in tetracycline susceptibility or the occurrence of the two species between the two groups	Martinez *et al.*, 2001
Similarly, Waites *et al.*, 2007, in their findings included evaluation of 45 tetM-positive ureaplasmas and also found no significant difference in its occurrence in *U. parvum* versus *U. urealyticum.*	Waites *et al.*, 2007,
However, earlier studies found more tetracycline resistance in the T960 biovar (*U. urealyticum*) than the parvo biovar (*U. parvum*)	(Mallard *et al.*, 2005).

Legends= BPD= bronchopneumonia dysplacia.

2.4.2 *Mycoplasma genitalium*

M. genitalium was first isolated in 1981 from urethral specimens of men with urethritis (Tully *et al.*, 1983). This mycoplasma has numerous similarities with *M. pneumoniae*, particularly the flask-shaped attachment organelle, terminal tip-like structure, as well as antigenic structures, and the ability to invade epithelial cells (Jensen, 2004). Understanding its role in human disease was greatly hampered by its slow growth, fastidious cultivation requirements, and serologic cross-reactivity with *M. pneumoniae* (Jensen, 2004).

A few subsequent reports of isolations of this mycoplasma in culture have been forthcoming, including some isolations from women (Jensen, 2004), but it was not until the availability of the PCR assay that investigation into the disease associations of this mycoplasma became fruitful. Recent evidence from studies utilizing the PCR assay, complemented by investigations that employed serology, and experimental studies in primates, indicates that *M. genitalium* is of aetiologic significance in approximately 25% of cases of nongonococcal urethritis and possibly prostatitis in men (Jensen, 2004), as well as cervicitis and pelvic inflammatory disease in women (Uno *et al.*,1997).

Serological evidence suggests indirectly that *M. genitalium* may play a role in some cases of tubal factor infertility, but this has not been confirmed by detecting the organism or its DNA directly in fallopian tubes of infertile women (Clausen *et al.,* 2001). *M. genitalium*, like the other genital mycoplasmas, may also be present in the lower urogenital tract in some healthy men and women. Jensen *et al.*, (2004) summarized the results of 19 clinical studies investigating the role of *M. genitalium* in 2,069 men with nongonococcal urethritis and 1,810 men without nongonococcal urethritis and determined that this mycoplasma was present in 21.1% of those with nongonococcal urethritis versus only 6.7% of those without nongonococcal urethritis.

M. genitalium was detected by PCR but not by culture in 11% of patients with urethritis or cervicitis. It was not detected by either the PCR assay or culture in the 232 amniotic fluid samples analyzed or by culture from the chorioamnion of 609 women. Its occurrence in extragenital sites, including the upper (Baseman *et al.*, 1988) and lower respiratory tracts (de Barbeyrac *et al.*, 1993) of adults, proven by the PCR assay, suggested it might colonize the respiratory tracts of neonates as well. A prospective study comparing culture and PCR to detect *Ureaplasma spp, M. hominis*, and *M. genitalium* in vaginal specimens of 47 high-risk pregnant women and from skin, throat, and endotracheal aspirates from eight neonates delivered to them found that *Ureaplasma spp* were the most common organisms detected with 31 of 47 (61.7%) women colonized in the vagina, in comparison to 7 of 47 (15%) for *M hominis* and 1 of 47 (2%) for *M. genitalium* (Luki et *al.*, 1998). These findings supported the concept that *M. genitalium* is much less common than either *M. hominis* or *Ureaplasma spp.* in the lower urogenital tract of women. There were two infants born to colonized mothers who became colonized with *Ureaplasma spp.* A mother whose vaginal specimen was positive by the PCR assay for *M. genitalium* delivered a 1,125-g male infant who developed acute respiratory distress and from whom *M. genitalium* was detected by PCR assay performed on tracheal secretions soon after birth, suggesting that vertical transmission occurred. Consistent with earlier experience, attempts to detect *M. genitalium* by culture in this study were unsuccessful, requiring the PCR assay to determine its presence. Labbe *et al.*, (2002) detected *M. genitalium* by the PCR assay in 6.2% of cervical specimens of 1,014 pregnant women in Guinea-Bissau. They were unable to relate the presence of *M. genitalium* in the cervix with stillbirth, spontaneous abortion, premature delivery, or small-for-gestational-age babies and concluded that this mycoplasma appears not to have any deleterious impact on outcome of pregnancy.

Two other studies found *M. genitalium* in the cervical or vaginal secretions of very few pregnant women (less than 5%) using the PCR assay and were unable to relate its presence to premature birth (Kovachev *et al.*, 2002). However, as documented earlier in this review, many studies attempting to relate the presence of *M. hominis* and *Ureaplasma spp* to adverse pregnancy outcome, neonatal infection, or diseases of the upper urogenital tract that limited their samples to the lower urogenital tract were unsuccessful, whereas studies examining their presence directly in the upper tract were sometimes able to show a relationship (Hillier *et al.*, 1988, Quinn, 1985 and 1987). Further investigations of *M. genitalium* as an agent of disease in pregnant women and neonates are warranted.

2.4.3 Mycoplasma *fermentans*

Attention was focused on *M. fermentans* in the late 1980s because of reports that it may be important as a mediator or cofactor in the development of AIDS (Lo *et al.*, 1989, Saillard *et al.*, 1990). Taken in aggregate, the preponderance of evidence in subsequent studies utilizing improved detection methods, including the PCR assay, suggests that this mycoplasma is not important in the development of AIDS in the large majority of patients. However, it apparently can play a role as an opportunistic pathogen in this setting and occasionally in persons who are human immunodeficiency virus negative (Lo *et al.*, 1989, Blanchard and Bebear *et al.*, 2002, Taylor-Robinson *et al.*, 1999).

The notoriety associated with the possibility that this mycoplasma may be involved in the pathogenesis of AIDS led to studies aimed at understanding how it may invade cells and produce disease in the human host. Unlike *M. pneumoniae, M. fermentans* lacks a well-defined terminal attachment tip to mediate attachment and cell invasion. Work by Yavlovichm *et al.*, (2001) demonstrated that *M. fermentans* binds plasminogen and converts it to plasmin, whereupon mycoplasmal cell surface proteins are altered to promote its internalization.

The role of plasminogen activation as a virulence factor and other aspects of *M. fermentans* pathogenesis, including the importance of membrane surface proteins that mediate cell fusion, cytadherence, and antigenic variation, are discussed at greater length by Rottem, (2003). *M. fermentans* can be detected in the upper and lower urogenital and respiratory tracts and bone marrow, and has been associated with a variety of systemic conditions in adults including inflammatory arthritis and pneumonia (Blanchard, and Bebear 2002). *M.*

fermentans has not been shown to have a pathogenic role in male urethritis (Totten *et al.*, 2001). It has been recovered from the throats of 16% of children with community-acquired pneumonia, some of whom had no other etiologic agent identified, but the frequency of its occurrence in healthy children is not known (Taylor-Robinson, 1996).

M fermentans has also been detected in adults with an acute influenza-like illness who developed respiratory distress syndrome (Blanchard, and Bebear 2002) and from bronchoalveolar lavage in AIDS patients with pneumonia, sometimes as the sole microbe, so it clearly has the potential to cause respiratory tract disease in susceptible hosts (Ainsworth, 2000). This mycoplasma is also known to colonize mucosal surfaces in healthy persons, complicating efforts to understand its role in disease (Ainsworth *et al.*, 2000). *M. fermentans* was not detected by culture or PCR in patients with urethritis or cervicitis but was detected by PCR in 4 of 232 amniotic fluid samples tested according to one study (Blanchard *et al.*, 1993). These results suggest that *M. fermentans* can be transferred transplacentally. Histological evidence of villitis and chorioamnionitis was present in two of the four patients, suggesting that *M. fermentans* may be a cause of chorioamnionitis (Blanchard *et al.*, 1993).

This *Mycoplasma* has also been detected in the placental chorionic villi, proving its ability to invade the upper reproductive tract (Cassell *et al.*, 2000). Inability to detect *M. fermentans* by culture or PCR assay in urine or cervical secretions in 94 men and 87 women who had urethritis or cervicitis further supports the fact that this mycoplasma is not an important cause of these conditions (Cassell *et al.*, 2001). We are unaware of any prospective studies in neonates to determine whether *M. fermentans* may be important as an agent of disease in this population. Waites and Talkington (2005) recently reviewed the importance of *M. fermentans* in human diseases and provided more detail on the conditions described above as well as others.

2.4.4 *Mycoplasma penetrans*

The attention focused on *M. fermentans* and its possible role in human immunodeficiency virus infection and AIDS led to the discovery of an additional mycoplasmal species from in humans and reevaluation of the possible significance of another. *M. penetrans* was first described in 1991, when it was detected in the urine of homosexual men infected with human immunodeficiency virus, but not from healthy age-matched volunteers (Lo *et al.*, 1991). It was later reported to be associated with Kaposi's sarcoma (Wang *et al.*, 1993), but further

studies have not supported a role for *M. penetrans* in this condition (Taylor-Robinson *et al.*, 1999) and it has since been detected in persons who are not infected with human immunodeficiency virus (Yanez *et al.*, 1999). *M. penetrans* may be found in the urethra, rectum, and throat of homosexual men (Taylor-Robinson *et al.*, 2003). Antibodies against *M. penetrans* were detected in up to 40% of human immunodeficiency virus-positive persons, in contrast to less than 1% of human immunodeficiency virus-negative persons (Wang *et al.*, 1992). Thus far there is no compelling evidence that this *Mycoplasma* causes significant disease in any population despite the fact it possesses some features present in other pathogenic mycoplasmas that might enable it to do so under favorable circumstances, such as a prominent terminal tip structure that confers its ability to invade epithelial cells (Baseman *et al.*, 1995, Blanchard and Bebear 2002). Rottem has discussed the interaction of *M. penetrans* with host cells in a comprehensive review (Rottem, 2003). No data are available for pregnant women or neonates.

2.4.5 *Mycoplasma pirum*

M. pirum was characterized in 1985 (Deguchi, 2004), but its natural host was unknown (Del Giudice *et al.*, 1985). Renewed interest in *M. pirum* came about during the early 1990s during the time when mycoplasmas were being actively studied as possible cofactors in human immunodeficiency virus-related disease, when Montagnier and Blanchard isolated this mycoplasma from peripheral blood lymphoid cells of human origin (Montagnier *et al.*, 1993). However, subsequent studies did not detect the organism in peripheral blood mononuclear cells in persons with or without human immunodeficiency virus infection (Kovacic *et al.*, 1996). It was not detected in the urethras of men with urethritis (Deguchi *et al.*, 1996); but by PCR in the rectums of five homosexual men (Taylor-Robinson *et al.*, 2003). *M penetrans, M pirum, M. fermentans* and *M. genitalium* were isolated from urine of patients with AIDS who had severe immunodeficiency more often than from that of persons who were human immunodeficiency virus negative (Hussain *et al.*, 1999). Despite the presence of *M. pirum* as a colonizer in the settings described above, no conclusive proof that this mycoplasma is independently pathogenic in humans has been offered thus far and no data for neonates or pregnant women are available.

2.4.6 *Mycoplasma pneumoniae*

M. pneumoniae is the best known and most intensely studied human mycoplasma. Its role in human disease has been reviewed recently by Waites, and Talkington. (2004). Since *M.*

pneumoniae is primarily a cause of respiratory tract infections in children and adults, it has been isolated also from the lower urogenital tract of women as reported by (Goulet, 1995) and infants sometimes experience mycoplasmal respiratory tract infection that is transmitted from person to person in the community setting. However investigators in Boston were unable to isolate this mycoplasma from the nose, throat, external ear canal, genitalia, conjunctivae, blood, urine, or CSF in 1,500 infants (Cassell *et al.*, 2001), but this was done in the days prior to the availability of more sensitive methods of detection, such as the PCR assay. Many women of childbearing age have respiratory infections caused by *M. pneumoniae*, and there has been one report (Ursi, 1995) of *M. pneumoniae* documented by PCR in the nasopharyngeal aspirate of a neonate with congenital pneumonia, suggesting transplacental acquisition may have occurred. This finding justifies the need for further study of *M. pneumoniae* as an agent of respiratory disease in neonates.

2.5 Mechanisms of pathogenesis

Mycoplasmas could be considered the optimal parasites, i.e. they rarely kill their host, and fulminant infections are uncommon, rather, they tend to follow a more chronic course (Bonnin *et al.*, 1995). *Mycoplasma* pathogenesis has been studied intensively for several years, but among the human mycoplasma species, *M. pneumoniae* has been more thoroughly studied than *U. urealyticum;* due to their close interactive and sites colonization relationship, some features can probably be generalised.

Mycoplasmas are primarily considered surface parasites of mucous membrane cells. The tissue damage caused by mycoplasmas can be accounted for only partially by mycoplasmal toxins and harmful metabolites such as hydrogen peroxide and superoxide metabolites known to be secreted by *U. urealyticum* (Bowman *et al.*, 1998). Much of the tissue damage seen by infection with *U. urealyticum* appears to be caused by the host cell response as seen in *M. pneumoniae* (Braun *et al.*, 1971). Mycoplasmas have been found to interact with many components of the immune system, inducing macrophage activation and cytokine production. Some mycoplasmal cell components may act as superantigens, and several autoimmune manifestations have been observed (Brown *et al.*, 1987).

2.5.1 Adhesion

The discovery of mycoplasma adhesins responsible for attachment to host cells has provided important new insights into the pathogenesis. Furthermore, within recent years, evasion of the

host immune system by antigenic variation of surface components has been intensively studied for a range of species. Demonstration of the ability of mycoplasmas to enter host cells and to cause cell fusion, apoptosis, and even oncogenic effects, has further stimulated research in this field. (Baseman *et al.*, 1984, Brown *et al.*, 1987, Razin *et al.*, 1992).

Adhesion of Mollicutes to host cells is a prerequisite for colonisation and subsequent infection. The lack of a cell wall and cell wall associated structures such as fimbriae often responsible for adhesion of other bacteria indicates that this process is mediated by membrane bound components, which have been termed adhesins (Baseman *et al.*, 1984). Adhesins have been intensely studied both in *M. pneumoniae* and in *Ureaplasma urealyticum*. Both species bind erythrocytes from a variety of species (Brus *et al.*, 1991) with their adhesin molecules clustered at the tip structure of the polar cell (Buckingham *et al.*, 2003). Both species also attach to eucaryotic cells such as Vero cells (Jensen *et al.*, 1994), but more importantly, *U. urealyticum* attaches to the epithelial cells of human fallopian tube, and lead to tubal infertility (Brown *et al.*, 1983).

2.5.2 Evasion of the host immune response by antigenic variation

Mycoplasmas are dependent on an intimate contact with the host cells. In order to evade the immune response of the host, and to adapt to changing environments, they have a need for phenotypic plasticity, such as variation in the antigenic composition of the surface components. Other mechanisms, such as mimicry of host antigens, and survival within professional phagocytes, may help the parasite to survive (Crouse *et al.*, 1998). The antigenic variation can be brought about by two basically different mechanisms. Either, the pathogen may regulate the expression of virulence factors in response to changes in the environment by signal transduction pathways, or the microbial population as a whole may spontaneously and randomly generate distinct new phenotypes that will survive the host response (Crouse, 1998). Since mycoplasmas have few regulatory genes that could serve as sensors to environmental stimuli, and a few genes encoding transcriptional factors, the most important means for phenotypic plasticity in mycoplasmas is antigenic variation caused by molecular switching events (Brown, 1987). Surface exposed membrane proteins are the major antigenic determinants of mollicutes, and a variety of mechanisms have evolved to generate high frequency intragenomic changes in nucleotide sequence or DNA conformation at selected chromosomal loci (Crouse, 1993; Cunliffe, 1996). The functional consequence is that these alterations in genotype can rapidly promote phenotypic heterogeneity, even in small, clonal

populations of bacteria, such as the limiting inoculum that initiates an infection (Da Silva, 1997).

2.5.3 Localization and Cytadherence

The Mollicutes are primarily mucosally associated organisms residing in the respiratory or urogenital tracts of their hosts in close association with epithelial cells. In some species, particularly *M. fermentans, M. penetrans, M. genitalium,* and perhaps even *M. pneumoniae* and *M. hominis* in some cases, invasion of host cells occurs and the organisms reside intracellularly. Such intracellular localization may contribute to the chronicity of infections and their ability to evade the host immune response (Taylor-Robinson, 1991). The cytoskeletal rearrangements, invasions, and receptors involved with mycoplasmal invasion of host cells and their intracellular survival are described thoroughly in Rottem's comprehensive review (Rottem, 2003).

Other mycoplasmas such as *M. genitalium, M. pirum,* and *M. penetrans* also have a flask-shaped morphology and terminal attachment organelles and knowledge of the cytadherence processes of these mycoplasmas is increasing, due to knowledge gained through study of *M pneumoniae* cytadherence (Jensen, 2004, Rottem, 2003). Ureaplasmas are known to adhere to a variety of human cells including erythrocytes (Yoon, *et al.,* 1991), spermatozoa (Carey, 1991), and urethral epithelial cells (Shepard and Masover. 1979). Ureaplasmas bind spontaneously to neutrophils and directly activate the first component of complement (Webster *et al.,* 1998). *Ureaplasma* adhesins are proteins expressed on the surface of the bacterial cell. There may be several of them involved in the cytadherence process, which has not yet been characterized in its entirety (Yoon, 1998). Pretreatment of HeLa cell monolayers or human erythrocytes with neuraminidase will reduce ureaplasmal adherence, suggesting that the receptors for ureaplasma adhesins are sialyl residues and/or sulfated compounds; similar to what has been observed with *M. pneumoniae* and other mycoplasmas (Waites, 2004).

2.5.4 Secretory Products

Arginine metabolism by *M. hominis* and urease activity in ureaplasmas has been suggested as potential virulence factors. More than 40 years ago Schimke *et al.,* (1963), proposed that *M. hominis* generates ATP by hydrolysis of arginine, a process that utilizes a three-enzyme pathway with end products of CO_2 and NH_3. Release of NH_3 in large amounts may deplete

arginine *in vitro,* resulting in a cytotoxic effect (Razin, 1998). However, direct evidence that arginine depletion by *M. hominis* causes toxic effects *in vivo* is still lacking. Release of NH_3 also occurs in *Ureaplasma spp* through hydrolysis of urea mediated by a very potent urease. Hydrolysis of urea is the predominant means by which these organisms generate ATP, making them unique in the class Mollicutes in this respect (Smith, 1993). Release of NH_3 in the urinary tract can cause elevation of urinary pH and precipitation of magnesium ammonium phosphate, also known as struvite. Inoculation of ureaplasmas into rat bladders results in the formation of struvite stones, popularly referred to as kidney stones (Grenabo, 1988). Clinically, *Ureaplasma spp* have been cultured directly from renal stones, and these organisms have been isolated from voided urine in 31 of 247 patients (13%) with metabolic stones, compared to 43 of 145 patients (30%) with metabolic stones ($P < 0.001$). In the patients for whom stone cultures were performed, ureaplasmas were found in 2 of 125 patients (2%) with metabolic stones, compared to 10 of 64 patients (16%) with struvite stones ($P < 0.001$). These observations strongly suggest that *Ureaplasma spp* is linked to the formation of struvite stones in the urinary tract, mediated by urease activity (Grenabo, 1988).

The potential pathogenic effect of ureaplasmal urease and its NH3 metabolic by-product was demonstrated in a mouse model by (Ligon, and Kenny 1991) in which they were able to demonstrate toxicity of Ureaplasmas injected intravenously that was prevented by injection of fluofamide, a potent urease inhibitor. The presence of phospholipases A and C in *Ureaplasma* spp has been suggested to be the means by which ureaplasmas may initiate preterm labor by liberating arachidonic acid and altering prostaglandin synthesis (De Silva *et al.*, 1999). Support for this hypothesis comes from studies that have demonstrated significant elevations of phospholipase A2 in serum and amniotic fluid specimens from women in preterm labor with chorioamnionitis than those undergoing term labor (Koyama *et al.*, 2000). De Silva *et al.*, (1999), identified and characterized phospholipase activities in multiple ureaplasma serotypes and reported that the specific activities of phospholipase A2 differed according to serotype. While the activities of phospholipases A1 and C were similar. They speculated that differences in phospholipase activity might cause differences in pathogenic potential for the various serotypes in terms of adverse pregnancy outcomes. However, Glass and colleagues (Glass *et al.*, 2000) were unable to identify phospholipase activity in the serotype 3 ureaplasma strain for which the complete genome was sequenced and gave the same possible explanations as those for the apparent lack of IgA1 protease activity.

The hemolytic activity of *M. pneumoniae* has also been reported and this is due to production of H_2O_2 and is inhibited by catalase (Somerson *et al.*, 1965). In contrast, the hemolytic activity of ureaplasmas is not inhibited by catalase, suggesting an alternative enzyme system may be responsible (Glass *et al.*, 2000).

2.5.5 Host Defenses in the Neonate

Very low birth weight infants are especially susceptible to bacterial and fungal infections (Stepan *et al.*, 1998). These infants have relative deficiencies in mucosal barrier function and in both the innate and adaptive immune responses. The immature host may generate limited type-specific antibody production in response to invading pathogens and the amount of secretory IgA may also be lower in mucosal surfaces than in more mature neonates (Fairchild, 2004). Deficiencies in serum complement components, defensins, fibronectin, and abnormalities in cytokine production contribute further to the relative immunodeficiency of the premature infant (Schelonka *et al.*, 1998). Clinically significant ureaplasmal infections rarely occur in infants born after 34 weeks of gestation. Data reported by Quinn *et al.*, (1985), also suggest that selective antibody to certain serotypes increases in women with pregnancy wastage and in infants with respiratory disease compared with control patients. Work done by Gallo *et al.*, (1983) suggests that the presence of *U urealyticum*-specific IgM antibody in infants is predictive of disease. Taken together, the available evidence suggests but does not prove that immunity to invasive infection by *Ureaplasma spp* is type specific. The mechanism of protection afforded by antibody seems to be mediated by metabolic inhibition of the organism rather than opsonization (Taylor-Robinson *et al.*, 1986, Webster *et al.*, 1988).

2.6 Molecular Epidemiology

The term "molecular epidemiology" involves both "molecular" – the use of the techniques of molecular biology – and the "epidemiology" – the study of the distribution and determinants of disease occurrence in human populations (Foxman and Riley, 2001). Molecular epidemiology can be used to examine disease patterns, investigate outbreaks, describe transmission and population dynamics, identify risk factors, understand evolution and disease pathogenesis, monitor the efficacy of control programmes (Foxman and Riley, 2001).Genotyping of microorganisms is the means to discriminate between and catalogue microbial nucleic acid molecules (van Belkum *et al.*, 2001). It is widely used in basic and applied microbiological research. In basic microbiological research, genotyping is used in the fields of taxonomy, phylogenetic relationships, evolutionary dynamics, population genetics,

and epidemiology (van Belkum *et al.*, 2001). In applied microbiological research, genotyping clearly affects several areas: the epidemiological investigation of outbreaks of infectious diseases and the measurement of genetic diversity in relation to relevant biological properties such as pathogenicity, drug resistance etc. (van Belkum *et al.*, 2001). Genotyping techniques are considered superior to phenotyping methods, which are often less reproducible and discriminatory. Genotyping of ureaplasmas also involves serotyping of ureaplasmas.

The distribution of species (previous biovars) and serovars differs in different populations, but generally the four serovars of *U. parvum* are more common than ten *U. urealyticum* serovars (Knox *et al.*, 2003). The phenotypic serotyping methods have contributed to our understanding of ureaplasma infections, but they are limited since they do not reveal information regarding genetic identity (Bidet *et al.*, 2003; Echahidi *et al.*, 2002). Many investigators now use genotypic techniques alone or in conjunction with certain phenotypic techniques (Martinez *et al.*, 2001). *Ureaplasma* studies demonstrate that more molecular epidemiological information is needed to understand the pathogenesis of disease and to design alternative prevention strategies (Bidet *et al.*, 2003; Zheng *et al.*, 1992).

2.7 Clinical Manifestations and Disorders

2. 7.1 Infertility

The initial associations of genital mycoplasmas with infertility came following reports that ureaplasmas could be isolated from the lower genital tract more commonly in infertile couples than in fertile couples, but this has not been found consistently in subsequent investigations (Matthews *et al.*, 1975 and Friberg *et al.*, 1972, Upadhyaya *et al.*, 1983). Additional studies that have utilized cultures from endometrial tissue obtained at laparoscopy have also shown that ureaplasmas can be recovered more commonly from infertile women than from fertile women, even when cervicovaginal isolation rates from the two groups are similar (Stray-Pedersen *et al.*, 1978, Stray-Pedersen *et al.*, 1982).

Ureaplasmas are known to attach to sperm and decrease motility, explaining the association with male factor infertility seen in some studies (Taylor-Robinson *et al.*, 1999). Elimination of ureaplasmas by antimicrobial treatment has been correlated with improvement in sperm motility, quantity, and appearance by some investigators (Toth and Lesser *et al.*, 1982). However, it has been stressed that the drugs used to treat ureaplasmas, such as tetracyclines,

have broad-spectrum activities that can affect other microbes and conception rates following antimicrobial treatment of infertile couples has improved (Taylor-Robinson *et al.*, 1999).

Because of its urealytic activity and subsequent release of ammonium ions, *U. urealyticum* induces cytotoxicity in a variety of established cell lines. Ammonia is capable of causing severe lesions in the ciliated epithelia of the trachea of chickens. When grown in the presence of bovine oviductal organ culture, human genital *U. urealyticum* caused ciliary activity to cease and caused severe lesions — deciliation and desquamation — of the epithelium. Decreases in both the cilia-stopping effect and nitrogen content by boiling at high pH values indicate that ammonia is the ciliostatic factor of *U. urealyticum* (Abele-Horn *et al.*, 1997).

U. urealyticum is able to hydrolyse urea to ammonia within a few seconds of contact with substrate. It is possible that such a strong local accumulation of ammonia might predict an *in vivo* pathogenic role of *U. urealyticum*. It has been found that *U. urealyticum* can attach massively to sperm, especially at the midpiece, thus producing marked hydrodynamic drag on the infested sperm. Both scanning electron microscopy and fluorescent light microscopy have dramatically shown that these bacterial hitchhikers can cause looped tangling of tails and multisperm agglutinations, both of which cause loss of motility (Acosta *et al.*, 1999). Clinically, sperm antibodies have been found in the semen of *Ureaplasma* carriers more often than in men with negative cultures. Thus the presence of these organisms might be one of a number of nonspecific stimuli that induce a form of autoimmune infertility (Ainsworth *et al.*, 2000).

Xu *et al.*, (2000), conducted one of the most informative studies of ureaplasma and infertility (In their study, 47 male Sprague-Dawley (SD) rats were infected artificially with *U. urealyticum* serotype 8 (T 960). Morphological changes in the seminiferous tubules were observed 3-5 weeks after inoculation in the killed animals. Dramatic impairment of spermatogenesis of both testes was found in 11 rats. Mating experiments confirmed infertility in 12 of 40 rats. Offspring of the infected rats were significantly smaller in both antenatal and birth weight than those of controls.

2.7.2 Postpartum Endometritis

The first studies that attempted to correlate genital mycoplasmas with postpartum endometritis were based on cervicovaginal cultures and caused much confusion with their

inconclusive results (Carey *et al.*, 1991). However, both *M. hominis* and *Ureaplasma spp* can be detected in the bloodstream of some women with postpartum or postabortion fever, with *M. hominis* being more common. This condition is usually self-limiting, but in some cases in which *M. hominis* is involved, dissemination to joints, resulting in arthritis, may occur. This topic has been reviewed in detail elsewhere (Cassell *et al.*, 2001, Taylor-Robinson *et al.*, 1999). Chorioamniotic colonization with *Ureaplasma spp* was associated with a threefold increased risk of post-Cesarean delivery endometritis and an eightfold higher risk in women in whom the onset of labor was spontaneous (Andrews *et al.*, 1995). The same investigators later provided indirect evidence that ureaplasmas may be involved in post-Cesarean delivery endometritis in a study in which 301 women who received doxycycline plus azithromycin were compared to 297 who received a placebo (Andrews *et al.*, 2003). The interesting finding in that investigation was that prophylaxis with antibiotics having activity against ureaplasmas reduced the length of hospitalization, frequency of endometritis, and wound infections.

2.7.3 Chorioamnionitis, Spontaneous Abortion, and Preterm Labor

The importance of genital mycoplasmas in prematurity, pregnancy loss, and chorioamnionitis have been topics of great interest in recent years. Isolation of *Ureaplasma spp* but not *M. hominis* from the chorioamnion has been consistently associated with histological chorioamnionitis and is inversely related to birth weight, even when adjusting for duration of labor, rupture of fetal membranes, and presence of other bacteria (Cassell *et al.*, 1993 and Cassell *et al.*, 2001). These organisms can invade the amniotic cavity and persist for several weeks when fetal membranes are intact and initiate an intense inflammatory reaction in the absence of labor (Gray *et al.*, 1994). Abele-Horn *et al.*, (1997) suggested that the density of ureaplasmal colonization is a factor that correlates with adverse pregnancy outcome, including development of chorioamnionitis and preterm delivery.

2.7.4 Bacterial Vaginosis

The first reported association of genital mycoplasmas with vaginitis occurred over 40 years ago. Since that time some evidence has accumulated that *M. hominis* may be of significance in the condition now known as bacterial vaginosis (BV). Symptomatic BV is characterized in part by a watery discharge with a fishy odor, but half of the women with this infection may be asymptomatic or experience only mild symptoms. Women with BV consistently have an increased prevalence of *Gardnerella vaginalis*, selected anaerobic bacteria, and *M. hominis* along with a decreased prevalence of *lactobacilli* (Martius and Eschenbach, 1990). *M*

hominis may act symbiotically with other BV-associated bacteria or as the sole pathogen (Taylor-Robinson and McCormack, 1980) based on the observation that this mycoplasma can be found in large numbers in the vagina of most women with BV but less often in healthy women. When present in healthy women, it is usually there in much lower numbers than in women with BV (Keane *et al.*, 2000).

2.7.5. Congenital and neonatal pneumonia

Respiratory disease remains the most common cause of perinatal morbidity and mortality, especially in preterm infants, despite many advances in neonatal intensive care and resuscitation, and the introduction of artificial surfactant in the early 1990s. In recent years, considerable attention has been given to the potential role of *M. pneumoniae* as a cofactor in development or exacerbation of asthma as summarized by Waites, and Talkington (2004). There is also some recent evidence that colonization or infection of the lower respiratory tract of infants with ureaplasmas may lead to somewhat similar outcomes as in the study of Waites, and Talkington (Benn *et al.*, 2002, Kundsin *et al.*, 1996). A Danish study Benn *et al.*, (2002) involving 2,927 women determined that maternal vaginal colonization with *Ureaplasma spp* during pregnancy was associated with infant wheezing but not with asthma, during the fifth year of life.

2.7.6. *Ureaplasma spp.* in the pathogenesis of bronchopulmonary dysplasia (BPD).

Multiple studies have specifically examined the radiographic course of infants with *Ureaplasma* colonization. Crouse *et al.*, (1993) evaluated chest radiographs of 44 preterm infants colonized in the lower respiratory tract by *Ureaplasma spp* in comparison to those who were culture negative and found that pneumonia was twice as common in the *Ureaplasma*-positive group (30% versus 16%). Importantly, precocious dysplastic changes in the lungs within 2 weeks of birth were significantly more common in the *Ureaplasma*-positive group, independent of gestational age, race, and sex.

2.7.7. Bacteraemia

The factor associated most significantly with sepsis due to any microorganism in the neonate is low birth weight (Soman *et al.*, 1985). Ureaplasmal bacteremia may accompany severe neonatal pneumonia (Waites, 1989). Two investigators have isolated *Ureaplasma spp* from the bloodstream of neonates in association with fatal pneumonia and persistent pulmonary hypertension of the newborn (Waites *et al.*, 1989), conditions with clinical manifestations

that were very similar to what is encountered with another well-known neonatal pathogen, group B streptococcus. Dan *et al.,* (1981) reported a case of *M. hominis* septicemia documented on two separate occasions 11 days apart in a 10-month-old infant who had suffered extensive burns.

2.7.8. Infections of the central nervous system

The first reports of meningitis due to an organism that was most likely *M. hominis* were published in the 1950s and since that time there have been numerous case reports, and multiple prospective studies that have identified cases of meningitis caused by this mycoplasma in both preterm and full-term neonates, some of whom had neural tube defects, though others were neurologically intact (Alonso-Vega *et al.*, 1997, Wang *et al.*, 1993). The fact that there have been more cases of *M. hominis* meningitis described than cases involving *Ureaplasma spp* is most likely due to the fact that many cases of *M. hominis* meningitis were discovered accidentally since this mycoplasma will often grow on routine bacteriological media whereas ureaplasmas require special media and growth conditions for their detection.

2.7.9. Other Infections

One of the very earliest reports of genital mycoplasmas in neonatal infection was a report from 1968 in which *M. hominis* was isolated from purulent drainage of 8 of 250 infants with conjunctivitis (Jones and Tobin, 1968). *M. hominis* has been isolated from pericardial fluid in an infant born with respiratory distress related to cardiac tamponade (Gelfand, 1993). In this case, the infant recovered after placement of a pleuropericardial window and a course of intravenous antimicrobials. This mycoplasma has also been shown to cause abscesses in infants, sometimes as a result of forceps delivery or intrapartum fetal monitoring, and has been isolated from purulent drainage from a lymph node in an infant with submandibular adenitis (Glaser, 1983, Powell *et al.,* 1979, Sacker *et al.,* 1970). Abscesses associated with fetal monitors have also been shown to be due to *Ureaplasma spp* (Hamrick, 1993). A case of fatal nonimmune *hydrops fetalis* was reported in which *Ureaplasma spp* was isolated in bronchial secretions, lung tissue and brain tissue, suggesting these organisms should be considered in the differential diagnosis of *hydrops fetalis* which may in some instances be caused by infections (Ollikainen, 1992). Since the normal habitat of genital mycoplasmas in adults is the urogenital tract, it is logical that this might also be a source of colonization and disease in neonates, but few studies have investigated the possibility.

2.8. Laboratory Diagnosis

2.8.1. Culture

The fastidious nature and susceptibility of these organisms to drying and other adverse environmental conditions mandate that careful attention be given to specimen collection, inoculation of transport medium at bedside whenever possible, and proper transportation and shipping conditions if organisms are to remain viable. Details of specimen requirements, collection, shipping, processing, and interpretation of results are described in detail by Waites (2003) and Waites, (2004). Waites, (2001) provides an up-to-date summary of all aspects of laboratory diagnosis of mycoplasmal and ureaplasmal infections. Although culture is considered the reference standard for detection of *M. hominis* and *Ureaplasma spp,* it is expensive and requires specialized media and expertise that are not widely available outside of larger medical centers or mycoplasma research or reference laboratories. Confirmed culture results can usually be available within 2 to 5 days, exclusive of specimen transport time and shipment if an offsite reference laboratory is used. Ureaplasmas can be positively identified to genus level by their characteristic fried egg colonial morphology on PPLO agar and urease production, (Waites *et al.*, 2003).

2.8.2. Serology

The unique susceptibility of hypogammaglobulinemic persons to invasive infections due to *Ureaplasma spp* testifies to the importance of the humoral immune response for protection against disease due to this organism (Taylor-Robinson *et al.*, 1986). Although it has been suggested that type-specific antibody titer rises against certain ureaplasmal serovars occur in women with pregnancy wastage and in infants with respiratory disease compared to control patients, more comparative data from well-characterized and carefully matched control populations are needed to fully appreciate the value of antibody determination in these settings. Serological tests for *M. hominis* and *Ureaplasma spp* using the techniques of microimmunofluorescence, metabolism inhibition, and enzyme immunoassay have been developed and used in research settings (Brown *et al.*, 1983), but such assays for genital mycoplasmas have not been standardized and made commercially available in the Western world. Thus, they remain primarily research tools and cannot be recommended for routine diagnostic purposes at present.

2.8.3. Antimicrobial Susceptibility Testing

Since mycoplasmas and ureaplasmas lack peptidoglycan, they are not affected by beta-lactams or vancomycin. They are not susceptible to sulfonamides or trimethoprim because they do not synthesize folic acid. However, they are generally susceptible to certain antibiotics that interfere with protein synthesis, such as tetracyclines. While ureaplasmas are generally susceptible to macrolides, they are resistant to lincosamides except in high concentrations. *M. hominis*, in contrast, is naturally resistant to erythromycin *in vitro*, but susceptible to 16-membered macrolides (josamycin and miocamycin) and lincomycin. Furneri *et al.*, (2000) determined that the genetic basis for macrolide resistance in *M. hominis* is due to mutations in genes corresponding to the loop of the peptidyl transferase (domain V) in 23S rRNA. Despite a report of high-level erythromycin resistance in ureaplasmas in the 1980s (Palu *et al.*, 1989). It is believed that such resistance to macrolides in these organisms is extremely uncommon, if indeed it occurs at all, and no mechanism for macrolide resistance in ureaplasmas has been verified at the ribosomal level.

2.8.4. Polymerase Chain Reactions – (Nucleic acid amplification)

The first Polymerase Chain Reactions (PCR) protocol was described by Kleppe *et al.*, (1971), the method employed the use of an enzymatic assay to replicate a short DNA template with primers *in vitro,* the work was published in the Journal of Molecular Biology. However, this early manifestation of the basic PCR principle did not receive much attention, and the invention of the polymerase chain reaction until 1983, when the Nobel idea was generally credited to Kary Mullis (Mullis, 1993).

The reaction is easy to execute. It requires no more than a test tube, a few simple reagents, and a source of heat. DNA polymerase occurs naturally in living organisms. In cells it functions to duplicate DNA when cells divide in mitosis and meiosis. Polymerase works by binding to a single DNA strand and creating the complementary strand. In the first of many original processes, the enzyme was used in vitro (in a controlled environment outside an organism). The double-stranded DNA was separated into two single strands by heating it to 94°C (201°F). At this temperature, however, the DNA polymerase used at the time was destroyed, so the enzyme had to be replenished after the heating stage of each cycle. The original procedure was very inefficient, since it required a great deal of time, large amounts of DNA polymerase, and continual attention throughout the process. However, in 1986, this original PCR process was greatly improved by the use of DNA polymerase taken from

thermophilic bacteria grown in geysers at a temperature of over 110°C (230°F). The DNA polymerase taken from these organisms is stable at high temperatures and, when used in PCR, does not break down when the mixture was heated to separate the DNA strands. Since there was no longer a need to add new DNA polymerase for each cycle, the process of copying a given DNA strand could be simplified and automated.

One of the first thermostable DNA polymerases was obtained from *Thermus aquaticus* and was called "Taq." Taq polymerase is widely used in current PCR practice. A disadvantage of Taq is that it sometimes make mistakes when copying DNA, leading to mutations (errors) in the DNA sequence, since it lacks 3'→5' proofreading exonuclease activity.
PCR has already replaced culture methods as the gold standard for diagnosis of *Chlamydia trachomatis* infections (Black, 1997). The commercial PCR procedure used for NGU detection has been recognized as a satisfactory alternative to culture (Crotchfelt *et al.*, 1997). PCR is certainly more practical than the inefficient culture methods that exist for *Mycoplasma genitalium* (Taylor-Robinson, 1995). Only one study has compared non-nested PCR with culture methods for *Trichomonas vaginalis* detection in men with urethral discharge, which concluded that PCR had a sensitivity of 89% and a specificity of 98% (Hobbs *et al., 1999*). Considerable attention has been given in recent years to the application of the PCR assay in primary detection of mycoplasmal and ureaplasmal infections in animals and humans.

PCR is very vital in fastidious and slow-growing mollicutes such as *M. genitalium or M. fermentans* and Ureaplasmas are sought and is also valuable in differentiating *Ureaplasma* species and serotypes, as described earlier. Gene targets for PCR assays used to detect Ureaplasmas have included the urease gene (Blanchard *et al.*, 1993), 16S rRNA genes (Robertson, *et al.*, 1993), and the multiple-banded (MB) antigen gene (Teng *et al.*, 1994). PCR assays for *M. hominis* have used 16S rRNA and ribosomal DNA as the gene targets (Grau *et al.*, 1994, Yoshida *et al.*, 2003). The theoretical advantages of the PCR assay for detection of genital mycoplasmas include the fact that no viable organisms are necessary, its limit of detection is much better than culture, and results can be available in 1 day.

Most studies (Blanchard *et al.*, 1993; Luki, 1998; Yoon *et al.*, 2000; Colaizy *et al.*, 2003) evaluating the PCR assay for detection of mycoplasmas in clinical specimens have compared the technique to culture for calculation of sensitivity and specificity. As has been the case

with other fastidious microbes such as chlamydiae, this approach may not be completely valid since culture is never going to be able to detect their presence as readily as nucleic acid amplification when performed properly using appropriate primers and in the absence of inhibitors. Several recent studies have compared culture with the PCR assay for detection of genital mycoplasmas in samples from pregnant women, neonates and a variety of other conditions, for instance Colaizy *et al.*, (2003) provides a timely critique of the application of the PCR assay in all facets of mycoplasmology. Blanchard *et al.*, (1993) identified 10 of 293 amniotic fluid specimens that were positive for *Ureaplasma spp* by PCR, four of which were also culture positive. There were no specimens that were PCR negative and culture positive.

The ability of the PCR assay to detect ureaplasmas in female genitourinary specimens, including cervices, amniotic fluid, and vaginal specimens, has been shown to be comparable or superior to that of culture according to studies (Luki, 1998, Yoon *et al.*, 2000). The PCR assay has also been evaluated as a diagnostic tool for rapid detection of ureaplasmal infection in neonates with lower respiratory infections as well as to aid in the elucidation of the possible role of these organisms in BPD. The first report of the PCR assay used for detection of ureaplasmas in endotracheal aspirates of neonates was by Scheurlen *et al.*, (1992). They were able to detect only one PCR-positive specimen among 36 ventilated neonates studied. Blanchard *et al.*, (1993) found 99% agreement between PCR analysis and culture in 95 endotracheal aspirate samples. Only one specimen was positive by culture and not by PCR assay. Other investigations have shown agreements of PCR to be 91 to 95% in comparison to culture (Nelson *et al.*, 1998). A major concern when considering the results of any study in which PCR is compared to the inherently less sensitive culture technique is how to interpret findings in which the PCR is positive and the culture is negative to evaluate the specificity of the PCR assay and ensure the results were not due to a false-positive reaction due to contamination. Use of a second gene target and/or assessing repeat specimens may help to resolve such cases. It is also important to emphasize that the sensitivity of culture in detecting ureaplasmas in tracheal aspirates may vary considerably with the experience of the laboratory performing the culture and the methods of culture used. Through a retrospective review of neonatal respiratory cultures, it was shown that limiting cultures to agar-based methods and omission of broth cultures and serial dilutions will result in a lower isolation rate (Waites and Cassell, 1995).

Kong, *et al* (2000), reported that enough evidence exists to prove that *U. urealyticum* could be divided into two species – *U parvum* (previously *U. urealyticum* biovar 1) and *U. urealyticum* (previously *U. urealyticum* biovar 2). In their study they designed a series of primers, targeting the 16SrRNA gene and 16SrRNA-23SrRNA intergenic spacer regions, the urease gene subunits, and the 5" end of the multi-banded antigen (MBA).genes to identify and subtype these *ureaplasma species*. They separated the 2 species into 14 serotypes, including 4 serovars 1,3,6 and 14 for *U. parvum* and 11 serovars 2 to 13, for *U. urealyticum*.

Multiplex PCR systems have also been described and applied to the detection of genital mycoplasmas in clinical specimens with favorable results in comparison to culture (Stellrecht *et al.,* 2004). Despite the overall favorability in studies comparing PCR with culture for detection of *M. hominis* and *Ureaplasma spp.*, this technique is not commercially available in the United States and has been limited to research laboratories or specialized molecular diagnostic reference laboratories. Until technology is advanced to the point that PCR assays for genital mycoplasmas can be purchased commercially in a kit format by diagnostic laboratories, it is unlikely to gain widespread usage for routine microbiological diagnosis.

Real-time PCR has been adapted for quantitation and characterization of ureaplasmal isolates agar and broth media for rapidly growing organisms such as *Ureaplasma spp* and *M. hominis* supports the concept that culture should remain an important part of the diagnostic process. Specimen types such as blood which may contain a very low concentration of microbes in a huge background of human DNA may not be ideal for PCR.

2.9. CONCLUSION

In humans more knowledge is required about the epidemiology and pathogenesis of several urogenital mollicutes such as *M. fermentans, M. genitalium, M. penetrans*, because they are raely isolated by culture. Considerable attention has been given in recent years to the application of the PCR assay in primary detection of mycoplasmal and ureaplasmal infections in animals and humans. PCR is very vital in fastidious and slow-growing mollicutes such as *M. genitalium or M. fermentans* and Ureaplasmas are sought and is also valuable in differentiating *Ureaplasmal species and serotypes*, as described earlier.

Gene targets for PCR assays used to detect Ureaplasmas have included the urease gene (Blanchard *et al.*, 1993), 16S rRNA genes (Robertson, *et al.,* 1993), and the multiple-banded (MB) antigen gene (Teng, *et al.*, 1994). PCR assays for *M. hominis* have used 16S rRNA and ribosomal DNA as the gene targets (Grau, *et al.*, 1994, Yoshida, *et al.*, 2003). The theoretical advantages of the PCR assay for detection of genital mycoplasmas include the fact that no viable organisms are necessary, its limit of detection is much better than culture, and results can be available in 1 day. The principal objective of the study under review, is to speciate and serotypes the Nigerian strains of human ureaplasmas as established by Kong, (1999). And to show the roles of risk factors in their transmission as well as the determination of the prevalence *M. genitalium* in infertility among patients attending STD Clinics in Nigeria.

CHAPTER THREE

EPIDEMIOLOGICAL SURVEY OF GENITAL MYCOPLASMAS WITH EMPHASIS ON *UREAPLASMA SPP*

3.1 INTRODUCTION

In developed world like Europe and United States, the average prevalence of human mycoplasmas is said to be within the range of 42 to 50 percent, but higher rates has been reported in developing worlds including 55% in south Africa 30% in Asia and 45 % in India (Taylor-Robinson *et al.*, 2003). Nongonococcal urethritis is the most common sexually transmitted infection. *Ureaplasma species* and *M genitalium* may account for a significant portion of cases that are not due to chlamydiae. More than 20% of live born infants may be colonized by *Ureaplasma*, and infants born preterm most likely harbor the organisms. Colonization declines after age 3 months. Less than 5% of children and 10% of adults who are not sexually active are colonized with genital mycoplasmal microorganisms. Surveys of a variety of human populations reveal widespread colonization of the genital tract with mycoplasma (Taylor-Robinson *et al.*, 2003).

Genital mycoplasma can be recovered from nearly 30% of newborn infants. It has been demonstrated that infants become infected with these organisms during the passage through the birth canal (Lein *et al.*, 1969). Infants delivered by caesarean section are less often infected than those delivered vaginally. Isolation rates are somewhat higher for female infants than males. After puberty colonization rates increase and are directly proportional to the extent of sexual activity, rising from 0% with no sexual contact to 45% with three or more partners. However, it has been shown that ureaplasma infection can be chronic, lasting several years at least, and is not necessarily the result of repeated sexual activity (Taylor-Robinson *et al.*, 2003).

The role of *Mycoplasma* in human disease is poorly understood in this part of the world, because only few laboratories are capable of handling *Mycoplasma* presently in Nigeria As the organism is more difficult to handle than typical bacteria and viruses. Hence, data generated from this study is expected to provide a strong basis for a comprehensive research on the role of *Mycoplasma* and *Ureaplasma* in the overall picture of STDs in Nigeria.

3.2 MATERIALS AND METHODS

3.2.1 Ethical Committee Approval

The research procedure was safe, simple, and free and subjects were not exposed to further risk of infections. Patient's consent was sought hence participation was voluntary and unanimous; furthermore patients' anonymity, confidentiality and integrity were maintained throughout the study and thereafter. There was an initial scrutinizing and vetting of the research proposal followed by the ethical approval granted by the ethical committees and managements of Jos and Lagos University Teaching Hospitals.

3.2.2 Study Population

The studied population was STD patients who presented with discharge, itching, ulcerations and sores in genital parts, currently or recurrent abortion, as well as patients with various degrees of primary and secondary infertilities in JUTH and LUTH. These are among the hospitals in Nigeria where one can have maximum level of representation of Nigerians attending STD clinics from the Northern and Southern parts of Nigeria. This is because of their geographical and political locations, commercial activities endowment, ethnic diversities and Federal Government Reference Teaching Hospitals for HIV/AIDS /STD patients in Nigeria. Swabs from 824 participants made up of equal number of male and female participants were examined. LUTH Site's breakdown included 70 males with STI symptoms, 180 females with STI symptoms, 70 asymptomatic males and 92 asymptomatic females amounting to 412 swabs. JUTH Site's Breakdown included, 70 males with STI symptoms, 180 females with STI symptoms, 70 asymptomatic males, 92 asymptomatic females amounting 412 swabs all together.

3. 2. 4 Study Sites

Federal Government of Nigeria Reference Centers for HIV/AIDS / Sexually Transmitted Infections (STI), Lagos University Teaching Hospital, (LUTH) and clinics in the South, Jos University Teaching Hospital, (JUTH) and clinics in the North.

Figure 3.1 Map of Nigeria showing the two study sites (Federal Government of Nigeria Reference Centers for HIV/AIDS / Sexually Transmitted Infections (STI), Lagos University Teaching Hospital, (LUTH) and clinics in the South, Jos University Teaching Hospital, (JUTH) and clinics in the North).

3. 2.5. Inclusion and Exclusion Criteria

Patients menstruating were excluded from the experimental group, but those that were apparently healthy were selected as the control. Swab specimens were also obtained from patients that were referred to submit pap smears. Pregnant and nursing mothers were also examined. Subjects were not selected based on when they urinated last, nor was their treatment with antimicrobial agents considered.

3.2.6. Controls (asymptomatic)

The control group in this study was the asymptomatic patients attending antenatal, prenatal, or postnatal clinics, as well as their accompanying husbands or relatives. Others included those of whom employers requested for medical tests and individuals referred by the religion leaders for HIV screening on marriage counseling.

3.2.7. Samples size and sampling method.

The qualitative case controlled study as described by Schlesselman (1982) was employed in the two metropolitan cities' hospitals in the Northern and Southern Nigeria. The study aimed at investigating the relationship among the genital mycoplasmas studied, and reproductive maladies such urethritis, cervicitis, pelvic inflammatory diseases, spontaneous abortion and various degrees of infertility among male and female patients attending STI clinics in Nigeria. A non-probability sampling method, known as convenience sampling method, was used. The sample size was determined using the formula recommended by WHO (1993), as shown below:

$$N = \frac{T^2 P Q}{D^2}$$

Where:

N = Required sample size (area under the curve)

T = Confidence Interval (CI) of 95%=1.96

P = Prevalence of genital Mycoplasmas in the study population, Nigeria (50%).

Q = 1-Prevalence of genital Mycoplasmas

D = Deviation amount according to the prevalence of genital Mycoplasmas Allowable/Tolerable/ Sampling Error=5% (0.05). 3.8416 x 0.5 x 0.5 divided by 0.0025= 50%=384,

The sample size was calculated according to Senocak, (1997) based on Nigerian's estimated prevalence of 50%, since the prevalence is unknown. The acceptable error margin used was 5% and 95% confidence interval. And to adjust for wastage factors such as contaminations, losses and other mishaps, the wastage rate was increased by 20% (WHO, 2003). From this formula a total of 824 subjects were used in the study. Of this, 500 were patients, while 324 individuals coming for routine checkup, and were considered under the asymptomatic group (Control). The symptomatic group (Patient), on the other hand, was those subjects with obvious discharge, itching with or without sores, urinary tract infections current or previously, abortions, primary or secondary infertilities.

3.2.8 Media preparation and primary isolation techniques.

Urogenital and urethral swabs obtained from patients were temporarily stored in cooler containing frozen ice packs during the sampling periods. This is because mycoplasmas are walless and very fragile, hence these organisms die rapidly on exposure to any form of temperature other than the minimal of 4 °C. On reaching the laboratory, the swabs were snapped off into sterile Bijou bottles containing PPLO media, used in culturing the organisms. Incubation was done under 37 °C and for a period of 24 to 72 hours. The methods used in our laboratory for the cultivation and identification of genital mycoplasmas have been described by Braun *et al.*, 1995. Immediately after collection, the vaginal swabs were broken into 4ml of screw-capped vials containing 2.5ml of basic mycoplasma broth (70% P.P.L.O. broth, 20% unheated horse serum, 10% yeast extract, 0.002% phenol red and 500 units per ml of benzyl penicillin), to which 50ug per ml of polymyxin B and 5ug per ml of amphotericin B had been added. The pH had been adjusted to 6.0-6.4 with 1M Hydrochloric acid. Broths were inoculated into 3 different PPLO substrates, because different species have selective affinity for a particular substrate for optimum metabolic activities. In all, 3 swab specimens were collected from each patient, one from urethral space in both male and female and 2 more obtained from the high vaginal space and endocervix in female subjects. The swabs were inoculated into PPLO media enriched with urea, for isolation of ureaplasmas; PPLO enriched arginine, for isolation of *M. hominis* and into PPLO enriched glucose, for the *M. fermentance* and *M. penetrance* isolation.

After a maximum of 72 hours of incubation, urea broth and arginine changed to pink in color indicating the presence of *Ureaplasma species* and *M. hominis* including *M. genitalium*, and

those that changed to yellow indicated the presence of *M. fermentance* and *M. penetrance.* The presumptive isolates were subjected to a specific biochemical test, urease agar test as recommended by Shepard, (1970). The reagents used for media preparation are as shown in Tables 4 and 5 below.

Table 3.1 PPLO Broth Protocol for primary mycoplasma isolation.

Pleuropneumonia-Like Organisms (PPLO) Media (Broth)	
PPLO Broth w/o crystal violent (Difco)	3.7g
Deionized/ultrapure water	175ml
Adjust pH to 6 using 1M HCL	1.5ml
Autoclave at	121 ^{o}C for 15 min
Cool to water bath temperature	60°C
Horse serum	50ml
Yeast extract (Difco)	25ml
l- Cysteine/Putricine dihydrochloride (Sigma)	1.25ml
Urea	1ml
Glucose	1ml
Penicillin/AmphotericinB/Thallium acetate	1ml
Phenol red (Eastman Kodak)	0.25ml
CVA enrichment (GIBCO Diagnostics)	1.25ml

Sterility test was carried out by taking 2ml of the broth in a test tube and incubated overnight. The next day, the tubes were certified sterile without any conramination before inoculation.

Positive patient by broth indicator were subcultured on PPLO agar prepared in a similar way as the broth except for bacteriological agar is added and phenol red is omitted, thus:

Table 3.2 PPLO Agar Protocol for primary isolation of mycoplasma.

Pleuropneumonia-Like Organisms (PPLO) Media (Agar)	
PPLO Broth w/o crystal violent (Difco)	3.7g
Trypticase soy broth (Difco)	1.5g
Distilled water	175ml
Agar bacteriological grade (Oxoid)	1g
Adjust pH to 6 using 1M HCL	1.5ml
Autoclave at	121°C for 15 min
Cool to water bath temperature	60°C
Horse serum	50ml
Yeast extract (Difco)	25ml
l- Cysteine	1.25ml
Urea	1ml
Isovitalex enrichment	1.25ml

Approximately 10ml were poured into sterile plates. Sterility test was carried out by incubating the plates overnight at 37 °C. Next day the plates were confirmed sterile before subculturing.

3.2.9 Identification of Ureaplasma colonies

Of the human mycoplasmas, *U. urealyticum* is the only species that is able to hydrolyze urea to ammonia. This property not only facilitates isolation of the organism but also provides a rapid and convenient way to identify the species in culture. The manganese II chloride ($MnCl_2$)-urea test provides a rapid visual method for detecting ammonia formation by *Ureaplasma urealyticum.*

The principle is that urease activity of Uu is detected by exposing the colonies to a solution of $MnCl_2$ in the presence of urea. The urease of the organism hydrolyses urea to ammonia, and then the following reaction occurs:

$2NH_3 + 2H_2O \rightarrow 2NH_4OH \rightarrow 2NH_4^+ + 2OH^-$

$MnCl_2 + 2OH \rightarrow MnO + 2Cl^- + H_2O$

The urease of the organism hydrolyses urea to ammonia, and manganese II oxide (black) formed in the reaction is insoluble and forms a dark brown precipitate around the colonies as recommended by (Smith, 1993). This reaction is then detected under a dissecting microscope. *Ureaplasma* sp was identified by the use of urease test, identifiable by its ability to produce urease on agar plate (Sheperd, 1954, and Sheperd 1970). Colonies of Ureaplasmas are usually minute (less than 100 μm in diameter); because of urea hydrolysis and ammonia liberation, the culturing medium becomes alkaline, after incubation for 48-72 hrs. When manganese tetra-oxo sulphate (VI) ($MnSo_4$) is added to such medium, the ureaplasma colonies stain dark brown. The pure mycoplasmas isolates in broth culture were lyophilized for the molecular studies abroad. The Lyophilization protocol is as shown below.

3.2.10 Lyophilization (Freeze Drying) Protocol.

It is procedures whereby pure isolates in slants or broth culture containing microorganisms are preserved remain alive and potent for years, without losing their pathogenic potency. Usually a stabilizer is included to ensure that the organism survives the very low temperature of -176 ^{o}C. The lyophilizer provided by National Veterinary Research Institute NVRI), Vom, Plateau state, was used to freeze dry urogenital broth culture. Approximately 2.5ml of the PPLO broth culture was lyophilized per patient, using 20% of sucrose and 6% of gelatin, reconstituted in deionized water as the preservative.

3.3 RESULTS.

3.3.1 Primary isolates from the 2 study sites (Plates 1 and 2)

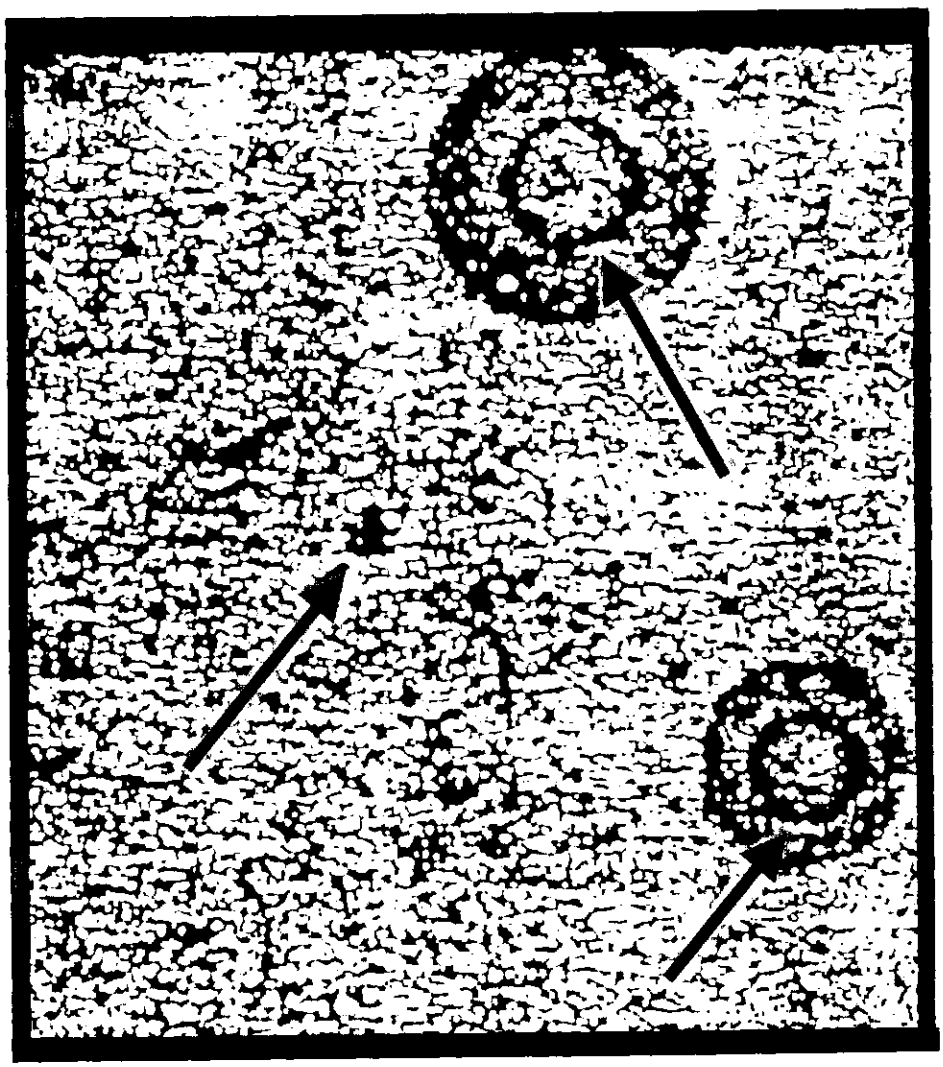

Plate 3.1 Colonies of *Mycoplasma spp* (X 100) isolated from urogenital specimen from one of the patients attending an STD clinic in LUTH. It shows the peripheral zone and the dense central zone as well as the fried egg characteristic colony. Arrow is showing Ureaplasma colony embedded with several colonies of MH (large colonies). It does not gram stain, because it has no cell wall. Colonies were viewed directly under a stereomicroscope/dissecting microscope (Fisher).

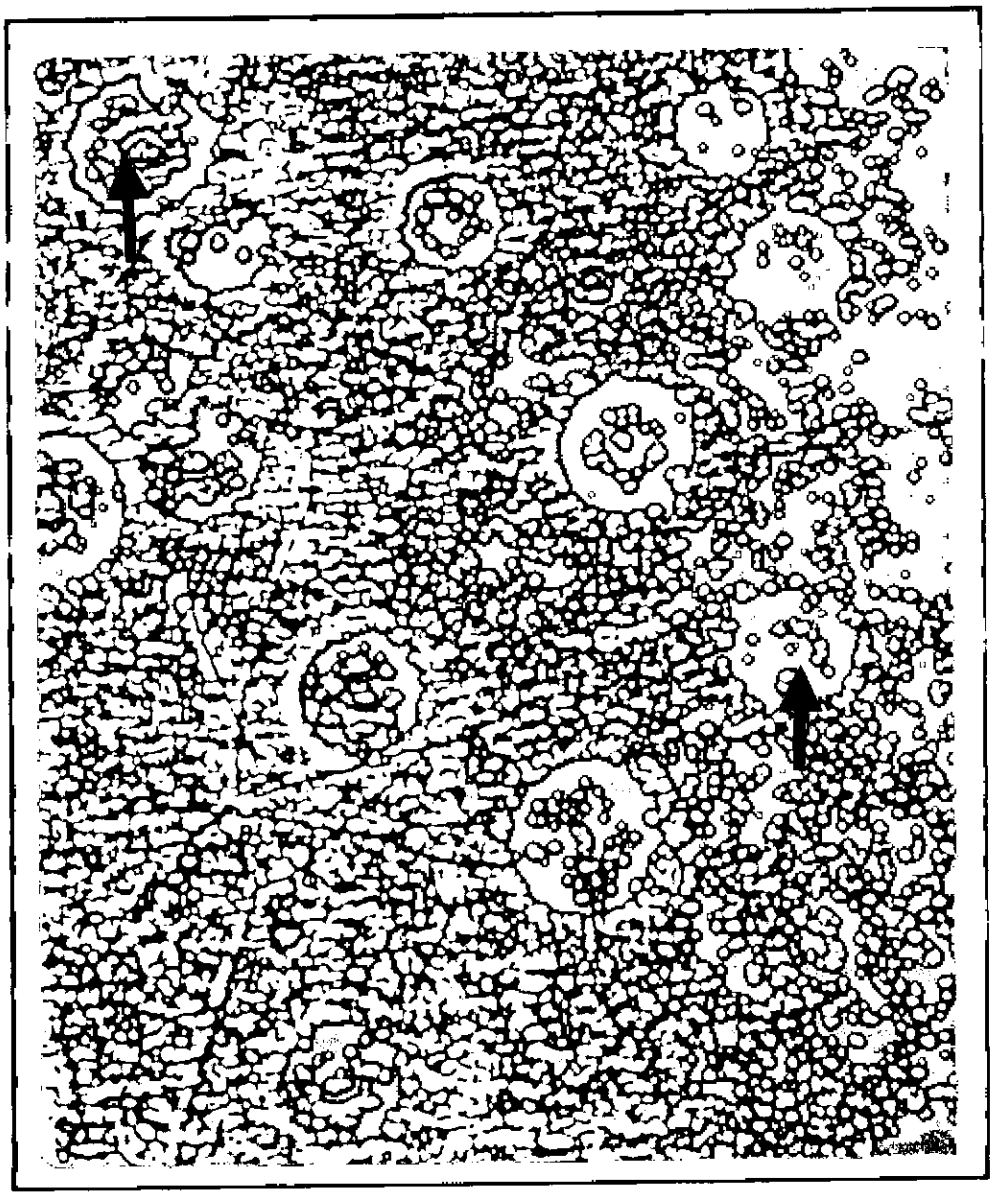

Plate 3.2 Colonies of *Mycoplasma spp* (X 100) isolated from urogenital specimen from one of the patients attending an STD clinic in JUTH. It shows the peripheral zone and the dense central zone as well as the fried egg characteristic colony. It does not gram stain, because it has no cell wall. Colonies were viewed directly under a stereomicroscope/dissecting microscope (Fisher).

Table 3. 4: The summary of the result of the primary isolation of mycoplasmas in males and females in JUTH and LUTH Nigeria.

Study Site	Study Group or Specimen	Number Examined	Number Positive	Prevalent Rate
		70	19	27.1%
LUTH and JUTH	Male patients	70	22	31.4%
TOTAL	**STD Males**	**140**	**41**	**29.3%**
		180	86	47.8%
LUTH and JUTH	Female patients	180	96	53.3%
TOTAL	**STD Females**	**360**	**182**	**50.6%**
		70	10	14.3%
LUTH and JUTH	Male controls	70	5	7.1%
TOTAL	Male STD controls	140	15	10.7%
		92	7	7.6 %
LUTH and JUTH	Female STD controls	92	6	6.5%
TOTAL	**Female STD controls**	**184**	**13**	**7.1%**

The results are summarized in Table 3.1. It shows the breakdown of the equal samples collected from males and females precipitants in either of the teaching hospitals. *Ureaplasma spp* was isolated from 22 of 70 males (31.4%) at the JUTH location, and from 19 of 70 males (27.1%) at the LUTH location. In females, 96 of 180 (53.3%) swabs were positive at JUTH, and 86 of 180 (47.8%) at LUTH. Overall prevalence was 44.6% (223 of 500) in the male and female patients. At the two locations, a total of 324 asymptomatic participants were sampled, and 28, representing 8.6% were positive.

3.4 DISCUSSION

Among all the Nigerian studies to date, non matches the geographical spread of this project. Covering two Federal Government Reference Centers for HIV/AIDS / Sexually Transmitted Infections (STI), Lagos University Teaching Hospital, (LUTH) and clinics in the South, Jos University Teaching Hospital, (JUTH) and clinics in the North. The total number of participants (824) was much larger than the previous studies, thus providing a fairly representative prevalent rate that can be used in demographic report of STD/STI in Nigeria, as far as genital mycoplasmosis are concern. The prevalence of genital mycoplasmas in Lagos (37.5%) and Jos (42.5%) were reported and compared. The prevalent rate of genital mycoplasma in Jos {in both Male (31.4%) and Female (53.3%)} was higher than that of Lagos {Male (27.1%) and Female (47.8%)} as shown in Tables 3.3. The overall prevalence of *Ureaplasma urealyticum* infection was 44.6% (223 of 500) in the male and female patients examined. The summary of the result of the primary isolation of mycoplasmas in JUTH and LUTH Nigeria is as shown in Tables 3.3, 3.4 and Figure 3.2. The present study provides further evidence in addition to Osoba (1972,) Ladipo *et al.*, 1978, Adegboye *et al.*, 1979, Bakare, (2000) and Bakare, (2002) that some Nigerian males and females do harbour mycoplasmas in their genital tract. Thus, the rate of isolation of *U. urealyticum* was higher in patients with clinical evidence of STI than in asymptomatic (control) groups, supporting previous studies in Nigeria and various other parts of the world. The previous study of Osoba in 1972 concluded that some Nigerians females do harbour *M hominis* (42%) and *U. urealyticum* (60%) in the genital tract in Ibadan. (Osoba, 1972) .Similarly Adegboye and colleagues reported 33.8% and 63% incidence respectively in Zaria (Adegboye *et al.*, 1979). Similarly the current study also confirmed the presence of these organisms in Nigerians suffering from obstetrics and gynaecological problems, utilizing the facilities at JUTH and LUTH, Nigeria (Table 1.2).

3.5 CONCLUSION

Since genital mycoplasmas recovered from participants were more prevalent in the group with specific STD problem; this implies that mycoplasmas might be responsible for cases of chronic STD in patients attending STD clinics in Nigeria. Looking at the diversities and the geographical spread of this study, one could infer that it is fairly representative of the country. The data generated could also serve as a base line data for epidemiological survey of the role of genital mycoplasmas in STD control in Nigeria.

CHAPTER FOUR

SPECIATION OF NIGERIAN UREAPLASMA *ISOLATES* BY POLYMERASE CHAIN REACTION

4.1 INTRODUCTION

Polymerase Chain Reaction (PCR) technology are applied in various genetic manipulations including cloning, sequencing of specific mutation, crime detection, forensic medicine, epidemiological disease surveillance, diagnosis, generic classification and speciation employed in this study. The theoretical advantages of the PCR assay for detection of genital mycoplasmas include the fact that no viable organisms are necessary, its limit of detection is much better than culture, and results can be available in 1 day. Most studies evaluating the PCR assay for detection of mycoplasmas in clinical specimens have compared the technique to culture for calculation of sensitivity and specificity. As has been the case with other fastidious microbes such as chlamydiae, this approach may not be completely valid since culture is never going to be able to detect their presence as readily as nucleic acid amplification when performed properly using appropriate primers and in the absence of inhibitors. Several recent studies have compared culture with the PCR assay for detection of genital mycoplasmas in samples from pregnant women, neonates and a variety of other conditions.

In this study, two hundred and thirty six (236) samples comprising of 119 patients and 117 controls were lyophilized and transported abroad to a host laboratory in the United States of America for Polymerase Chain Reaction (PCR) technology. Lyophilized samples were soaked in 2X in phosphate buffer solution PBS and pooled into Sigma tubes for DNA extraction, using Sigma protocol (Sigma GenElute Bacterial Genomic DNA Kit). The target primers included UU (Serotype 2, ATCC #27814) and UP (Serovar 3, genome sequencing strain, ATCC # 700970) were purchased from the American Type Culture Collection, Bethesda.

The aim is to speciate for the first time the Nigerian strains of *Ureaplasma spp* into the 2 biovars *U. parvum* (parvo biovar) and *U. urealyticum* (T-960) by PCR technology. And to compare the sensitivity and specificity of culture, Polymerase Chain Reaction techniques in the rapid detection of genital mycoplasmas from Nigerian STD patients. In this chapter, speciation of Nigerian strains of UU by PCR technology was carried out and reported.

4.2 MATERIALS AND METHODS

4.2.1 Selection of specimen

From the 824 overall samples, 238 samples comprising of 117 controls and 119 patients with history of urethritis and infertility were selected for the PCR assay. The breakdown is as shown in the Table 4.1 below:

Table 4.1: Nigerian Urogenital Specimens for PCR Studies

	PATIENTS	JUTH	LUTH	TOTAL
1	No of males with infertility	21	11	32
2	No of females with infertility	56	31	87
	Total infertility for male and females	77	42	119
	CONTROLS	**JUTH**	**LUTH**	**TOTAL**
3	Males referred for medical check up	21	11	32
4	Females referred for medical check up	56	31	87
	Total males and females referred	77	40	117
	TOTAL SAMPLED	**154**	**82**	**236**

The table above is showing the breakdown of patients from the 2 teaching hospitals with clinical cases of urethritis as well as primary and secondary infertility. Equal number of males and females were selected for the assays.

4.2.2 DNA Extraction materials:

1. Lyophilized Ureaplasma urealyticum culture –ATCC (Serotype 2, ATCC #27814).
2. Lyophilized Ureaplasma parvum culture –ATCC (Serovar 3, genome sequencing strain, ATCC # 700970). These were purchased from the American Type Culture Collection, Bethesda, MD., and were propagated in ATCC urea broth (10B).
3. Sigma GenElute Bacterial Genomic DNA Kit.
4. Samples series J1-L119 and JC1-LC119.

4.2.3 DNA Extraction methods using Sigma GenElute Bacterial Genomic DNA Kit.

Ureaplasma urealyticum and *Ureaplasma parvum* ATCC strains ordered were reconstituted in laminar flow room with 1.5ml X PBS each. Samples J1-L119 and JC1-LC119, were reconstituted in batches of 20 in this order ($A1_{-20}$, B_{1-20}, C_{1-20}, D_{1-20}, E_{1-20}, F_{1-20}, G_{1-20}, H_{1-20}, I_{1-20}, J_{1-20}, K_{1-20}, L_{1-20}, M_{1-20}, N_{1-20}). Making a total of 236 samples comprising of 119 patients and 117 controls from both teaching hospital sites. Samples spun on centrifuge 5417C Eppendorf at 14,000 rpm for 2 min. Supernatant discarded and drained dry with 200 µL pipette. Samples spun on centrifuge 54170 Eppendorf at 14,000 rpm for 2 min. Supernatant discarded and with a 200 µL pipette drained dry. Pellets were treated with 200 µL of the reconstituted lysozyme, incubated for 30 min. Then 20 µL of proteinase K solution added to each of the samples after reconstitution. Preparation of proteinase K stock involved addition of 500 µL of sterile water into the centre content of the long proteinase K container to dissolve the 10mg proteinase K, before dispensing 20 µL into each of the lysates. This was followed by the addition of 200 µL of lysis solution C. Vortexing the lysates thoroughly for about 15 sec each.

Preparation incubated at 55oc for 10 min. Add 500 µL of column preparation solution to each of the preassembled relabeled tubes, centrifuge at 12,000 for 1 min, and discard the eluate ie the liquid residue from the column, but the jacket tube retained. Add 200 µL of ethanol to the lysates and mix thoroughly by vortexing for 5-10sec. Transfer the entire tube content into the binding column using a wide bore tube centrifuge at 7,000 for 1 min/ Discard the collection tube containing the eluate and place the naked binding column in relabeled new tubes. First wash by adding 500 µL of wash solution 1 to the column and centrifuge at 7,000 for 1min. Discard tube and replace for the second washing. Second washing was by adding 500µL of wash solution 1 to the column and centrifuge at 16,000 for 3 min. Discard eluate

and retain the tube if you need to spin again for 1 min to remove the enduring alcohol, otherwise you discard and elute. Elute by adding 200 µL of the elution solution directly into the centre of the column. Incubation at 37°c for 5min and spinning at 7,000 for 1min. The final 200uL DNA was splitted into two 100 µL aliquots – one for PCR screening of *U. urealyticum, U. parvum,* studies, and the other 100 µL for *M. genitalium,* real-time PCR studies.

4.2.4 Polymerase Chain Reaction Assays

Test trials conducted prior to PCR assays included: Sensitivity, optimizing tests on primers and standardization of PCR programmes. Polymerase Chain Reaction test trials on 10 culture positive sample and PCR tests on the overall Nigerian DNA urogenital samples. Optimizing the sensitivity of *Ureaplasma urealyticum* and *Ureaplasma parvum* on standard strains obtained from ATCC used as positive controls for each organism. Various pairs of Primers were tested for their species sensitivity and specificity.

Table 4. 2: Development and test trials of PCR programs used in speciation of UU into UU and UP.

PCR RUNS	PROGRAMME	TEMPLATE/PRIMERS	RESULTS
I	TD50	Uu/U8/UUA	Positive +++
		Uu/UUS2/UUA2	Positive +++
		Up/UPS1/UPA	Positive +
		Up/UPS2/UPA2	Negative -
II	TD50	Uu/U8/UUA	Positive ++
	TD58	Uu/U8/UUA	Positive +++
III	TD50	Uu/UUS2/UUA2	Positive +++
	TD55	Uu/UUS2/UUA2	Negative -
IV	TD58	Up/UPS1/UPA	Positive +++
	TD50	Up/UPS1/UPA	Positive ++
V	TD50	Up/UPS2/UPA2	Negative -
	TD55	Up/UPS2/UPA2	Negative-

Table 4.2, above displayed the results of the species-sensitivity and specific Tests on primer mix for *U. urealyticum* (U8/UUA) and *U. parvum* (UPS1/UPA). These primers pairs gave satisfactory results with TD 58 program.

4.2.5 Selection of primers

In this study 2 sets of primer pairs were used, including U8 primer in the forward direction and UUA primer in the reverse direction. The two primers were included to bind with the target gene located at a 650-bp (well conserved region) a fragment of the structural UU, 16S rRNA urease gene located on the small segment of the DNA template for the identification of *ureaplasma urealyticum* biovar. While the primers UPS2 in the forward direction and UPA2 in the reverse direction were also included to bind with the target gene at a 400-bp (well conserved region) fragment of the structural UP, UB urease gene located on the small segment of the DNA template for the identification of *Ureaplasma parvum* biovar. This set of primers used was published by Kong *et al.*, (2000). The nomenclature of the primers was procured from the Integrated DNA Technologies and the detailed nomenclature is as shown Table 4.3, below:

Table 4.3: Development and test trials of primer used in speciation of UU (UU and UP)

Oligo name	Size (kb)	Length (DNA Bases)	Source	Forward (Sence5') and Reverse (Antisence3') Sequence (5'->3')
U8	650	24	Uu 16S rRNA	GAA GAT GTA GAA AGT CGC GTT TGC
UUA	650	22	Uu 16S rRNA	CTA CAA CAC CGA CTC GTT CGA G
UUS2	400	22	Uu urease gene	CAG GAT CAT CAA ATC AAT TCA C
UUA2	400	21	Uu urease gene	CAT AAT GTT CCC CTT CGT CTA
UPS1	650	24	Up 16S rRNA	ATG AGA AGA TGT AGA AAG TCG CTC
UPA	650	23	Uu 16S rRNA	TTA GCT ACA ACA CCG ACC CAT TC
UPS2	400	22	Up urease gene	CAG GAT CAT CAA GTC AAT TTA G
UPA2	400	23	Up urease gene	AAC ATA ATG TTC CCC TTT TTT ATC

Table 4. 4: PCR Experimental lay out for the speciation of *Ureaplasma sp* into UU and UP using specific primers.

SPECIATION	UREAPLASMA COMPLEX																							
Spp		*Ureaplasma urealyticum*												*Ureaplasma parvum*										
Master mix	Qty	1	2	3	4	5	6	7	8	9	10	11	12	Qty	1	2	3	4	5	6	7	8	9	10	11	12
10x buffer	2											+C UU	-C H_2O	2											+C UP	-C H_2O
2Mm DNTPs	2													2												
25Mm MgCl2	2													2												
Taq Polymerase	0.15													0.15												
Template	2													2												
5 ' primer, 5uM	2													2												
3' Primer, 5uM	2													2												
H_2O	7.85													7.85												
Total	20													20												

This is a combined experimental lay out for *Ureaplasma spp* speciation, using the species sensitivity and specificity primers pairs of ATCC positive strains of known Uu and Up using TD 58 cycling program. The experiment was run concurrently and its layout is as shown in Table 4. 4 above.

The reaction mixture shown below was purchased as a kit from a company, except for the template which was the DNA extracted from the Nigerian urogenital specimens.

19 x buffers	2 µL
2mMdNTP	2 µL
25Mm $MgCl_2$	2 µL
Taq Polymerase	0.15 µL
Template	2 µL
5' Primers	1 µL
3' Primers	1 µL
H_2O Ultra pure i.e. free of DNAse and RNAse	9.85 µL
Total	20 µL

The above constituted the 20 µL master mix volume, which contains the buffers that cushions the reactants enriched with dinucleotide triphosphate as a building block for the elongation stage catalyzed by the activities of the Taq polymerase heat stable enzyme. The primers bind with the corresponding DNA single strands in the forward and the reverse pairs. After this primary mixing, templates were added outside the PCR room to avoid contamination.

4.2.6 PCR Dynamics

There is a block of DNA sequence on the gene that makes up the urease gene on the *Ureaplasma* genome. It is unique, specific, peculiar and characteristic. It is very important too, for the survival of this organism. It contains about 15-20 nucleotides sequence. All the PCR reactants and reagents were assembled, including, 19 x buffers, 2mM dNTP, 25mM $MgCl_2$, Taq Polymerase, Template, 5' Primers, 3' Primers and H_2O Ultra pure i.e. free of DNAse and RNAse. These were set in the PCR machine, the DNA of the unknown specimen was the first target. It was a double strand polymer, and by the time the PCR machine raised the temperature to 95°c the DNA double strand was broken into single strands with forward and reverse pairs freed, once this was accomplished, the machine automatically lowered the temperature to 65°c for the complimentary binding of the forward primer with the reverse DNA pair of the unknown, similarly, the reverse pair of the primer sought for the forward of the unknown DNA to bind with. The machine now raised the temperature to

72°c to activate the Taq polymerase into catalytic reactions. Taq polymerase being a heat stable enzyme enhances the complimentary binding of DNA molecules binding and specific pairing, for instance, when the Taq polymerase recognizes that 'A' nucleotide is alone it then looks out for 'T' and binds it with 'A' forming 'A-T' pairing base. He sees 'C' alone, he pairs 'G' with it to form 'A-G' pairing bases. In another space 8 'A' that may be alone, it looks for 8 'T's to pair with the 'A's. This is elongation stage following the annealing stage.

The machine now raised the temperature again to 95°c the DNA double strand is broken into single strands with forward and reverse pairs freed and the cycle continues giving rise to PCR products that were further subjected to agarose gel electrophoresis. The PCR dynamics in summary consist of 3 steps involving denaturation, primer annealing and elongation, the 3 steps were repeated in several rounds in various temperature range and time to achieve the set objectives. The summary of the steps and cycling Turn Down program is as shown below:

TD 58	
STEP 1	95 °C -9 min
STEP 2	94 °C 1 min
STEP 3	58 °C 1 min
STEP 4	72 °C 1 min
STEP 5	Repeat steps 2-4 x 34 times
STEP 6	72 °C 5 min
STEP 7	4 °C forever.

4.2.7 Agarose Gel Electrophoresis

1.5% agarose in 1x TAE buffer was prepared each round. The tank size was 150ml therefore, 2.25g in 150ml. Microwave for 2mins without covering –keeping an eye on the gel, while boiling, microwave was left opened intermittently until agarose melt. Fix up the tank, while the red rubber linings help seal the edges. Inserts the 2 combs, pour gel and allow to set. Remove tank with gel. Place in running tank ensuring the wells are well positioned to run from negattive to positive pole. Add 1xTAE buffer as the runing buffer. Ready to load, inactivate samples at 65^{oc} for 10min.Take out 4 µL gel loading buffer into 6 spots on parafilm. Add 15 µL of each sample and mix 3 times.Load 4ul of QX 174 DNA Hae III Digest visualized by ethidium bromide stainning marker. Load each sample. Sigma life Science Research SYBR Grade 1 nucleic acid gel strain 2 µL/1000 µL, 1500 µL of the gel loading buffer. Ran at 80V for about 1hr 15min. Polymerase Chain Reaction (PCR) products were separated on a 1.5% ethidium bromide-stained agarose gel electrophoresis.

4.2.8 Photography

Deoxyribonucleic Acid (DNA) bands were first visualized with an ultraviolet illuminator with goggles on, in order to check the migration patterns of the DNA bands. Consequently, photography of the DNA bands was taken using a photographic computer programme. The migration patterns of ampliers were compared with those of the positive controls (standard) of known molecular weight. The photographs of ampliers were scanned, as shown in the Tables of PCR results.

4.3.1 Amplification

The PCR was performed with thermostable Taq DNA polymerase (Amplitaq, Perkin Elmer Cetus, Nor-walk, Conn) in an automated DNA thermal cycler (PHC-1; Techne LTD., Cambridge, United Kingdom). The 50- µL sample to be analyzed was adjusted to a total volume of 100 µL in 1 x PCR buffer containing final concentrations of 125 µM each dATP dCTP, dTTP and dGTP; 0.2 µM each primer; and 1 U of *Taq* polymerase per 100 µL. Each sample was overlaid with 4 drops of mineral oil to prevent evaporation. Samples were denatured at 95°C for 1 min, and primers were annealed at 65°C for 1 min extended at 72°C for 1 min. A total of 35 cycles were performed. In the 35th cycle, the extension time was increased to 6 min.

4.3 RESULTS

4.3.3 Polymerase Chain Reaction Results and monographic pictures of ampliers

4.3.3.1 Sensitivity and specificity optimizing, of primers and standardizing of PCR Programmes for Nigerian strains of *Ureaplasma spp*

a. Sensitivity testing of primers and standardizing of PCR programmes (Fig 4.1)
b. Specificity testing of primers and standardizing of PCR programmes (Fig 4.2)

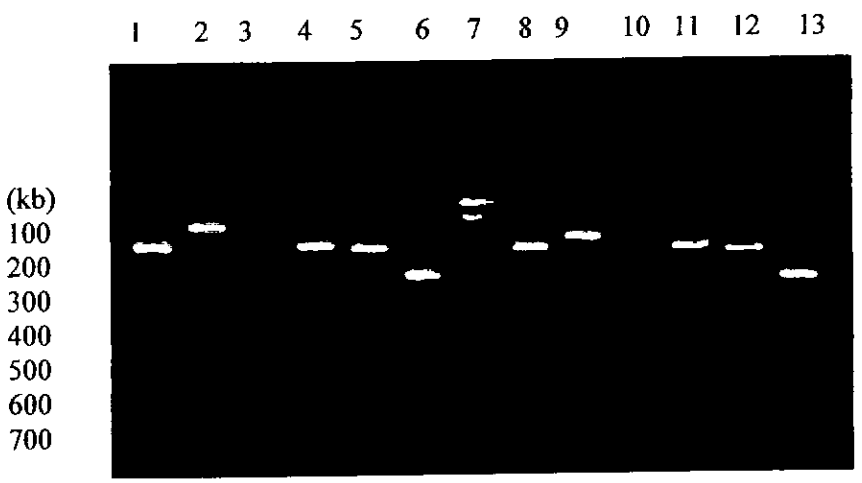

Figure 4. 1 Sensitivity testing of primers and standardizing of PCR programmes.

Legend= Lanes 1-13 contained 19 x buffers (2 µL), 2mMdNTP (2 µL) 25Mm $MgCl_2$ (2 µL), Taq Polymerase (0.15 µL), 5' Primers (1 µL), 3' Primers (1 µL), and Ultra pure H_2O (9.85 µL) as the reaction mixture in the PCR microtubes.

Table 4.5.: Test of species-specificity of primers for UU and UP, speciation.

Test of species-sensitivity of primers for Uu and Up using 6 pairs of primers			
Controls	**Lane**	**Template/Primer**	**Interpretation**
ATCC type strain	1	U8+UUA	Amplification
	2	UUS2+UUA2	Amplification
Ureaplasma	3	UU16f+UU16r	No amplification
urealyticum	4	UPS1+UPA	Amplification
	5	UP16f+UP16r	Amplification
Undiluted DNA	6	US2+UPA	Amplification
Marker	7	O X 174	Amplification
ATCC type strain	8	U8+UUA	Amplification
	9	UUS2+UUA2	Amplification
Ureaplasma parvum	10	UU16f+UU16r	No amplification
	11	UPS1+UPA	Amplification
Undiluted DNA	12	UP16f+UP16r	Amplification
	13	US2+UPA	Amplification

Legend= 2 ATCC positive controls were ran against 6 sets of primers for sensitivity testing.

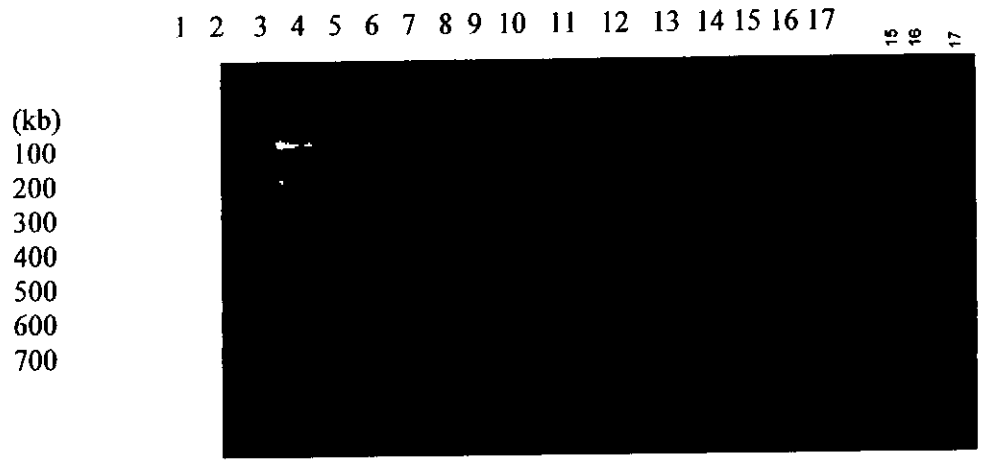

Figure 4.2 Specificity testing of primers and standardizing of PCR programmes.

Legend= Lanes 1-17 contained 19 x buffers (2 µL), 2mMdNTP (2 µL) 25Mm $MgCl_2$ (2 µL), Taq Polymerase (0.15 µL), 5' Primers (1 µL), 3' Primers (1 µL), and Ultra pure H_2O (9.85 µL) as the reaction mixture in the PCR microtubes.

Table 4. 6: Test of species-specificity of primers for UU and UP

TEST OF SPECIES-SPECIFICITY OF PRIMERS FOR UU AND UP			
	LANE	**TEMPLATE/PRIMER**	**INTERPRETATION**
Marker	1	O X 174	Amplification
ATCC type strain *Ureaplasma urealyticum* Undiluted DNA	2	U8+UUA	Amplification
	3	UU16f+UU16r	No amplification
	4	UPS1+UPA	No amplification
	5	UP16f+UP16r	Amplification
ATCC type strain *Ureaplasma urealyticum* Undiluted DNA	6	U8+UUA	Amplification
	7	UU16f+UU16r	Amplification
	8	UPS1+UPA	Amplification
	9	UP16f+UP16r	No amplification
Mycoplasma genitalium G 37 DNA undiluted	10	U8+UUA	Amplification
	11	UU16f+UU16r	No amplification
	12	UPS1+UPA	No amplification
	13	UP16f+UP16r	No amplification
Human DNA	14	U8+UUA	No amplification
	15	UU16f+UU16r	No amplification
	16	UPS1+UPA	No amplification
	17	UP16f+UP16r	No amplification

Legend= 2 ATCC positive and 2 negative controls were ran against 4 sets of primers for specificity testing.

4.3.3.2 Polymerase Chain Reaction Tests on Nigerians urogenital DNA templates

a. Test trials on 10 culture positive samples from LUTH (Fig 4.3)

b. Test trials on 10 culture positive samples from JUTH (Fig 4.4)

c. PCR tests on 238 Nigerian DNA urogenital samples. (Fig 4.5)

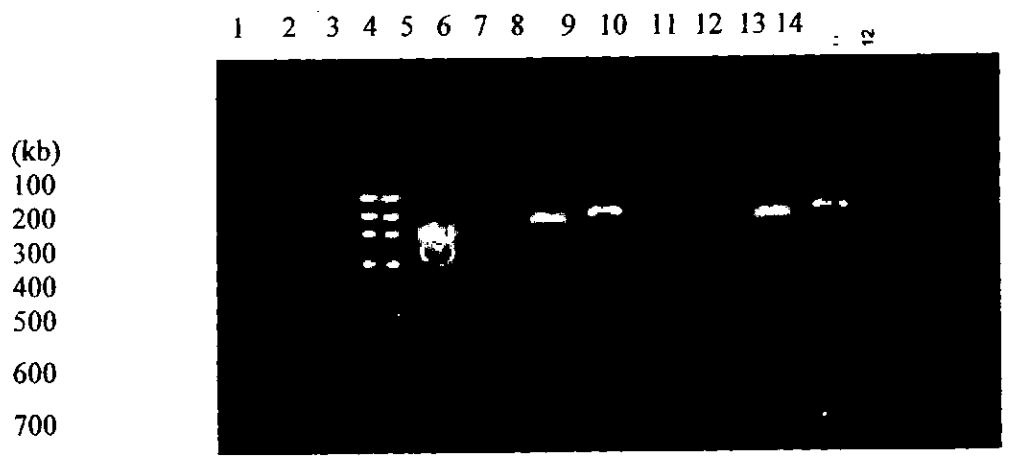

Figure 4.3 Test trials on 10 culture positive samples, from LUTH.

Legend= Lanes 1-12 contained 19 x buffers (2 µL), 2mMdNTP (2 µL) 25Mm $MgCl_2$ (2 µL), Taq Polymerase (0.15 µL), 5' Primers (1 µL), 3' Primers (1 µL), Ultra pure H_2O (9.85 µL) and the 10 DNA targets from the culture positive samples from patients and controls from LUTH site. Composed the reaction mixture in the PCR microtubes.

Table 4.7:Test trials on 10 culture positive samples from LUTH

Test trials on 10 culture positive samples			
Test	**Lane**	**Patient's case No**	**Results**
PCR-TD 58	1	Marker	Amplification
	2	L44	Amplification
	3	LC56	No amplification
	4	L77	Amplification
	5	LC77	Amplification
	6	L78	Amplification
	7	LC99	Amplification
	8	LC81	Amplification
	9	L4	No amplification
	10	L85	No amplification
	11	L101	Amplification
	12	UP + control (ATCC)	Amplification
	13	UP + control (Host)	Amplification
	14	-ve control (water)	No amplification

Legend=ATCC Up positive control ran in parallel with Adegboye's Up positive control to test the presence of *Ureaplasma parvum* from Nigerian DNA samples. L and LC means LUTH patients and LUTH controls respectively.

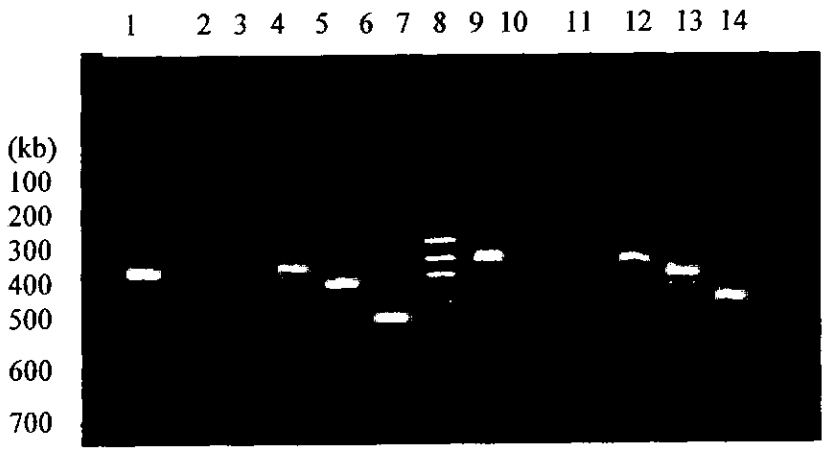

Figure 4.4 Test trials on 10 culture positive samples from JUTH.

Legend= Lanes 1-12 contained 19 x buffers (2 µL), 2mMdNTP (2 µL) 25Mm $MgCl_2$ (2 µL), Taq Polymerase (0.15 µL), 5' Primers (1 µL), 3' Primers (1 µL), Ultra pure H_2O (9.85 µL) and the 10 DNA targets from the culture positive samples from patients and controls from JUTH site. Composed the reaction mixture in the PCR microtubes.

Table 4.8: Test trials on 10 culture positive samples from JUTH.

TEST TRIALS ON 10 CULTURE POSITIVE SAMPLES			
TEST	**LANE**	**PATIENT'S CASE NO**	**RESULTS**
PCR	1	J10	AmplificationUU,UP
TD 58	2	J46	AmplificationUU,UP
	3	JC50	No amplification
	4	J71	Amplification
	5	JC77	No Amplification
	6	J78	Amplification UP
	7	Marker	Amplification
	8	JC80	Amplification UU
	9	J84	No amplification UU,UP
	10	JC85	No amplification UP
	11	J100	Amplification UP
	12	UU + control (ATCC)	Amplification
	13	UU + control (Host)	Amplification
	14	-ve control (water)	No amplification

Legend=ATCC Uu positive control ran in parallel with Nigerian Uu positive control, kindly supplied by Adegboye to test the presence of *Ureaplasma urealyticum* from Nigerian DNA samples. J and JC means JUTH patients and JUTH controls respectively.

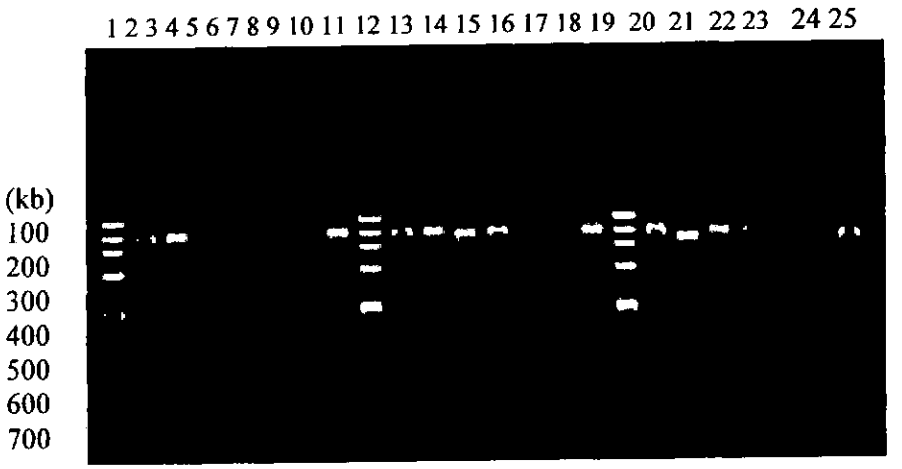

Figure 4.5 PCR tests model on 238 Nigerian DNA urogenital samples

Legend= Lanes 1-25 contained 19 x buffers (2 μL), 2mMdNTP (2 μL) 25Mm $MgCl_2$ (2 μL), Taq Polymerase (0.15 μL), 5' Primers (1 μL), 3' Primers (1 μL), Ultra pure H_2O (9.85 μL) and the DNA of target from the 238 participants, as the reaction mixture in the PCR microtubes.

Table 4.9: PCR tests model on 238 Nigerian DNA urogenital samples

Program	A Cross section of PCR results using ATCC standardized controls			
	Lane	Patient's Case No	UU	UP
TD50	1	Marker	Amplification	=
	2	J54	Amplification	=
	3	J75	Amplification	=
	4	L13	Amplification	=
	5	L33	Amplification	=
	6	L39	No amplification	=
	7	ve control water	No amplification	=
	8	UU +ve	No amplification	=
TD54	9	Marker	Amplification	=
	10	J54	Amplification	=
	11	J75	Amplification	=
	12	L13	Amplification	=
	13	L33	Amplification	=
	14	L39	No amplification	=
	15	-ve control water	No amplification	=
	16	UU +ve	Amplification	=
TD58	17	Marker	=	Amplification
	18	J54	=	Amplification
	19	J75	=	Amplification
	20	L13	=	Amplification
	21	L33	=	Amplification
	22	L38	=	Amplification
	23	ATCC UU	=	No amplification
	24	-ve control water	=	No amplification
	25	UP + control	=	Amplification

Legend=ATCC Uu positive control ran in parallel with Adegboye's Uu positive control to test the presence of *Ureaplasma urealyticum* from Nigerian DNA samples. J and JC means JUTH patients and JUTH controls respectively.

Table 4.10. Speciation of Ureaplasmas into the 2 biovars Up and Uu and prevalent rates of culture with PCR

Subjects (Patients with the history of NGU and infertility)	Prevalent rate of UU by culture	Prevalent rate of UU by PCR	Prevalent rate of UP by PCR	Prevalent rate of UP+UU (coinfection with the both biovars)	Prevalent rate of UP/UU (infection with either of the biovars)
Patient (n=119)	44 (36.97 %)	26 (21.85%)	49 (41.18%)	19 (15.97%)	66 (55.45%)
Control (n=117)	18 (15.39%)	15 (12.82%)	20 (17.09%)	3 (2.56%)	32 (27.35%)
Total (n=236)	62 (26.27%)	41 (17.37%)	69 (29.24%)	22 (9.32%)	98 (41.53%)

Out of the 119 infertile patients examined, 26 (21.85 %) patients were positive for *Ureaplasma urealyticum* by culture. Uu was detected in 44 (36.97%), while 49 (41.18%) Up isolate was detected out of the 119 cases by PCR technology. But of the 117 controls examined, 18 (15.39%) was positive by culture, whereas PCR assay revealed 15 (12.82%) and 20 (17.09%) of Uu and Up respectively.

4.4 DISCUSSION

Of the 119 infertile patients examined 44 (36.97%) patients were positive for Uu by culture, while 66 (55.45%) Uu were positive by PCR. The Uu and Up specific PCR assays distinctively detected *U. urealyticum and U. parvum* type strains, with an analytical sensitivity of 10 genomes per reaction. Uu was detected in 26 (21.85 %), while 49 (41.18%) Up isolate was detected out of the 119 cases. 16 (14.0 %) of 117 controls, only 18 (15.39%) was isolated by culture. PCR revealed 15 (12.82%) and 20 (17.09%) of Uu and Up respectively. In all, *Ureaplasma parvum* biovar was detected more from both patient and control groups, (Table 4.10). This might implies that Up biovar is more common among Nigerians attending STD clinics than Uu. The presence of the 2 biovars coinfection, in both patients (15.97%) and controls (2.56%) subjects have been reported by some earlier authors in similar studied population (Harasawa, 1999, Kong *et al.*, 1999 and 2000, Robertson *et al.*, 1986, 1993 and 2004). The last category involving subjects been infected with either Uu or Up at any point in time amounted to 55.45% in patients and 27.35% in controls. Speciation has been the limitations of my predecessors in this field; they have not been able to utilize high molecular technique such as PCR as employed in this study. Hence, they reported their prevalent rates based on culture which is not sensitive enough to differentiate ureaplasmal species in mixed infections (Table 1.2).

4.5 CONCLUSION

In conclusion, this result indicates that PCR is more sensitive and more specific than culture. Diagnostic technique by culture is unable to detect the biovar mixtures of *Ureaplasma spp* isolated; hence PCR technology was employed. Up Moreover, was detected more than Uu from both patients and controls, attending STD clinics in Nigeria. Therefore the parvo biovar might be responsible for the cases of chronic NGU and infertility seen in Nigeria, and not Uu as reported by the early authors limited to cultural isolation technique only. Therefore the findings from this project have paved ways for further use of high technology in mycoplasmology and other related fields.

CHAPTER FIVE

RAPID DETECTION OF *MYCOPLASMA GENITALIUM* BY REAL TIME –POLYMERASE CHAIN REACTION

5.1 INTRODUCTION

Mycoplasma genitalium has been incriminated in cases of urethritis, cervicitis and infertility in males by attaching to the spermatozoa (Busolo,1984) and tubal infertility in females (Collier *et al.*, 1990) and generally responsible to many cases of reproductive failures in couples (Gnarpe, and Friberg 1972). Mycoplasma genitalium is highly fastidious, cultural isolation technique is time wasting, unreliable, and serological methods lack sufficient sensitivity and specificity in acute phase of disease. An accurate detection is often available using vero cell line tissue cultures after several days of disease onset.

One of the more recent developments in diagnostic PCR is the homogeneous real-time PCR. The major advantage of this type of assay is the closed format, where amplification is detected without opening the PCR tube. Thereby, the risk of amplicon contamination is drastically reduced. Furthermore, real-time assays can be performed quantitatively with a wide dynamic range; the probe-based assays provide additional specificity to the assay, and the subjective reading of an ethidium bromide stained agarose gel is no longer needed. The first real-time PCR assay for *M genitalium* was reported by Yoshida *et al.*, 2002. This assay was employed for the qualitative and quantitative detection of *Mycoplasma genitalium* in a closed system reducing the risk of contamination by amplicon carry-over. I

The trend now is molecular diagnostic approach which is faster, more specific, permits biovar/serovar determination of Uu, and affords detection of highly fastidious mycoplasmas such as *Mycoplasma genitalium* (Ma *et al.*, 2004). This high PCR and RT-PCR molecular techniques are not yet available in Nigeria. This chapter is devoted for the account of the prevalence of *Mycoplasma genitalium* by RT-PCR technology. The record of the possible association of *Mycoplasma genitalium* with disease among patients with clinical cases of urethritis and infertility attending STD clinics in Nigeria was also enumerated.

5.2 MATERIALS AND METHODS

5.2.1 REAL-TIME-POLYMERASE-CHAIN REACTION ASSAY

Mycoplasma genitalium specific genes were aligned, primers selected and probes designed and used in a RT-PCR detection assay. This protocol was previously developed in the host laboratory at LSUHSC (Ma *et al.*, 2004). It was adapted to rapidly detect, and identify *Mycoplasma genitalium* from the two hundred and thirty six (236) DNA samples processed for the Real time-PCR technology. *Mycoplasma genitalium* specific genes were aligned, primers selected and probe designed and used in a RT-PCR detection assay. Analytical specificity of the assay was evaluated using representative type strains from ATCC and sensitivity was determined by 10-fold serial dilutions of *Mycoplasma genitalium* genomic DNA. Real-time PCR was performed in a Smart Cycler instrument (Cepheid) with Taman propes R-PCR version (TaKaRa Bio., Japan). Amplification was achieved with 40 cycles of denaturation at 94°C for 10 sec, renaturation at 55°C for 30 sec, and elongation at 72°C for 30 sec, after the initial denaturation at 94°C for 30 sec. The experimental lay out were shown in Table 5.1 below:

Table 5.1: Real-Time-Polymerase Chain Reaction Experimental lay out.

	1	2	3	4	5	6	7	8	9	10	11	12
A	H_2O	H_2O	J	JC	J	JC	J	JC	L	LC	L	LC
B	3 STD	3 STD	J	JC	J	JC	J	JC	L	LC	L	LC
C	10STD	10STD	J	JC	J	JC	J	JC	L	LC	L	LC
D	30STD	30STD	J	JC	J	JC	J	JC	L	LC	L	LC
E	300STD	300STD	J	JC	J	JC	L	LC	L	LC	L	LC
F	3000STD	3000STD	J	JC	J	JC	L	LC	L	LC	L	LC
G	30000STD	30000STD	J	JC	J	JC	L	LC	L	LC	L	LC
H	300000STD	300000STD	J	JC	J	JC	L	LC	L	LC	L	LC

A1 and A2 are negative controls. B-H constituted the positive controls and they were all duplicated in that order. Columns 3-12 made up the real experimental lay out consisting of J and JC for JUTH patients and controls respectively, while L and LC connotes LUTH patients and LUTH controls respectively. The plate in this format is inserted into the RT-PCR machine along with the master mix shown in Table 5.2 below.

Table 5.2: Real-Time-Polymerase Chain Reaction Master Mix lay out.

PCR mixture (25ul)	
1. Taqman master mix (2x)	12.5
2. MG190F (5' primer, 20Um)	1.25
3. 190R3 (3' primer, 20Um)	1.25
4. MG190P (probe, 5Um)	1.25
5. IPC probe (5Um)	1.25
6. IPC (150 copy)	2.5
7. STD/DNA	5
PCR cycling program: MG190 (50^{O}C, for 2min; 95^{O}C for 10min; and 50 cycles of 95OC, for 15 sec and 60^{O}C, for 1min).	

This was the composition of the master mix for the RT- PCR work. Most paramount were the primers which bind to the corresponding target DNA and the probes that signify the binding reaction by fluorescence indicating the real time the binding occurred. The reactions occurred all at the same time in all the 96 wells per reaction cycles.

5.3 RESULTS

Fluorescence readings were taken throughout the experiments. The results were linear over a 3-log range, from 104 to 10 cfu/assay, demonstrating a strong linear relationship ($r2 > 0.995$) between the Ct values and the log10 of the input number of mycoplasma cells. Real-time monitoring of the release of fluorescence several times during each cycle allows collection of abundant data. After 40 cycles, data were processed by the software within seconds. Data can be viewed in an "amplification window" in the analysis program (Figure 5.1). This allowed the operator to check the fluorescence from each reaction at each cycle. The linearity of the fluorescence response for each sample at each cycle and the baseline could be checked for each tube.

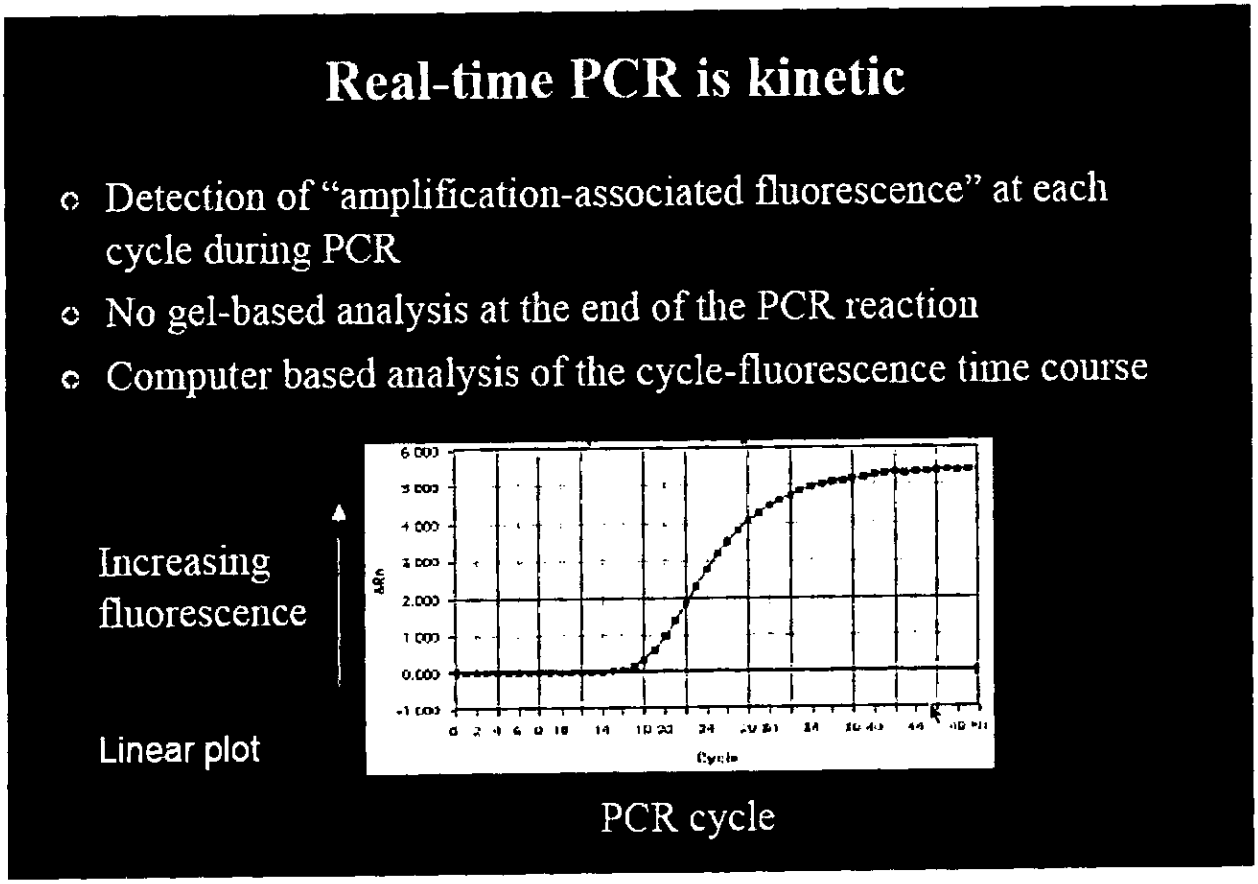

Figure 5.1 Mycoplasma Real-Time PCR Kinetic

This is a typical example of the graphical representation of RT-PCR kinetic operations. As shown, the chart showed that fluorescence is directly proportional to PCR cycle. And since the reaction process and results were feasible as it was happening, there was no need for carrying out the agarose gel electrophoresis post RT-PCR operation as the practice with conventional PCR.

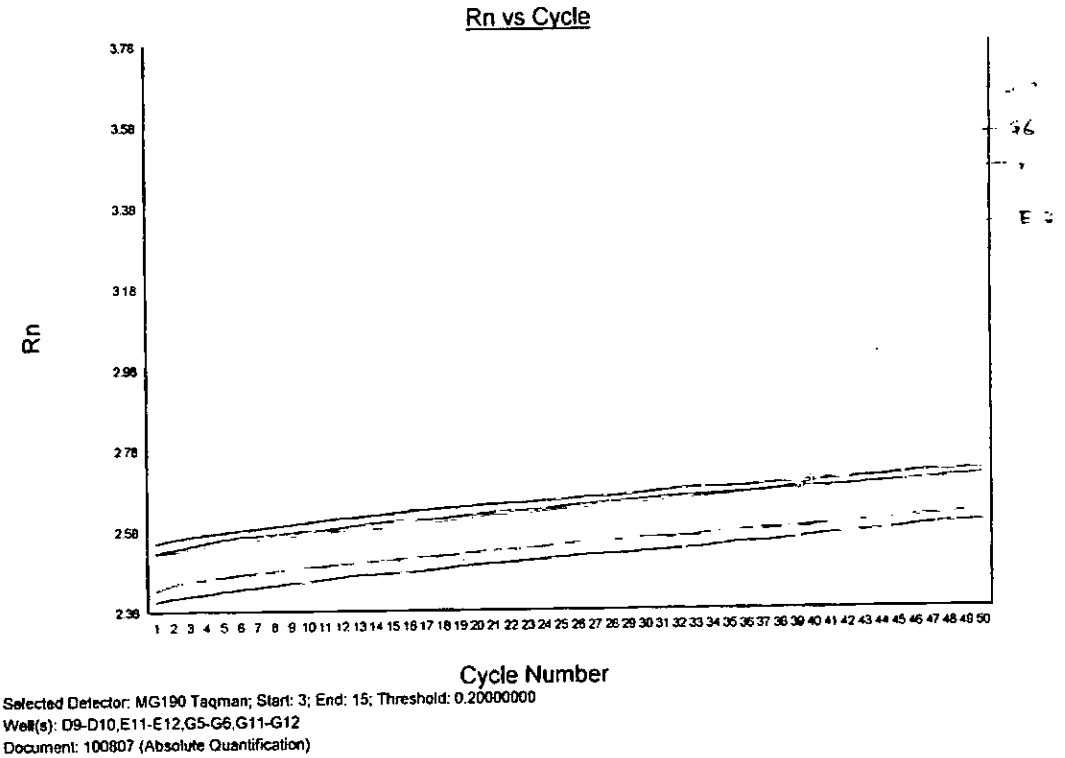

Figure 5.2 Real-Time PCR result showing the computer generated data outputs, post fluorescence time course.

This was the amplification curve of each sample depicted by lines E12, G12, G6 and D10.All negative samples were parallel to the horizon and never crossed the base line called the threshold cycle (C_T) ie the cycle at which the amplification curve of target positive sample crossed the base line. As the binding occured, migration of the amplicons continued to show at the real time per round as shown in Fig. 5.2 above.

Table 5.3.1: Prevalent rate of *Mycoplasma genitalium* in the overall participants

	MG by RT-PCR		
	Males	**Females**	Total
Patient	6 (18.75%)	11 (12.64%)	17 (14.29%)
Control	1 (3.13%)	6 (7.05%)	7 (6.0%)
Total	7 (10.94%)	17 (9.88%)	24 (10.17%)

As depicted in Table 5.3.1 above, the prevalent rate of *Mycoplasma genitalium* (Mg) in patients is higher than those recorded against the controls. Hence Mg might be responsible for the cases of urethritis and pregnancy complications such as infertility in the group of patients examined in this study.

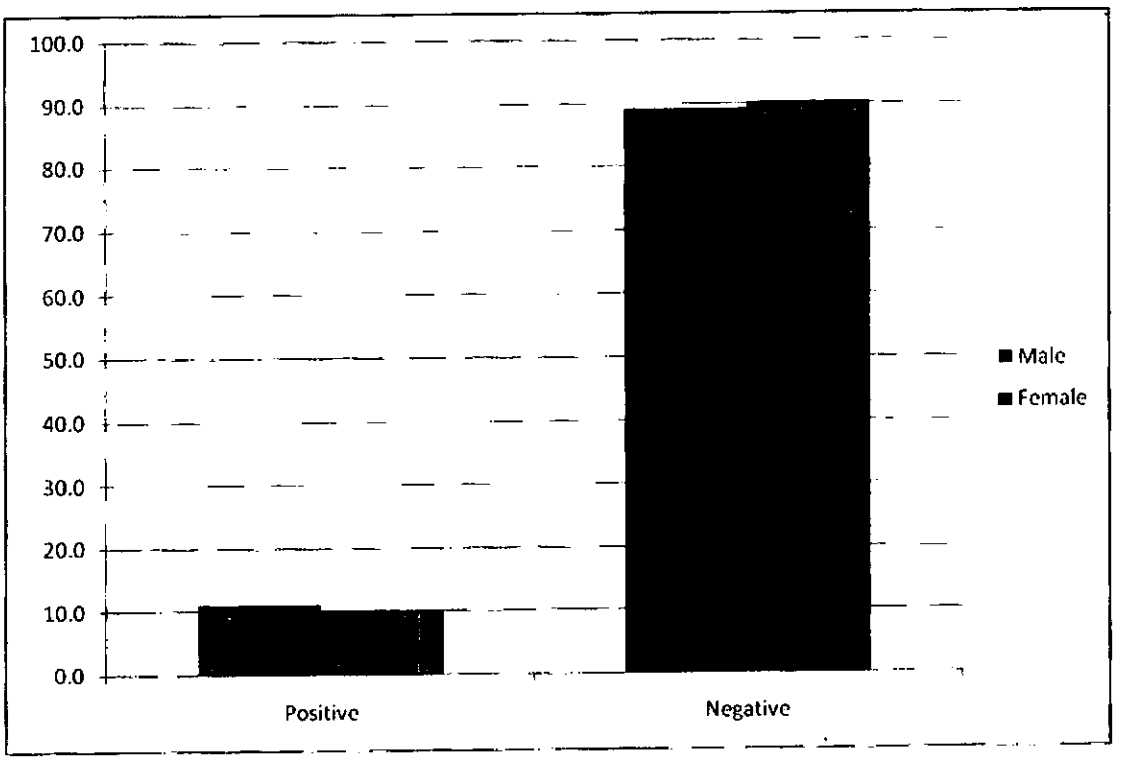

Figure 5.3.1 Prevalent rate of *Mycoplasma genitalium* in male and female patients

As shown in Fig. 5.3.1 above, the prevalent rate of *Mycoplasma genitalium* in male patients is higher compared to those of the female counterparts.

5.3.5 Overall Summary of *Mycoplasma genitalium* prevalence in the participants

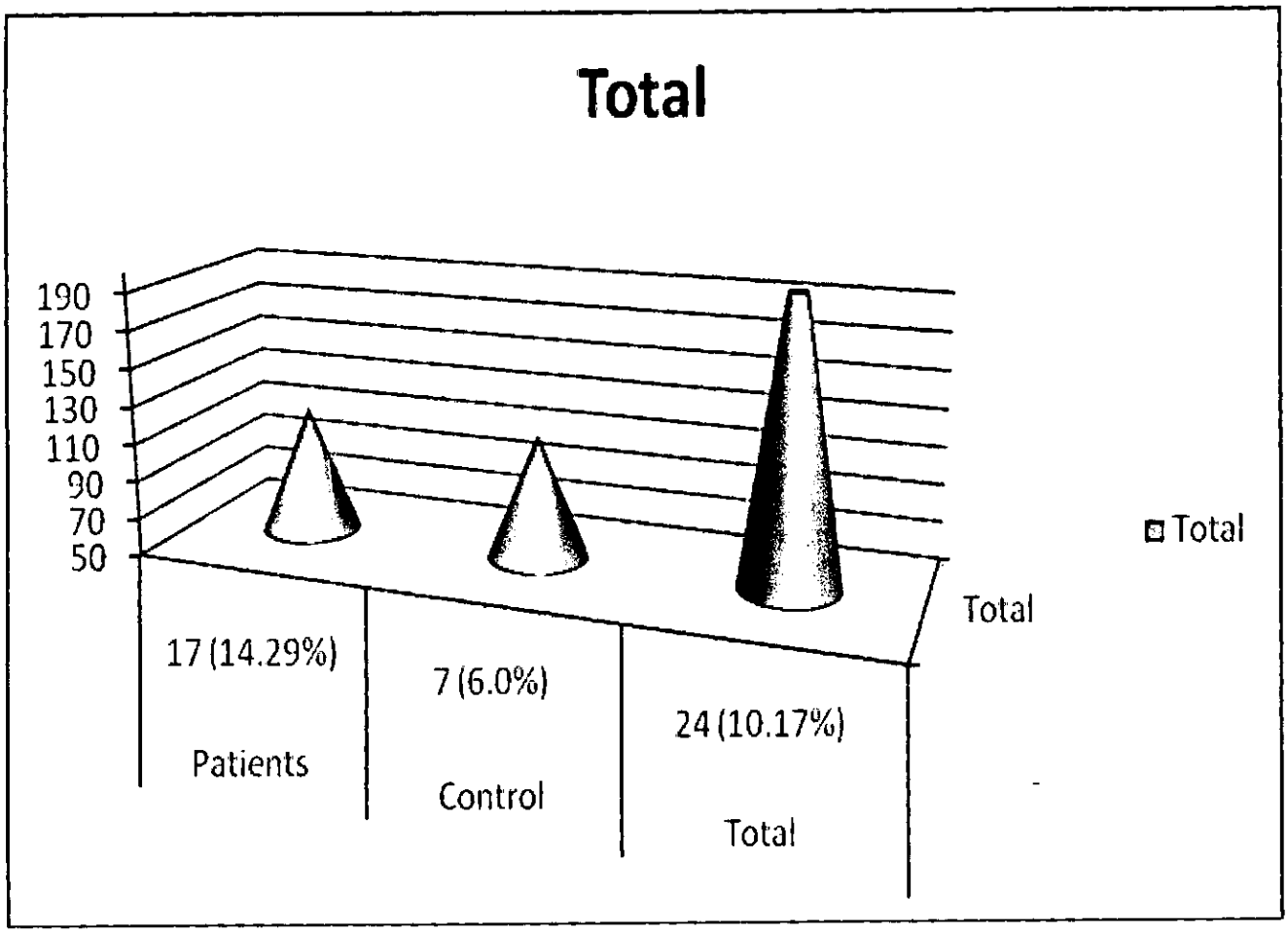

Figure 5.3.2 Prevalent rate of *Mycoplasma genitalium* in the overall participants

The prevalent rate of *Mycoplasma genitalium* in patients is higher than those recorded against the controls (Figure 5.3.2). Hence Mg might be responsible for the cases of urethritis and pregnancy complications such as infertility in the group of patients examined in this study.

5.4 DISCUSSION

As shown above, *Mycoplasma genitalium* was detected in 11 (12.64 %) of 119 cases and 6 (7.05 %) of 117 controls, in the female subjects (Table 5.3.1). While in male subjects, *Mycoplasma genitalium* was detected in 6 (18.75 %) of 119 cases and 1 (3.13 %) of 117 controls. The overall prevalence amounted to was 7 (10.9%) in males and 17 (9.9%) in females males and females respectively (Figure 5.3.1).. RT-PCR technology was specifically employed for the rapid detection of *Mycoplasma genitalium* and results showed a prevalent rates of 11 (12.64%) in patients and 6 (7.05%) in controls respectively. Of the 119 patients studied, only 11 (12.64%) harboured Mg and only 6 (7.05%) of the control subjects harboured the organism respectively (Figure 5.3.2). The higher rate in patients than in controls supported previous studies in Nigeria and various other parts of the world including. World prevalence rate 10%-45%. West Africa, 10%, Ibadan, 14% Current studies, 14% Jos, 18%, Lagos, giving an average of 16% in Nigeria in men with NGU. More people were found with NGU than in asymptomatic controls being a common cause of NGU in men and more frequently isolated from sexual partners of infected women. RT-PCR technology was specifically employed for the rapid detection of *Mycoplasma genitalium* and results showed prevalent rates of 11 (12.64%) in patients and 6 (7.05%) in controls respectively (Figure 5.3.2). However, culture remained the fundamental procedure in clinical diagnosis, where an organism could be morphologically pinned down.

5.5 CONCLUSION

The overall prevalence detected in the 236 sampled population amounted to 24 (10.17%) in the males and females altogether. The association of *Mycoplasmas genitalium* and NGU was strongest in subjects <35 years of age ($P \leq 0.050$). This project reports *M genitalium* in Nigerian patients for the first time, *Mycoplasma genitalium* may be responsible for cases of non gonococcal urethritis and infertility in males and females Nigerians.

CHAPTER SIX

STATISTICAL CORRELATION OF STD RISK FACTORS WITH EPIDEMIOLOGY OF HUMAN GENITAL MYCOPLASMOSIS

6.1 INTRODUCTION

Infants presumably become colonized with genital mycoplasma during passage through the birth canal, infants delivered though cesarean procedure are colonized to a lesser extent than those delivered vaginally (Cassel *et al.*, 2001). Ureaplasmas have been isolated from the genitalia of up to one third of infant girls and from a smaller proportion of infant boys. (Cassel *et al.*, 2001).Neonatal colonization tends not to persist. There is a progressive decrease with age in the proportion of infants who are colonized. Genital mycoplasma is often recovered from urine or genital specimens from prepubital boys (Taylor-Robinson *et al.*, 2003). Whereas 9-22% of prepubital girls have been found to be colonized with *U. urealyticum*, and 8-95 with *M.. hominis* (Taylor-Robinson *et al.*, 2003).

After puberty colonization occurs primarily as a result of sexual activity (Cassel *et al.*, 2001 and Taylor-Robinson *et al.*, 2003). Sexually matured persons with no history of sexual contact are infrequently colonized with genital mycoplasmas. Among those who are sexually experienced, colonization increases with increase in the number of different sexual partners. It is interesting to note that colonization increases more rapidly with increasing sexual experience in women than in men, suggesting that women are more susceptible to colonization with these organisms. Infections of the adult male genitourinary tract by ureaplasmas may be transient (Taylor-Robinson *et al.*, 2003) with the organism disappearing spontaneously, or it may be persistent.

Genital Mycoplasmas are more frequently isolated from black men and women (Centers for Disease Control and Prevention, (2000) than in than from whites. The extent to which this differences are due to differing sexual experience was backed up by a carefully controlled study for sexual experience of the subjects, black women were colonized more often with *M. hominis* and *U.urealyticum* than were other women.

Socioeconomic status is also related to genital colonization with Mycoplasmas. One group (Bonnin *et al.*, 1995) isolated *M. hominis* from 53.6% and Ureaplasmas from 76.3% of patients treated at clinics at a municipal hospital as compare with the isolation of *M. hominis* from 21.3% and ureaplasmas from 52.9% of patients visiting private obstetricians and gynaecologists in the same area. Whether socioeconomic differences or other environmental factors could be involved abound, but it appears that the prevalence of colonization with *M. hominis* (Taylor-Robinson *et al.*, 2003) and *U. urealyticum* decreases after the menopause.

Assessing morbidity and mortality for diseases specifically caused by genital mycoplasmal infections is difficult because few studies systematically evaluate them and some conditions with which they are involved can be polymicrobial (e.g., pelvic inflammatory disease, urethritis). Difficulty in detecting the more fastidious species, such as *M.→ genitalium* and *M. fermentans*, further complicates such assessments. In adults with an intact and functional immune system, infections associated with genital mycoplasmal organisms are usually localized and do not result in severe illness, attesting to their relatively low virulence and perceived status as opportunists. In the western world *Ureaplasma species* have been isolated from cervicovaginal specimens in 40-80% of women who are asymptomatic and sexually active. *M. hominis* has been isolated from cervicovaginal specimens in 21-53% of women who are asymptomatic and sexually active. These rates are somewhat lower in males. Only subgroups of adults who are colonized in the lower urogenital tract develop symptomatic illness from these organisms.

In Nigeria several risk factors namely age, occupation, gender, number of sexual partners, sexual frequency, level of education, the use of barrier cautions, such as condom and IUDs have been associated with STD prevalence. These risk factors have been studied in relation with the sources, incidence, prevalence, course and transmission of STD pathogens and none specifically addressing human mollicutes in Nigeria. In addition, recent studies have associated some mycoplasmas in HIV/AIDS pandemic. Moreover, W.H.O.,(2001) has also expanded the curable STD pathogens to include non specific agents of NGU such as mycoplasmas. These constitute the statement of problems that led to the study on the risk factor assessment and demographic profile of human genital mycoplasmas in Nigeria. This chapter covers the record of the statistical correlations of some risk factors in the prevalence rates of genital mycoplasmas in Nigeria.

6.2 MATERIALS AND METHODS

There was an initial briefings on the purpose of the research and health education on the transmission and public health significance of genital mycoplasmas and its relationship with other STD pathogens. A questionnaire covering demographic information, health status, number of sexual partners, history of STI, use of contraceptives, primary and secondary clinical conditions was administered to the 824 participants. Swabs stick and biros were distributed to the participants for labeling of samples and filling of the forms. In assisting participants in completing the forms more appropriately, colloquial languages were employed where required. The research procedure was safe, simple, and free and subjects were not exposed to further risk of infections. Patient's consent was sought hence participation was voluntary and unanimous; furthermore patients' anonymity, confidentiality and integrity were maintained throughout the study and thereafter.

6.2.1 Study design

Study design employed was a descriptive epidemiological study involving 5 study centers (3 in LUTH and 2 in JUTH). LUTH, Departments of Obstetrics Gynecology and Surgery (Cytology and Urology units) and LUTH's Medical Microbiology laboratory diagnostic unit. JUTH, Special Treatment Clinics and JUTH's Medical Microbiology laboratory diagnostic unit. Federal Government of Nigeria Reference Centers for HIV/AIDS / Sexually Transmitted Infections (STI), Lagos University Teaching Hospital, (LUTH) and clinics in the South, Jos University Teaching Hospital, (JUTH) and clinics in the North.

6.2.2 Study population

The studied population was STD patients who presented with discharge, itching, ulcerations and sores in genital parts, currently or recurrent abortion, as well as patients with various degrees of infertilities in LUTH and JUTH. These are among the hospitals in Nigeria where one can have maximum level of representation of Nigerians attending STD clinics from the Northern and Southern parts of Nigeria. This is because of their geographical and political locations, commercial activities endowment, ethnic diversities and being the Federal Government Reference Teaching Hospitals for HIV/AIDS /STID patients in Nigeria. The control group in this study was the asymptomatic patients attending antenatal, prenatal, or postnatal clinics, as well as their accompanying husbands or relatives. Others included those of whom employers requested for medical tests and individuals referred by the religion

leaders for HIV screening on marriage counseling. Infection rates and proportions of genital mycoplasmas among the symptomatic and asymptomatic patients attending STD clinics in JUTH and LUTH were compared. Males and Females data were also compared to see the concordance in statistical association using some demographic and clinical variables. These include the different STD clinics used, state of origin, corresponding LGAs, ages, occupation, level of education, sexual history (i.e. when debut, when married, no of children, no of abortion, sexual frequency, whether pregnant or nursing), no of sexual partners in the past 3 months, history of STI, use of IUDs, occupation, marital status, gender, primary illness (NGU) and secondary illness (Infertility) involved. The statistical analysis employed was the SPSS (statistical package) version 11 and Microsoft Excel.

6.2.3 Samples size and sampling method.

The qualitative case controlled study as described by Schlesselman (1982) was employed in the two metropolitan cities' hospitals in the Northern and Southern Nigeria. The study aimed at investigating the relationship among the genital mycoplasmas studied, and reproductive maladies such urethritis, cervicitis, pelvic inflammatory diseases, spontaneous abortion and various degrees of infertility among male and female patients attending STI clinics in Nigeria. A non-probability sampling method, known as convenience sampling method, was used. The sample size was determined using the formula recommended by WHO (1993), as shown chapter 2, since the participants are the same. Questionnaires were administered to 824 participants made up of equal number of male and female subjects from either of the teaching hospitals. JUTHLUTH Site's breakdown included 70 males with STI symptoms, 180 females with STI symptoms, 70 asymptomatic males and 92 asymptomatic females amounting to 412 participants. JUTH Site's Breakdown included, 70 males with STI symptoms, 180 females with STI symptoms, 70 asymptomatic males, 92 asymptomatic females amounting 412 participants all together. The participants through the questionnaires were drawn from various states in Lagos and Jos, Nigeria. Lagos as a metropolitan city, typically representing the entire nation, in population diversities, ethnicity, religion, culture and industrial nuclei of the country. Similarly Jos, an urban city in the north mimicking Lagos in all its endeavours, except, of her lower socioeconomic status and industrialization. The respondents residing in these cities from the 37 states of Nigeria make use of these facilities for their health problems. This study seized the advantages of the location of these teaching hospitals for this research.

6.3 RESULTS

The completed questionnaires bearing the case no, specimen no were separated into JUTH and LUTH and handled confidentially. The results were collated and analysed using the SPSS (statistical package) version 11 and Microsoft Excel. Students T-test was used to compare the prevalence of genital mycoplasmas and the risk factors examined. The X^2 Chi-square test and the Yates correlation co-efficient values were used to compare proportions. Mean values were expressed as mean +/- standard deviation. P values ≤ 0.05 were considered as significant Chi square analysis. The data generated against the risk factors examined in the study was tested statistically and it was discovered that history of STI, number of sexual partners, use of condon, age, occupation and level of education, had their P values ≤ 0.05. When data outputs were further subjected to Chi square analysis those that their p values were less than or equal to 0.05 were considered to be significant (Tables 6.1 and 6.2) and (Figs 6.1 to 6.8).

The frequency of mycoplasma infection is more in females than in males except for *Mycoplasma genitalium*. It was also discovered that the use of condom against MG was statistically significant, (P = 0.024) apart from the integrity of the object, this organism is known to be motile; hence it could migrate away from the reach of the barrier caution employed. I then compared the responses from the questionnaires of the symptomatic and asymptomatic subjects from whom Uu, Up and Mg were recovered (n = 119) to those obtained from the normal subjects (n = 117) from whom no history of sexually transmitted pathogens reported. Both cohorts averaged 21 years of age and were almost entirely heterosexual (99%). In addition, the participants were sexually active for at least the previous 3 months and were with similar numbers of partners. However, low income occupations (P = 0.0001), less educated and past diagnoses of STD (P = 0.0377) were significantly more common among subjects infected with genital Mycoplasmas. In addition, barrier methods to prevent STD, e.g., condoms, were not used consistently by either group, though significantly (P = 0.0024) more symptomatic subjects reported occasional use. More notably, mycoplasmas were recovered from six (29%) and five (16%) symptomatic men and women, respectively, despite their claims that condoms were always used. The most significant in this study is the history of sexually transmitted infections, since patients attending STD clinics were the sampled population.

All Respondents

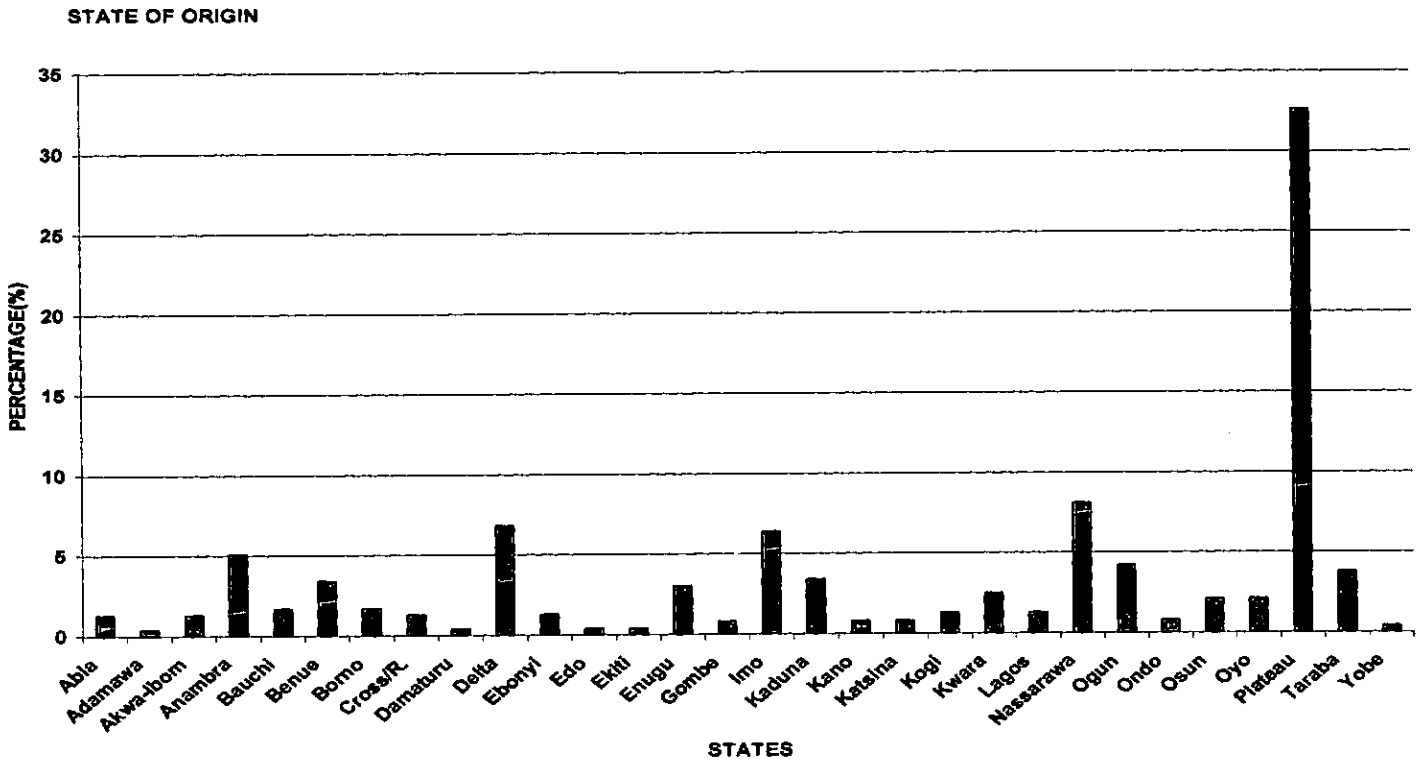

Figure 6.1 Distribution of respondents by state of origin in Nigeria.

The figure 6.1 above depicts the distribution of respondents by state of origin in Nigeria. The geographical spread of the participants drawn from 30 out of the 37 states of Nigeria. When it was analysed as shown above Delta and Imo states were bracketed among the states in the southern part of the country. While Plateau state has the highest no of participants, seconded by Nassarawa state.

Table 6.1: Demographic profile and disease distribution among subjects examined

(Patients and Controls)

AGE (yr)	**Patient (%)**	**Control (%)**
11-15	6.7	2.6
16--20	8.5	8.4
21--30	33.6	35.9
31--40	41.2	40.2
41--50	12.6	11.1
51--60	2.5	2.6
71+	1.7	1.7
Age of debut (yr) age of first sexual experience	**Patient (%)**	**Control (%)**
11--15	14.3	6.8
16--20	41.2	35.9
21--25	18.5	16.2
26--30	5.9	10.3
31--35	0.8	0
36+	3.4	0

Table 6.1 above displays the demographic pictures of participants including, age as in birth, and age of first sexual experience(age of debut). These factors were compared between patients and controls, and the outcome depicted the fact that prevalence rates were higher within factors and patients more than the control groups. The tables and histogram displayed the compared data outputs of the respondents' based on the questionnaire administered at the clinic sites.

Table 6. 2: Risk factors data on age-matched subjects from the study sites (JUTH and LUTH)

FACTOR	HOSPITAL (%)	
Age (yr)	**JUTH**	**LUTH**
11--15	13.0	0
16--20	41.2	35.9
21--30	35.7	32.9
31--40	39.0	43.9
41--50	8.4	18.3
51--60	3.9	0
71+	0	4.9
Age of debut (yr) age of first sexual experience	**JUTH**	**LUTH**
11--15	13.0	6.1
16--20	45.5	25.6
21--25	16.2	19.5
26--30	7.1	9.8
31--35	0	1.2
36+	0	4.9

Table 6.2 shows the demographic picture including, age as in birth, and age of first sexual experience. These factors were compared between patients and controls, and the outcome depicted the fact that prevalence rates were higher within factors are higher among patients from JUTH than the patients from LUTH. The tables and histogram displayed the compared data outputs of the respondents' based on the questionnaire administered at the clinic sites.

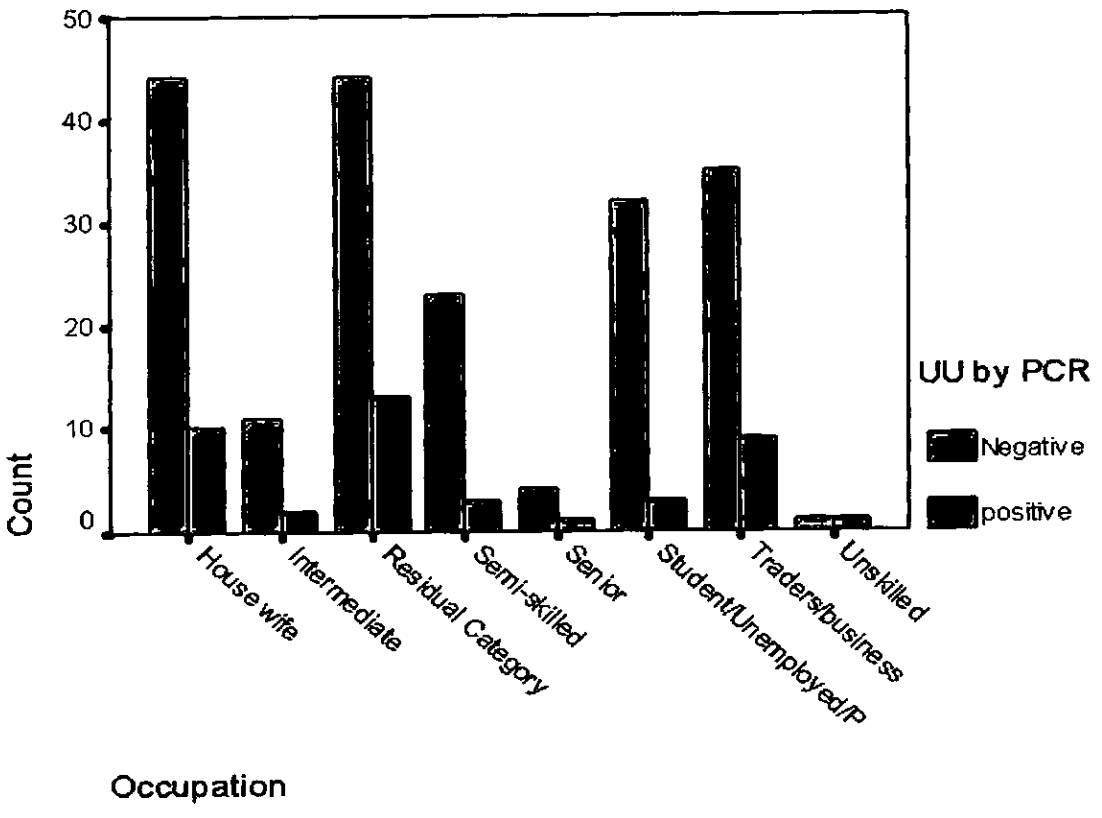

Figure 6.2: Risk factor by occupation

The histogram above (Figure 6.2) compared the prevalence of STI among the occupational groups regarded as risk factors in STI transmission. These factors include occupations such as long distant drivers, guards, police, soldiers (residual group); others are the petty traders, tailors, hoteliers, and commercial sex workers groups, which are found to be more prone to STI. This is because these groups are likely to engage in multiple sexual acts due to the nature of their vocations. Chi-Square Tests revealed, Chi-square =16.562, DF = 7, P = 0.020. Occupation is one of the socioeconomic parameters considered in this study, which is statistically significant, P value of 0.020, for this risk factor proved that the occupational status of an individual has influence on the prevalent rates of genital mycoplasmas as shown in Figure 6.2. in which the frequency was highest in the residual category. This might be due to poor income, the level of knowledge on sex education, hygiene and general life styles that enhances the transmission of the organism.

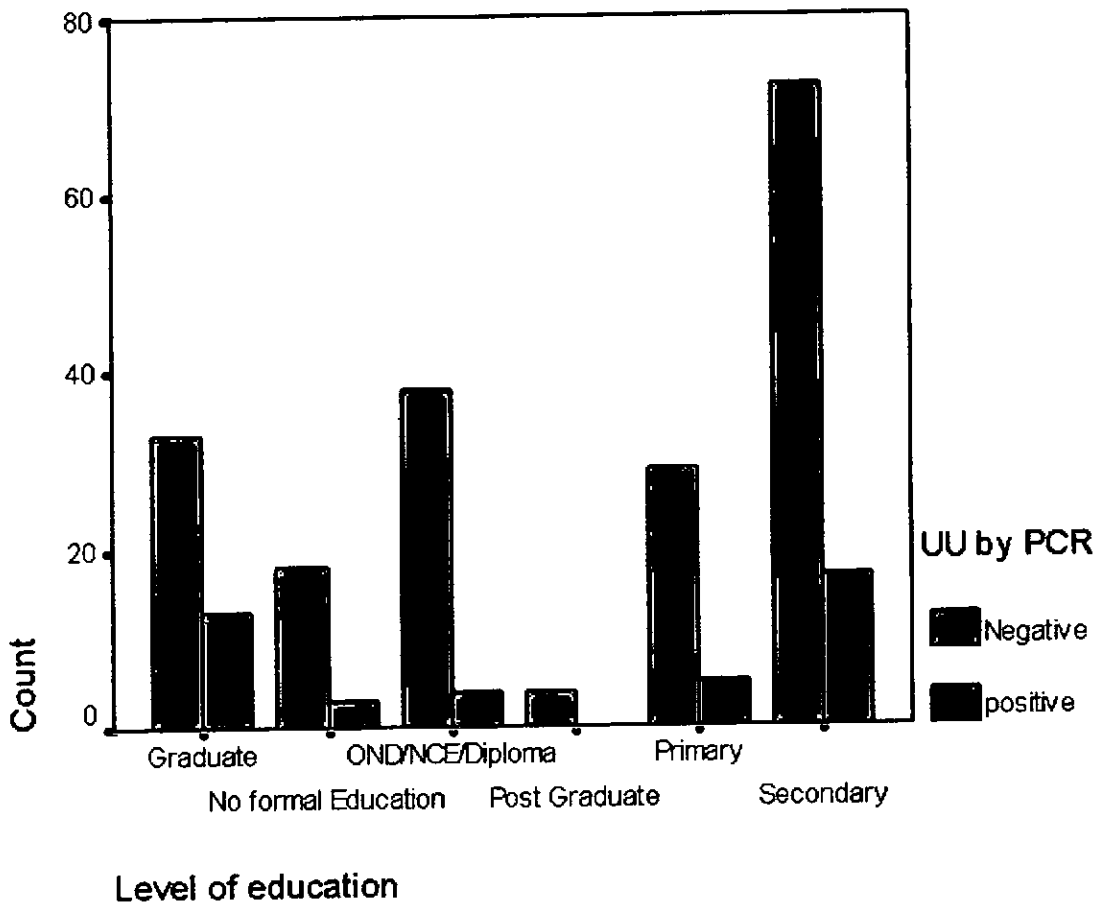

Figure 6.3 Risk factor by education

Chi-Square Tests revealed, Chi-square =12.853, DF = 5, P = 0.025. One of the socioeconomic parameters examined was level of education, the P value of 0.025; for this risk factor proved that the level of education in an individual/group has influence on the epidemiology trends of genital mycoplasmas and spread, as shown in Figure 6.3.

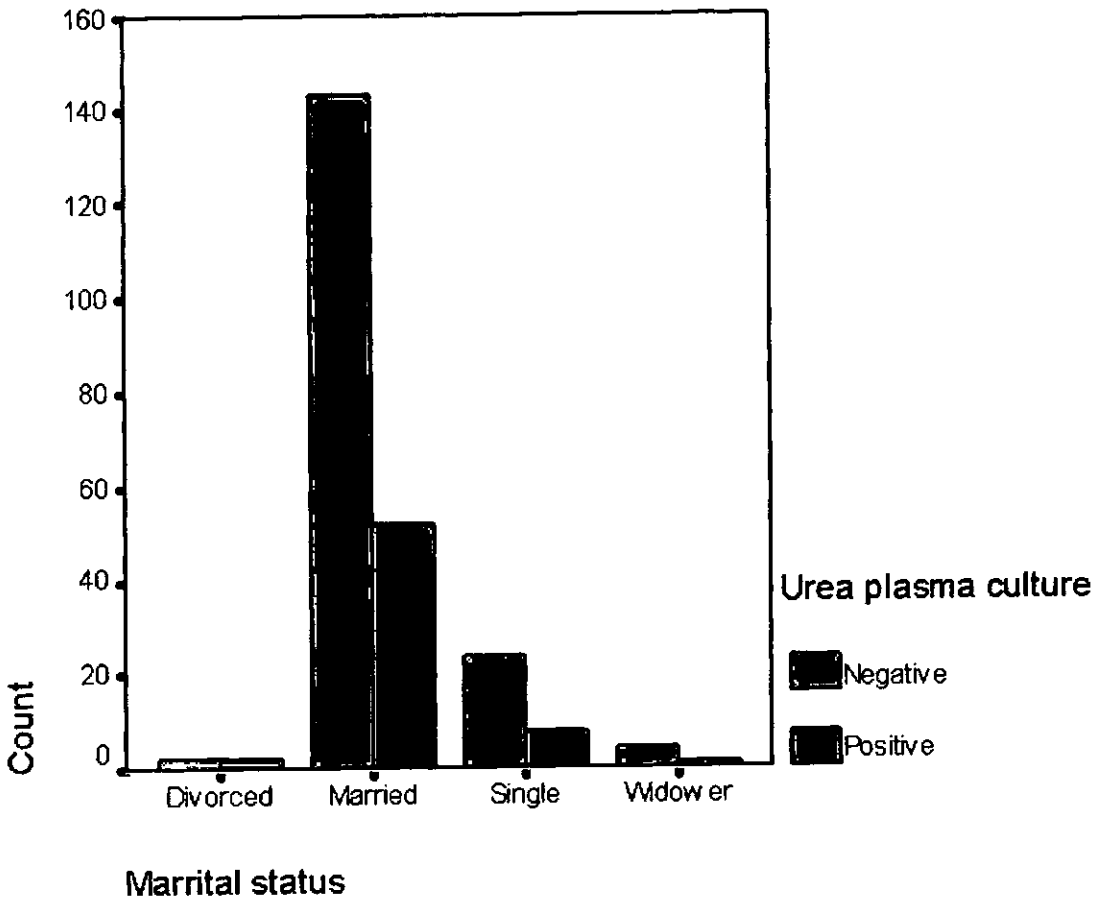

Figure 6.4 Risk factor by marital status

Chi-Square Tests revealed that Chi-square =6.024, DF = 1, P = 0.014

As presented in Figure 6.4 the participants that were married were acquiring the disease from sexual partners, whereas, singles had lower prevalent rates, P value is 0.014. One may therefore infer that sexual activities could have some relations with prevalence of genital mycoplasmas in marriage and outside marriage.

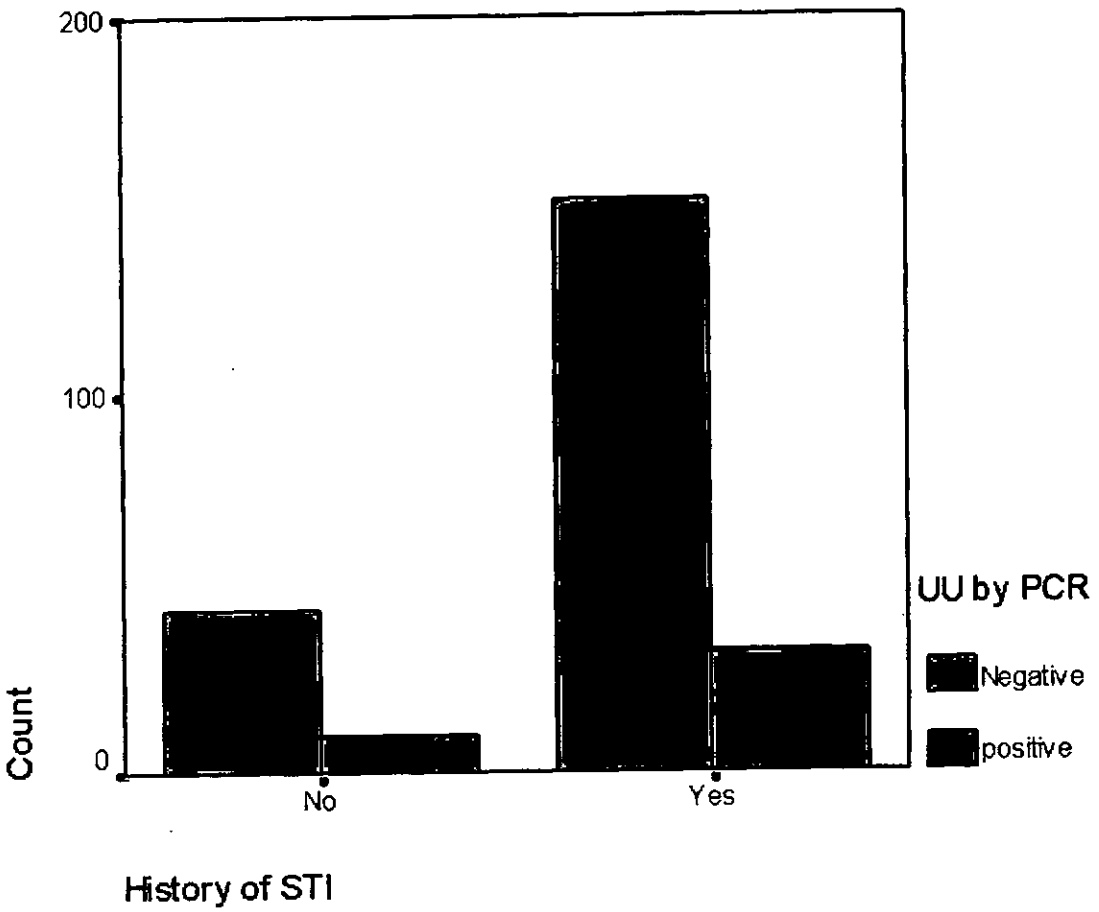

Figure 6.5 Risk factor by STI

As shown in the histogram (Figure 6.5) above, the prevalence of Uu infections were higher in those with the patients with records of STI and low in those patients without STI. This implied that mycoplasmas could exhibit a chronic course and when all had been taken care of, regarding the management of other co-infections, such as STT; infection could still remained if organism were not susceptible to the antibiotics used. Chi-Square Tests revealed that Chi-square =4.700, DF = 1, P = 0.030. The association of ureaplasma infections with history of sexually transmitted infections was statistically significant in this study P value being 0.030, ie STI has to do with mycoplasmas spread it implied that sexual act could enhance its spread, as shown in Figure 6.5 above. Mg prevalence on the other hand revealed that Chi-Square Tests=0.040, DF = 1, P = 0.841. The prevalence of Mg in patients without the history of STI was higher than those with STI. This implied that the urethritis seen or infertility might be solely caused by Mg order than the other agents of NGU/infertility.

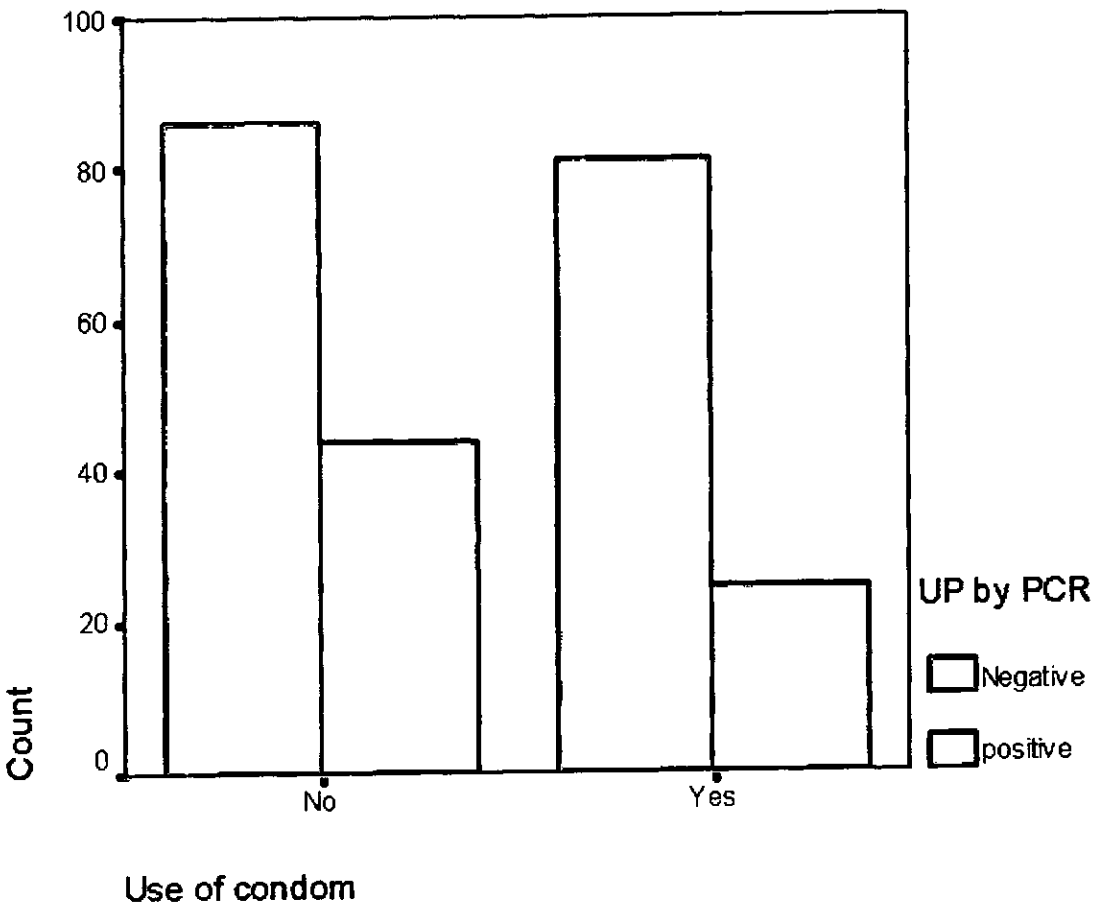

Figure 6. 6 Risk factor by Condom

Chi-Square Tests revealed that Chi-square =5.109, DF = 1, P = 0.024. The use of condom as a barrier caution devise was tested and found truly protective in the transmission of Up, P value being 0.024 hence it was statistically significant, as shown in Figure 6.6

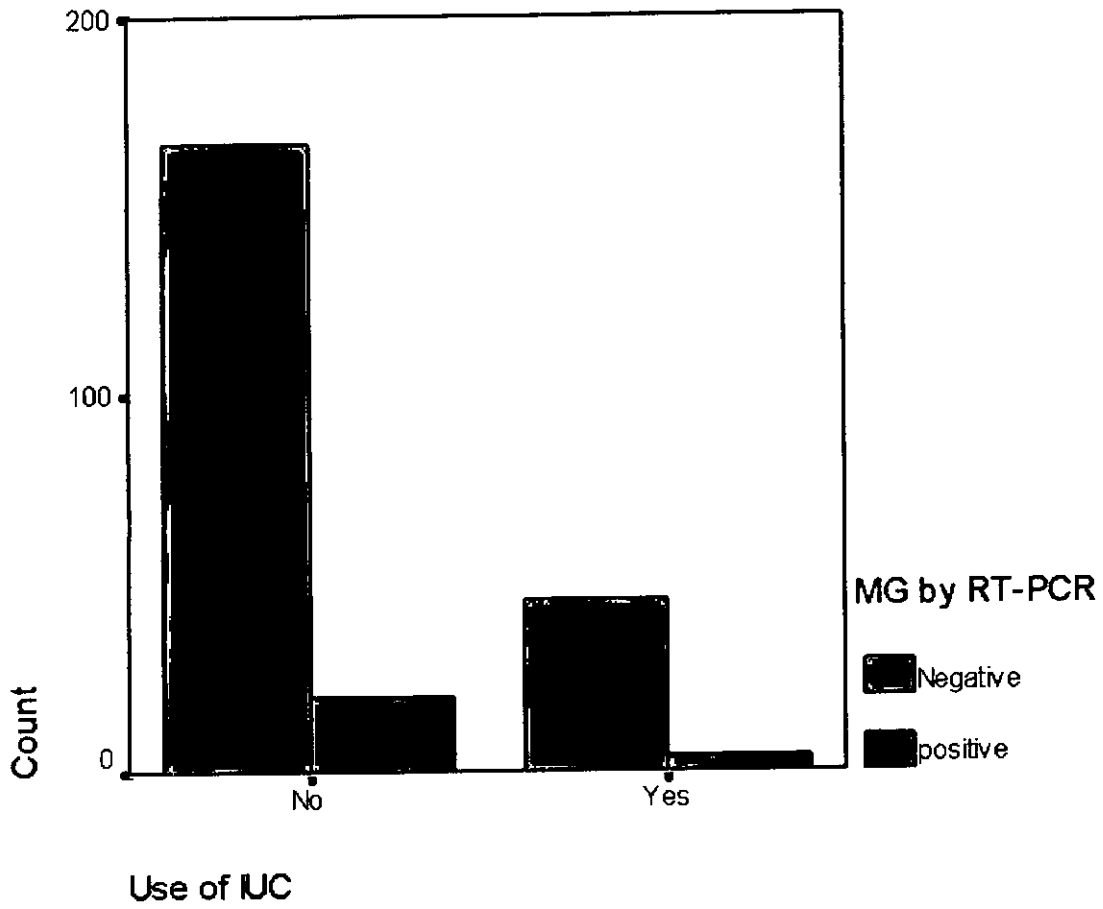

Figure 6. 7 Risk factor by IUD

Chi-Square Tests revealed that Chi-square =3.552, DF = 1

P = 0.059. The use of intra uterine devices as a barrier caution was tested and found truly protective in the transmission of Mg, P value being 0.024 hence it was statistically significant, as shown in Figure 6.7

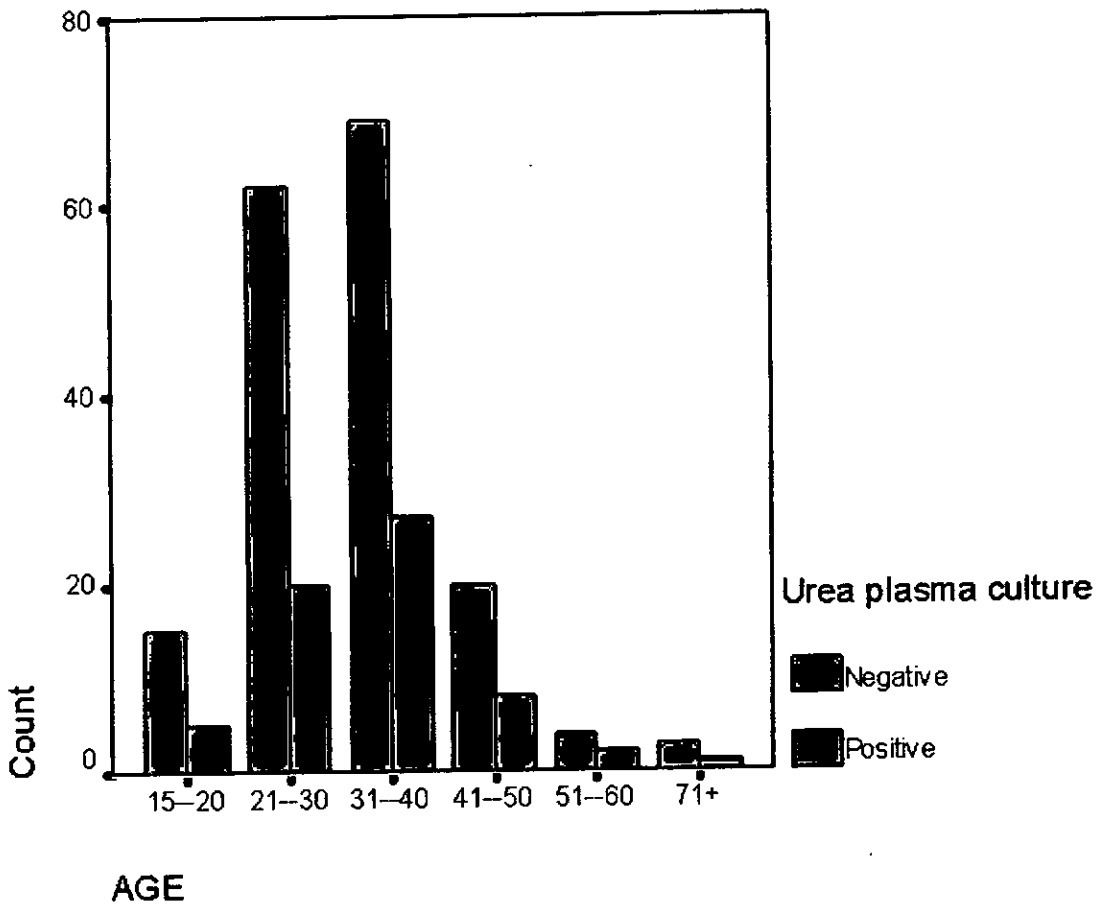

Figure 6.8 Risk factor by Age.

As shown above, the association of genital mycoplasma with NGU was strongest in subjects <35 years of age ($P \leq 0.005$). Mycoplasma is acquired from birth evidence of none prevalent in children born by C.S. And being an optimal parasite infection rate increases with age until an individual reaches the peak of sexual activities. This picture is portrayed in this study whereby infection rates increases from age bracket (15-20yrs) to (21-30yrs) to (31-40yrs) the peak and infection rates declining from 41-50 and to 51-60 when sexual activities must have come to a stop as displayed in Figures 6.8 above.

6.6 DISCUSSION

Generally, several risk factors have been implicated in the literature regarding the course and transmission (epidemiological status) of human mollicutes namely age, occupation, gender, number of sexual partners, sexual frequency, level of education, the use of barrier cautions, such as condom and IUDs, were also evaluated in this study (Tables 6.1 and 6.2) and (Figs 6.1 to 6.8). The most statistically significant risk factor in this study is the history of sexually transmitted infections (Figure 6.5) This is because in Nigeria the use of antibiotics are not as regulated in use as experience in the developed world. And mycoplasmas are resistant to penicillin and its groups, because they have no cell wall on which these antibiotics attack. Hence most of the cases of NGU and infertility managed are not cured and remained chronic. The studied population been STD patients presented with discharge, itching, ulcerations and sores in private parts currently or recurrent abortion as well as patients with various degrees of infertilities in JUTH and LUTH, these are about the two hospitals in Nigeria whereby one can have maximum level of representations of Nigerians attending STD clinics from various parts of the country. This is because of their strategic locations, commercial activities endowment, ethnic diversities and Federal Government Reference Teaching Hospitals for HIV/AIDS /STID patients in Nigeria. Completed questionnaire covering demographic information, distribution of respondents by state of origin health status, No of sexual partners, history of STI, use of contraceptives, primary and secondary clinical conditions from various states of the federation, were analysed. Beneath is further discussion of the risk factors implicated in STD pathogens, as well as enhancing prevalence in genital mycoplasmas.

Distribution of respondents by state of origin in Nigeria.

Figure 6.1 displayed the state distribution of correspondents that participated in the epidemiological survey of the Nigerians attending STD clinics in JUTH and LUTH during the period under review. The data generated and graphically depicted were from the statistically analysed research questionnaires administered to all the 824 participants. Population outputs were compared graphically among participants drawn from the 37 states of Nigeria. It was discovered that 30, out of the overall 37 states of the nation participated in this research. The subjects from these various states were actually residing in Jos or Lagos and their environs during the research. The figure 6.1 displayed the distribution of correspondence from participants by state of origin in Nigeria. When it was analysed as shown above participants from subjects from Delta and Imo states were bracketed among the states in the southern part of the country. While Plateau state has the highest no of isolates,

seconded by Nassarawa state. In Lagos majority of the participants were not an indigenes of Lagos to buttress the fact that the city is cosmopolitan. Jos on the other hand combed the entire rural areas making use of the facility at JUTH, in addition, majority of the participants claimed Plateau state Jos, as their state of origin either of ignorance, education, religion hence its highest number of participants recorded in this survey.

Age by birth

The association of genital mycoplasmas with NGU and infertility in this study was strongest in subjects ≤ 35 years of age (P ≤ 0.005). Mycoplasma is acquired from birth evidence of none prevalent in children born by C.S, and being an optimal parasite infection rate increases with age until an individual reaches the peak of sexual activities, as shown in this study. This picture is portrayed in this study whereby infection rates increases from age bracket (15-20yrs) to (21-30yrs) to (31-40yrs) the peak and infection rates declining from 41-50 and to 51-60 when sexual activities must have come to a stop (Tables 6.1 and 6.2). These results imply that prevalence of genital mycoplasmas is higher in the working class; this might affect the economy of a nation in the phase of development. Hence government must put in place enough machinery in the control of this disease among this age range (31-40yrs) In Nigeria, the media prevalence of HIV among STI patients was found to be 11.5% (FMOH Technical report, 2001). Aside from the risk of acquiring HIV infection, consequences of STI can be grave resulting in conditions such as infertility, ectopic pregnancy, cancer, neonatal infections and death. In America the highest rates of gonorrhea are found in young 15-30 yrs unmarried persons and in groups of low educational and socioeconomic status (CDC, 2006). In this study the age range at which the prevalence of *Mycoplasma* infection was at the peak was 31-40 (35yrs), and this was slightly higher in patients (41.2%) than in controls (40.2%). This age range falls within the working class and during which the sexual activities are maximum in both males and females. Above this age range, sexual activity reduces and hence the prevalence of STD pathogens including mycoplasma falls (Waites *et al.*, 2005).

Age debut

This is the age at which an individual was first exposed to sexual act, when a male or female first had a sexual experience. In this study, the recorded average age at which an individual was first exposed to sexual activities was 18 years (16-20yrs), that is, as teenagers. It was also observed that teenagers from the Northern part were exposed to sexual activities earlier at an average age of 13 years (11-15yrs), whereas, in the southern part of the country teenagers attained 18 years (16-20yrs), of age before being exposed to sexual activities (Tables 6.1 and 6.2). This was in conformity with the earlier reports of WHO, 1995 and CDC, 2000. STD, chancroid and trichomoniasis occurred in men and women within the age range of 15 to 49 years (Waites, 2005). This might be due to the low socioeconomic status in this part of the country and the result imply that transmission of genital mycoplasmas, would continue to be high in this age group, except control measures such has improved sex education is instituted.

Gender

An asymptomatic carrier state can occur in both sexes, but is believed to occur more frequently in females than in males. Serious sequelae are much more common in women than in men. PID in women may lead to ectopic pregnancy or infertility and women are more likely to develop disseminated gonococcal infection. The risk of transmission of STD from an infected woman to the urethra of her male partner is approximately 20% per episode of vaginal intercourse and rises to 60-80% after 4 or more exposures.

In contrast, the risk of male-to-female transmission approximates 50-70% per contact, with little evidence of increased risk with more sexual intercourse. Transmission through penile rectal contact is fairly efficient. Persons who have unprotected intercourse with new partners frequently enough to sustain the infections are defined as core transmitters (CDC, 2006).

In this study, the prevalence of genital mycoplasma was higher in females (73.1%) than in males (27.4%), although the prevalence in females control (22.6%) and male controls (16.9%) were very close, implying that males and females Nigerian harbors theses organisms in their reproductive organs as earlier on reported by Osoba in 1972 (Table 2).. Transmission could still be sustained in lieu of this fact, hence control measures like the use of barrier cautions such as condon and IUD might be very useful in prevention and control of this disease.

Occupation

Among the occupational categories examined in this study, those of residual group including the long distant drivers, security, guards and police had the dominant prevalence (33.6%). The least prevalence was recorded against the professionals. This is because the long distant drivers, security, guards and police categories might be more prone to casual sex activities than the stable professionals. Residual occupational was statistically significant (P=0.020). This risk factor proved that the occupational status of an individual has influence on the prevalent rates of STD and genital mycoplasmas (Figure 6.2). This might also be due to poor income earning, the level of knowledge on sex education, multiple sexual partners, hygiene and general life styles or behavioral attitudes that enhance the transmission of these organisms. This finding of occupational roles in STD prevalence has been reported earlier through a similar study conducted by Bakare *et al.,* (2000) among commercial sex workers in Ibadan. Further evidence for the association of Mg with cervicitis was provided in recent studies of female STD clinic attendees in Sweden (Falk *et al.,* 2005) and female sex workers in west African (Pepin *et al.,* 2005).

Level of Education

In this study those subjects that stopped their educational career at secondary school scored the highest prevalence (41.9%) as opposed to the post graduate category (2.5%). Chi-Square Tests, revealed: Chi-square =12.853, DF = 5 and P = 0.025 (Figure 6.3). One of the socioeconomic parameters examined was level of education, the P value of 0.025, for this risk factor proved that the level of education in an individual has influence on the epidemiology trends of genital mycoplasmas and spread as shown in the Table 28 above.

Marital status

The prevalence of genital mycoplasma is highest among the married category (79%) and lowest with the widower and divorced (25%). This finding proves the correlation between sexual activities and STD, this is the only way these organisms could be spread and lower prevalence has also been reported when barrier cautions were employed (Sloane, 1983). Although in some circumstances transmission could be higher among the singles, unmarried, widows and widowers, especially where such individuals or groups are engaged in casual sexual activities. Chi-Square Tests revealed that Chi-square =6.024, DF = 1, P = 0.014 As presented in Figure 6.4 the participants that were married were acquiring the disease from sexual partners, whereas, singles had lower prevalent rates, P value is 0.014. One may

therefore infer that sexual activities could have some relations with prevalence of genital mycoplasmas in marriage and outside marriage. (Figure 6.4).

Sexual partner in the past 3 months

All sexually active populations are at risk, and the level of risk rises with the number of sexual partners and the presence of other STDs (CDC, 2006). In this study, the prevalence rates of genital mycoplasmas increases as the number of sexual partner increases, thus patients with the highest number of sexual partner 4 had 74.8% whereas, patients with no sexual partner in the last 3 months had 1.7% prevalence. The higher the number of sexual partners the higher the prevalence, as females constituted a reservoir while the females were the carriers and hence the distributors. Prevalent rates of ureaplasma spread increases with multiple sex partners in this study and it was truly significant P value being 0.076. Aside from the increased possibility of contracting a STD, it is now known that some women may develop an immune response to sperm over time, especially in women exposed to multiple partners (CDC, 1983 and CDC, 2000). In addition, seropositivity increases with people with multiple sex partners. Fawole *et al.,* (2000) in a seroprevalence survey among the sex workers in Ibadan recorded a prevalence rate of 19.1% for *Haemophillus ducreyi* infection by culture method, and believed the rate could be higher if a more sensitive technique were used.

History of STI

The individual participants in this study with the clinical records of prior infections with STD or symptoms and signs suggestive were investigated in this study. There were numerous of these with cervical or urethral discharges, urethritis, infertility. Most patients were revisiting the STD clinics after they had undergone treatments for this ailment in the past. Some could just be ones, while others had been coming to STD clinics on several occasions. Among these categories, those with the history of STD/STI had the dominant prevalence (80.7%) as opposed to the control group prevalence (19.3%) that had no history of STD before. Chi-Square Tests revealed: Chi-square =0.040, DF = 1 and P = 0.841. The prevalence of Mg in patients without the history of STI was higher than those with STI (Figure 6.5). This implied that some of the cases of urethritis or infertility seen, might be solely caused by Mg order than the other agents of NGU/infertility in the patients examined in this study as been earlier reported in a similar studies in other African countries. For the Mycoplasma genitalium data analysis in which the Chi-Square Tests showed: Chi-square =3.552, DF = 1 and P = 0.059 (Figure 6.5). The history of patients with previous STI was truly significant in

Ureaplasma parvum biovar transmission, P value being 0.059. Patients might have been treated with antimicrobial agents that the organisms were not susceptible to or resistant to, *in lieu* of the unfamiliarity of some physicians/self therapist/traditional healers with the antibiogram patterns of this pathogen. Subjecting this to Chi-Square Tests, Chi-square =4.700, DF = 1 and P = 0.030. The association of ureaplasma infections with history of sexually transmitted infections was statistically significant in this study P value being 0.030, ie STI has to do with mycoplasmas spread it implied that sexual act could enhance its spread, as shown in (Figure 6.5). Appropriate counseling of infected individuals must be performed, to avoid reinfection from the sexual partner by facilitating treatment of the contact prior to sexual responses. Counsel patients to use latex condoms to prevent reinfection (CDC, 2006).

Use of condom

The use of condom as a protective barrier against STD/STI has diversities of school of thoughts as in its effectiveness, but in this study condom was protective, since those that employed the use had less prevalence (29.4%) than those that had regular sex without the use of condom (70.6%). When this was Chi-Square Tested, Chi-square =5.109, DF = 1 and P = 0.024. The use of condom as a barrier caution devise was tested and found truly protective in the transmission of Mg, P value being 0.024 hence it was statistically significant, as shown in Table 33. Chi-square =2.972, DF = 1 and P = 0.085 The use of condom, as a barrier caution in the spread of ureaplasma among sexual partners might be protective, since prevalence was less with the use of condom, than those having sex unprotected although the P value was 0.085, as shown in Figure 6.6. Chi-square = 6.024, DF = 1 and P = 0.014.As presented in Figure 6.6. The participants using condom as a barrier method against acquiring the disease from sexual partners had a low prevalent rates than those groups that had sex without condom, P value is 0.014. (Figure 6.6).

Use of IUD

Intra-Uterine Devices are usually employed by female subjects to prevent pregnancy and such was found in addition to be protective against genital mycoplasma infections in this study. Since the prevalence was less (18.5%) in those with IUD and higher (81.5%) in those without the use of IUD as reported ealier by the Technical Report Team of the Federal Ministry of Health (FMHO), Nigeria (1999). Chi-Square Tests revealed that Chi-square =3.552, DF = 1, P = 0.059. The use of intra uterine devices as a barrier caution was tested and

found truly protective in the transmission of Mg, P value being 0.024 hence it was statistically significant, as shown in Figure 6.7

Number of children

In this study it was discovered that the number of children owned by individual participants were scored along with genital mycoplasma infection rates, since the sampled population involved infertility cases and indeed, the prevalence increases with the decreased number of children as shown in Figure. With the highest prevalence (89.9%) in subjects that had no children and least prevalence (6.8%) recorded against with those with highest number of children.

6.7 CONCLUSION

In all, infections with mycoplasmas, especially the ureaplasmas (57%), were common and the organisms were the only potential sexually transmitted pathogen detected in 40 (62%) symptomatic patients. The subjects were sexually matured persons, presented with discharge, itching, ulcerations and sores in genital parts, currently or recurrent abortion, as well as patients with various degrees of primary and secondary infertilities in JUTH and LUTH. These are among the hospitals in Nigeria where one can have maximum level of representation of Nigerians attending STD clinics from the Northern and Southern parts of Nigeria. This is because of their geographical and political locations, commercial activities endowment, ethnic diversities and Federal Government Reference Teaching Hospitals for HIV/AIDS /STD patients in Nigeria. The controls were the group with no history of STD and are infrequently colonized with genital mycoplasmas.

Among those who are sexually experienced, colonization increases with increase in the number of different sexual partners. It is interesting to note that colonization increases more rapidly with increasing sexual experience in women than in men, suggesting that women are more susceptible to colonization with these organisms. Additionally, the participants rarely used protection devices during sexual intercourse and some symptomatic subjects apparently acquired their infections despite using condoms regularly. The findings demonstrated a strong association between abnormal urogenital findings, some certain risk factors and detection of mycoplasmas, particularly ureaplasmas, and suggest the infections will remain endemic, if control measures are not in place (Tables 6.1 and 6.2) and (Figs 6.1 to 6.8).

CHAPTER SEVEN

CONCLUDING DISCUSSION AND RECOMMENDATIONS

7.1 General Discussion

Nongonococcal urethritis is the most common sexually transmitted infection. *Ureaplasma species* and *M genitalium* may account for a significant portion of cases that are not due to chlamydiae. More than 20% of live born infants may be colonized by *Ureaplasma*, and infants born preterm most likely harbor the organisms, which could be acquired vertically (Sanchez, and Regan 1990) and cases of transmission of genital mycoplasmas from mother to neonate in women with prolonged membrane rupture has been reported also by Dinsmoor, (1989). Colonization declines after age 3 months. Less than 5% of children and 10% of adults who are not sexually active are colonized with genital mycoplasmal microorganisms. Surveys of a variety of human populations reveal widespread colonization of the genital tract with mycoplasma. Indeed the ubiquitous nature of this organism constitutes part of the evidence for low-order pathogenicity and has complicated the problem of delineating the role of the organism in disease causation. Genital mycoplasma can be recovered from nearly 30% of newborn infants.

In the epidemiological survey component of this study, Plates 1 and 2 showed the typical colonies of mycoplasmas isolated from one of the patients having NGU from each of the study sites. While Table 3.4 compared prevalent rates between the 2 teaching hospitals. JUTH had a higher prevalent rate than LUTH in males and females in the patient and control groups. The prevalent rate of genital mycoplasma in Jos {in both male (31.4%) and female (53.3%)} was higher than that of Lagos {Male (27.1%) and Female (47.8%)}. There were no data available in these locations prior to this study (Table 2). The higher prevalence in the Northern part of Nigeria, might be due to some epidemiological/risk factors including, socioeconomic status, early marriage and exposure to sex, level of education, sex education, (hygiene) associated with the transmission of genital mycoplasmas which are predominating in the North than in the Southern part of Nigeria. Sex education and hygiene is very poor at this location with polygamous marriage settings and higher number of sexual partners. Mycoplasmas being an optimal parasite, stays for a long period undiagnosed with the host still suffering from itching and urethral discharge. Even after being treated with potent antimicrobial agents and proven aetiological roles in several experimental animal models, most scientists still pay less attention to their roles in non-gonococcal urethritis and infertility.

Considering the population distribution of all the respondents from both study sites, again more STD patients patronized JUTH than LUTH. This might be due to the fact that more people attend private hospitals in Lagos; most residents in Lagos are non-indigenes, more educated, but in the North, JUTH being central is a good catchment site for more cases of STD. Moreover, it used to be more peaceful than all other states in the North for larger population, in addition, there are less private hospitals that can compete with the quality of services obtainable at JUTH (Figure 6.1). All over the world, scientists and family planners are beginning to realize that early marriage is quite common in Africa, hence increased rate of divorce, multiple sex partners, STD and pregnancy out of wed-lock, has become an issue of great concern (CDC, 1983 and CDC, 2000). African women marry young, compared with women in the rest of the world. It has also been reported that marriage close to the age of menache is common in many Islamic areas. According to the data drawn from 28 African countries, about 55% of women aged 15-19 years were married as seen in this study, especially from the Northern part of Nigeria. This data is based on figures ranging from 82% in Guinea, 81% in Niger, 79% in Mali, 74% in Ivory Coast, 73% in Chad, 72% in Nigeria to 32% in Ghana, 22% in Lesotho, 18% in Rwanda, 14% in Somalia, 14% in Botswana and 12% in Burundi (Pepin, 2005, CDC, 1983 and CDC, 2000).

The amplification patterns of the target DNAs (amplicons) and standard controls are shown in Figures 4.1 to 4.5. Of the 119 infertile patients examined using the PCR technology, only 44 (36.97%) patients were positive for Uu by culture. Whereas, 66 (55.45%) infertile patients were detected by PCR from the same 119 patients (Table 4.10), indicating that PCR is more sensitive than culture, as ealier reported in other studies (Harasawa, (1999), Kong *et al.*, (1999 and 2000), Robertson *et al.*, (1986, 1993 and 2004). With the use of specific primers, *Ureaplasma spp,* was speciated into Up and Uu in this study, with the prevalence rates being 21.85% and 41.18% respectively. This finding is in contrary with the findings of Harasawa, (1999), Kong *et al.*, (1999 and 2000), Robertson *et al.*, (1986, 1993 and 2004), who previously reported Uu to be more common than Up. But in conformity with their claims that co-infections of the 2 biovars may also occur (Harasawa, 1999, Kong *et al.*, 1999 and 2000, Robertson *et al.*, 1986, 1993 and 2004), as also discovered in this study. Similarly, Uu was discovered to be more common than Up in young males with NGU in a study conducted by Povlsen *et al.*, (2002); and Deguchi *et al.*, (2004). In this study only 19 (15.97%) patients harboured both species as confections; this is in agreement with Povlsen *et al.*, (2002); and Deguchi *et al.*, (2004), while 66 (55.45%), and out of the 119 patients was infected with

either biovars. The findings provide strong evidence that genital mycoplasmas specifically Up, could be responsible for some cases of chronic urethritis and various degrees of infertility in Nigeria; rather than *Ureaplasma urealyticum* as reported by the previous authors, who were limited by application of high molecular technology.

In this study, PCR and real-time PCR technology were employed, to study the prevalence of the two *U. urealyticum* biovars in Nigerian males and females patients attending STD clinics in 2 major Hospitals in Nigeria. The patients had history of infertility, urethritis with symptoms of genital discharge. In all, 238 cervical, vaginal and urethral swab samples were screened by culture and PCR for infection with *U. urealyticum, ureaplasma parvum and Mycoplasma genitalium* by real-time PCR. Of the 119 patients studied, only 11 (12.64%) harboured Mg and only 6 (7.05%) of the control subjects harboured the organism respectively. The higher rate in patients than in controls supported previous studies in Nigeria and various other parts of the world. As depicted in Table 5.3.1, the prevalent rate of *Mycoplasma genitalium* (Mg) in patients is higher than those recorded against the controls. Hence, Mg might be responsible for the cases of urethritis and pregnancy complications such as infertility in the group of patients examined in this study. Prevalent rate of *Mycoplasma genitalium* in male and female patients as shown in Figure 5.3.1. The prevalent rate of *Mycoplasma genitalium* in male patients is higher compared to those of the female counterparts. Figure 5.3.2 displayed the prevalent rate of *Mycoplasma genitalium* in the overall participants The prevalent rate of *Mycoplasma genitalium* in patients is higher than those recorded against the controls (Table 5.3.1 and Figure 5.3.2). Hence Mg might be responsible for the cases of urethritis and pregnancy complications such as infertility in the group of patients examined in this study. The Mg prevalence result is in conformity with the world prevalence rate (10%-45%) including, in West Africa (10%), in Ibadan (14%), and current studies 14% in Jos, and 18%, in Lagos respectively, giving an average of 16% in Nigeria subjects with NGU. More people were found with NGU than in asymptomatic controls being a common cause of NGU in men and more frequently isolated from sexual partners of infected women.

Table 7 and Figure 7. displayed the overall data outputs of genital mycoplasmas detected by either culture, Polymerase chain reaction or real time Polymerase chain reaction. Out of the 119 infertile patients examined, only 44 (36.97%) patients were positive for *Ureaplasma urealyticum* by culture. In PCR assays, two primers were used to bind with either the target

gene located at a 650-bp conserved region of Uu 16S rRNA gene for the identification of *U urealyticum* or the target gene at a 400-bp conserved region of Up urease gene for the identification of *U. parvum* biovar. Indeed, Uu was detected in 26 (21.85 %), while 49 (41.18%) Up isolate was detected out of the 119 cases by PCR technology. But of the 117 controls examined, only 18 (15.39%) was positive by culture, whereas PCR assay revealed 15 (12.82%) and 20 (17.09%) of Uu and Up respectively. In all, *Ureaplasma parvum* biovar was detected more from both patient and control groups. RT-PCR technology was specifically employed for the rapid detection of *Mycoplasma genitalium* and results showed a prevalent rates of 11 (12.64%) in patients and 6 (7.05%) in controls respectively (Table 7 and Figure 7).

The sensitivity and specificity of all the techniques employed in this study were compared in terms of prevalent rates and the distribution patterns of the genital mycoplasma species identified among patients, (Table 7 and Figure 7), and it was discovered that RT-PCR was most specific, fastest and most accurate, it was the only technique that could be employed to identify *Mycoplasma genitalium* from lyophilized broth culture. In this study PCR technique was faster and more specific than cultural technique. However, culture remained the fundamental procedure in clinical diagnosis, where an organism could be morphologically pinned down. This primary isolation technique constituted a basic tool for further molecular studies utilizing PCR and RT-PCR technology.

Generally, several risk factors have been implicated in the literature regarding the course and transmission (epidemiological status) of human mollicutes namely age, occupation, gender, number of sexual partners, sexual frequency, level of education, the use of barrier cautions such as condom and IUDs and most significant in this study is the history of sexually transmitted infections, since patients attending STD clinics were the sampled population. The population spread across the geopolitical zones of Nigeria involving 30 out of the 37 states of the federation targeting the two cosmopolitan reference teaching hospitals for HIV/AIDS pandemic. When the data outputs were subjected to chi square statistical data analysis those that their p values were less than or equal to 0.05 were considered to be significant as shown in (Tables 6.1 and 6.2) and (Figs 6.1 to 6.8).. The data generated against the risk factors examined in the study was tested statistically and it was discovered that the following had their P values </= 0.05. This includes history of STI, number of sexual partners, use of condon, age, occupation and level of education. That is, people with history

of STI had more of genital mycoplasmas infections as seen in other factors. The frequency of mycoplasma infection is more in females than in males except for Mg that had a relatively lower prevalence compared with *ureaplasma spp* biovars, but there were more of Mg in male patients than in females. It was also discovered that the use of condom against Mg was statistically significant, (P = 0.024) apart from the integrity of the object, this organism is known to be motile; hence it could migrate away from the reach of the barrier caution employed.

In this study, the prevalence of Up compared with Uu was higher in patients than in control group. This finding implies that Up may be more relevant in the pathogenesis of infertility and urethritis than Uu in Nigeria. In addition it might be that the Nigerian strains of Uu are more susceptible to the tetracycline being one of the commonest antibiotics employed to cure other agents of NGU and infertility in Nigeria (Ladipo *et al.*, 1978) (Table 2). Hence, the chronic cases of STI encountered in this study might be as a result of Up infection, (P=0.059), but the particular serotypes under this biovar needs to be further investigated to ascertain if serovar 4 could be most pathogenic among the 4 serovars under the Up.

The newer innovation and data generated from the present study could be associated to the following: wider study population, better environments and advanced technology employed. More people participated from the North, especially Jos, where JUTH is located and as a Federal Government Reference centre for HIV/AIDS and serves as a pool for the groups of patents examined in this study. However, the no of participants was very low compared to JUTH, because most people residing in Lagos are non Lagosians, while most people also attended private hospitals rather than coming to LUTH. Moreover *in lieu* of their levels of educational status, sex education and awareness, as well as good health practices, make them to be less prone to mycoplasma infections. It is believed that, data outputs of this study, were fairly representative of the current rate of infection with the organism in Nigeria.

Table 7: Sensitivity and specificity of the diagnostic tests used for study

Subjects (Patients with the history of NGU and infertility)	Prevalent rate of UU by culture	Prevalent rate of UU by PCR	Prevalent rate of UP by PCR	Prevalent rate of UP+UU (coinfection with the both biovars)	Prevalent rate of UP/UU (infection with either of the biovars)	Prevalent Rate of MG by RT-PCR
Patient (n=119)	44 (36.97 %)	26 (21.85%)	49 (41.18%)	19 (15.97%)	66 (55.45%)	17 (14.29%)
Control (n=117)	18 (15.39%)	15 (12.82%)	20 (17.09%)	3 (2.56%)	32 (27.35%)	7 (6.0%)
Total (n=236)	62 (26.27%)	41 (17.37%)	69 (29.24%)	22 (9.32%)	98 (41.53%)	24 (10.17%)

Tables 7 displayed the account of the sensitivity of all the techniques employed in this study. Culture, PCR and RT-PCR techniques were compared in terms of prevalent outputs among the patients and controls as well as the role of each in the diagnosis of genital mycoplasmas. Out of the 119 infertile patients examined, 44 (36.97%) patients were positive for *Ureaplasma urealyticum* by culture. Up was detected in 26 (21.85 %), while 49 (41.18%) Uu isolate was detected out of the 119 cases by PCR technology. But of the 117 controls examined, 18 (15.39%) was positive by culture, whereas PCR assay revealed 15 (12.82%) and 20 (17.09%) of Up and Uu respectively. In all, *Ureaplasma urealyticum* biovar was detected more from both patient and control groups. RT-PCR technology was specifically employed for the rapid detection of *Mycoplasma genitalium* and results showed prevalent rates of 11 (12.64%) in patients and 6 (7.05%) in controls respectively.

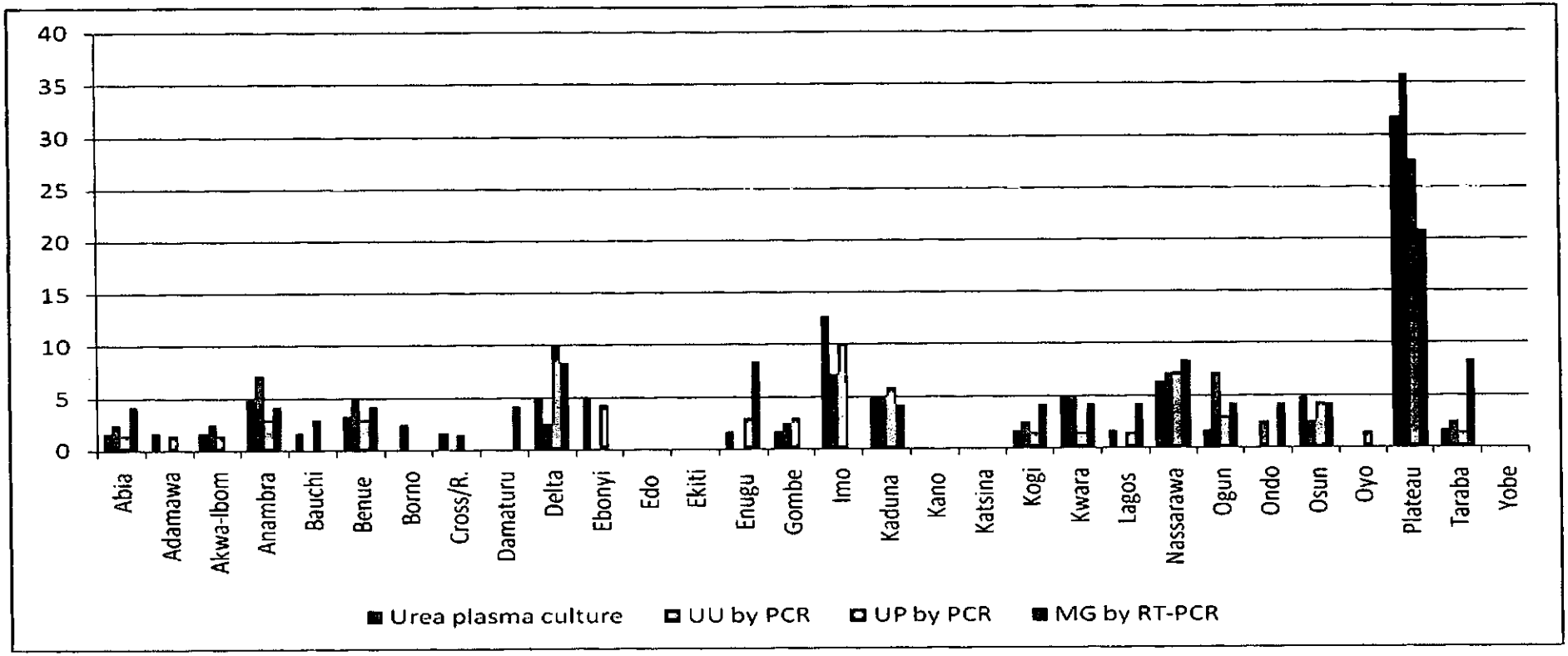

Figure 7 State distributions of mycoplasma isolates by Culture, PCR and RT-PCR techniques among patient attending STD clinics in Nigeria

Figure 7 displayed the state distribution of mycoplasma isolates by Culture, PCR and RT-PCR techniques among patients attending STD clinics in Nigeria. Prevalence outputs were compared graphically among participants drawn from 30, out of the overall 37 states of the nation. The figure 7 above depicts the distribution of genital mycoplasmas isolated from participants by state of origin in Nigeria. When it was analysed as shown above isolates from subjects from Delta and Imo states were bracketed among the states in the southern part of the country. While Plateau state has the highest no of isolates, seconded by Nassarawa state.

7.3 CONCLUSION

Until technology is advanced to the point that PCR and RT-PCR assays for genital mycoplasmas can be purchased commercially in a kit format by diagnostic laboratories, it is unlikely to gain widespread usage for routine microbiological diagnosis. Real-time PCR has been adapted for quantitation and characterization of genital mycoplasma isolates to species level (Mallard *et al.*, 2003). The high yield and relative simplicity of cultivation in agar and broth media for rapidly growing organisms such as *Ureaplasma spp.* and *M. hominis* supports the concept that culture should remain an important part of the diagnostic process. Specimen types such as blood which may contain a very low concentration of microbes in a huge background of human DNA may not be ideal for PCR.

In modern diagnostic work, however, culture has great limitations as far as speed, specificity, and scope of capability are concerned. The trend now is molecular diagnostic approach which is faster, more specific, permits biovar/serovar determination of Uu, and affords detection of highly fastidious mycoplasmas such as *Mycoplasma genitalium* (Mg). Mg could be very difficult to grow in culture, and serological methods lack sufficient sensitivity and specificity in acute phase of disease. An accurate detection is often available using vero cell line tissue cultures after several days of disease onset. However, conventional and real-time PCR approaches have been found to be useful for the rapid, sensitive and specific detection of these microorganisms from urogenital tract specimens (Table 7 and Figure 7).

The sensitivity and specificity of all the techniques employed in this study were compared, and it was discovered that RT-PCR was most specific, fastest and most accurate; it was also the only technique that could be employed to identify *Mycoplasma genitalium* from lyophilized broth culture in this study. PCR technique was faster and more specific than cultural technique. It has also enabled the speciation of Nigerian Uu isolates into Uu and Up biovars for the first time, in Nigeria. Genital mycoplasma including Uu, Up and Mg may play some roles in cases of non gonococcal urethritis and infertility in males and females attending STD clinics in LUTH and JUTH, Nigeria (Table 7 and Figure 7).

In Nigeria, high technology molecular techniques in mycoplasmology are either at her teething stage or completely lacking in our hospitals and medical research institutes. This has made the previous data obsolete compared to other studies from various part of the world. However, this study has paved way for future studies that could focus on determining

Ureaplasma serovars in human subjects in Nigeria, and develop PCR diagnostic facilities for *Ureaplasma spp, Mycoplasma genitalium*, and related microorganisms. The analysis of the questionnaire administered revealed that more participants responded from JUTH than LUTH for STI screening. Participants from the North also had early exposure to marriage and sexual activities more than in the south. It was also observed that prevalence and transmission of mycoplasmas are sexually related and that prevalence could be attributed to increasing number of sexual partners, occupational lifestyle, and level of education, use of barrier cautions and history of sexually transmitted infections (Tables 6.1 and 6.2) and (Figs 6.1 to 6.8).

The following has been established through this study: (1) Among all the Nigerian studies to date, non matches the geographical spread of this project. Covering two Federal Government Reference Centers for HIV/AIDS / Sexually Transmitted Infections (STI), Lagos University Teaching Hospital, (LUTH) and clinics in the South, Jos University Teaching Hospital, (JUTH) and clinics in the North. (2) The total number of participants (824) was much larger than the previous studies, thus providing a fairly representative prevalent rate that can be used in demographic report of STD/STI in Nigeria, as far as genital mycoplasmosis are concern. (3) The speciation of Nigerian strains of Ureaplasma into 2 biovars (*Ureaplasma urealyticum* and *Ureaplasma parvum*), for the first time in Nigeria, using Polymerase Chain Reactions (PCR) technology.(4) The prevalence of *Mycoplasma genitalium* by Real Time-Polymerase Chain Reactions (PCR) was determined and reported for the first time in Nigeria. Finally, the way has been paved for the future studies that would focus on determining. (5) *Ureaplasma serovars* in human subjects in Nigeria, and develop PCR diagnostic facilities for *Ureaplasma spp, Mycoplasma genitalium*, and related microorganisms, has been paved. It is believed that, data outputs of this study were fairly representative of the current rate of infection with genital mycoplasma organisms in Nigeria.

7.4 Recommendations

Physician and researchers need more awareness on the clinical relevance of mycoplasmas in STD/STI control. There is need to establish a modern laboratory for PCR and RT-PCR analysis of these fastidious pathogens, for purposes of routine diagnosis and research. Data generated from this study could be used by the Federal Ministry of Health/WHO as a baseline data for formulating policy and planning for health care delivery in Nigeria. There is need for complete molecular typing of the 14 serovars of *Ureaplasma urealyticum* and their differential pathogenic roles in STD patients in Nigeria.

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APPENDENCES

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MD., FRCPI, FRCPCH, FMC Paed., FWACP, FICP DCH

Director of Administration:
O. A. O. ONABOWALE, B.Sc. EPA, ADMIN, ARAN.

Chief Medical Director:
PROF. ONATOLU ODUKOYA, *B.D.S. (Lagos) M.M.Sc (Harvard). Cert. Oral Path.,(Harvard) FMCDS(Nig.), FWACS.*

Chairman, Medical Advisory Committee:
PROF. S. O. ARIGBABU, M.B.B.S. (Lagos), FRCS (Edinburgh), FWACS, FICS

Cable and Telegrams: UNIHEALTH, YABA, LAGOS Telex No: 27636 Telephone: Lagos, 5453760 - 74 (15 Lines)

7th January, 2004

Ref. No. ADM/DCST/221/Vol.8

Dr. I. D. Olorunshola,
PhD Student,
u.f.s. Prof. & Head,
Department of Medical Microbiology & Parasitology,
Lagos University Teaching Hospital,
Idi- Araba,
Surulere, Lagos.

APPROVAL FOR RESEARCH AND ETHICS COMMITTEE

I wish to refer to your request in respect of the above stated subject matter.

Approval has been granted you to continue with the study titled "Studies on Human Urogenital Mycoplasmosis and Ureaplasmosis in Nigeria".

Wishing you all the best in the study.

Miss. A. O. Haastrup
For: Chairman, Research & Ethics Committee.

JOS UNIVERSITY TEACHING HOSPITAL
JOS, NIGERIA

Ref. No: JUTH/ADM/DCS/127/XXIV/541 Date: 11th July, 2006

Dr. Olorunshola I. Dayo
Microbiology and Parasitology
JUTH - Jos.

RE: APPLICATION FOR ETHICAL CLEARANCE

I am directed to refer to your application dated ..... on the research proposal titled:

"Human Urogenital Mycoplasmosis and Ureaplasmosis in patients attending STD Clinics in JUTH, Jos.

and your appearance before the Ethical Committee on 12th May, 2006

Following recommendation from the Ethical Committee, I am to inform you that Management has given approval for you to proceed on your research topic as indicated.

You are however required to obtain a separate approval for use of patients and facilities from the Department(s) you intend to use for your research, please.

Hajia R. Danfillo
for: Chairman, JUTH Ethical Committee

DISPATCHED
13 JUL 2006

cc: Chief Medical Director
" Director of Administration
" The Chairman, Ethical Committee

Department of Medical Microbiology and Parasitology
(SCHOOL OF BASIC MEDICAL SCIENCES)
COLLEGE OF MEDICINE OF THE UNIVERSITY OF LAGOS
P.M.B. 12003, LAGOS, NIGERIA

Cables and
Telegrams: UNIMED. YABA, LAGOS
Telephone: LAGOS 801500 (20 Lines)
Ext. 2230

Prof. A. I. Adetosoye
Dept. of Vet. Microbiology
U.I. Oyo state.

Dear Prof. Adetosoye.

Re: OLORUNSHOLA, ISAAC DAYO [DVM, MSc]

This is to introduce Dr Olorunshola; a PhD student under the supervision of Prof.A.O.Coker. He wishes to embark on studies on human Mycoplasmosis in Nigeria. He requires some skills to carry out his research work in this area of microbiology, which is not routinely done in this department for now.

I will be grateful if you can allow the candidate for an attachment of 3-months duration in your laboratory.

Your kind consideration will be highly appreciated.

Yours truly,

Professor Tolu Odugbemi,
Professor and Head.

Prof. Adetosoye
This is approved
12/11/2003

H.O.D.

It will be appreciated if you could kindly permit me to assist Dr Olorunshola to go through the techniques needed for the isolation, identification besides methods of culturing of mycoplasma as requested by Prof. Odugbemi.

SOUTHERN UNIVERSITY AT NEW ORLEANS
6801 PRESS DRIVE
NEW ORLEANS, LA 70126
PHONE: (504) 286-5325 OR 286-5101 FAX: (504) 284-5400

Academic Affairs

November 12, 2008

TO WHOM IT MAY CONCERN:

Ph.D. Program of Isaac Dayo Olorunshola, D.V.M., M.Sc.

It has been a great pleasure working with Professor A. O. Coker to guide the Ph.D. project of Isaac Dayo Olorunshola titled *"Studies on human urogenital mycoplasmosis in Lagos and Jos, Nigeria"*. Apart from the support I was able to provide during the epidemiological studies in Nigeria, with the cooperation of my institution, I am highly delighted that the candidate was able to visit my laboratory at Southern University at New Orleans (SUNO), Louisiana, and my research collaborators' laboratory at the Louisiana State University Health Sciences Center (LSUHSC), Infectious Disease Unit, Department of Medicine, to apply molecular biology technology to his research.

Specifically at SUNO, where he spent approximately six weeks in 2007 before moving to LSUHSC, he did intensive laboratory exercises to acquire basic skills in:

- Principles and practice of agarose gel electrophoresis
- Restriction enzyme usage (for cutting and analyzing DNA fragments)
- Polymerase Chain Reaction
- Size determination of DNA restriction fragments
- DNA fingerprinting
- DNA paternity testing
- Isolation of chromosomal DNA from bacteria (*Escherichia coli*)
- Gel photography

At LSUHSC, he worked intensively on:

- DNA extraction from his Nigerian human urogenital specimens.

- Application of PCR technology to screen Nigerian specimens for *Ureaplasma* spp.

  He was able to compare *Ureaplasma* spp. prevalence by culture and identification (done in Nigeria) with PCR detection. This enabled him to speciate *Ureaplasma* spp of Nigerian origin into *U. urealyticum* and *U. parvum*.

- He applied a modified PCR technique known as Real-Time PCR (RT-PCR) to screen Nigerian human urogenital specimens for *Mycoplasma genitalium* for the first time ever, to the best of our knowledge.

Isaac was extremely hard-working. What he accomplished in a three-month visit could have taken at least six months.

I believe his contribution to knowledge could be easily acknowledged as follows:

1. Among all Nigerian studies to date, including our study at Ahmadu Bello University, Zaria, none matches the geographical spread of this project. The choice of two important sites in Nigeria (LUTH and JUTH) made this possible.

2. The total number of participants (824) was much larger than previous studies, thus providing a fairly representative prevalent rate that can be used in demographic reports of STD/STI in Nigeria, as far as the organisms he worked on are concerned.

3. Using PCR technology, he confirmed that *Ureaplasma* spp. isolates from Nigerian patients could belong to either of the two known species - *U. urealyticum and U. parvum.*

4. He confirmed *M.genitalium* in Nigerian patients for the first time.

5. He paved the way for future studies that could focus on determining *Ureaplasma* serovars in human subjects in Nigeria, and develop PCR diagnostic facilities for *Ureaplasma* spp, *Mycoplasma genitalium*, and related microorganisms.

If there are other specific information required from me, please feel free to contact me on e-mail address, dadegboye@suno.edu. I have had a great personal joy working with the University of Lagos on this project, using the technology available here to assist scientific research in my home country.

Thank you.

Yours truly,

David S. Adegboye, DVM, Ph.D.
Professor of Microbiology, and
Associate Vice Chancellor for Academic Affairs

8.2.2 HUMAN UROGENITAL MYCOPLASMOSIS AND UREAPLASMOSIS PhD RESEARCH QUESTIONAIRE [STI CLINIC FORM 001]

1] INSTITUTION.....

2] UNIT.....

3] CASE NO.....

4] SPECIMEN NO.....

5] DATE.....

6] STATE OF ORIGIN .....

7] LOCAL GOVERNMENT AREA.....

8] ADDRESS.....

9] DATE OF BIRTH.....

10] SEX.....

11] OCCUPATION.
.....

12] LEVEL OF EDUCATION.....
.....

13] MARITAL STATUS.....

14] NO OF WIVES/PARTNERS.....

15] Reason[s] for attending clinic
a] Comment.....
b] Asymptomatic check up.....
c] Symptomatic self-reporting.....
e] Others.....

17] SOURSE OF REFERRAL.....
.....

18] SEXUAL HISTOR/SOURSE OF INFECTION.
.....
1] First contact, how old.....
2] When married.....
3] No of children
4] No of abortion/MC.....
5] Sexual frequency per week.....
6] Pregnant or nursing.....

19] NO OF SEXUAL PARTNERS FOR HE PAST 3 MONTHS [SPECIFY NO]

a] Wives
b] Husband
c] Girl friend
d] Boy friend
e] Casual

20] PREVIOUS HISTORY OF
STI.....
.....
.....

21] HOW OFTEN BARRIER [CONDON] USED

a] Always
b] Usually
c] Sometimes
d] Never

22] OTHER CONTRACEPTIVES USED [name them]

a] Type.....
b] How often do you use them.....

23] SPECIFIC PRIMARY ILLNESSES

a] Discharge
b] Dysuria
c] Urinary problems [specify]
d] Ulcer
e] Pain
f] Swelling
g] Rash
h] Itching
I] Abdominal pain
J] Eyes

24] SPECIFIC SECONDARY ILLNESSS

a] Infertility
b] Pelvic inflammatory disease
c] Pneumonia
d] Spontaneous abortion
e] Prostitis
f] Non-gonococci urethritis
g] Low birth weight in children
h] Others

25] SPECIFIC ORGANS INVOLVED

a] Mouth
b] Eyes
c] Skin
d] Abdomen
e] Groin

f] Lymph nodes
g] Perineum
h] Penis
I] Scrotum
J] Vulva
K] Vagina
L] Cervix
M] Others

26] SYNDROME BASED DIAGNOSIS.....
.....

27] SPECIMEN [S] TAKEN
a] Urethral swab
b] Cervical swab
c] Urine
d] Semen
e] Blood
f] Discharge

28] SAMPLING STRATEGY
a] Broth inoculation
b] Agar inoculation
c] Broth to agar
d] Agar to agar
e] Use of transport media

29] ISOLATE [S].....

30] CONSULTANT.....

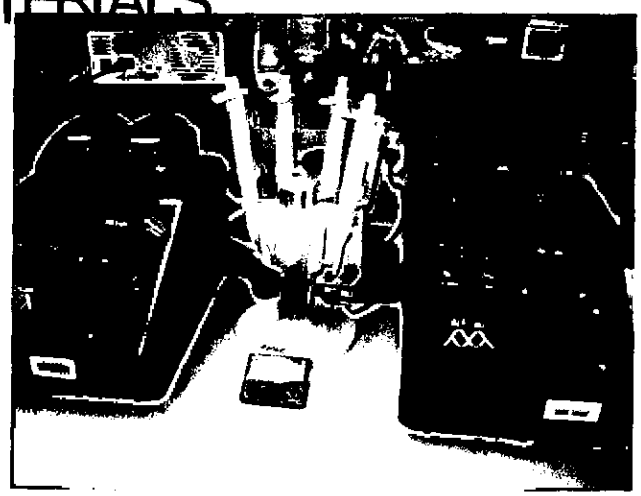
31] THERAPY.....

BY:
Dr I.D.OLORUNSHOLA
DEPARTMENT OF MEDICAL MICROBIOLOGY AND PARASITOLOGY
COLLEGE OF MEDICINE UNIVERSITY OF LAGOS, LAGOS NIGERIA.

SUPERVISOR
Prof A. O. COKER, FAS.

8.2.3 POLYMERACE CHAIN REACTION **MACHINE**

MATERIALS



8.2.4 REAL-TIME POLYMERACE CHAIN REACTION MACHINE

Real-Time PCR Machine

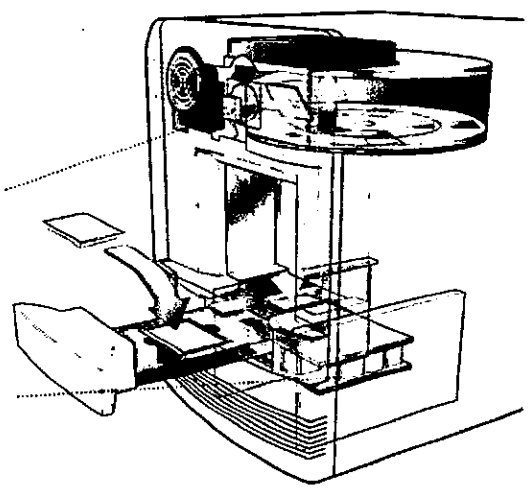
How it Works: Real Time PCR

Brendan Maher

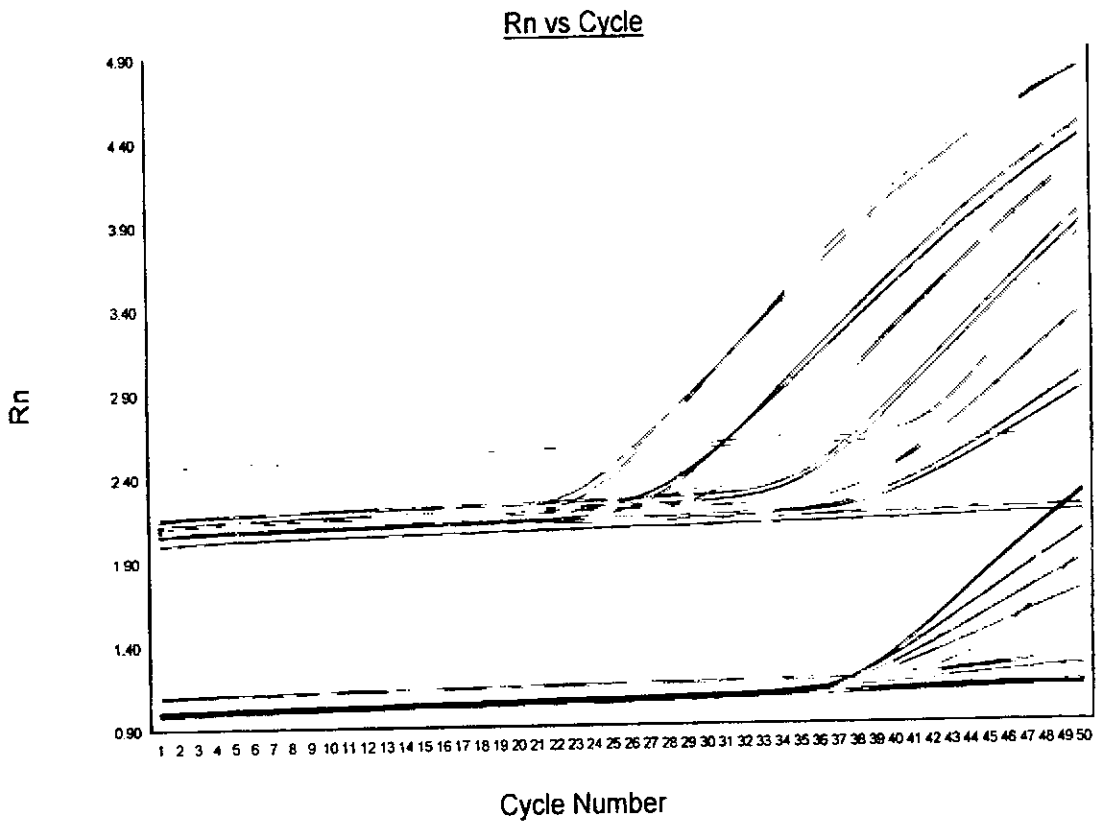
The instrumentation is basic: a thermal cycler for amplification, a light source for excitation of fluorescent probes (see chemistries below), a camera for recording, and a computer to control the instrument and record data. Increasingly sophisticated instruments, such as those capable of multiplex experiments, are becoming affordable in academic labs.

The light source in the Applied Biosystems 7500 (represented here) is a simple halogen lamp shone through one of five different excitation filters over the entire sample. A CCD camera positioned above the sample records fluorescence from behind one of five emission filters. Some makes and models use a scanning head that moves over the plate, exciting and reading fluorescence in the wells individually.

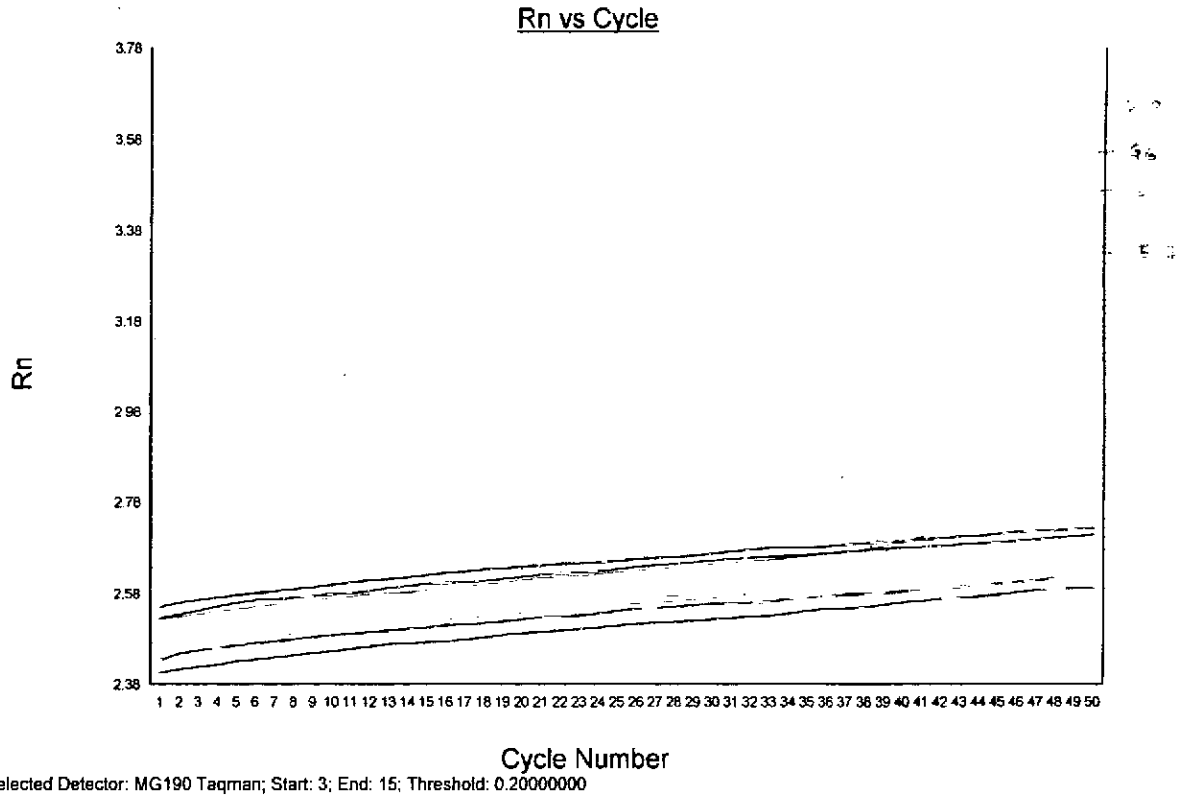
Many qPCR instruments including the ABI 7500 use a Peltier element for heating and cooling. Peltier coolers use electron flow between semiconductor couples to heat or cool one side of a plate depending on the direction of current. Other systems use liquid or air flow or mechanical transition between blocks of different temperatures to cycle the samples.



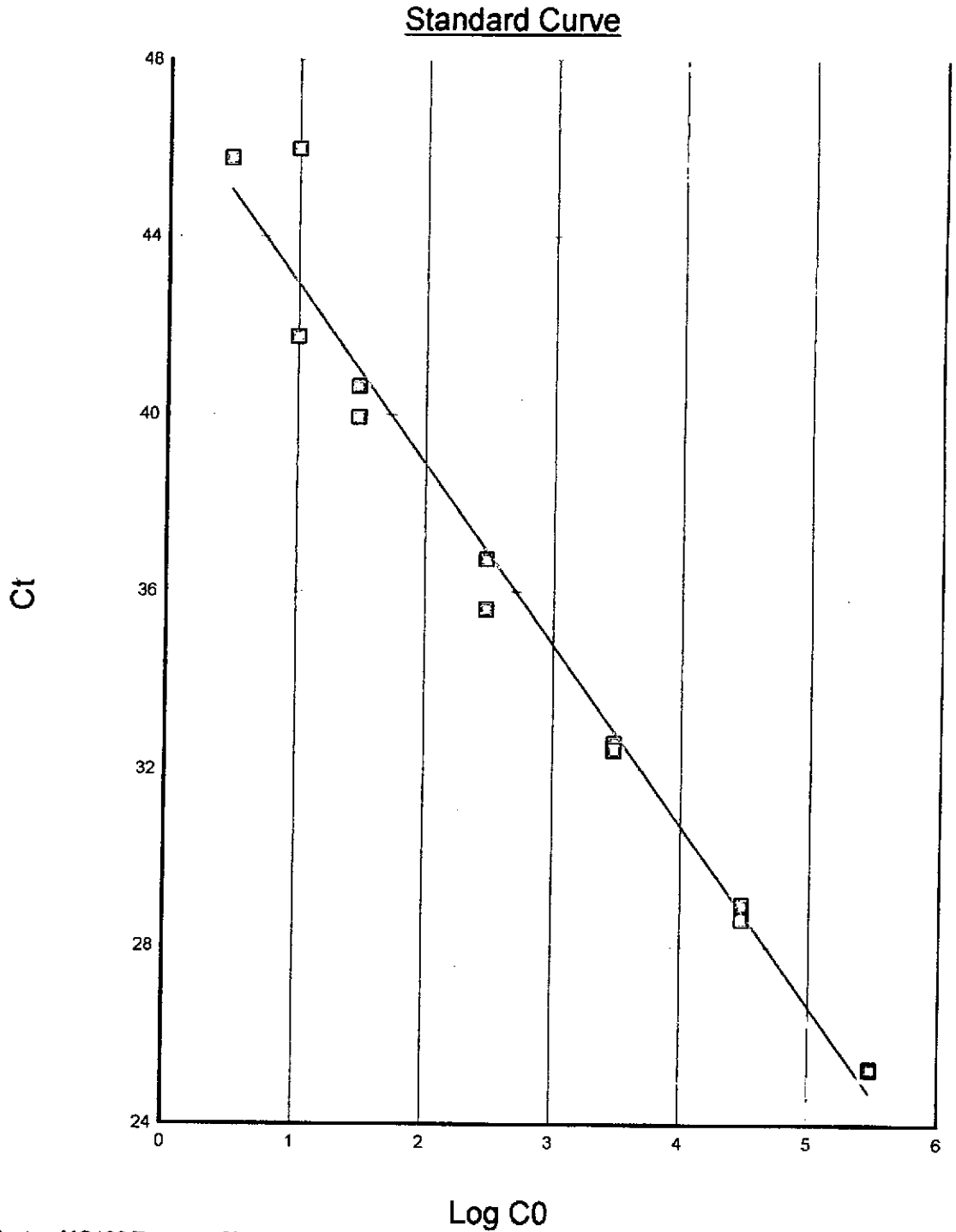
8.2.5 REAL-TIME POLYMERACE CHAIN REACTION



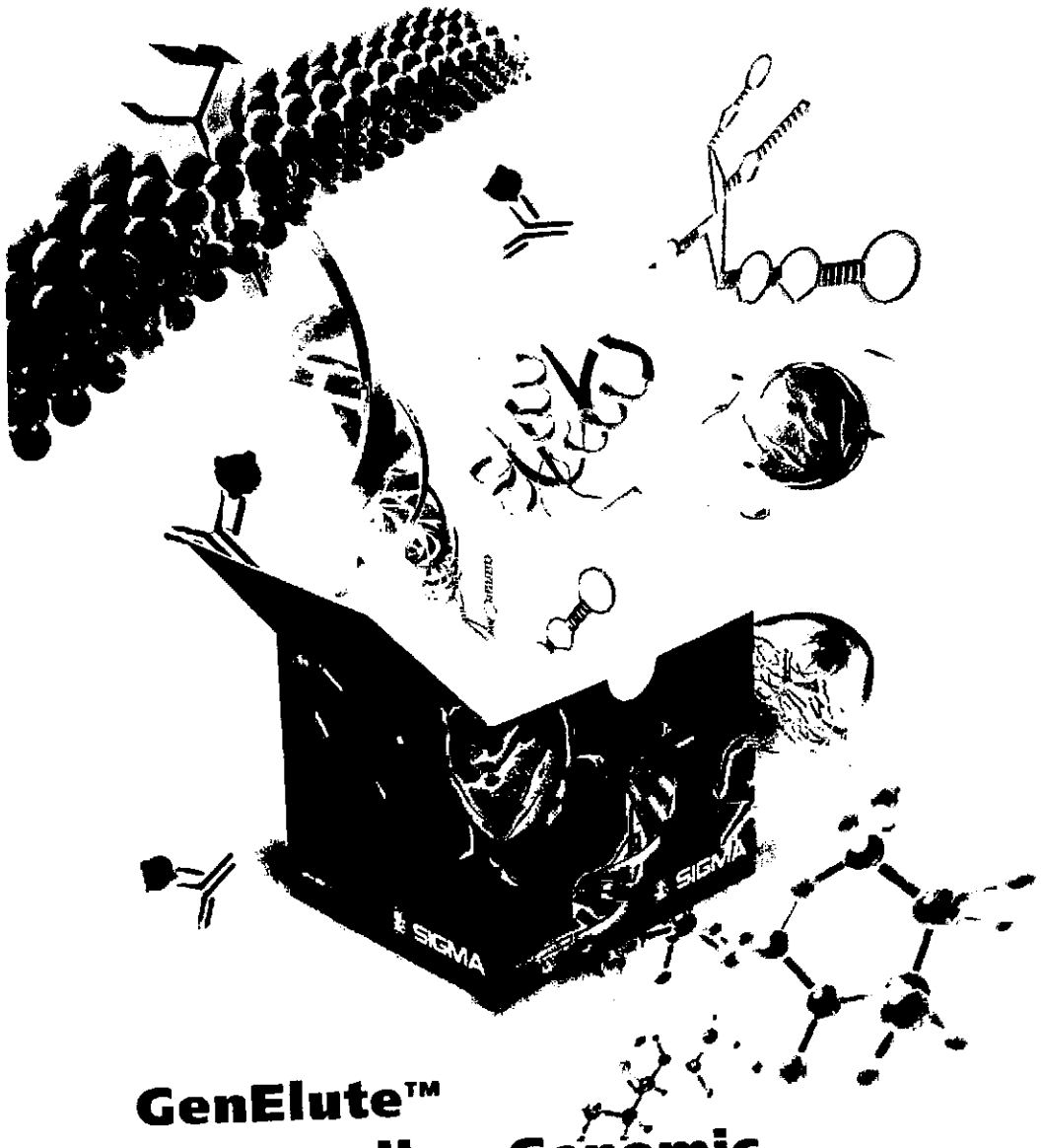
Selected Detector: All
Well(s): A1-A2,B1-B2,C1-C2,D1-D2,E1-E2,F1-F2,G1-G2,G7-G8,H1-H2
Document: 100907 (Absolute Quantification)



Selected Detector: MG190 Taqman; Start: 3; End: 15; Threshold: 0.20000000
Well(s): D9-D10,E11-E12,G5-G6,G11-G12
Document: 100807 (Absolute Quantification)



Detector: MG190 Taqman, Slope: -4.073076, Intercept: 47.022846, R2: 0.975730
Document: 100807 (Absolute Quantification)



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Catalog Numbers
G1N10, G1N70, and G1N350

SIGMA-ALDRICH

8.2.7 Samples size and sampling method.

The qualitative case controlled study as described by Schlesselman (1982) was employed in the two metropolitan cities' hospitals in the Northern and Southern Nigeria. The study aimed at investigating the relationship among the genital mycoplasmas studied, and reproductive maladies such urethritis, cervicitis, pelvic inflammatory diseases, spontaneous abortion and various degrees of infertility among male and female patients attending STI clinics in Nigeria. A non-probability sampling method, known as convenience sampling method, was used. The sample size was determined using the formula recommended by WHO (1993), as shown below:

$$N = \frac{T^2 P Q}{D^2}$$

Where:

N = Required sample size (area under the curve)

T = Confidence Interval (CI) of 95%=1.96

P = Prevalence of genital Mycoplasmas in the study population, Nigeria (50%).

Q = 1-Prevalence of genital Mycoplasmas

D = Deviation amount according to the prevalence of genital Mycoplasmas Allowable/Tolerable/ Sampling Error=5% (0.05). 3.8416 x 0.5 x 0.5 divided by 0.0025= 50%=384,

The sample size was calculated according to Senocak (1997) based on Nigerian's estimated prevalence of 50%, since the prevalence is unknown. The acceptable error margin used was 5% and 95% confidence interval. And to adjust for wastage factors such as contaminations, losses and other mishaps, the wastage rate was increased by 20% (WHO, 2003). From this formula a total of 824 subjects were used in the study. Of this, 500 were patients, while 324 individuals coming for routine checkup, and were considered under the asymptomatic group (Control). The symptomatic group (Patient), on the other hand, was those subjects with obvious discharge, itching with or without sores, urinary tract infections current or previously, abortions, primary or secondary infertilities. For molecular studies 238 samples were selected, comprising of 119 patients and 119 controls.

GLOSARY

Agarose

A long chain polysaccaride isolated from seaweed. It is similar to collagen (used in Jello) in that when it is heated (to dissolve it) and then cooled it forms a matrix (gel) with buffer solution trapped inside.

Amplicon or amplimer

The specific DNA product generated by PCR using one pair of PCR primers.

Anneal

When primers anneal to the template, they stick to the DNA strand in a specific way due to base pairings.

Base pairs

Since double stranded DNA is a very large molecule, it's awkward and relatively uninformative to talk about its size in terms of molecular weight (i.e., g/mole or kilodaltons). However, an excellent understanding of the size of the molecule can come from knowing how many consecutive base pairings (base pairs or bp) are lined up in the molecule. A piece of double stranded DNA that is 10 bp has one strand with 10 consecutive bases paired with another strand with the complimentary bases:$^{5'}$A-G-G-T-CA-A-T-T-G$^{3'}$ $^{3'}$T-C-C-A-G-T-T-A-A-C$^{5'}$

DNA polymerase

This is an enzyme that catalyze the synthesis of new DNA strand alongside a template DNA strand. All DNA polymerases synthesize DNA in the 5' to 3' direction and can add a nucleotide only onto preexisting 3'-OH group. For this reason, DNA polymerase needs a primer at which it can add the first nucleotide. DNA polymerase is widely used in PCR for various research applications. Also see Taq DNA Polymerase.

Hybridization	Complimentary bindings between probe and target DNA or RNA. It is the annealing of two complementary nucleic acid strands to form a double-stranded molecule; a technique for detecting specific nucleic acid sequences.
Homogenate	The solution made when cells are lysed in a buffer. This involves grinding the tissue up to break (lyse) all the cell walls and homogenize the entire mixture.
Kilobases	One thousand base pairs mRNA for cleavage.
Marker	Something that is experimentally measureable. This could be an enzyme activity, a radioactive label, a protein that you have an antibody against, etc.
Molecular Inversion Probe (MIP)	A single-stranded DNA molecule containing several features enabling target identification, detection, and quantification. Typically it contains two regions complementary to adjacent regions in the target DNA sequence that are allowed to anneal directly to the genomic DNA, followed by enzyme-assisted detection of the SNP alleles in the Molecular Inversion Probe assay.
Negative Control	This is an experiment that you expect to fail (like using water instead of a protein sample in a protein assay). This shows that you have the conditions controlled and a positive result is truly a positive result.

Nucleotides.	You'll need to add nucleotides (dNTPs) so the DNA polymerase has building blocks to work with.
OD600	**O**ptical **D**ensity (light scatter by suspended cells) at 600nm - a spectrophotometric measure of the number of cells/ml
Overgo	Overlapping oligonucleotide. Overgo probes are produced using two oligos that are complementary to each other and anneal to form a double-stranded region. For 36-mer overgo probes, two 22-mers create the initial template for DNA synthesis, overlapping in 8 bp, leading to the production of a labeled probe.
PCR	Polymerase Chain Reaction. A technique for amplifying DNA sequences in vitro by separating the DNA into two strands and incubating it with oligonucleotide primers and DNA polymerase. It can amplify a specific sequence of DNA by as many as one billion times.
Primer	A short oligonucleotide complementary to target DNA and acts as the leader for DNA extension. Or Short strands of DNA that adhere to the target segment. They identify the portion of DNA to be multiplied and provide a starting place for replication.
Probe	DNA or RNA molecules of specific base sequence, often labeled either radioactively or immunologically, that are used to detect the complementary base sequence by hybridization. Or A flourescent or radioactively-tagged piece of DNA or RNA used to bind and detect complimentary sequence.

PAGE

polyacrylamide gel electrophoresis functions similarly to agarose gel electrophoresis, except it uses polymerized acrylamide to produce the lattice. Acrylamide produces a lattice with smaller size holes and provides better separation of small length fragments. For example, in agarose electrophoresis, the best separations are in the 3000 bp and 300 bp and the resolution is, at best, 50 bp. In PAGE, the best separation is in the 20 base pair to 300 base pair range and the resolution is 1 bp. This kind of resolution is necessary for sequencing.

Positive control

This is an experiment that should definitely work. It's important to set one of these up so that if your test experiment doesn't work, you know that it's because of the thing that you're testing and not because of some other factor (like the cells being dead).

Quantitative RT- PCR

First commercialized in 1995. This method capitalizes on the fact that there is a quantitative relashionship between the amount of starting target sample and the amount of PCR product at any given PCR cycle number. This chemistry exploits the exonuclease activity of Taq DNA polymerase by using a cleavable labeled probe that hybridizes with target sequence in combination with forward and reverse PCR primers.

RT-PCR

Reverse Transcription Polymerase Chain Reaction. This is a PCR preceded with conversion of sample RNA into cDNA suitable for amplification by DNA polymerase enzyme.

Real-Time PCR

The polymerase chain reaction (PCR) allows a single, small, specific area of DNA to be copied numerous times. In Real-time PCR, the accumulation of PCR product is detected in "real-time" (as it happens) by using a fluorescently labeled probe and the 5'-3' exonuclease activity of Taq polymerase. The technique is highly sensitive and can be used quantitatively.

Taq polymerase. This is the enzyme that is in charge of replicating DNA. This is the polymerase part of the name polymerase chain reaction. Or heat resistant DNA polymerase isolated from Thermus aquaticus. This is one of most popular DNA polymerase used for PCR.

LIST OF ACRONYMS

ATCC:	American type culture collection
BAL:	Bronchoalveolar lavage
BV:	Bacterial vaginosis=
CSW:	Commercial sex worker
ELISA:	Enzyme linked immunosorbent assay
FVU:	First void urine geq Genome equivalent
hpf:	High power field
IHA:	Indirect haemagglutination
LAMP:	Lipid associated membrane protein
MIC:	Minimal inhibitory concentration
MIF	Microimmunofluorescense
MPC:	Mucopurulent cervicitis
NCNGU:	Non-chlamydial non-gonococcal urethritis
NGU:	Non-gonococcal urethritis
PAGE:	Polyacrylamide gel electrophoresis
PID:	Pelvic inflammatory disease
PMNL:	Polymorphonuclear leucocytes
SARA:	Sexually acquired reactive arthritis
SDS:	Sodium dedocyl sulphate
STD:	Sexually transmitted diseases
STI:	Sexually transmitted infections
LGV:	Lymphogranuloma venerum
PPLO	Pleuropneumonia-Liked Organism
WHO	World Health Organization
HIV	Human Immunodeficiency Virus
AIDS	Acquired Immune Deficiency Syndrome

JUTH	Jos University Teaching Hospital
LUTH	Lagos University Teaching Hospital
CBPP	Contagious Bovine Pleuropneumonia
PAP	Primary Atypical Pneumonia
CSF	Cerebro Spinal Fluid
CRD	Chronic Respiratory Disease
NVRI	National Veterinary Research Institute
TD	Turn Down
dNTP	di-Nucleotide Triphosphate
PBS	Phosphate Buffered Saline
CNS	Central Nervous System
C_T	Threshold Cycle
UGA	Uracil, Guanine, Adenine
J	JUTH patient
JC	JUTH Control
L	LUTH patient
LC	LUTH Control
Vaa	Variable adherence-associated antigens
MB	multi-banded antigens
UU	Ureaplasma urealyticum
UP	Ureaplasma parvum
MG	Mycoplasma genitalium
MH	Mycoplasma hominis
MP	Mycoplasma penetrance
MF	Mycoplasma fermentance
MPe	Mycoplasma pneumoniae
ATP	Adenosine Tri- Phosphate
T-strain Mycoplasma	Tiny-strain(Ureaplasma)