STUDIES ON UREAPLASMA SPP OF HUMAN AND CATTLE ORIGIN WITH REFERENCE TO PREVALENCE, CHARACTERIZATION AND PATHOGENICITY IN NIGERIA

BY

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TO GOD BE THE GLORY

D E C L A R A T I O N

I hereby declare the originality of this piece of work which was carried out by me in the Laboratory of the Department of Veterinary Pathology and Microbiology, Ahmadu Bello University, Zaria, under the supervision of Professor D.S. Adegboye.

The work of other investigators is duly acknowledged and referred to in this thesis. No part of this thesis has been presented elsewhere for a degree or diploma.

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THESIS CERTIFICATION

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ABSTRACT

A strong association has been reported between the occurrence of Ureaplasma in human and cattle and various infertility problems. In Nigeria, not much has been done to determine the role of these Ureaplasma organisms in urogenital conditions of man and animals. Also the subsequent effect on the livestock industry is not known. This work was therefore designed to determine the prevalence of the organism in human and cattle population. In addition attempts were made to determine their susceptibility to antimicrobial agents, study the various serotypes of these organisms and also to establish the pathogenicity of some of the isolates.

Five media preparations comprising a conventional urea broth (UB) and four buffered media N-tris (Hydroxymethyl) methyl-2- aminoethane-sulfonic acid (MES), N-2-Hydroxyethyl piperazine-N'-2-ethanesulfonic acid (HEPES), Tris (Hydroxymethyl) aminomethane (TRIS), and N-tris (Hydroxymethyl) methyl-2-aminoethane-sulfonic acid (TES) buffers were compared for ability to support the growth of human strains of Ureaplasma spp. Swabs from the cervix and urethra of 116 patients attending the sexually transmitted disease clinic with gonococcal and non gonococcal infections were propagated in each of the

five media. Moreover, five arginine-supplemented preparations, namely arginine broth, MES, HEPES, TRIS, and TES-supplemented arginine broths and the urea broth (UB) were compared for the detection of Mycoplasma hominis, a mycoplasma frequently encountered in human genital tract. A high recovery rate of U. urealyticum was obtained with the UB which detected 98.3% of the ureaplasmal positive samples. The UB solidified with 1% agar, apart from its suitability for culturing Ureaplasma spp. also compared favourably (100%) with arginine agar (AA) in supporting the growth of M. hominis. Improved viability evident by a higher growth titre, was obtained with HEPES and TRIS buffered media than with the UB. The best growth profile was obtained with the HEPES - buffered medium.

Using the UB media, swabs from the cervix or posterior vaginal fornix of 929 women comprising 283 attending the family planning clinic, 100 pregnant women attending antenatal clinic, 500 with various infertility problems attending the gynaecology clinic, and 46 women attending the venereal disease clinic were cultured for U. urealyticum and M. hominis. In addition, urethral swabs from 70 men with gonococcal urethritis were similarly cultured. The recovery rate of U. urealyticum from all the 500

infertility cases was 60% while from the controls (family planning group and pregnant group) the recovery rate was 37%. Amongst venereal disease patients the recovery rate was 51.7%. Data show a strong association between Ureaplasma or mixed Ureaplasma and M. hominis infection and secondary infertility. No relationship was observed between M. hominis infection alone and any of the condition. Two hundred bovine samples consisting of 150 vaginal swabs, 30 preputial swabs and 20 bull semen were also cultured in UB medium and glucose medium (G-medium) for Ureaplasma and mycoplasma-like organisms respectively. The recovery rate of Ureaplasma from these specimens was 70%, 83%, and 65% from vaginal swabs, preputial swabs and semen respectively. Also a recovery rate of 42%, 76% and 30% was obtained for mycoplasma-like organism from the bovine vagina, preputial and semen samples respectively.

One hundred and seventy five Ureaplasma isolates consisting of 60 from the family planning group, 50 from the infertility patients and 35 from the sexually transmitted disease patients and also 30 from bovine sources were compared in certain biochemical tests. All strains were phosphatase positive, catalase negative, and did not reduce tetrazolium dye. Weak zones of B-haemolysis were

observed with all the strains on UB medium. Ureaplasma were inactivated by their toxic metabolite in broth.

When the growth inhibition test was compared with the metabolic inhibition test using 134 human isolates, a high degree of agreement between the two tests was obtained in serotyping the isolates. The growth inhibition test was easier to conduct and was highly specific but lacked sensitivity; the metabolic inhibition test was less specific but was more sensitive. No particular correlation between patient status and serotypes were observed in this study. Overall, serotype 8(15%) was encountered most frequently, followed by serotypes 2(12%) and 4(12%). Other serotypes were encountered in lesser degrees except serotype 7 which was however not encountered in this study. In cattle, 80% of the strains were typed and classified into the existing three groups using only the growth inhibition test.Of 100 bovine isolates typed, 39% were in group A, 33% in group B, and 8% in group C.

Eighteen strains of Ureaplasma species consisting of 15 isolated from humans and 3 from bovine sources and one of M. hominis were tested for their responses to various antimicrobial agents. The strains exhibited three susceptibility patterns. Firstly, togamycin, chloramphenicol, and

chlorletracycline inhibited the organism at much lower concentrations. A tetracycline resistant strain, STC 145U (serotype 4), was encountered. This strain, however, was highly sensitive to togamycin - a spectinomycin. Secondly, erythromycin, streptomycin, and gentamicin susceptibility pattern was observed. The third susceptibility pattern was that of clindamycin, cotrimoxazole, nalidixic acid and nitrofurantoin. Clindamycin was generally ineffective (MIC = I500ug/ml) on the Ureaplasma strains, but was highly effective on M. hominis (MIC = 2.9ug/ml). A reverse pattern was observed with erythromycin. No overt differences between human and bovine isolates of Ureaplasma were observed in their general suceptibility to the various antimicrobial agents.

The pathogenic potentials of local strains of Ureaplasma BS 2112 from bovine semen and STC 145U, ACC 80, FP 81 obtained from human urogenital tract were compared. The mammary glands of 17 lactating goats were inoculated with these local isolates. Strain differences in the response of the glands were observed. Bovine strain BS 2112 elicited severe mastitic response. The human strain STC 145U caused some degree of clinical mastitis which was

resolved by the 14th day post-inoculation. Similar but milder responses were obtained with strains ACC 80 and FP 81.

It is concluded that the mammary gland of the Savannah Brown goats can be used to study the pathogenesis of the various Ureaplasma strains.

Intravenous inoculation of Ureaplasma strain BS 2112 did not cause any increase in rectal temperatures of the goat. Mild vulvitis was, however, observed a few hours after intravaginal inoculation of the Ureaplasma. Abortion occurred in one of two goats inoculated intravaginally and Ureaplasma was isolated from the genitalia of the doe and from the lungs of the aborted fetus.

This study therefore highlights the development of media for Ureaplasma cultivation in this environment, is the first to serotype Ureaplasma specie in Nigeria, and successfully used the goat mammary gland as a pathogenicity marker.

CHAPTER ONE

INTRODUCTION

Until recently, one of the most neglected areas of research in Microbiology in Nigeria and indeed Africa has been the study of genital mycoplasmosis. A renaissance of interest in these micro-organisms has recently occurred as a result of various reports by research scientists in other parts of the world implicating Mycoplasma and more specifically Ureaplasma in various human and animal urogenital conditions.

Ureaplasma are grouped together because of a common biological property which they possess - their small colony size and the ability to hydrolyse urea (Shepard et al., 1974). The number of antigenically distinct species within the genus Ureaplasma is not known. Mouches, Taylor-Robinson, Stipkovits and Bove (1981) in their studies reported the existence of two groups - A and B of human ureaplasma.

It was also clear from the study of Mouches et al. (1981) and that of Howard, Pocock, and Gourlay (1981) that bovine Ureaplasma can be divided into three groups. With these differences it is not known whether the human and bovine ureaplasmas should be separated into species or not. However, the basic

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distinct differences in the protein properties between the human and bovine ureaplasma have compelled these authors to press for a formulation of at least one species name for the bovine strain. They subsequently proposed Ureaplasma diversum for a strain from cattle (Howard and Gourlay, 1982).

Ureaplasma have been isolated from the genital tract of a wide variety of animals and have been observed to have the potential for causing disease in this anatomical site (Shepard, 1967; Koshimizu and Magaribuchi, 1978; Ruhnke, Doig, Mackay, Gagnon and Kierstead, 1978). The occurrence of Ureaplasma in the genitourinary tract of cattle particularly in the semen which is probably responsible for the impairment of sperm motility has led to the observation that these may be a likely cause of infertility (Jurmanova and Sterbova, 1977). The association between Ureaplasma and vulvovaginitis, a condition which reduces fertility, has been reported (Doig and Ruhnke, 1977). Other reports of reproductive failure with which Ureaplasma have been associated include endometritis, salpingitis (McCormack, Braun, Lee, Klein and Kass, 1973), and abortion (Stalheim, Proctor and Gallagher, 1976).

Under experimental condition, Ureaplasma spp has produced mastitis in cattle, goats, and mice, and is responsible for a portion of bovine cuffing pneumonia (Taylor-Robinson, 1979).

Experimental infection of human volunteer reveal that Ureaplasma undoubtably cause urethritis (Taylor-Robinson, Csonka and Prentice, 1977) but whether these associations are indeed causal is still a subject of controversy.

If the associations are indeed causal, clearly, the significance of an indepth study of this organism cannot be overemphasized.

In a preliminary investigation into the isolation of this organism in humans in association with infertility in Nigeria, Osoba (1972) reported a prevalence rate of 60% while a rate of 65% was reported by Adegboye, Briggs and Lister (1979). Moreover, investigation has revealed the presence of Ureaplasma at a high rate in the Nigerian cattle (Adegboye and Hassan, 1976; Adegboye, Hassan, Bwala and Fasanya, 1980).

In our quest for improved livestock production in Nigeria, emphasis is shifting towards the use of high quality bulls for semen production. But with the ubiquitous distribution of Mycoplasmas in general and the high rate of Ureaplasma isolation

already observed, this organism could be a threat to the livestock industry.

These problems have led to the following specific research objectives:

(i) To determine the prevalence of infection by various serotypes in humans and cattle in Zaria area; Serotyping of Ureaplasma has not been done previously on isolates from Nigeria.

(ii) To characterize Ureaplasma isolated from cattle and humans as to their biochemical properties and antibiograms.

(iii) To determine the pathogenicity of selected local serotypes of Ureaplasma in an animal model.

CHAPTER TWO

LITERATURE REVIEW

A. Mycoplasma and Related Organisms in General

Mycoplasmas are the smallest organisms capable of autonomous living and replication (Anon, 1974). Mycoplasmas lack cell wall, and this single most important characteristic distinguishes them from other prokaryotes. This anatomical feature is responsible for most of the peculiar properties of the mycoplasmas as a group, notably their sensitivity to lysis by osmotic shock, alcohols and organic solvents, detergents, antibody and complement; their filterability through 0.45um pore diameter membrane filters; their "fried egg" colony shape, and their total resistance to penicillin and other antibacterial substances that degrade or specifically inhibit peptidoglycan synthesis (Razin, 1983). However many of these features are shared by the L-phase variants (L-forms) of bacteria, in which the cell wall is either defective or is totally missing. These L-phase variants can usually revert to the wall covered bacterial forms once the inducing substance is lacking or removed from the growth medium. Edward and Freundt (1956) pointed out

that important differences exist between mycoplasmas and L-phase bacteria in metabolism and growth requirements. While both Mycoplasma and the L-phase bacteria require serum-enriched media, the serum supplied cholesterol, an essential nutrient for the mycoplasmas and rather only appears to neutralize a growth inhibitor for the L-phase. Although Dienes and Weinberber (1951) admitted the existence of an independent Mycoplasma group, they suggested that the organisms had evolved in the past from bacteria by variation to an L-phase which had become stable and persistent. To dispel this theory, Edward (1954) reported that if mycoplasmas had been derived from different bacteria as was thought, it would be expected that their fermentative capacity for various carbohydrates would vary from organism to organism, but he found that all Mycoplasma organisms if they ferment carbohydrate at all fermented the same carbohydrate.

Mycoplasmas resemble viruses by being able to pass through 0.45um membrane filters, but unlike viruses, can be cultivated in cell-free, though highly complex media.

The lack of a cell wall constitutes the basis for the inclusion of the mycoplasmas in a separate

class called Mollicutes (mollis, soft; cutis skin). A summary of the main characteristics of the class Mollicutes, its subdivisions and properties used for differentation within each taxonomic category is given in Table 2.1.

The family Mycoplasmataceae to which the genus Mycoplasma belongs includes two other closely related genera, viz: genus Acholeplasma which produces colonies similar to those of the genus Mycoplasma but whose members do not require sterol for growth, and the genus Ureaplasma, originally known as T-mycoplasma, because of their characteristic tiny colonies. The renaming of the latter as Ureaplasma was based on their ability to hydrolyse urea (Shepard, Lunceford, Ford, Purcell, Taylor-Robinson, Razin and Black, 1974).

Table 2.1: Taxonomy of the Class Mollicutes

Class: Mollicutes

Order: Mycoplasmatales

Family I: Mycoplasmataceae

1. Sterol required for growth
2. Genome size approximately 5.0×10^8 daltons
3. NADH oxidase localized in cytoplasm

Genus I: Mycoplasma (64 species current) Do not hydrolyse urea

Genus II: Ureaplasma (2 species with serotypes) Hydrolyse urea

Family II: Acholeplasmataceae

1. Sterol not required for growth
2. Genome size approximately 1.0×10^9 daltons
3. NADH oxidase localized in membrane

Genus I: Acholeplasma (8 species current)

Family III: Spiroplasmataceae

1. Helical organisms during some phase of growth
2. Sterol required for growth
3. Genome size approximately 1.0×10^9 daltons

4. NADH oxidase localized in cytoplasm

Genus I:Spiroplasma (3 species current)

Genera of uncertain taxonomic position

Thermoplasma (1 species: apparently belongs to Archaebactoia)

Anaeroplasma (2 species)

From Freundt (1983)

B. Ureaplasma

(a). History and Nomenclature

Ureaplasmas constitute a group of mycoplasmas which were first isolated from the human genito-urinary tract by Shepard (1954). They were originally designated "Tiny-form pleuropneumonia-like organisms", and subsequently designated as T-mycoplasmas or T-strains because of the tiny colonies they produce on agar medium (Shepard, 1954).

The subcommittee on the Taxonomy of Mycoplasmatales (1974) concluded that the presence of urease in the T-mycoplasmas is sufficiently unique to warrant their assignment to a new genus within the family Mycoplasmataceae. The name Ureaplasma was proposed for this new genus. This together with the works of Shepard and coworkers (1974) culminated in the formulation of a new genus and species Ureaplasma urealyticum for the human strains.

Ureaplasmas have similarly been isolated from various animal species such as cattle (Taylor-Robinson, Haig and Williams, 1967; Gourlay 1968, Adegboye et al., 1980), dogs (Taylor-Robinson, Martin-Gourgon, Watanabe, and Addey, 1971), cats

(Harasawa, Yamamoto and Ogata, 1977), monkey (Taylor-Robinson et al., 1971), birds (Koshimizu and Magaribuchi, 1978; Stipkovit and Rashwan, 1976), pigs (Stipkovit, Rashwan, Takacs and Lapis, 1978) and sheep and goats (Livingstone and Gauer, 1975; Doig and Ruhnke, 1977; Kotani, Nagatomo and Ogata, 1980).

Using the one and two dimensional protein analysis on polyacrylamide slab gel procedures, Mouches et al. (1981) observed that the ureaplasmas of human origin can be separated into two groups, A and B. This had earlier been revealed by Howard et al. (1981) who used a one dimensional polyacrylamide gel electrophoresis (PAGE). This division of human ureaplasma into two groups (Mouches et al, 1981; Howard et al., 1981) was consistent with the result of DNA-DNA hybridization studies by Christiansen Black and Freundt (1981) and Howard, Pocock and Gourlay (1978) in which the eight serotypes of Black (Black, 1972) were subdivided in the same way. It was however not stated whether this difference means that human ureaplasma should be separated into two species. It was also clear from the study of Mouches et al. (1981) that bovine unreaplasma can be divided into three groups using the PAGE analysis. Group A

comprised of a cluster of related strains (Mmbi 167, Bu2, A417, T45, T95 and T288) which they revealed could further be subdivided into three subgroups. A distinct group B comprising strains T74 and T44 was observed. Another clustering of strains was reported for group C comprising strains T315 T71 and D48. These divisions of the bovine ureaplasmas was essentially the same as those described independently by Howard et al., (1981). Both studies confirmed that bovine ureaplasma strains were similar to each other, but were distinct from the human strains based on the marked differences in their protein profiles. These combined with the known differences of the guanine + cytosine (G+C) content of the bovine and human ureaplasma strains compelled these authors to press for a formulation of at least one species name for the bovine strains. Thus a new name Ureaplasma diversum was proposed for the bovine strains (Howard and Gourlay 1982).

On the other hand, the ureaplasmas isolated from talapoin monkey, chimpanzee and chicken were found to be similar to the human ureaplasmas of group A, the most closely related being the chimpanzee strains.

(b). Morphology and Cultural Characteristics

Ureaplasma urealyticum in broth cultures occur as spherical to ovoid elements, approximately 300nm in diameter, growing singly, in pairs, triads, tetrads and in short chains as well as in flocs or aggregates of organism (Shepard & Masover, 1979). In agar cultures, U. urealyticum occur as coccoidal organism growing in loosely-bound masses downward into the agar medium as visualized under the light microscope.

Individual cells are spherical, 0.2-1.0um in size with a bounding trilaminar membrane 10nm thick and containing 7.5 - 12.5nm particles and a layer of pilus-like projections on the outer surface (Whitescarver and Furness, 1975). During replication, these elongate slightly and the ribosomes migrates to the ends of the cell leaving a ribosome-free area into which the bounding membrane invaginates to form a bud. The bud eventually separates by the completion of the process of invagination.

Williams (1967) presented a few pictures of negatively stained Ureaplasmas. The presence of

heavily stained material around the cells made interpretation difficult, but organisms varying in size from 150 to 800nm and frequently possessing an envelope with spike-like structures were described.

In the same year Clyde and Kim (1967) published a micrograph of sediments of Ureaplasma prepared by the shadowing technique. It revealed clusters of spherical elements without filaments. Shepard (1969) using osmic acid fixation and chromium shadowing described the ureaplasmas as round to ovoid cells embedded in a tenacious gelatinous matrix. In an earlier study, Shepard (1967) using the fluorescence microscopy after acridine staining observed that these mycoplasmas were rich in RNA and DNA. Work on electron microscopy (EM) by this author further confirmed these observations and also showed that the cells divide primarily by budding in different directions producing poly-directional growth ramifications (Shepard, 1969).

Taylor-Robinson et al. (1968) published electron micrographs of thin sections of one bovine and one human ureaplasma using uranyl acetate and lead citrate for staining. They observed that the cells were spherical and varied in size from 80 to

420nm; were bounded by an 8-9nm thick triple-layered membrane. The cytoplasm contained electron-dense and transparent spheres in some of which fibrils were 30-40A^{o} in diameter. Using the same preparation and staining technique, Rottem, Pfendt, and Hayflick (1971) confirmed the observations of Taylor-Robinson et al. (1968) and in addition, found filamentous forms which were more than 2nm long and 200um in diameter. A more extensive study by Black, Birch-Andersen and Freundt (1972a) revealed that the basic characteristics of the cells such as morphology, size, membrane, ribosomes and DNA strands were in accordance with previous findings. In addition Black et al. (1972a) observed that in the interior of some larger cells, vacuole-like structures were seen, always bounded by a triple-layered membrane and possessing an additional layer of interior surface. Further, Black (1974) felt that the membrane bounded 'vacuoles' described for ureaplasmas and in other mycoplasmas may properly be cross-cuts of invaginations. His interpretation is supported by the finding of an extra-membraneous layer on the inside surface of these structures. The calculated G + C contents of Ureaplasma were in the range of 26.9 to 28% (Black, Christiansen and Askaa, 1972b).

(c). **Growth Requirements**

Ureaplasmas are fastidious in their nutritional requirements, (Shepard and Lunceford, 1967), and thus require complex growth medium. The complete growth requirements of ureaplasmas are not fully known except for cholesterol and possibly urea.

The commonly used broth base of most Ureaplasma medium are trypticase soy broth Hartley digest broth, tryptic soy broth and PPLO broth (Shepard and Lunceford, 1970; 1976.). These basal media often contain sodium chloride which helps in adjusting the required medium tonicity.

(i) **Sterol:**

Rottem et al. (1971) who found that sterol which could be supplied by cholesterol as well as B-stilbesterol was needed for growth of Ureaplasma. This finding was confirmed by Black (1974) who tested human ureaplasma serotypes for their susceptibility to digitonin.

In most media, the sterol is provided by addition of horse serum to the medium.

Ureaplasmas as in most mycoplasmas have limited biosynthetic abilities due to their limited genetic information (Razin, 1983). Serum has been known to provide fatty acids and cholesterol for membrane synthesis in an assimilable and non-toxic form. The serum in addition to providing a non-toxic source of lipids may be supplying essential nutrients (Rodwell, 1983).

(ii) **Urea**

Shepard and Lunceford (1967) and Ford and MacDonald (1967) concluded that Ureaplasma require urea for growth. Their findings were based on the observation that urea in low concentration with an optimum of about 0.05% had a growth enhancing effect. Also they observed that urease treated and dialyzed horse serum failed to support growth unless urea was added and that exhausted media could be reactivated by the addition of urea. Furthermore, Ford (1972) reported the inhibition of growth of ureaplasmas by

hydroxamic acids which are known as specific inhibitors of urease. In a related study, Ford (1973) claimed that the urease activity of Ureaplasma strains was a direct requirement for multiplication by the organism. On the other hand, Masover, Benson, and Hayflicks (1973) claimed that ureaplasmas replicated in media prepared from dialysed components with added urea.

In another related experiment, Razin et al., (1977) reported the inclusion of putrescine in agar media containing dialyzed calf serum and dialyzed horse serum. It was found that putrescine by itself did not support the growth of ureaplasma. However, some workers (Shepard and Combs, 1979) agree that addition of putrescine to the media together with urea improved growth considerably over that observed with urea alone. The reason for the growth enhancing property of putrescine was not stated. However since putrescine are polyamines, and these are known to be stimulatory agents for a variety of bacterial, mammalian tissue cell lines,

viruses and plants, it could be speculated that the growth enhancing quality might be related to this polyamine property. It has been shown that there was no indication of incorporation of either nitrogen or carbon from urea into the ureaplasmal cells (Ford and MacDonald, 1967; Ford et al., 1970). However, Romano, Tolone, Ajello and Lalicata (1980) gave some preliminary evidence supporting the hypothesis that ATP is generated in the ureaplasmas through an electrochemical ion gradient produced by the ammonia liberated during the intracellular hydrolysis of urea by urease produced by the organisms. This might probably explain why Ureaplasma show that unique requirement among living organisms for urea.

(iii) **Temperature**

The influence of temperature on growth of Ureaplasma was first reported by Ford (1962) who found that Ureaplasma could grow at 30°C as well as at 33°C but remained viable longer at the lower temperatures. This report was later confirmed by Black (1974) who showed that eight human Ureaplasma serotypes could

grow on solid medium at temperatures from 22°C to 40°C and some even at 20°C with the optimum at 37°C. Growth in liquid medium gave similar results. At 37°C the peak of the growth curve was normally reached after 12-16 hours and viability lost after 30-40 hours. Ford (1962) showed that ureaplasmas could remain viable at room temperature for three days, at 4°C for at least 16 days and at -20°C for more than 90 days. Addey et al. (1970) extended these studies and found that human ureaplasma, like other mycoplasmas, could be stored at -30°C for more than 27 months and at -70°C for more than 42 months.

There is a reported disagreement in the literature on the resistance of ureaplasma to heating at 56°C. Taylor Robinson (1968) found that only one of the ureaplasmas tested could withstand heating at 56°C for 5 minutes in liquid medium but Black (1974) reported that some serotypes of Ureaplasma could survive in liquid medium at 60°C for 10 minutes but none for 30 minutes. The differences in heat resistance as reported by these two workers may be attributed, in part, to the types of liquid media and the serotypes used.

(iv) **Culture Media**

Since its discovery by Shepard (1954) the cultivation of Ureaplasma urealyticum has presented problems to laboratory workers. This difficulty in growth detection resulting in the absence of turbidity evident with Ureaplasma isolation was lessened when Shepard and Lunceford (1970) introduced the U-9 broth. This broth has an in-built indicator system based on the ability of U. urealyticum to degrade urea to Carbon-dioxide and ammonia (Shepard and Lunceford, 1967). The resultant accumulation of ammonium ions increases the pH to change the colour of the phenol red indicator from yellow to red (Shepard and Lunceford, 1970). This change is not evident, however, until stationary growth phase is reached and the cultures lose viability soon thereafter.

The urease colour test broth 10C developed by Shepard and Lunceford (1978) was said to be good for primary isolation and general cultivation of ureaplasmas of human and bovine origin. The broth base consists of PPLO broth as compared to Trypticase soy broth

of medium U9. Both these media incorporated penicillin G. potassium at a final concentration of 1000 U/ml as bacteriostat and both used phenol red as pH indicator. A urease color test broth and agar U4 was employed by Howard et al. (1978) for serotyping bovine ureaplasma isolates. All the broth media described have been successful in their various laboratories for the isolation of Ureaplasma spp. from humans or bovine but have the problem of retaining viability upto the pH change.

Although the rapid decline characteristic of ureaplasma cultures have been overcomed through the use of a vacuum flow system, (Hendley and Allred, 1972), dialysis culture (Masover and Hayflick 1974) and an ion-exchange resin with pH adjustment (Windsor & Trigwell, 1976), none of these methods is applicable to the routine laboratory handling of cultures. More lately, Robertson (1978) reported the use of a bromothymol blue broth which was an improvement on the U9 broth both in counts and viability of organisms. However

the U9 broth is still the most widely used broth for the detection and cultivation of U. urealyticum.

Several agar and broth media have been described for the isolation of Ureaplasma, each an improvement over the other in an attempt to improve recovery, isolation and survival of this delicate organism. The use of a number of different media and culture conditions may explain, in part, the wide variation in the reported isolation rates of ureaplasmas in various human and animal populations.

Yajko et al. (1984) concluded after a media evaluation study that a combination of the agar medium A7B with a broth medium like U9B both devised by Shepard and Combs (1979) can be used for the simultaneous isolation of Ureaplasma with a high recovery rate. A new differential agar medium designated A8 was formulated by Shepard (1983). The author replaced manganous sulphate indicator with equimolar calcium chloride ($CaCl_2$) with a resultant 10% gain in colony numbers.

Robertson and Chen (1984) recently reported that strains of U. urealyticum isolated from humans were inhibited by manganese (Mn) and this was reflected in a reduction in the rate of growth as well as morphological alteration of colonies. However they reported that the Mn inhibition could be blocked by magnesium (Mg) suggesting that the latter was required for an essential cellular function although the blocking effect of the Mg is only partial. This Mn inhibition of ureaplasmas was strain specific. The differential response has led to the thought of a possibility of the existence of biotypes among strains of Ureaplasma isolated from humans.

(d). Biochemical Reactions

(i) Urease Activity

Purcell, Taylor-Robinson, Wong, and Chanock (1966) were the first to show that ureaplasmas are able to metabolize urea. Later, more detailed studies on the occurrence

of urease activity in ureaplasmas were reported (Shepard and Lunceford, 1967; Ford and MacDonald, 1967).

This activity was found both in cultures with multiplying cells and those at the stationary growth phase, with the maximal activity reported at pH 6.0 $\pm$ 0.5. Ureaplasmas are the only mycoplasmas so far known to possess urease enzyme; thus, demonstration of urease activity is specific for their identification. This urease activity, and consequently the presence of Ureaplasma, can be demonstrated by adding 0.05% urea and a pH indicator to the growth medium resulting in an alkaline shift in pH, or by the direct test of Shepard and Howard (1970) in which a solution of urea and manganous chloride is applied directly on to the plate with growth of Ureaplasma resulting in the formation of manganese dioxide causing a dark colouring of the colonies. The hydrolysis of urea leading to the liberation of ammonia plays a significant role in the manganese dioxide formation. The water insoluble manganous dioxide precipitates on or around the Ureaplasma colonies tinting them dark brown.

(ii) Phosphatase Activity

The occurrence of phosphatase activity in human and bovine ureaplasma was reported by Black (1973a). Both acid and alkaline phosphatase can be demonstrated on both liquid and solid medium containing phenolphthalein diphosphate by alkalinization of the medium after growth had occurred. However in contrast to urease, phosphatase activity is common to most mycoplasmas (Aluotto, Wittler, Williams and Faber, 1970; Burger et al., 1970).

(iii) Toxic Product

Furness (1973) reported the production of an inactivating toxic substance during the growth of ureaplasma in broth. This toxic substance which was catalase resistant, heat-stable, and dialysable was produced by all the ten strains tested and each inactivated not only the strain producing it but all other strains. The results of this study showed that the production of toxic substance is a characteristic of the ureaplasmas and that all strains produce the same inactivating factor, although this remains to be substantiated.

The author observed that the ability of spent culture media to support growth was not restored by adding the media constituents like yeast extract, horse serum, or the trypticase soy broth. That the limiting factor was not an essential metabolite was confirmed by increasing the nutrients in the urea broth.

(iv) **Other Activities**

Test for glucose and arginine breakdown, hydrolysis of aesculin, reduction of tetrazolium and methylene blue, lecithinase activity, and serum digestion were all found to be negative (Black, 1974; Woodson, McCarty and Shepard, 1965).

Vinther and Black (1974) observed that U. urealyticum possess amino-peptidase activity. This adds to the limited knowledge of the biochemical activity of these organisms. This proteolytic activity is a well known property of some Mycoplasma species. However the possible taxonomic significance of this peptidase activity is not known.

Robertson et al. (1984) reported that U. urealyticum possesses the ability to cleave

human IgA into at least two fragments. All the 14 serotype standards and 34 of the 35 wild type strains isolated demonstrated this immunoglobulin A protease activity. This property has so far been known to occur in only three of the many bacterial genera that have been examined, and only species within these genera exhibit this activity. This activity had generally been associated with pathogenicity, although there are a few inconsistencies where the activity was observed in organisms considered saprophytic. The possibility of the potentials for pathogenicity in these saprophytic strains can, however, not be ruled out. Ureaplasma urealyticum is the only member of the Class Mollicutes known so far to possess this property. Whether the bovine ureaplasma would possess this property is not known.

(v) Haemolysis

The human ureaplasma was shown by Shepard (1967) to produce a soluble B-type haemolysin which lysed guinea-pig erythrocytes but only small haemolytic zones were seen. Similarly Black (1974) confirmed that eight serotypes of human ureaplasma all produced clear B-haemolysis when guinea-pig or rabbit

erythrocytes were employed but no effect were seen on human erythrocytes. The haemolysis was observed both when the plates were incubated under aerobic and anaerobic conditions. No inhibition of the haemolysis was observed when catalase was incorporated into the blood agar, but only when it was added in a high concentration in a well in the plate. So far the nature of the haemolysin of this group is still uncertain. Although peroxide production was not reported by a direct test, the inhibition observed by Black (1974) of the haemolysis by catalase may indicate that the haemolysin or at least part of it is a peroxide.

(vi) **Lipoglycans**

Lipoglycans are polysaccharides containing covalently attached lipids. They are found as surface structures on several Acholeplasma, Anaeroplasma, Mycoplasma, and Thermoplasma species. Recently, Smith (1985) reported finding membrane-associated polymers which he called lipoglycans based on their component parts.

He observed that most of these lipoglycans are an intergral part to the cytoplasmic membrane and that each serotype possesses a distinctive lipoglycan. The surface location suggested that structural differences among lipoglycan could contribute to the antigenic specificity of the different serotypes. The significance of this new finding is not stated, but it is unlikely to be responsible for haemadsorption, since only one serotype 3 exhibits this activity with guinea-pig erythrocytes. This finding however offers another cellular involvement in pathogenicity.

(e). Antibiotic Sensitivity

Ureaplasmas are very susceptible to tetracycline, chloramphenicol, streptomycin, kanamycin and gentamicin (Stewart et al., 1969) but only slightly so or not at all to penicillin and ampicillin. Shepard et al. (1966), first demonstrated that erythromycin inhibited Ureaplasma in-vitro while lincomycin and clindomycin inhibited M. hominis and M. fermentans in much lower concentrations than required for the Ureaplasma

(Braun, Klein and Kass, 1970). MOst of these studies were carried out in liquid medium and a high degree of variation between these different strains were observed.

Using the tablet-diffusion test for the study of possible major differences in susceptibility between the serotypes of human Ureaplasma, Black (1974) observed no significant differences and his results were in agreement with those obtained in liquid medium.

Selective inhibition of ureaplasmas by thallium acetate in primary isolations and by 5-iodo-2'de-oxyuridine (idu) were first reported by Shepard (1967) and later confirmed by Taylor-Robinson et al., (1977) and Black (1974).

It has been taken as an indication of a causative role of Ureaplasma in non-gonococcal urethritis (NGU) that a good correlation is seen between the in-vitro sensitivity of Ureaplasma and the effectiveness of antimicrobial agents in the treatment of the disease. Confirming this assumption, Csonka et al. (1966) found that 78% of their NGU patients who responded to tetracycline treatment haboured Ureaplasma in the genito-urinary tract as against only 36% of patients who did not

respond. Csonka and Spitzer (1969) also observed that the cure rate for NGU patients treated with lincomycin (26%) was similar to that found after giving a placebo confirming the non-effectiveness of the drug, and that Ureaplasma did not disappear after unsuccessful treatment.

Gnarpe and Frieberg (1972, 1973) observed that 29% of women from Ureaplasma infertile couples became pregnant after a course of treatment with doxycycline, although no controls were included in this study making any conclusions questionable.

However, recently Quinn et al. (1983b) reported a reduction in pregnancy loss from 96% to 49% after treating these group with doxycycline.

They reported that treatment with doxycycline was more effective in reducing the loss rate for women with previous first trimester abortions than for women with previous second trimester abortions. In the same study it was reported that treating couples to clear Ureaplasma with doxycycline prior to conception and treating with erythromycin from second month of pregnancy reduced the loss rate further to 17%. This latest study confirms earlier reports on the efficacy of doxycycline on ureaplasmas.

Ford and Smith (1974) first reported the isolation of a tetracycline-resistant strain of U. urealyticum from an NGU patient who failed to respond to treatment with tetracycline.

Stimson, Hale and Bowie (1981) studied the prevalence of tetracycline resistance and found that 7% of the 82 viable isolates from NGU cases were resistant. The mechanism of resistance, though, is unknown.

(f). Serotypes

The human ureaplasmas are antigenically distinct from all other recognized mycoplasmas and consist of a heterogenous group of at least 14 recognized serotypes (Robertson and Stemke 1982). The existence of antigenic differences among ureaplasmas was first reported by Ford (1962, 1967). Black (1970, 1973b, 1974) proposed the presently recognized classification of ureaplasma serotypes and utilized four different serological methods i.e. the growth inhibition test, an indirect immunofluorescent test, an indirect hemagglutination test, and a metabolic inhibition test (MIT) in which they proposed 8 serotypes. Robertson and Stemke (1979), using a modification of a metabolic

inhibition test originally designed by Purcell et al. (1966) described a ninth serotype, and, subsequently by extending Black's classification, a tenth serotype was identified by Robertson and Stemke (1982). A different serological classification of the human ureaplasma employing a complement - dependent mycoplasmacidal test was proposed by Lin et al. (1972) and they developed a separate 11 member serotyping scheme for U. urealyticum.

Robertson and Stemke (1982) attempted to correlate the two independent schemes using the modified MIT and colony epifluorescence (CEF) to establish the existence of 14 member serotyping scheme.

Howard et al. (1978) identified eleven serological types of bovine ureaplasma and later Howard Pocock and Gourlay (1981) showed that these could be assigned to three major groups with some degree of relatedness amongst the members.

These groupings were further confirmed by the work of Mouches et al. (1981) using two dimensional protein analysis on polyacrylamide gel slabs.

Truscott (1983) using the immunoperoxidase test observed that the system, though laborious,

could be a satisfactory means of typing bovine ureaplasmas. Using the method he observed that group B strains were isolated with the greatest frequency from herds with infertility and or vulvovaginitis followed by group A strains.

From the male members of the group he reported that group A members predominate from both semen and preputial washing.

(g). Infection in Human and Animals

Ureaplasmas have been isolated from the genital tract of man and a wide variety of animals and have been observed to have the potential for causing disease in this anatomical site. The data to support the role of Ureaplasma as a causal agent in this anatomical site can be explained from studies in patients with these different conditions.

Role of Ureaplasma in Diseases of Male Genital Tract

(i) Non-gonococcal Urethritis

The term non-gonococcal urethritis (NGU) means urethral inflammation in the absence of Neisseria gonorrhoeae. There is an impressive

evidence in the literature suggesting that U. urealyticum causes or is associated with NGU, although it is still shrouded with controversy.

Since Ureaplasma was first reported from the urethra of men with NGU (Shepard, 1954) many attempts have been made to show their etiologic significance by comparing rates of isolation from patients with and without NGU. In some of these studies, U. urealyticum was isolated more often from patients with NGU (Bowie et al., 1977, Holmes et al., 1977, Prentice et al., 1976) while in other cases there was no difference in the isolation rate (Taylor-Robinson and Csonka 1981, Hunter et al., Hunter et al., 1981). In a matched case control study reported by Lee et al. (1976) and Piot (1976) no significant differences were observed between NGU patients and their controls.

The most successful experimental inoculation to prove the association of ureaplasmas and NGU was performed on two human subjects (Taylor-Robinson et al., 1977). Strains of U. urealyticum which were derived

from patients with NGU were used after being cloned and passaged. Both subjects received an inoculum of about 5 x 10^4 ureaplasma organisms and both developed orogenital symptoms. Treatment of one subject with minocycline was followed by a prompt recovery. This was a classical experiment incrimating Ureaplasma as a causative factor in NGU complex. This experimental infection of human volunteers reveals that Ureaplasma undoubtedly causes urethritis, but whether these are responsible for a major or small proportion of naturally occurring NGU is still controversial.

(ii) **Prostatitis**

Studies of the role of mycoplasmas in prostatitis have been inconclusive while Meares (1973) found no ureaplasma in the genital tracts of patients with chronic, nonbacterial prostatitis, Hofstetter (1977) found ureaplasmas in great number in the secretions of patients with urethroprostatitis than in those of healthy persons. In a recent study, Brunner et al. (1983) reported that U. urealyticum was obtained in high numbers in

expressed prostatic secretions and urine voided after prostatic massage from patient with chronic prostatitis. Because numbers of ureaplasmas in first voided urine and midstream urine were significantly lower, they concluded that the source of the organisms in these patients were assumed to be the prostate. These coupled with data on the results of tetracycline treatment in their study provided sufficient evidence they claim for the etiologic importance of ureaplasmas in chronic prostatitis.

(iii) **Epididymitis**

This is another condition with conflicting reports on the aetiologic role of Ureaplasma. Harnisch et al. (1977) suggested that U. urealyticum might cause epididymitis, however the result of subsequent studies by the same workers failed to support their initial hypothesis.

Role of Ureaplasma in Diseases of Female Genital Tract

(i) Vaginitis and Cervicitis

Romano and Romano (1968) observed that the organisms were as prevalent in both patients and controls. Similar studies by Mardh & Westrom (1970) also observed isolation rates of 55 and 46% for cases and control respectively.

More lately, Waites et al. (1983) reported a case of exudative vaginitis in a 10 year old which was associated with M. hominis and U. urealyticum as cultures for all other agents were negative, while high numbers of M. hominis and U. urealyticum were present in the vagina, cervix and urethra. Treatment of the patient with tetracycline resulted in the resolution of the symptoms.

Kundsin et al. (1973) isolated Ureaplasma from 80% of women with genitourinary tract infections including cervicitis and vaginitis but from only 51% of women with other reproductive problems who were otherwise asymptomatic. In the same study, these authors found that the occurrence of metabolism

inhibiting antibody to several ureaplasmas was greater in women with lower genital tract infections than in other groups of women.

(ii) **Acute Urethral Syndrome**

In a study by Stamm et al. (1983) on the etiologic role of Ureaplasma in acute urethral syndrome (AUS), data obtained did not clearly indicate that U. urealyticum causes AUS. The finding did however suggest that U. urealyticum may be an etiologic agent in some women based on their finding that U. urealyticum was isolated from 64% of women with AUS of unknown aetiology versus 51% of women with AUS due to coliforms or chlamydia. These workers also observed that suprapubic urine specimens from 27% of the former group versus 13% of the later group yielded U. urealyticum. While these data may suggest a possible etiologic role for U. urealyticum they certainly do not prove such a role.

Role of U. Urealyticum in Reproductive Disorders

(i) Foetal Wastage

In a controlled study, Caspi et al. (1972) showed a statistically significant difference between the presence of Ureaplasma in the cervix of women with induced abortion (17%) and with spontaneous abortion (51%). However, Kundsin and Driscoll (1970b) and Braun et al. (1971) reported only a tendency towards a correlation between U. urealyticum and spontaneous abortion and stillbirth. Many reports have been published on the isolation of Ureaplasma from the products of conception (Kundsin et al., 1967, Romano et al., 1968, Kundsin and Driscoll, 1970a, Romano et al., 1971, Caspi et al., 1972), but in none could secondary infection or simple contamination from the lower genital tract be excluded. Recently Kundsin et al. (1984) were able to show a positive association between colonization with U. urealyticum and chorioamnionitis - a frequent concomittant of premature birth and perinatal morbidity and mortality.

(ii) **Low Birth Weight**

Klein et al. (1969) demonstrated a statistically significant correlation between colonization of new-born infants with Ureaplasma and low-birth weight. Braun et al., (1971) confirmed this by demonstrating that women harbouring Ureaplasma in their lower genital tract gave birth to babies with a mean birth weight 202g less than that of infants by Ureaplasma negative mothers. While agreeing with other workers, Naessens et al. (1983) indicated that an association between colonization by Ureaplasma and low birth weight exist, but that cervical or placental colonization with U. urealyticum has no major influence on birth weight as far as a well to do population is concerned.

That the isolation of U. urealyticum was postively associated with low birth weight and with short gestational periods was recently confirmed by Kundsin et al. 1984). They observed that the highest isolation rate occurred among infants with the lowest birth weight and the odd's ratio of the association between U. urealyticum isolation and low birth weight was 2.1. This study, though convincing,

did not offer any evidence that Ureaplasma organisms did indeed incite the inflammatory reactions seen in the fetal membranes.

(iii) **Infertility**

Gnarpe and Frieberg (1972) reported a significantly higher isolation rate of Ureaplasma from cervical secretions (91%) and seminal fluid (85%) from married couples with primary sterility than from control groups consisting of pregnant women and their husbands. These workers in another study observed that 29% of women from Ureaplasma infertile couples became pregnant after a course of treatment with doxycycline although no controls were included in this study which leaves it open to several interpretations.

Infections in Bovine

(i) **Urogenital Infections**

The isolation of Ureaplasma from the bovine urogenital tract has earlier been documented (Taylor-Robinson et al., 1967, 1968; Howard Gourlay and Brownlie, 1973; Stalheim et al., 1974). The occurrence of Ureaplasma in the genitourinary tract

of cattle particularly in the semen, which is probably responsible for the impairment of sperm motility has led to the observation that these are a likely cause of infertility (Jurmanova et al. 1977). Doig et al. (1979) have reported an association between Ureaplasma infection and bovine granular vulvitis. In an earlier study, Ruhnke et al. (1978) had isolated Ureaplasma from 23.5% of clinically normal cows and from 75% of cows with moderate vulva hyperemia with no discharge, while the isolation rate was 100% from cows with acute vulva hyperemia accompanied by purulent discharge. They were able to show statistical significance in the isolation of these different groups and they suggested that Ureaplasma should be viewed as one of the causes of bovine granular vulvitis. In a related study, Doig & Ruhnke (1977) had observed poor conception rate among a group of sheep with purulent vulvitis which was attributed to Ureaplasma. Ureaplasma was isolated from significantly more sheep with vulvitis than without. Subsequently experimental transmission studies were carried out on some three-month-old lambs and on an aged ewe which had negative pre-inoculation culture. Hyperaemia and granularity with isolation of the organism was reported.

Previous studies by Langford (1975) and Panangala (1975) however failed to show an association between the recovery of Ureaplasma and bovine genital disease or infertility. Pirvulescu et al. (1972) reported the isolation of unclassified strains of mycoplasmas from samples of vaginal secretion collected from cows. These produced three morphologically distinct types of colonies whether any of these strains cause disease in the lower genital tract is unknown.

(ii) Pneumonia

Gourlay (1968) isolated Ureaplasma from the lungs of cattle with pneumonia in the UK, and these organisms have likewise been isolated from cattle in U.S.A. (Livingstone, 1972) and Japan (Shimizu et.al, 1975). To confirm that Ureaplasma might be a cause of the pneumonia observed, a series of studies were conducted. In one such study, Gourlay and Thomas (1970) produced pneumonia in conventionally raised calves by inoculating them endobronchially with Ureaplasma isolated from pneumonic lung. Later, Gourlay and coworkers (1976) showed that inoculation of pneumonic lung suspension containing Mycoplasma

dispar and Ureaplasma, produced wide spread pneumonia whereas a similar suspension treated with ampicillin produced less lesions. This study showed that not all the pneumonia was due to mycoplasmas, but the possibility that some might be due to M. dispar, the Ureaplasma or both was indicated by the fact that a tylosin-treated suspension caused negligible pneumonia. Further experiments with gnotobiotic calves undertaken by the same group of workers (Howard, Gourlay, Thomas and Scott, 1976) showed that endobronchial inoculation of sterile medium was without effect, whereas isolated cloned ureaplasmas produced a cuffing pneumonia.

It was suggested that the cuffing pneumonia formed a basis upon which superinfection by other micro-organism resulted in more severe disease.

(h). Pathogenicity

No consistently successful animal model for urethral mycoplasma infection has so far been developed. In a few experiments macaques and chimpanzees that had received intraurethral inoculation with strains of U. urealyticum have been colonized, but with little evidence of urethritis

(Taylor-Robinson et al., 1978, Bowie et al. (1978).

Swenson and O'Leary (1978) inoculated an 11-year-old female chimpanzee by vaginal instillation of a fresh culture of human U. urealyticum from a patient with idiopathic infertility. Ureaplasmas were recovered in cultures from cervical swabs throughout a period of 21 days. The organisms were successfully eliminated by treatment with a single dose of spectinomycin injected 4.0g intramuscularly.

This study thus demonstrates that the chimpanzee is susceptible to experimental infection by ureaplasmas from the human genitourinary tract.

A pathogenic role for U. urealyticum in human NGU was established following the result of experimental intra-urethral inoculation of ureaplasmas into human volunteers. Jansch (1972) was the first to inoculate himself with a pure culture of Ureaplasma and after 3 days developed urethritis with dysuria and urethral discharge consisting of large numbers of leukocytes, few epithelial cells with no bacteria. A rather large number of Ureaplasma were isolated from the urethral exudate.

In another study, Taylor-Robinson et al. (1977) reported the results of two authors who

inoculated themselves intraurethrally with 5 x 10^4 ccu/ml. This study was similar to that of Jansch (1972) except that the source, time of original isolation, purification, number of Ureaplasma inoculated, number of passages and freedom of chlyamydia from original source were not ascertained by Jansch (1972). Both subjects developed urethritis characterized by dysuria, frequent urethral discomfort, and pyuria. In addition to these the second subject developed urinary mucous threads with polymorphonuclear leukocytes which persisted for six months after treatment with minocycline, suggesting that the prostate became infected. Both patients displayed an eight-fold rise in titre showing active infection thereby establishing the pathogenic role of U. urealyticum for the human genital tract.

While experiments involving human volunteers would provide very useful information about the pathogenic effect of the Ureaplasma, it is most likely that the problem of ethics and repeatability will be encountered. Thus an animal model reproducing the human disease would be very useful. The use of non-human primates has been observed to overcome this difficulty (Taylor-Robinson, 1979)

because of their phylogenetic closeness to man and thus results from such an experiment can be extrapolated to the human situation. For some aspects of the pathogenicity trials, the use of mice has been suggested. Mice mammary glands are readily infected with human U. urealyticum (Howard et al., 1975). Earlier experiments designed to infect animals with U. urealyticum had variable success. Attempts in cattle to induce U. urealyticum mastitis failed probably because of host resistance (Howard and Gourlay, 1973), but goats mammary glands were infected with some human Ureaplasma. The injection of 10^6 - 10^8 ccu of Ureaplasma into the urinary bladders of rats resulted in bladder stone formation (Friedlander and Braude, 1974). Mice have been used by Kraus et al. (1977) as an experimental animal model for subcutaneous tissue cavity infection with Ureaplasma.

They felt that the advantage of using this animal model lies in that large numbers of mice can be studied in contrast to the situation where larger animals such as non-human primates are used thus making for a meaningful statistical analysis.

Gourlay et al. (1972) were able to produce clinical mastitis in cows inoculated with bovine

Ureaplasma, while human ureaplasma strains were isolated from milk only on the day 1 after inoculation with the strain.

These authors using the mammary gland system to induce mastitis in another study (Gourlay et al., 1972) were able to establish the existence of both virulent and avirulent strains of Ureaplasma isolated from cattle. In this study they were able to confirm their earlier finding that none of the human ureaplasma strains were able to cause mastitis in cattle.

However, in goats, Gourlay et al. (1973) showed that human Ureaplasma strains can cause a subclinical mastitis, as shown by an increase in cellular response which occurred in the glands. They were able to establish that Ureaplasma actually multiplies in the glands of the goats and thus suggested that goats could be used as a suitable animal model for studying the pathogenicity of Ureaplasmas using the mammary gland.

(i). Serology

(i) Growth Inhibition Test (GIT)

The growth inhibition test, based on the finding of Edward and Fitzgerald (1954) that mycoplasmas are specifically inhibited by hyperimmune serum incorporated in solid medium, is commonly used for the Mycoplasma and Acholeplasma species.

Black (1973b) observed that several variables influence the GIT. These are that, low incubation temperatures increased the growth inhibition zones - and 27°C was found to be optimal. Moreover, low pH levels of the agar medium gave the best results, and application of antiserum in wells instead of on filter-paper discs increased the inhibition zones.

Shepard and Lunceford (1978) further modified Black's method by use of a differential agar medium in place of a standard agar medium used by Black (1973b).

This differential medium contained a urea-manganese indicator which facilitate recognition of U. urealyticum colonies and

demonstration of the inhibition zones. Also Shepard (1967) used 37°C incubation temperature in contrast to 27°C used by Black (1974).

(ii) Metabolic Inhibition Test (MIT)

The method described by Purcell et al. (1966) is a slight modification of those applicable to other mycoplasmas (Jensen, 1964, Taylor-Robinson et al. 1966a).

This test is based upon the observation that specific antibodies inhibit the ability of ureaplasmas to hydrolyse urea to ammonia and carbondioxide thereby preventing a shift in the alkaline direction of the pH of the medium.

Black (1973b) reported that when recorded at the proper time, the test was found to be independent of the number of organisms used. However Robertson et al. (1979) disagreed with this assertion.

The MIT has been instrumental in both the initiation (Purcell et al. 1966) and the development of the presently recognized serotyping scheme for strains of U. urealyticum of human origin (Shepard et al.,

1974). Robertson et al. (1979) introduced some changes in the conduct of this test by replacing phenol red with bromothymol blue indicator which allows the use of a lower medium pH and one which yields better growth in terms of both generation time and cell yields. Also the presence of complement in this test was said to result in higher titres.

(iii) Immunofluorescence Test (IMF)

The introduction by Del Giudice et al. (1967) of an immunofluorescence technique applicable directly to unfixed mycoplasma colonies made the IMF a simple and very useful technique in the serological studies of mycoplasmas (Taylor-Robinson et al. 1966b, Barile et al. 1970; Lind, 1968; Rosendal and Black, 1972; Barile, 1973).

Application of immunofluorescence to Ureaplasma was first reported by Black (1970) and later by Black and Krogsgaad-Jensen (1974). The indirect test was found to be more sensitive and just as specific as the direct one. This was in consonance with the reports of Rosendal and Black (1972).

When compared to the GIT, Piot (1977) observed that the indirect IMF was highly sensitive and could detect mixed cultures and uses less serum. After staining, the colonies retained their fluorescence for more than six months.

(iv) **Mycoplasmacidal Test**

Lin and Kass (1970) described a modification of the MIT, which was based on the observation previously made by Taylor-Robinson et al. (1966a) and Purcell et al. (1966) that in addition to the initial inhibition recorded in the MIT reaction, a final inhibition which does not diminish with duration of incubation occurs. The mycoplasmacidal test in constrast to the MIT was reported to be dose dependent and also dependent on the presence of complement.

(j). Seroepidemiology

A variety of serological tests have been developed for the detection of specific antibodies to Ureaplasma but many are limited by technical

difficulties and lack the ability to distinguish responses among immunoglobulin classes. Probably this latter test capability may be of utmost importance in ultimately determining the etiological significance of Ureaplasma in various diseases.

Antibodies to M.hominis and U. urealyticum are present in sera from many healthy adults. For this reason it has not been possible to use seroepidemiologic surveys to study the natural history of infection by these organisms (Oriel, 1983).

At least 14 types of U. urealyticum have been described and it has been suggested that perhaps some types cause morbidity whereas others are part of the normal flora (Taylor-Robinson & MacCormack, 1980).

Demonstration of four-fold antibody titre between acute and convalescent phase serum specimens or detection of IGM antibody to Ureaplasma would be most helpful in firmly establishing this agent as the cause of infection.

Most serological methods developed thus far are relatively type-specific which limits their usefulness as seroepidemiologic tools. Previous investigators demonstrated no correlation between

Ureaplasma serotype and NGU or other genito-urinary tract disorders (Black, 1970, 1973b; Piot, 1976; Piot, 1977). However the number of clinical isolates of Ureaplasma serotyped in each case were relatively small.

Shepard and Lunceford (1978) employing an agar growth inhibition test serotyped a total of 338 clinical isolates of Ureaplasma from male patients with NGU, symptomatic patients with other genitourinary tract infections and disorders and isolates from a symptomatic carriers of Ureaplasma in the genital tract. From their study, among the 122 men with NGU, serotype 4 was most frequently (52%) associated with this disease. Seventeen percent of the isolates were type 2. The remaining isolates consisted of types 1, 3, 6 and 8 and accounted for 6 to 9% each of the serotypes isolated from men of the NGU group. They did not detect types 5 and 7. Among the 91 symptomatic patients with other genitourinary tract infections, and disorders, type 4 was also associated most frequently (37%) within this group. However among 125 asymptomatic genitourinary tract carriers of Ureaplasma (i.e. controls) the predominant type was 8, with type 4 the next most frequently encountered serotype.

Using a modification of his earlier growth inhibition test, Black (1973b) serotyped 10 different strains of Ureaplasma isolated from NGU patients. Four isolates were type 3 and three were type 1. One each of types 5, 7 and 8 were demonstrated, while types 2, 4 and 6 were not found.

Piot (1976) used the modified growth inhibition method of Black (1973b) to type 46 Ureaplasma isolates from 19 NGU patients, 12 gonorrhoea patients and 15 asymptomatic patients. No difference in distribution of eight serotypes among the 46 isolates was demonstrated and no particular serotype could be correlated with disease symptoms. Also Piot's study could not confirm the high incidence of serotypes 1 and 3 in NGU cases reported by Black (1973) in ten isolates.

In another study, Piot (1977) extended his serotyping studies to include a comparison of agar growth inhibition and indirect immunofluorescence test in serotyping 39 clinical isolates of Ureaplasma. Using the agar growth inhibition test, 27 of 39 isolates could be serotyped with the available eight antisera, while 12 isolates (30.8%) could not be serotyped. The indirect immunofluorescent test was more sensitive but

yielded more cross-reactions. Thirty-four of the 39 isolates were typed by the immunofluorescent method and five isolates (12.8%) could not be typed. Based on these results, Piot (1977) recommended that clinical isolates be screened with the indirect immunofluorescence test and all isolates with strong cross-reactions or doubtful results be examined additionally by the agar growth inhibition method.

Recently attention has been drawn towards the possible application of the enzyme-linked immunosorbent assay (ELISA) test in the measurement of antibody to Ureaplasma responses in patients with NGU. No significant cross-reaction was observed among the rabbit antisera to a variety of mycoplasmas of human origin and ureaplasma antigen used. This demonstrated the specificity of the ELISA test (Brown et al., 1983).

When 17 acute phase and 19 convalescent phase sera from male NGU were tested for the presence of Ureaplasma antibody by both MIT and by ELISA, an overall agreement of 82 and 95% for acute and convalescent-phase respectively were obtained. The sensitivity of ELISA was said to be comparable to that of the MIT or greater.

The ELISA in contrast to the MIT detected class-specific immunoglobulin changes. But the response as measured by the ELISA was independent of the serotype of the antigen used.

This apparent lack of serotype specificity in the ELISA observed by Brown et al. (1983) is compatible with the results of Stemke and Robertson (1981) who found that removal of surface antigens by treatment with Triton X-100 caused the Ureaplasma serotype characteristic to become indistinguishable.

This means that if the ELISA is to be effective in determining serotype specific responses, a purified antigen must be developed.

This serotype - specific antigen when developed will undoubtedly be of importance from an epidemiological standpoint.

Gallo et al. (1983) applied the indirect immunofluorescence test using acetone fixed U. urealyticum to measure antibody responses in sera from adults with genital infections and from symptomatic infants. This test method had earlier been reported by Stemke and Robertson (1981). However these authors employed Triton x-100 treated antigen which appeared to be group specific. In the Gallo et al., (1983) trial, it was reported that the

acetone-fixed antigen also presumably exposed the group - specific components within the organism.

Quinn et al. (1983) reported that 80% of the local population whether fertile or infertile can carry serotype 3 compared to 20% or less for the other serotype. Thus a comparable antibody response would be expected in all groups. However, these authors observed that the respiratory distressed neonates had elevated titres to serotypes 4, 7 and 8, while their mothers did not show any significant titre response. Among Ureaplasma positive women with history of pregnancy loss, the maternal titres to types 4 and 8 were elevated. In the neonates, titres to types 6 and 8 were elevated. It was thus concluded that since the significantly elevated antibody response was not to serotype 3, rather it was to types 4, 6 and 8 then serotype 3 may exist as a commensal, while serotypes 4, 6 and, particulary, 8 may be more pathogenic.

CHAPTER THREE

MEDIA EVALUATION AND BIOCHEMICAL STUDIES

INTRODUCTION

There is growing interest in the isolation of Ureaplasma and M. hominis from the urogenital tract due to the numerous reports on the possible roles these organisms are assuming in non-gonococcal urethritis (Shepard, 1980), infertility (Jurmanova and Sterbova, 1977; Cassell et al., 1983), premature birth and low birth weight of infants (Braun et al., 1971, Kundsin et al., 1981).

Several agar and broth media have been described for the isolation of these organisms. Shepard et al., (1976) and Shepard and Combs (1979) devised a differential medium called A7B agar which supports the growth of both M. hominis and U. urealyticum. Some selective media have been devised to differentiate the two organisms during isolation.

The need to develop a medium with a high degree of sensitivity and specificity in its isolation studies cannot be overemphasized.

The objective of this study was therefore to compare the isolation rates of U. urealyticum and M. hominis from clinical specimens using specially formulated media and to determine whether any one medium used non-selectively would yield good recovery of both organisms from urogenital tract specimens.

MATERIALS AND METHODS

(a). Media Evaluation

(i) Specimen Collection

Specimens were obtained using sterile swabs from the cervix or posterior vaginal fornix of 46 women and from the urethra of 70 men with gonococcal urethritis, attending the sexually transmitted disease (STC) clinic of the Ahmadu Bello University Teaching Hospital, Zaria. Each swab was expressed in 1.8ml of sterile PPLO broth base (Difco) containing 1000 units per ml of penicillin G, 10% of horse serum and 10% of yeast extract. These were stored at -70^{o}C until cultured within 8 weeks of collection.

(ii) Preparation of Horse Serum

A number of horses were bled separately using a Donafix(R) bleeding set into a sterile receiving bottle. The blood was allowed to clot at room temperature and serum was tapped by the use of sterile pipettes into sterile bottles and stored at -20°C.

The sera were pooled and filter-sterilized using 0.45u and then 0.22u Millipore filter membranes. The filtered serum was dispensed in 100ml amounts, appropriately labelled, and stored at -20°C until used. The quality control testing was performed as described by Tully and Rose, (1983) by comparing growth of a known titre Ureaplasma in a medium with known horse serum to its growth in a medium formulated with the test horse serum. A difference of 10-100 colour changing units between control and test serum media were not considered significant.

(iii) **Preparation of Yeast Extract**

This was prepared by dissolving 250g of active dry yeast (Engedura^{R}) into 750mls of double-distilled deionized water and brought to boiling under continuous stirring using a magnetic stirrer for about 20 minutes. The boiled product was cooled and frozen overnight at -20^{o}C. This was then thawed and centrifuged at 3015 x g for 15 minutes. The resultant supernatant was filter - sterilized using 0.8u and then 0.45u and finally 0.22u millipore membrane.

The yeast extract so prepared was quality tested by the methods of Tully and Rose (1983) for its ability to support growth of Ureaplasma. Thereafter this particular brand of yeast and method of preparation were adopted throughout this study.

(iv) **Culture Media**

Conventional Ureaplasma broth (UB) was used for the general cultivation of strains of U. urealyticum and for cloning of freshly isolated strains from clinical specimens. This medium consisted of PPLO broth without crystal

violet (Difco Laboratories, Detroit, Michigan USA). The broth powder was weighed according to the protocol specified by manufacturers, i.e. 1.47gms of powder was completely dissolved in 73mls of deionized distilled water. The pH of the basal broth was adjusted to 5.5 with 1N HCl. Sterilization was accomplished by autoclaving at 121°C for 15 minutes at a pressure of 15lb/sq in. This broth was cooled to 37°C and converted to standard UB medium by the addition of 20mls of filter - sterilized normal horse serum, 10mls of sterile yeast extract, 0.2mls of a 50% urea and 1ml of 100,000 units/ml of stock penicillin G potassium. The medium was aseptically dispensed in 0.9ml aliquots into 13 x 100mm screw-capped tubes and stored at -20°C until used.

The buffered medium was formulated from Trypticase soy broth (BBL no.11768) according to the method of Shepard and Lunceford (1978). The basal broth consisted of 2.4g of the broth powder, 0.5g sodium chloride, 0.05g of magnesium chloride and 0.05M of the respective buffer, all dissolved in 100mls of double distilled deionized water. Buffers used were:

N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid (HEPES), 2-(N-Morpholino)-ethane-sulfonic acid (MES), N-tris (Hydroxymethyl) methyl-2-aminoethane-sulfonic acid (TES) and Tris (hydroxymethyl) aminomethane (TRIS). The various components were dissolved and the pH adjusted to 6.0 with either 2N sodium Hydroxide for MES buffered medium or 2N Hydrochloric acid for HEPES, TRIS, and TES media. These basal media were sterilized at 121^{o}C for 15min by routine autoclaving. After cooling, 85mls of each of the sterile broth was converted to complete medium by the addition of 10ml horse serum, 0.5ml of a 2% L-cysteine hydrochloride, 5ml yeast extract, 0.05ml of 50% urea, 1ml of 100,000u/ml of stock penicillin G potassium, and 0.1ml of 1% stock sterile phenol red solution. These were dispensed in 0.9ml aliquots and stored at -20^{o}C until used.

Arginine supplemented media (AB) were prepared in a similar manner to that of the conventional urea broth (UB) media with a few modifications: the 0.2mls of a 50% urea (ultrapure grade urea No. 9200. Schwarz Mann Division of Becton Dickinson and Co. Rockville

Md.) was replaced with 0.1ml of 10% arginine (sigma Chemical Co. London). Moreover, 0.02% of thallium acetale was added to this medium and the pH adjusted to 6.5. Buffered arginine broth media were also prepared as for buffered urea broth, except that urea was replaced with arginine and thallium acetate was added as described above.

Corresponding solid media of urea or arginine - supplemented preparations were *prepared by incorporating* 1% *Ionagar No.2* (Oxoid Ltd. London, S.E.I) to the basal broth recipes before autoclaving after which the same sterile supplements as in UB or AB were added. Urea agar had in addition a 0.5ml of a 3% manganous sulphate. The complete agar medium was carefully mixed and poured into sterile 60 by 15mm or the standard (100 by 15mm) plastic petri dishes. After solidifying, the poured plates were allowed to dry at room temperature and subsequently stored in plastic bags at 4°C and used within 2 weeks.

(v) **Cultural Procedure for Clinical Specimens**

The cervico-vaginal swabs or the urethral swabs were cultured for <u>Ureaplasma</u> and

Mycoplasma spp. All frozen specimens were allowed to thaw at room temperature and 0.1ml amount of each was inoculated into 0.9ml of broth (UB and AB). In addition, a 10-fold serial dilution in each broth was made for each specimen. Uninoculated medium was included as medium control and all tubes were incubated at 37°C. Cultures were examined every 8 hours for evidence of colour change from light yellow to light pink compared with medium control. Dilutions that showed colour change without precipitates indicating bacterial contamination were frozen at -70°C as soon as the change became apparent to avoid excessive alkalinity. All cultures were passaged at least once and incubated for 7 days before being designated as negative.

(vi) **Assessment of Growth Pattern of Ureaplasma in the Experimental Media**

Three known strains of Ureaplasma, namely, STC 145U isolated from a male patient with gonococcal urethritis from preliminary studies, strain 2312 supplied by Dr. H.L. Ruhnke of the Veterinary Services Laboratory, Guelph, Canada, initially obtained from cows

with clinical acute granular vulvitis, and a human strain SU3 also obtained from Dr. H.L. Ruhnke were used. These were grown in broth and their titre recorded at 8-hourly interval to observe the pattern of growth and titre in the 5 urea-supplemented broths.

(vii) **Determination of Pattern of Hydrogen ion Concentration (pH) Change**

The pH determination study was performed delivering 5ml of culture of Ureaplasma strain SU3, SU4 and STC 145U from human, and a bovine strain 2312 into 45ml amount respectively of UB, HEPES, TRIS, TES and MES. The pH values were determined for each medium and for each strain by withdrawing 5mls into a test vial at 8-hourly interval and measuring the pH with a pH meter 7010 (Electronic Instruments Limited).

(viii) **Toxic Product Determination**

This test was performed using 3 known Ureaplasma strains SU3, STC 145U, and 2312 in three different media, namely UB, TRIS and HEPES broth. Five millilitre broth of the respective Ureaplasma strain was inoculated

into 45.0ml each of UB, TRIS, or HEPES broth and incubated for 48-hours to produce overgrown cultures. After plating to ascertain that no viable Ureaplasma was present, the overgrown media were further incubated and tested for viable Ureaplasma on agar again after 60 hours. The 60hour-cultures were dispensed in 1.8ml aliquots in tubes and re-inoculated with 0.2ml of respective test strain. Control fresh broth were also inoculated at the same time. At 8 hourly interval, 0.1ml aliquots were withdrawn and plated from each of the cultures and from the inoculated control broth onto agar medium. Qualitative assessment of growth was made. Various (100%, 90%, 50% and 10%) titrations of the overgrown (spent) medium and the control medium were made to assess the level of inactivating factor present. In another experiment overgrown broth cultures were heated at 56^{o}C for 30 minutes and then inoculated with Ureaplasma and incubated further for growth of Ureaplasma.

(b). Biochemical Tests

A total of 175 Ureaplasma isolates consisting of 60 from the family planning group (FP), 50 from the infertile patients (ACC), 35 from the sexually transmitted disease group (STC), and 30 from bovine sources were tested for catalase, phosphatase, tetrazolium reduction, and haemolytic activities.

(i) **Catalase Test**

The Ureaplasma strains were each tested for production of catalase by the addition of few drops of 3% hydrogen peroxide onto marked colonies. Absence of effervescence was interpreted as lack of production of catalase by the isolate.

(ii) **Phosphatase Test**

This was performed according to the methods of Aluotto et al. (1970). Agar media used for the test had 0.01% ($^{W}/_{V}$) sodium phenolphthalein diphosphate incorporated. The serum and yeast extracts used were heated at 60^{o}C for 1 hour and phenol red was omitted from the agar plates. Each test organism was grown

in 2mls of broth and at the sign of first colour change, phenolphthalein diphosphate agar plates (60 x 15mm) were inoculated with 0.02 - 0.05ml of each culture. The inoculated plates were incubated in an anaeobic jar using the Gas-Pak H_2 and CO_2 generator envelopes (BBL, Division of Becton Dickinson and Co. Cockeysville MD 21030 USA) for 48 hours. Plates were then flooded with 5M NaOH and examined for a reddish colouration around the colonies compared with control plates without colonies.

(iii) **Tetrazolium Reduction Test**

This test was conducted on solid medium as reported by Senterfit (1983). Agar plates containing the various Ureaplasma colonies were used. Four millilitres of a mixture of 0.21% sterile aqueous 2,3,5 - triphenyl - tetrazolium chloride and 1.3% ionagar in equal parts was poured onto each Ureaplasma plate and incubated anaerobically as above at 37°C. These agar test plates were examined for colour change after 15min to 1 hour of incubation. For comparison, uninoculated controls were incubated simultaneously.

(iv) **Test for Haemolysis**

The agar overlay technique of Clyde (1963) with some modifications was used. Guinea-pig red blood cells (rbc) was washed three times in phosphate buffered saline (PBS) pH 7.2 and a 20% suspension in the buffer was made. A stock solution of 3.4% ureaplasma agar base was made by dissolving the medium component through heating in distilled water. This was dispensed in 2ml aliquots in screw - capped tubes and autoclaved. Approximately 0.8ml of the rbc suspension was then added to the 2ml molten agar after it had cooled to 50^{o}C. This was well mixed and poured immediately over plates of viable Ureaplasma colonies. These plates were then sealed and incubated at 37^{o}C. They were observed 8 hourly for zones of haemolysis over a 24-48-hour incubation period.

RESULTS

Tables 3.1 and 3.2 show the recovery of U. urealyticum and M. hominis from the respective media used. The recovery rate of U. urealyticum in both urea broth and HEPES broth from 116 specimens was

51.0%. The recovery rates with the other media were TRIS broth, 50%; TES broth, 41.4%; and MES, 34.5%.

The total recovery rate of M. hominis as obtained from the arginine broth (AB) and the arginine supplemented buffered media and also from urea agar (UA) are presented on Table 3.2. When compared, the 5 media gave similar recovery patterns for both Ureaplasma and or M. hominis.

Agar plates cultured directly with the known positive clinical specimens were seriously deficient in the isolation of Ureaplasma when compared with their respective broth preparations (Table 3.3). However, the difference between broth and agar were not as marked in the recovery of M. hominis. For both organisms when the broth positive cultures were plated onto agar, colonies were obtained.

Results indicate that the UB detected all the Ureaplasma and all the M. hominis isolates in the specimens. Table 3.1 shows the percent isolations on each medium. The MES and TES medium were clearly the poorest for isolating U. urealyticum and M. hominis.

Data indicate that the HEPES medium gave a higher titre over a prolonged time period (Table 3.4). Under standard conditions of growth in the UB, all the strains tested had attained maximal titres

and were in the stationary phase by 20 hours of incubation and colony counts on agar showed a decline in colony forming units (CFU) by 23-24 hours of incubation.

For the buffered media, the growth curve was gradual and the stationary phase was attained by the 32nd hour of incubation.

There was a high degree of correlation between the growth pattern of the Ureaplasma strains and the pH of the growth medium (Table 3.5).

The buffered medium with a 0-0.8 range of pH over a 48 hour period had a higher growth titre, while the UB with a pH range of between 1.8 to 2.4 gave a significantly lower titre.

On re-inoculation and subsequent incubation of the test strains for 18 hours in 100% overgrown broth, no growth was obtained on agar. Similarly 90% overgrown culture added to 10% fresh culture yielded no growth of the test strain. On subjecting the overgrown medium to 56^{o}C inactivation for 30 mins, no change occurred in the growth pattern of the strains indicating that the toxic metabolites were not inactivated at 56^{o}C for 30 minutes.

All the Ureaplasma strains from human and bovine hosts were phosphatase positive, catalase negative, and did not reduce tetrazolium. The haemolysin test was not conclusive as the zones of hemolysis were not distinct.

Table 3.1 Rate of Recovery of Ureaplasma urealyticum and Mycoplasma hominis from 116 Sexually Transmitted Diseases Patients (STC) in Five Experimental Media.

	U. urealyticum in broth and agar		Mixed U. urealyticum & M. hominis confirmed on agar	
Medium*	No. of Positive Specimen	Percent[a] Positive	No. of Positive Specimens	Percent Positive
UB	60	51.0	18	15.5
TES	48	41.4	14	12.1
TRIS	58	50.0	16	13.8
HEPES	60	51.0	17	14.7
MES	40	34.5	12	10.4

(a) Percent of total number of specimens taken i.e. 116.

* UB = Conventional urea broth
TES = Urea broth buffered with TES buffer
TRIS = Urea broth buffered with TRIS buffer
HEPES = Urea broth buffered with HEPES buffer
MES = Urea broth buffered with MES buffer

Table 3.2 Recovery Rate of Mycoplasma hominis from 116 Specimens Using Six agar Formulations.

Medium	No. of Positive Specimens*	Percent Positive[a]
Arginine	32	27.6
TES	28	24.1
TRIS	28	24.1
HEPES	30	25.9
MES	25	21.6
UB	32	27.6

a Percent of total number of specimens taken i.e. 116

* Subculturing from corresponding broth formulations.

Table 3.3 Isolation of Ureaplasma urealyticum and Mycoplasma hominis by direct Plating of Clinical Specimens onto agar Plates.

Medium*	No (%) of Positive Isolates of: U. urealyticum (n= 60)		M. hominis (n= 32)	
Arginine	0	(0.0)	28	(87.5)
UB	52	(86.7)	26	(81.3)
Tes	40	(66.7	18	(56.3)
Tris	50	(83.3)	21	(65.6)
Hepes	51	(85.0)	22	(68.8)
Mes	39	(65.0)	16	(50.0)

* As described on Table 3.2.

Table 3.4 Comparative Growth Pattern of Strains STC 145U, 2312, and SU3 of Ureaplasma in four Selected Media.

Strain 145u

	0	8	16	24	32	40	48HRS
Hepes	-	10^1	10^3	10^5	10^8	10^{11}	10^{11}
Tris	-	10^1	10^3	10^4	10^5	10^5	10^6
UB	-	10^3	10^4	10^4	10^4	10^4	10^4
Tes	-	10^1	10^2	10^2	10^3	10^3	10^3

Strain 2312

	0	8	16	24	32	40	48HRS
Hepes	-	10^1	10^2	10^4	10^8	10^8	10^8
Tris	-	10^1	10^1	10^4	10^6	10^7	10^7
UB	-	10^2	10^4	10^5	10^5	10^5	10^5
Tes	-	10^1	10^2	10^2	10^2	10^3	10^3

Strain SU3

	0	8	16	24	32	40	48HRS
Hepes	-	10^2	10^2	10^4	10^6	10^7	10^8
Tris	-	10^1	10^1	10^3	10^4	10^4	10^5
UB	-	10^2	10^3	10^6	10^6	10^6	10^6
Tes	-	10^1	10^1	10^2	10^3	10^3	10^3

Table 3.5 Comparative pH Values of Strains SU3, SU4, 2312 and STC 145U of Ureaplasma in five Media.

HEPES MEDIUM

	Incubation (Hrs)							
	0	4	8	12	16	20	24	48HRS
SU3	6.0	6.0	6.2	6.1	6.3	6.4	6.4	6.6
SU4	6.0	6.0	6.1	6.2	6.4	6.4	6.4	6.8
2312	6.1	6.1	6.2	6.2	6.4	6.4	6.4	6.9
145U	6.0	6.0	6.2	6.2	6.4	6.4	6.4	6.8
Mean pH	6.0	6.0	6.2	6.2	6.4	6.4	6.4	6.8

TES MEDIUM

	0	4	8	12	16	20	24	48HRS
SU3	6.0	6.0	6.0	6.2	6.3	6.4	6.6	6.8
SU4	6.0	6.0	6.0	6.1	6.1	6.3	6.6	6.7
2312	6.0	6.0	6.0	6.0	6.2	6.4	6.7	6.7
145U	6.0	6.1	6.1	6.0	6.2	6.4	6.8	6.9
Mean pH	6.0	6.0	6.0	6.1	6.2	6.4	6.7	6.8

UB MEDIUM

	0	4	8	12	16	20	24	48HRS
SU3	6.0	6.2	6.6	6.8	6.8	7.8	7.8	8.4
SU4	6.0	6.0	6.2	6.2	6.4	7.4	7.6	8.2
2312	6.0	6.0	6.1	6.2	6.2	6.4	6.8	7.6
145U	6.0	6.4	6.6	6.6	6.8	7.0	7.4	7.8
Mean pH	6.0	6.1	6.4	6.4	6.6	7.2	7.4	8.0

(Con'td)

TRIS MEDIUM

	Incubation (Hrs)							
	0	4	8	12	16	20	24	48HRS
SU3	6.0	6.0	6.1	6.2	6.4	6.4	6.5	6.7
SU4	6.0	6.0	6.1	6.2	6.4	6.4	6.4	6.6
2312	6.0	6.1	6.1	6.2	6.4	6.4	6.5	6.8
145U	6.0	6.0	6.2	6.2	6.4	6.5	6.6	6.9
Mean pH	6.0	6.0	6.1	6.2	6.4	6.4	6.5	6.7

MES MEDIUM

	0	4	8	12	16	20	24	48HRS
SU3	6.0	6.0	6.0	6.1	6.1	6.2	6.2	6.4
SU4	6.0	6.0	6.1	6.1	6.1	6.2	6.2	6.3
2312	6.0	6.0	6.0	6.1	6.1	6.1	6.1	6.3
145U	6.0	6.0	6.0	6.2	6.2	6.3	6.4	6.6
Mean pH	6.0	6.0	6.0	6.1	6.1	6.2	6.2	6.4

DISCUSSION

The urease colour test medium (Shepard and Lunceford, 1970), though the most widely used broth for the detection of U. urealyticum, was not designed initially as a growth medium. To suit the laboratory condition under which the work was done, this medium modified and used as conventional urea broth (UB), was selected as the basis of comparison for this study.

A higher recovery rate of Ureaplasma was observed in the UB than in TES, TRIS, MES and HEPES buffered media. Several factors may be responsible for this higher recovery rate. Amongst these factors, the serum concentration may have played an important role. In a separate study (Abdulrahman, 1985) it was observed that the level of horse serum from 5% to 20% did not affect the recovery rate of the Ureaplasma but it affected the titre of the organism from clinical samples. It was reported in that study that lower horse serum media gave higher growth titres than the 20% horse serum medium.

This is in agreement with the findings of Rodwell (1983). The difference in titre was attributed to the probable presence of certain inhibitory substances in

higher concentration in the medium when a higher volume of serum was used.

Also the improved growth observed in the UB broth might be due to an increase in the amount of urea which is further supplied by the increased horse serum (Shepard, 1983). Improved viability evident by the higher titre was obtained with the buffered medium than with the UB. The lower urea content of these buffered media coupled with the low serum content and the limited pH changes controlled by the buffering capacity of the buffers must have accounted for the prolonged viability and resultant higher titre.

A comparison of the four buffered media shows that HEPES medium gave the best growth profile and the highest titre over the study period. This finding is in agreement with the report of Manchee and Taylor-Robinson (1969), who observed an enhanced growth with the HEPES buffer. However, although these authors did not compare HEPES to the other types of buffers used in this study, extrapolating from Shepard and Lunceford (1976), it could be speculated that TES and MES buffers, like phosphate ions, are probably toxic to ureaplasmas and that the optimal concentration needed for media formulation especially

when used for primary isolation from clinical materials is crucial. If this assertion is accepted, then the concentration used in this preparation was perhaps not optimal.

The presence of cysteine hydrochloride, a reducing agent, could also account for the improved viability of ureaplasmas in the buffered medium, as this could have removed all heavy ions in the medium which tend to depress the growth of Ureaplasma in broth media preparations.

The superior performance of UB in the recovery of M. hominis is not surprising since the pH range of this medium within a few hours of incubation shot to the optimal pH (6.5) for M. hominis, while the buffered media took longer time period to attain that optimal value (Shepard and Lunceford, 1965). The time lag could have resulted in death of the M. hominis which was manifested by the poor recovery rate in the buffered media.

It is apparent that ureaplasmas produce a heat-stable, catalase resistant factor, that inactivates them in broth. This probably accounts for the self-limiting nature of this organism in broth. The factor was toxic for the organisms at the concentration present in the broth and inhibitory at

lower concentration. This factor was present in both the animals and human Ureaplasma strains. The presence of this factor might explain the poor keeping quality of Ureaplasma which declines rapidly after cultures must have attained the stationary phase of growth and then stored.

Furness (1973) had earlier suggested that this toxic substance may be responsible for the death of Ureaplasma during storage.

Depletion of the medium of its essential nutrients by Ureaplasma during its growth does not appear to be responsible for death observed since addition of up to 50% or more of fresh broth medium only resulted in minimal growth. It therefore appears that growth of this organism is accompanied by the production of inhibitory factor in broth medium. This study further agrees with previous reports (Black, 1973a) that ureaplasmas are phosphatase-positive and catalase - negative and are unable to reduce the tetrazolium dye.

The haemolysin effects of the strains used here were not very distinct, but it was evident that the weak effects noticed were those of B-lysin.

In conclusion, for isolation studies it appears that the UB is the most suitable while for studies

that desire a high titre of growth, the use of the HEPES or TRIS buffered media is advocated.

SUMMARY

A higher recovery rate of Ureaplasma was obtained with the urea broth than with the other broth media used in the isolation trial. However, improved viability evident by the resultant higher titre was obtained with the HEPES buffered media than with the conventional urea broth. Both the human and bovine Ureaplasma isolates produce heat-stable catalase resistant factor which is probably responsible for the self limiting nature of these organisms in broth. Ureaplasmas are phosphatase positive and catalase negative.

CHAPTER FOUR

ISOLATION STUDIES IN HUMAN PATIENTS

INTRODUCTION

The frequency of isolation of Ureaplasma urealyticum and Mycoplasma hominis from the genitourinary tract has been reported as indicating their commensal rather than pathogenic role (McCormack et al., 1973). However a number of qualitative and quantitative evidence exist associating these organisms with various disease conditions of the genitourinary system both in human and animal systems (Kundsin and Driscoll, 1970 a and b; Taylor-Robinson and coworkers, 1977; Doig and Ruhnke, 1977; Brunner et al., 1983; Kundsin et al., 1984).

There is rarity in literature on the role the organisms play in various infertility problems in both animals and human in Nigeria, although Osoba (1972) and Adegboye et al. (1979) have reported some findings. This study was therefore designed to determine the frequency of isolation of human ureaplasmas and mycoplasmas from various case presentations at the Ahmadu Bello University Teaching Hospital, Zaria.

MATERIALS AND METHODS

(a) Patients

Five hundred women attending the gynaecology clinic at the Ahmadu Bello University Teaching Hospital for various problems were included in this study as cases. Data obtained included history of previous pregnancies, miscarriages, still birth, and neonatal deaths. A second group of cases consisted of 116 patients attending the sexually transmitted disease clinic (STC) of the hospital. 70 were males and 46 females with acute, uncomplicated gonorrhoea. The control group consisted of 283 women attending the family planning clinic and 100 pregnant women attending routine antenatal clinic. Thus a total of 999 samples were analysed for the presence of *U. urealyticum* and *M. hominis*.

(b) Evaluation of Patients

A standardized history with information on contraception and fertility history of the patients were obtained by the attending physician from the gynaecology patients. Classification into primary and secondary fertility patients was therefore made by the physician based on parity/number of abortions, miscarriages and still-birth. Primary infertility

patients were those who had never been pregnant while those with secondary infertility were broadly those who had one or more pregnancies previously.

The family planning patients were those who had come for counseling and loop insertion. Patients from this group who from available history had not experienced any overt infertility problem manifested by lack of or early termination of their pregnancies were considered a control group. Similarly, pregnant women with no prior history of abortion or miscarriages formed another control group.

(c) **Specimens**

Cervicovaginal swabs were collected from females using sterile cotton-tipped swabs through the aid of a vaginal speculum. Endourethral samples from male patients with venereal diseases were collected by the Venereologist using sterile cotton tipped swabs.

Each swab was snapped into 2.0mls of transport medium comprised of PPLO broth containing 1000 units of benzyl penicillin, 5% horse serum and 10% yeast extract. The pH of the transport medium was adjusted to 6.0 at the laboratory and the samples were frozen at -20^{o}C until they were tested for the presence of U. urealyticum and M. hominis usually within 1-2 weeks of collection.

(d) **Culture Media**

The culture media used were the conventional urea broth (UB) and arginine broth (AB) and their respective agar preparation earlier described (Chapter three).

Each medium consisted of PPLO broth supplemented with 0.1% urea for UB or 0.1% arginine for AB, enriched with 20% horse serum and 10% fresh yeast extract. Each medium contained 1000 units benzyl penicillin per ml as bacterial inhibitor. The AB medium contained, in addition 0.02% of thallium acetate, another bacterial inhibitor. Their respective agar media were also prepared routinely. Urea agar (UA) contained in addition 0.5ml of a 3% stock solution of manganous sulphate per 100ml of the medium

(e) **Propagation of Cultures**

Frozen specimens were thawed and 0.1ml amount of each specimen was inoculated into 0.9ml of fresh broth. A serial ten-fold dilution of each specimen was made in the range of 10^{-1} to 10^{-5} or more in UB or AB before incubating at $37^{o}C$. Cultures were examined every 8 hours for colour change of broth

from yellowish (acid) to pinkish (alkaline). Any suitable dilution with clear colour change without obvious bacterial contamination was further diluted down to 10^{-3}. A drop or two of each dilution was plated (without streaking) and allowed to dry. Plates of UA were incubated as earlier described in a GasPak anaerobic system, and plates of arginine agar were incubated in a glass anaerobic jar under a 5-10% carbon dioxide provided by the addition of IN hydrochloric acid to marble chips in an enclosed beaker. Plates were examined every 2 days using an Olympus stereomicroscope (NAAFCO Scientific Supplies,40 Niger Road, Kano) at a magnification of x100.

(f) **Identification of Ureaplasmas and Mycoplasmas**

Ureaplasmas assumed a dark colouration on growth on the plate due to the reaction with manganous sulphate incorporated into the medium (Shepard and Lunceford, 1976). Colonies on urea agar and arginine agar with "fried egg" appearance typical of mycoplasmas were noted and cloned according to Adegboye *et al*., (1980). Single, well separated representative colonies from each specimen were transfered with a sterile Pasteur pipette into 0.1ml of the arginine broth for *Mycoplasma* or urea broth in the case of *Ureaplasma*, and incubated and subcultured

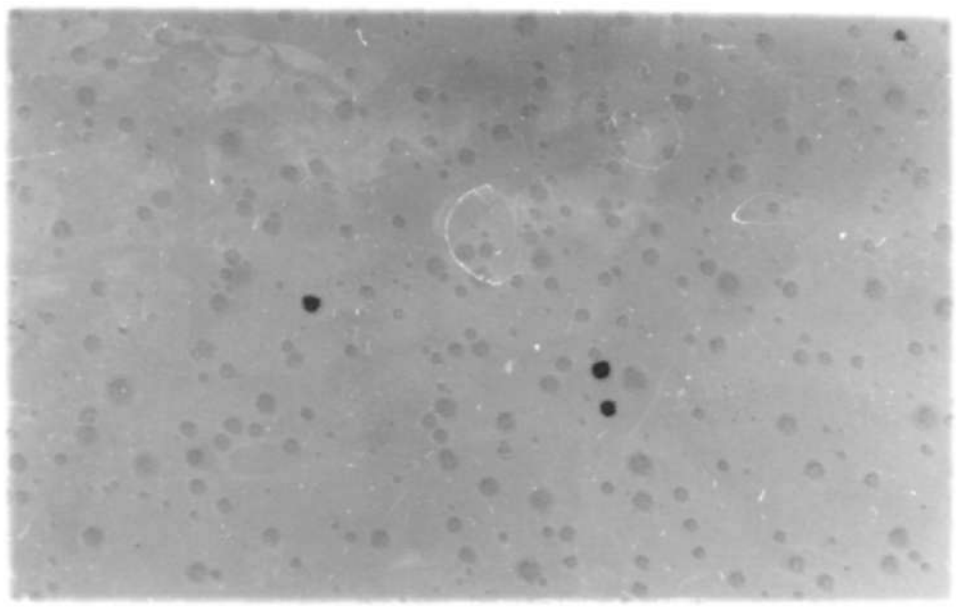


Plate 4.1a Ureaplasma colonies on Urea agar

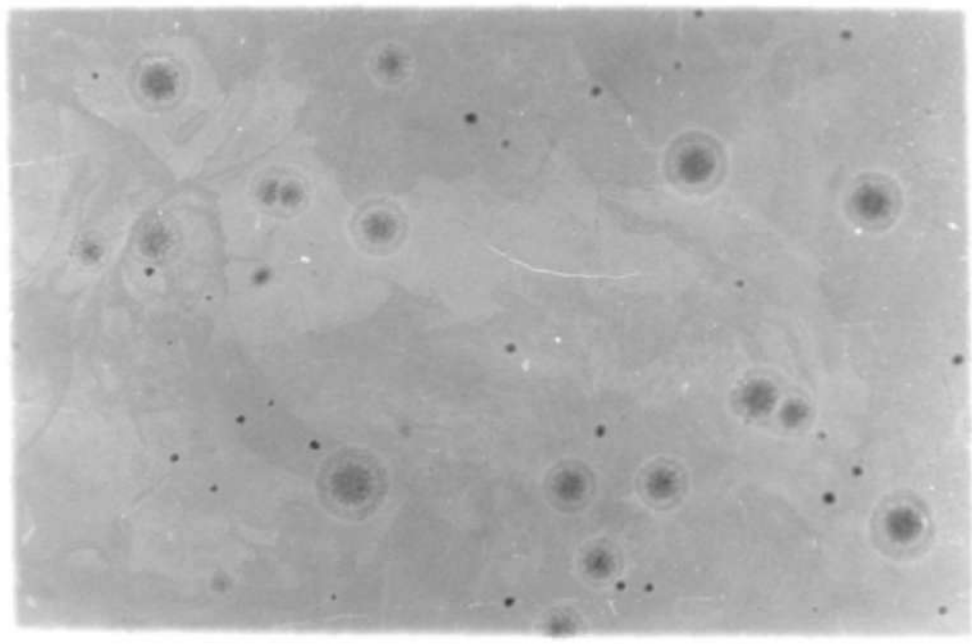


Plate 4.1b Mycoplasma hominis on Urea-agar showing a typical "fried egg" appearance. The arrow head indicates colonies of Ureaplasma

again after 2 to 3 days onto agar. This cloning process was often repeated twice before the isolate was typed using the growth inhibition test with M. hominis antiserum PG 21 (Adegboye et al., 1979). A similar cloning procedure was performed on individual well separated colonies of Ureaplasma spp into urea broth.

(g) **Statistical Analysis**

The chi-square (x^2) test described by Fleiss (1973) was used to analyse the significance of association between isolation of mycoplasmas/ureaplasmas and the health status of the patients.

An Odd's Ratio (OR) was considered to be an estimate of the rate of positive isolations among subjects within a given health status relative to the control subjects.

A value greater than unity (1) indicates a strong positive causal relationship between isolation of Ureaplasma or Mycoplasma or both and the condition tested for.

The critical value of the X^2 test at 1 degree of freedom and a p at 0.05 level of significance is 3.84 (from X^2 distribution tables). If the calculated X^2 value was greater than this criteria value, then the association was significant.

RESULTS

The rate of recovery of U. urealyticum alone, M. hominis alone and these organisms mixed from the various groups are presented in Tables 4.1, 4.2 and 4.3.

The pregnant women study group had the least isolation rate for Ureaplasma (36%) as shown on Table 6. Among these pregnant women, 52% of them haboured either or both micro-organisms while 54% of women attending the family planning clinic haboured either Ureaplasma, M. hominis or both organisms. (Table 4.1). Ureaplasmas were most frequently isolated from the secondary infertility group than in the primary infertility groups (Table 4.2). A comparison of primary infertility with the secondary infertility showed a significant difference ($p=0.05$; x^2) in the isolation of Ureaplasma either singly or in combination with M. hominis between the two groups. However with regards to M. hominis isolation, the difference was not statistically significant ($p=0.05$; x^2) (Table 4.2).

Table 4.4 shows the recovery of either or both micro-organisms from two sites commonly used for sample collection. The organisms were more frequently isolated from the lower vaginal site than from the cervix.

The Chi-square values and the OR values of all the various association are presented in Tables 4.5, 4.6 and 4.7. When all the cases (Gynaecology patients and sexually transmitted disease patients) were compared with the controls there was a significant difference ($p=0.05$) in the rate of isolation of Ureaplasma in patients and control subjects. No significant difference ($p=0.05$) however was observed with M. hominis isolation. A similar trend occurred when STC patients were excluded from the cases and a matched case control approach was adopted. When the STC patients alone were compared alongside controls, the isolation of U. urealyticum was significantly associated with the STC condition (Table 4.5).

The primary infertility group was compared each with the family planning (FP) control and the pregnant control group. In both instances, no significant difference was observed for total Mycoplasma or U. urealyticum isolation (Table 4.6). The secondary infertility group however exhibited high significance ($P \leq 0.01$) when compared with FP and pregnant control groups for general mycoplasmal and U. urealyticum isolation (Table 4.7). The OR were correspondingly significant with the total mycoplasmal isolation and with U. urealyticum isolation but not with M. hominis isolation.

Table 4.1 Recovery of *Ureaplasma urealyticum* and *Mycoplasma hominis* from two Control Groups

Patient Status	No. of Samples Collected	Frequency of Isolation of: *Ureaplasma* only(%)	*M.hominis* only (%)	Mixed *M.hominis* + *Ureaplasma*	Total Recovery of: *Ureaplasma*	*Mycoplasma*	Negative[a]
Control Group 1 (Family Planning)	283	78(27.9)	47(16.6)	28(9.9)	106(37.8)	75(26.5)	129(45.6)
Control Group 2 (Pregnant Women)	100	25(25)	16(16)	11(11)	36(36)	27(27)	48(48)
Total	383	104(27.1)	63(16.5)	39(10.2)	143(37.3)	102(26.6)	177(46.2)

( ) Figures in parentheses indicate Percent of total sample in each group

a Negative for both *Ureaplasma* spp and *Mycoplasma hominis*

Table 4.2 Distribution of *Ureaplasma urealyticum* and *Mycoplasma hominis* among 500 Gynaecology (Infertility) Patients

Patient Status	No. of Samples Collected	Frequency of Isolation of: *Ureaplasma* only(%)	*M.hominis* only (%)	Mixed *M.hominis* + *Ureaplasma*	Total Recovery of: *Ureaplasma*	*Mycoplasma*	Negative[a]
Primary Infertility	188	66(35.1)	20(10.6)	12(6.4)	78(41.5)	32(17.0)	90(47.9)
Secondary Infertility	312	200(64.1)	30(9.6)	51(16.4)	251(80.1)	81(26.0)	31(9.9)
Total	500	266(53.2)	50(10.0)	63(12.6)	329(65.8)	113(22.6)	121(24.2)

( ) Figures in Parentheses indicate percent of total samples

a Negative for both *Ureaplasma* spp. and *M. hominis*

TABLE 4.3 Distribution of *Ureaplasma urealyticum* and *Mycoplasma hominis* Among Sexually Transmitted Diseased (STC) Patients

Age(Years)	No. of Patients Screened	Sex Distribution		Frequency of Isolation of:			Total Recovery of		Negative for Both Organisms
		Female	Male	Ureaplasma	Mycoplasma	Mixed Mycoplasma Ureaplasma	Ureaplasma	Mycoplasma	
< 16	1	0	1	1	0	0	1	0	0
16 - 20	35	11	24	10	2	4	14	6	19
21 - 25	37	15	22	11	6	6	17	12	14
26 - 30	26	9	17	15	2	3	18	5	6
31 - 35	8	7	1	2	1	4	6	5	1
36 - 40	2	0	2	1	0	0	1	0	1
Total	116	46	70	42	18	12	60	30	44

TABLE 4.4 Recovery of Ureaplasma urealyticum and Mycoplasma hominis from two sites in 44 Selected Women Attendingthe Gynaecology Clinic

Site	Frequency of Isolation of		
	U urealyticum	M. hominis	Mixed M. hominis U. urealyticum
Lower Vagina	30 (68.2)	14 (31.8)	18 (40.9)
Cervix	22 (50.0)	10 (22.7)	13 (28.6)

( ) Figures in Parentheses indicate percent of total sample.

Table 4.5 Chi-square and Odd's Ratio Values of the Association Between Isolation of Mycoplasmas from Cases and Controls.

Organisms Isolated	All Patients (Gynae+STC) against all Controls		Patients (Gynae only) against all Controls		Patients (STC only) against all controls	
	X^2	OR**	X^2	OR	X^2	OR
U. urealyticum and M. hominis	38.7	2.4	46	2.7	2.15	1.41
U. urealyticum	62.2	2.9	69.5	3.2	7.05	1.8
M. hominis	1.3	0.8	1.70	0.8	0.002	0.96

*X^2 = Chi-square

**OR = Odd's Ratio

TABLE 4.6 Chi-square and Odd's Ratio Values of Association Between Isolation of Mycoplasmas and Primary Infertility - Using Different Controls

Organisms	Primary Infertility/ Family Planning Control		Primary Infertility/ Pregnant Control	
	X2	OR	X2	OR
Mycoplasmas in general	0.16	0.91	0.01	1.01
Ureaplasma urealyticum	0.5	1.17	0.6	1.26
Mycoplasma hominis	5.25	0.57	3.4	0.56

TABLE 4.7 Chi-square and Odd's Ratio Values of the Association of Isolation of Mycoplasmas and Secondary Infertility.

Organisms Isolated	Secondary Infertility/ Control (Family Planning)		Secondary Infertility/ Control (Pregnant)	
	X^2	OR	X^2	OR
All Mycoplasmas	94.1	7.6	68.4	8.4
Ureaplasma urealyticum	110.8	6.8	68.7	7.3
Mycoplasma hominis	0.003	0.97	0.006	0.95

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DISCUSSION

An association between the isolation of U. urealyticum and secondary infertility was demonstrated in this study.

Gnarpe and Friberg (1972) reported a singificantly higher isolation rate of U. urealyticum from cervical secretion (91%) of couples with primary infertility when compared with controls consisting of pregnant women (23%) indicating a causative role. This obvious association with primary infertility was not observed in this study and the reason for this is not known. Similarly, Archer (1968), Taylor-Robinson and Furr (1974) also failed to demonstrate such a correlation.

A causal association evident by the OR was established between the cases (the gynaecology + STC patients and the gynaecology patients alone) and the controls (the family planning and preganant patients) for U. urealyticum in most of the test case control combinations except with the primary infertility case. With the primary infertility, however, while mycoplasmas in general and U. urealyticum failed to show any statistical significance in the frequency of isolation, M. hominis did, although without a back up causal relationship established. This indicates that

there was no causal difference between M. hominis presence in the control group and those in the primary infertility group Although this study clearly showed that Ureaplasma is unlikely to have a major role in primary infertility based on the data presented, the possibility cannot be completely ignored because of the complexity of factors involved.

The high prevalence of U. urealyticum and M. hominis in the control patients, although relatively low when compared with other studies, is a cause for concern. Mardh and Westrom (1970) had reported an isolation rate of U. urealyticum in pregnancy to be as high as 68.4% and assumed that local changes accompanying pregnancy might offer favourable environment for the growth of the organism. Lately, Kundsin et al., (1984) did establish a positive association between colonization of the mother with U. urealyticum and chorioamnionitis - a frequent concomittant cause of fetal premature death. If this is the situation, it is therefore debatable to classify pregnant women as controls in studies involving U. urealyticum.

The ubiquitous nature of U. urealyticum evident

in this study does not support a possible etiologic role for it. Oriel (1983) postulated that the development of disease condition under a Ureaplasma colonization may be related to the dose of inoculum received, or may be determined by host factors such as impaired local immunity or that some serotypes may be more pathogenic. From this, it is evident therefore that isolation alone is clearly not sufficient evidence for final diagnosis of a causal role. Probably a quantitative determination of the Ureaplasma should be performed to resolve this impasse. However, the very high level of significance ($P \leq 0.001$) observed in this study for most of the associations gives credence to the role of isolation in determining causal relationships.

The finding here that 56% of the subjects who presented with gonococcal urethritis were positive for Ureaplasma and M. hominis is important. The significance of this high rate of isolation of Ureaplasma and Mycoplasma spp. among these urethritis patients lies in the fact that during the course of this study, a tetracycline resistant strain was identified. In Zaria, tetracycline has been used as a drug of choice in the treatment of gonorrhoea (Bello et al., 1987). Also Bello (1986) had observed that

tetracycline was useful in only about 70% of urethritis cases in Zaria. It is presently not known if tetracycline - resistant Ureaplasma spp. are responsible for some of the persistent urethritis. This aspect certainly deserves further investigation.

While it could be presumed that U. urealyticum and M. hominis found in the vagina should equally be present in the endocervix, this was not the case in the present study as the frequency of isolation were different. A similar finding was reported by other workers (McCormack et al., 1972). However the reason for this difference in anatomic site in the isolation is not known.

In conclusion, a general low isolation rate was observed in this study for cases and controls when compared with most other studies. It could be argued that this could be a true reflection of the prevalence of the infection in this environment. Although this difference may in part be due to variations in the methods used for transporting clinical specimens, the poor storage conditions encountered, and in the general different isolation technique.

SUMMARY

An association between isolation of Ureaplasma and M. hominis and secondary infertility was established. No such association was observed between isolation of these micro-organisms and primary infertility. A relatively high rate of isolation of Ureaplasma was observed with the control group, although a statistical difference was observed between the isolation of the Ureaplasma and M. hominis in cases and controls.

CHAPTER FIVE

ISOLATION STUDIES IN CATTLE

INTRODUCTION

Interest in ureaplasmal infection in bovine reproductive disease has lately increased. Once considered a part of the flora of the lower reproductive tract, the organism has now been associated with field cases of vulvitis (Ruhnke et al., 1978), endometritis, and salpingitis (Hirth, Plastridge and Tourtellotee, 1967) seminal vesiculitis and impaired sperm motility (Jurmanova and Sterbova, 1977).

In a preliminary study of cattle in Nigeria, Adegboye and Hassan, (1976) and Adegboye et al. (1980) obtained a 73% isolation rate for Ureaplasma. This high rate of isolation was observed for both local and exotic breeds.

In view of this, this study was designed to determine the frequency of isolation of the bovine Ureaplasma from semen, preputial and vaginal samples with the view to further characterize them in future studies.

MATERIALS AND METHODS

(a) Specimens

Two hundred specimens consisting of 150 cervico-vaginal, 30 preputial, and 20 bull semen samples respectively were collected and cultured for the presence of Ureaplasma spp. and for mycoplasma - like organisms. Swabs of cervico-vaginal secretions were collected after exposing the cervix with a speculum.

The cervico-vaginal specimens were collected from cows from local Fulani herd, the University Farm (UF), and from the National Animal Production Research Institute (NAPRI) herd. Preputial swabs were obtained from bull at the UF, NAPRI and a few from slaughtered local bulls at the abattoir. Semen samples from bulls used for artificial insemination from the NAPRI herd were collected aseptically with the aid of an electro-ejaculator.

Cervico-vaginal and preputial swabs were collected in duplicate. One of each sample was snapped into 2.0mls of PPLO broth with pH adjusted to 6.0 while the other was snapped into PPLO broth with pH adjusted to 7.6. These samples were transported to the laboratory in a cold pack and kept frozen at $-70^{o}C$ until tested for the presence of Ureaplasma spp and mycoplasma - like organisms within 3 weeks of

collection. The semen was collected in about 2ml amounts and stored frozen.

(b) **Culture Media**

The media used for Ureaplasma and Mycoplasma isolation consisted of Difco PPLO broth (Difco Laboratories, Detriot, Michigan) supplemented with 0.1% urea or 0.1% glucose respectively. The respectivbe media were then enriched with 20% horse serum and 10% yeast extract. Each medium contained *1000 units of benzyl penicillin sodium or potassium* per ml as bacterial inhibitor, while the glucose supplemented medium contained, in addition, 0.02% thallium acetate as bacterial inhibitor (Adegboye, 1977). The pH of the urea-medium and the glucose medium were adjusted to 5.5 and 7.6 respectively. Both media contained 0.002% phenol red as the pH indicator, while the agar medium for urea substrate had incorporated into it 0.5ml of 3% manganous sulphate per 100ml of medium.

(c) **Cultural Procedure**

This was performed in the same manner as described by Adegboye and Hassan, (1976) and Adegboye (1977). Briefly cultures were examined 8 hourly for

colour change and those without obvious turbidity, i.e. bacterial contamination, were frozen at -70°C. To check for growth, 0.05 - 0.1ml of cultures were inoculated onto the appropriate agar i.e. Urea-agar (U-agar) or glucose-agar (G-agar) and allowed to dry. Plates of U-agar were incubated in Gas-pak anaerobic system (BBL, Division of Becton, Dickinson and Co., Cockeysville MD). Plates of G-agar were incubated in a glass anaerobic jar under a 5-10% CO_2 tension provided by addition of 1N HCl to marble chips in an enclosed beaker. Plates were examined every 2 days using a stereomicroscope.

(d) **Identification of Ureaplasmas**

With incorporated manganous sulphate, Ureaplasma assumed a dark coloration on U-agar plates as earlier described by Shepard and Lunceford (1976).

(e) **Identification of Mycoplasmas**

Single colonies of microorganisms with typical 'fried-egg' appearance on G-agar were transferred with a Pasteur pipette into 0.1ml of G-broth and were further incubated. This was diluted down serially to a 10^{-3}. After about 3 to 5 days or when colour change was evident, a suitable dilution of such specimens

was sub-cultured onto G-agar and observed to re-produce the typical mycoplasma colonies. No further identification was carried out on such colonies.

RESULTS

The result is summarized in Table 5.1. Of the 150 cervico-vaginal samples, 105 (70%) were positive for Ureaplasma spp., while 63 (42%) were positive for mycoplasma - like organisms. Of the 30 preputial samples, 25 (83.3%) were positive for Ureaplasma spp., and 23 (76.7%) positive for Mycoplasma - like organisms, while of the 20 semen samples, 13 (65%) were positive for Ureaplasma, and 6 (30%) positive for mycoplasma - like organisms.

Some of the mycoplasma - like organisms exhibited wrinkling on surface of agar while some had both spots and wrinkling, a biochemical reaction referred to as "film and spots" often noticed with cattle urogenital mycoplasmas such as M. bovigenitalium (Adegboye and Kumi-Diaka, 1979),and M. bovis (Gourlay and Howard, 1983).

TABLE 5.1 Isolation of *Ureaplasma* and Mycoplasma - Like Organisms from Various Bovine Specimens.

Specimen	No. of Samples Collected	Isolations	
		Ureaplasma	Mycoplasma-like organisms
Cervico-Vaginal sample	150	105 (70%)	63 (42%)
Preputial sample	30	25 (83.3%)	23 (76.7%)
Semen	20	13 (65%)	6 (30%)

DISCUSSION

It has been observed that local breeds of cattle are not only susceptible but are naturally colonized by ureaplasmas and mycoplasmas (Adegboye, Hassan, Bwala and Fasanya, 1980). Although no emphasis was placed on speciation of Mycoplasma - like organisms in this study, deducing from the earlier work of Adegboye et al. (1980), M. bovigenitalium, M. bovis, Acholeplasma laidlawii and M. bovirhinis are commonly encountered in this environment with a higher frequency of isolation of M. bovigenitalium. This could have been the case in this study as results indicate that most of the mycoplasma-like organisms produced a wrinkling (characteristic of M. bovigenitalium) of the surface of the agar.

There was a high degree of agreement in the level of isolation of Ureaplasma in this study and that reported by Adegboye and Hassan (1976) and Adegboye et al. (1980). However, while generally the level of mycoplasma-like organisms agree with Adegboye et al. (1980), the level of mycoplasma-like organism from semen in this present study was significantly higher ($p \leq 0.05$) than that earlier

reported which was 15.6%. The implication of this finding is not yet known. However, that mycoplasmas could play a role in bovine infertility is shown by the ability to cause impaired sperm motility (Jurmanova and Sterbova, 1977; Chang, Choi, Matsuo, and Yoshii, 1984).

The high level of Ureaplasma in the preputial swab observed in this study is cause for concern moreso that Little et al. (1985) in their study had observed that most of the semen Ureaplasma were of the same type as that found in the preputial washing. They also observed that a reduction as a result of disinfection of the prepuce before semen collection resulted in a corresponding reduction of Ureaplasma in semen.

The significance of this high level of isolation of Ureaplasma in this study though not known, cannot be overemphasized. It is of concern that bulls being used for artificial insemination harboured the organism and even mycoplasma-like organisms at a high rate.

This study further confirms the high occurrence of Ureaplasma and mycoplasma-like organisms from the urogenital tract of Nigerian cattle.

SUMMARY

A total of 200 specimens consisting of 150 cervico-vaginal, 30 preputial and 20 bull semen samples respectively from cattle mainly of local breeds were cultured for Ureaplasma and for mycoplasma like organisms. Ureaplasma spp. was isolated from 70% of the vaginal samples, 83% of the preputial samples and 65% of the semen samples. While, mycoplasma-like organisms were obtained from 42%, 77% and 30% of vaginal, preputial and semen samples respectively. No attempts was made to speciate the mycoplasma-like organisms.

There was a high degree of agreement in the level of isolation of Ureaplasma in this study and an earlier study from the same environment. This study therefore confirms the existence of a high rate of occurrence of Ureaplasma and Mycoplasma from the urogenital tract of the Nigerian cattle.

CHAPTER SIX

ANTIBIOTIC SUSCEPTIBILITY STUDIES

INTRODUCTION

Ureaplasma spp. were first isolated from the human urogenital tract (Shepard, 1954). These organisms have also been isolated from urogenital tracts of cattle (Taylor-Robinson et al., 1967), and from the eyes of cattle with kerato-conjunctivitis (Gourlay and Thomas, 1969). These organisms are believed to be associated with infertility in cattle (Ruhnke et al., 1978), sheep (Livingstone et al., 1978) and man (Gnarpe, 1974).

Several reports have been published on the sensitivity of Ureaplasma strains isolated from human urogenital tracts (Braun et al., 1970) and bovine respiratory and genital tracts (Kishima and Hashimoto, 1979) to antimicrobial drugs. Although, Ureaplasma has been isolated from cattle in Nigeria, previous studies did not include antimicrobial sensitivity (Adegboye and Hassan, 1976, Adegboye et al., 1980) This study makes the first report of in vitro antimicrobial susceptibility tests on strains of Ureaplasma of human and bovine origin in Nigeria.

MATERIALS AND METHODS

(a) **Test Isolates**

A total of eighteen isolates of Ureaplasma species isolated from human and from cattle and one of M. hominis from studies earlier reported (Chapters Four and Five) were used. The Ureaplasma were identified by the urease spot test (Shepard, 1973) while the M. hominis was typed with antisera PG 21 by the growth inhibition test method. The isolates were cloned (purified) by a process of filtering their broth culture through 0.45u Millipore filters onto appropriate agar media and re-passing individual colonies back into the corresponding broth. This process was repeated 3 times for each isolate used. The final cloned isolate was then grown in 5ml quantity and stored in aliquots of 1ml at -70^{o}C until used. The 18 Ureaplasma isolates consisted of 6 isolates from normal patients attending the family planning clinic (FP), 6 from infertility cases attending the gynaecology clinic (ACC), and 3 from cases of proven gonococcal urethritis attending the sexually transmitted diseases clinic (STC). Two isolates were from bull semen and one from cervico-vaginal samples of a cow. The M. hominis isolate used in the study was obtained from a male gonococcal urethritis patient.

All isolates were stored at -70°C, duplicate sets of the cultures were stored in liquid nitrogen.

(b) **Antimicrobial Drugs**

A total of 10 antimicrobial agents were used. They were streptomycin, clindamycin, gentamicin, togamycin, chloramphenicol, chlortetracycline, nitrofurantoin, nalidixic acid, co-trimoxazole and erythromycin. Stock solutions of the antimicrobial agents were prepared in sterile, double distilled, deionized, water in concentrations varying from 400ug/ml to 2500ug/ml. Each solution was then sterilized by passing through first 0.8u then 0.45u sterile millipore filter. The stock solutions were dispensed in 5ml aliquots, appropriately labelled and stored at -20°C until used.

(c) **Media**

The medium of Taylor-Robinson as described by Kishima and Hashimoto (1979) was used for the seed culture. It consisted of PPLO broth $^{W}/_{O}$ crystal violet (Difco laboratories, Detroit Michigan), 70mls; 0.4% phenol red solution, 0.25ml; horse serum, 20mls; 10mls of fresh yeast extract, 1ml of 10% urea solution, and 1000 units per ml of penicillin G

potassium. For the sensitivity test, the medium mentioned above was modified by omitting penicillin G. potassium.

(d) **Sensitivity Testing**

The minimum inhibitory concentration of each drug was determined by the liquid medium-dilution method (Kishima and Hashimoto, 1979) using screw cap tubes in a total volume of 1ml. Each drug was serially diluted two-fold in a row of tubes as follows: into each tube, 0.5ml of broth was dispensed; to the first tube in each row, 0.5ml of one of the antimicrobial stock solution was then added. Thereafter a sterile 1ml pipette was used to make doubling dilutions of the antimicrobial agent. This was done for each drug for the respective number of isolates to be tested. The 0.5ml of the broth medium containing 10^3 to 10^4 colour-changing units per ml of ureaplasmal culture was inoculated into each of the diluted antimicrobial agent. Two sets of controls were included for each experiment, one being a broth or medium control and the other an inoculum control (i.e. inoculated without any antimicrobial agent added). The tubes were capped and incubated aerobically at 37°C.

For the M. hominis isolate, arginine broth which was prepared by substituting the urea with arginine and adjusting the pH to 6.5 was used in the test and in the control sets.

The tubes inoculated with ureaplasmas were observed after 18 and 24 hours incubation for colour change from yellow to pink. The test was read as soon as colour change was detected in the inoculum control tube.

The minimal inhibitory concentration (MIC) was defined as the lowest concentration of a drug that inhibited the colour change of the medium by metabolism of the urea for Ureaplasma or arginine for M. hominis and was determined twice for each isolate tested. The initial MIC was determined when control wells containing organisms in medium without antimicrobial drug showed colour change usually within 18 hours and the final MIC was determined after 24 hours of incubation for Ureaplasma and after 48 hours for M. hominis.

After the initial MIC reading, all tubes with higher concentrations of the antimicrobial agent starting from the MIC were subcultured by mixing 0.5ml of the content of each tube with 0.5ml of plain broth. The minimal lethal concentration (MLC) was

taken as the first well that on subculture failed to change to pink after 24 hours incubation (Truscott et. al. 1975).

RESULTS

Data on the MIC and MLC of the ten antimicrobial agents to 18 isolates of Ureaplasma and one of M. hominis are presented in Tables 6.1 and 6.2.

For the Ureaplasma isolates the pattern of inhibitions observed fell into three groups. In the first group were the togamycin, chloramphenicol and chlortetracycline which were effective and inhibited growth at much low concentrations. One exception was observed with isolate STC 145U which was resistant to all dilutions of chlortetracycline (>25 ug/ml) used. In the second group were erythromycin, streptomycin, and gentamicin, which were effective at relatively higher concentrations than those of group one. In the third group were clindamycin, co-trimoxazole, nalidixic acid, and nitrofurantoin, which were not quite as effective, and higher concentrations were needed to inhibit growth of the organisms.

For the M. hominis strain STC 145H, a similar pattern of susceptibility was generally observed as that of the Ureaplasma grouping with the exception of its reaction to clindamycin. While clindamycin was generally ineffective (MIC $\geq$ 1500 ug/ml) on the Ureaplasma strains, it was highly effective on the M. hominis strain (MIC <2.9ug/ml).

A reverse pattern was observed with erythromycin. It was highly effective with MIC between 2-32 mcg/ml against Ureaplasma but not against M. hominis (MIC >128).

There was no overt difference observed with the sensitivity pattern between the human ureaplasma isolates and the bovine strains.

A high degree of correlation was observed between the inhibitory and the cidal effects at higher concentrations amongst the highly effective group. However with decrease in concentrations there was a slight discord between inhibitory and cidal effects. At a concentration of 3.125ug/ml, togamycin inhibited 90% of the strains tested, whereas chlortetracycline and chloramphenical inhibited 58% and 53% of the strains, respectively. With togamycin at $\geq$ 6.25ug/ml a complete cidal effect was obtained on all strains.

Using the McNemar's chi-square statistic, no significant differences were obtained between cidal and inhibitory effect for togamycin and chlortetracycline at all the concentrations used. However, a significant difference ($P \leq 0.05$) was observed for chloramphenicol at a concentration of 3.125ug/ml between cidal and inhibitory response.

When MIC was compared to MLC at various dilutions for gentamicin and streptomycin, significance was obtained at $p \leq 0.05$ for both at a concentration of 6.25ug/ml but not at other concentration.

In the third group, no definite susceptibility pattern was observed. Co-trimoxaxole and Nalidixic acid did not show any significant difference at each concentration between inhibitory and cidal effects. Whereas nitrofurantoin exhibited strong significant difference ($P \leq 0.05$) between MIC and MLC at concentrations of 62.5ug/ml and 125ug/ml. Similarly, clindamycin at 750ug/ml showed significant difference between MIC and MLC. Also in this group, nalidixic acid and nitrofurantoin did attain 100% inhibitory and cidal effect at the concentrations of 500ug/ml used. Clindamycin and cotrimoxazole, however, were highly ineffective even at the highest concentration

TABLE 6.1: Minimal Inhibitory Concentration (MIC) of 10 Antimicrobial Agents to 18 Isolates of *Ureaplasma* and one of *Mycoplasma hominis*

Number	Source	Sex	Species	Serotype	Antimicrobial agents and their MIC (ug/ml)									
					G	CTE	C	NF	SXT	NA	TG	CL	S	E
FP80	Human	F	*U. urealyticum*	1	3.1	3.13	6.25	62.5	1200	250	3.12	1500	6.3	2.0
FP81	Human	F	"	10	6.3	1.56	6.25	62.5	4800	250	1.56	375	6.3	2.0
FP51	Human	F	"	4	3.1	1.56	3.13	62.5	4800	250	1.56	>1500	3.1	4.0
FP77	Human	F	"	1	3.1	3.13	3.13	>500	4800	<31.25	3.13	1500	12.5	2.0
FP50	Human	F	"	8	6.3	1.56	3.13	62.5	>4800	31.25	0.78	750	12.5	2.0
FP128	Human	F	"	8	3.1	1.56	6.25	62.5	4800	31.25	0.78	750	6.3	2.0
STC145U	Human	M	"	4	3.1	>25.0	3.13	>500	1200	250	0.78	>1500	6.3	2.0
STC145H	Human	M	*M. hominis*	PG 21	25.0	12.5	12.5	62.5	4800	125	3.13	2.9	25.0	>128.0
STC2	Human	M	*U. urealyticum*	4	6.3	0.78	3.13	62.5	2400	125	3.13	187.5	6.3	4.0

(con'td)

Number	Source	Sex	Species	Serotype	Antimicrobial agents and their MIC (ug/ml) G	CTE	C	NF	SXT	NA	TG	CL	S	E
STC12	Human	F	"	8	6.3	0.78	3.13	125	>4800	125	1.56	>1500	3.1	2.0
ACC209	Human	F	"	2	6.3	6.25	3.13	62.5	2400	62.5	0.78	750	12.5	4.0
ACC237	Human	F	"	4	6.3	3.13	6.25	62.5	2400	62.5	0.78	750	6.3	4.0
ACC210	Human	F	"	4	3.1	3.13	1.56	125	4800	62.5	1.56	750	<3.1	2.0
ACC223	Human	F	"	11	3.1	0.78	12.5	62.5	4800	62.5	1.56	750	3.1	4.0
HCC144	Human	F	"	1	3.1	12.5	6.25	62.5	1200	62.5	6.25	1500	6.25	8.0
ACC229	Human	F	"	1	3.1	1.56	3.13	125	4800	62.5	3.13	375	<3.1	8.0
B2112	Bovine	M	Ureaplasma spp	2312	3.1	1.56	3.13	125	4800	125.0	3.13	750	6.3	8.0
B 2354	Bovine	M	"	A417	12.5	6.25	6.25	500	4800	125.0	6.25	750	25.0	8.0
B 1411	Bovine	F	"	A417	6.3	1.56	6.25	500	1200	125.0	3.13	750	12.5	16.0

G = gentamicin
CTE = chlortetracycline
C = Chloramphenicol
NF = Nitrofurantoin
SXT = Co-Trimoxazole
NA = Nalidixic acid
TG = Togamycin
CL = Clindamycin
S = Streptomycin
E = Erythromycin

TABLE 6.2: Minimal Lethal Concentration (MLC) of 10 Antimicrobial Agents to 18 Isolates of *Ureaplasma* and one of *Mycoplasma hominis*

Number	Source	Sex	Species	Serotype	Antimicrobial agents and their MIC (ug/ml)									
					G	CTE	C	NF	SXT	NA	TG	CL	S	E
FP80	Human	F	*U. urealyticum*	1	12.5	3.13	6.25	250	1200	250	6.25	>1500	6.25	4.0
FP81	Human	F	"	10	6.25	1.56	6.25	125	4800	500	3.13	1500	12.5	4.0
FP51	Human	F	"	4	12.5	12.5	12.5	500	4800	500	3.13	>1500	6.25	8.0
FP77	Human	F	"	1	6.25	12.5	6.25	>500	4800	<31.25	6.25	>1500	25.0	4.0
FP50	Human	F	"	8	25.0	6.25	6.25	250	>4800	125	3.13	750	25.0	4.0
FP128	Human	F	"	8	3.13	6.25	6.25	125	4800	62.5	3.13	750	6.25	2.0
STC145U	Human	M	"	4	6.25	>25	12.5	500	2400	250	1.56	>1500	12.5	4.0
STC145H	Human	M	*M. hominis*	PG 21	50.0	12.5	12.5	62.5	4800	500	3.13	<93.75	50	>128.0
STC 2	Human	M	*U. urealyticum*	4	12.5	6.25	6.25	125	4800	125	3.13	750	12.5	8.0

(Con'td)

Number	Source	Sex	Species	Serotype	Antimicrobial agents and their MIC (ug/ml)									
					G	CTE	C	NF	SXT	NA	TG	CL	S	E
STC12	Human	F	"	8	25.0	12.5	6.25	250	>4800	250	3.13	>1500	12.5	8.0
ACC209	Human	F	"	2	25.0	12.5	12.5	250	4800	125	1.56	1500	25.0	8.0
ACC237	Human	F	"	4	12.5	6.25	12.5	250	2400	125	3.13	1500	12.5	4.0
ACC210	Human	F	"	4	6.25	6.25	3.13	250	>4800	250	1.56	1500	<3.13	2.0
ACC223	Hunam	F	"	11	3.13	6.25	12.5	250	4800	125	1.56	>1500	12.5	8.0
ACC144	Human	F	"	1	3.13	12.5	6.25	250	1200	62.5	6.25	>1500	6.25	16.0
ACC229	Human	F	"	1	3.13	1.56	6.25	250	4800	62.5	3.13	>1500	3.13	8.0
B 2112	Bovine	M	Ureaplasma spp	2312	12.5	1.56	3.13	125	4800	125	3.13	750	12.50	32.0
B 2354	Bovine	M	"	A417	25.0	12.5	12.5	500	>4800	250	6.25	>1500	25.0	16.0
B 1411	Bovine	F	"	A417	25.0	3.13	6.25	500	2400	125	6.25	750	12.5	32.0

*G = gentamian CTE = Chlortetracycline C = Chloramphemicol
NF = Nitrofurantoin SXT = Trimoxazite NA = Nalidixic Acid
TG = Togamycin CL = Clindamycin S = Streptomycin
E = Erythnomycin

of 1500ug/ml and 4800ug/ml, respectively. One exception was observed for clindamycin with strain STC 145H. This antimicrobial agent inhibited this organism at a very low concentration of $\geq$ 2.9ug/ml.

DISCUSSION

The minimal inhibiting concentration (MIC) is defined as the lowest concentration of antimicrobial agent that inhibits growth, while the minimal lethal concentration (MLC) is the lowest concentration of an antimicrobial drug that kills the organism (Mardh, 1975; Harwick and Fekety, 1969).

Since the presence of ureaplasmas in semen constitutes a hazard with respect to the transmission of these agents and a concomittant problem that might ensure from their presence in the female reproductive tract (Doig et al., 1980a, 1980b), it is desirable to eliminate them. It is generally known that tetracyclines, aminoglycosides, macrolides, chloramphenicols, and nitrofuran derivative and others have a growth inhibiting activity on mycoplasmas (Kishima and Hashimoto, 1979). The results of this study are in agreement with previous

reports on antimicrobial susceptibilities, although in varying degrees. Bygdeman and Mardh (1983) did indicate that the MIC and MLC of various antimicrobial compounds are influenced by the pH of the test media, the inoculum size, and the incubation time, and that these might be responsible for differences in the optimal drug concentration needed for activity.

This, in part, might account for the relatively higher MIC and MLC values obtained in this study which are mostly 2-3 fold higher than the reported MIC values for Ureaplasma by Kishima and Hashimoto (1979), but lower than those reported by Truscott et al. (1975).

When the incubation time for the Ureaplasma was increased from 18 hours to 24 hours, the difference in titre was usually one dilution higher or in most instances the same. It therefore implies that the initial MIC and final MIC readings were not significantly different and thus either reading could be taken. For Ureaplasma, this static titre phenomenon might be due to the self-sterilizing or limiting effect of this organism in broth due to toxic metabolites it liberates during growth (Chapter Three). Generally it was observed that the MIC and MLC at various concentrations of antimicrobial agents

used did not differ significantly, although for a few concentration for chloramphenicol, gentamicin, nitrofurantoin and clindamycin, significant statistical differences were observed.

There is however a need to interprete these findings with caution, because of the rather small sample size used in this study.

However, the results of this study indicate that the concentration needed for inhibitory effect and that for lethal effect of these antimicrobial agents were significantly different. The significance of this might be that higher doses beyond the inhibitory doses might be required for therapeutic effect in a clinical case.

The susceptibility of most strains of Ureaplasma and that of the M. hominis to tetracycline, chloramphenicol, and togamycin are in agreement with previous studies (Truscott et al., 1975, Truscott, 1981, Kishima and Hashimoto, 1979). These drugs are known to interfere with protein synthesis, although the difference in activity of each drug might depend on the different binding sites to the ribosomes.

The resistance in this study of isolate STC 145U to tetracycline is however not surprising. Ford

and Smith (1974) first reported the isolation of a tetracycline - resistant strain of U. urealyticum from a non-gonococcal urethritis patient whose urethritis was unaffected by tetracycline therapy. Strain STC 145U was traced to a patient who had presented a case of gonococcal urethritis and was placed on tetracycline. With the persistence of the urethritis the case was represented and was placed on togamycin - a spectinomycin, which is an aminoglycoside. The sensitivity of this tetracycline-resistant strain to togamycin in this gonococcal urethritis patient is significant since this drug is recently largely used for Neisseria gonococcal infections in Nigeria (Bello personal communication).

Erythromycin on the other hand is not primarily widely used to treat urethritis but is used mainly in cases of re-presentation of urethritis (Bello, Personal Communication), thus the sensitivity of the Ureaplasma to this drug is significant when viewed against Ureaplasma urethritis.

From this study, the level of tetracycline-resistant strains of Ureaplasma and possibly M. hominis in this environment is not known. A detailed antibiogram study of more isolates from various NGU patients and other patients would be

required to establish this level. It is however known that tetracycline resistant strains exist in this environment.

The mechanism of resistance of mycoplasma to tetracycline is unknown. It has been suggested that the resistance could be plasmid- mediated as is the case in many bacteria (Christiansson and Mardh, 1983).

Aminoglycosides which interfere with protein synthesis at the 30_s ribosomal unit as tetracycline did show activity against the Ureaplasma spp. and M. hominis. However, of the three aminoglycosides used in this study, togamycin a spectinomycin was most effective against the organisms. Truscott and Ruhnke (1984) found a lincomycin-spectinomycin combination quite active against bovine Ureaplasma and Mycoplasma and further found that this combination is well tolerated by bovine spermatozoa and thus could be used in semen stored for insemination. This study further comfirms this superior activity of togamycin (a spectinomycin) against both Ureaplasma from animal and human sources and also against M. hominis. That gentamycin did not appear very effective also corroborates the findings of Truscott and Ruhnke (1984).

Co-trimoxazole - a trimetoprim whose mode of action is that of indirectly interfering with the nucleic acid synthesis did not show much activity against both genera of organisms in this study, a finding in consonance with those of Bygdeman and Mardh (1983). Clindamycin on the other hand showed a good activity against M. hominis but not Ureaplasma. This is understandable because this antimicrobial agent is a derivative of lincomycin which has been reported by several authors to be highly and selectively effective against M. hominis (Mardh, 1975; Harwick and Fekety, 1969; Braun et al., 1970). The reason for this selective mode of action is unknown although the general mechanism of action is that of interfering with protein synthesis at the 50_S ribosomal unit like the macrolides.

Nitrofurantoin and Nalidixic acid are antibiotics used for the treatment of urinary tract infections and have been reported to be mycoplasmacidal (Braun et al., 1970). A good correlation was obtained with the report of Braun et al. (1970) who reported an MIC of 500ug/ml against both groups of organisms.

The fact that ureaplasmas or M. hominis can be involved in serious infections such as pelvic

inflammatory disease (Mardh and Westrom, 1970), and septicaemia (Harwick and coworkers 1967) and the emergence of tetracycline-resistant strains (Ford and Smith, 1974) as also recorded in this study, points to the need for correct diagnosis of infections due to this agent. The need to check isolates for their antibiotic susceptibility, particularly in cases of therapeutic failure, cannot be over emphasized.

SUMMARY

Eighteen isolates of Ureaplasma spp. obtained from human and from cattle and one of M. hominis were tested for their susceptibility to ten commonly used antimicrobial agents in hospitals in this environment. Three susceptibility patterns to these antimicrobial agents emerged. In the first group were the togamycin, chloramphenicol, and chlortetracycline which were highly effective and inhibited growth of the Ureaplasma at much lower concentrations. The second susceptibility pattern was with erythromycin, streptomycin, and gentamicin. These antimicrobial agents were inhibitory to the Ureaplasma spp. at

relatively higher concentration than those of group one. In the third group were clindamycin, co-trimoxazole, nalidixic acid and nitrofurantoin, which were not quite as effective, and higher concentrations were needed to inhibit growth of the organisms. There was no overt difference observed with the sensitivity pattern between human ureaplasma strains and those of bovine strains. Also the M. hominis isolate exhibited generally a similar susceptibility pattern as was observed with Ureaplasma except in its reaction to clindamycin.

A tetracycline resistant Ureaplasma strain, which was however sensitive to togamycin was encounted.

CHAPTER SEVEN

SEROLOGICAL TYPING OF LOCAL ISOLATES OF BOVINE AND HUMAN UREAPLASMA BY THE GROWTH INHIBITION AND METABOLIC INHIBITION TESTS

INTRODUCTION

Since the first description of Ureaplasma by Shepard (1954), there have been numerous attempts to provide the evidence for its aetiological role in a number of conditions. Some report suggests a causal relationship (Shepard et al., 1978; Csonka, Williams and Corse, 1966) while others do not support this view (Ingham et al., 1966; Black and Rasmussen, 1968).

Some authors have tried to correlate particular serotypes to disease conditions (Black, 1973b; Shepard and Lunceford, 1978) but the small number of samples tested do not allow for a definite conclusion.

A few test procedures have been used for the study of the serotyping scheme, (Black, 1973b; Shepard and Lunceford, 1978; Robertson and Stemke, 1979; Quinn et al. 1981; Piot, 1977). Using the bovine Ureaplasma isolates, Ogata et al., (1979) found that the metabolism inhibition test gave

essentially the same result as the growth inhibition test. However, some previous investigators (Black, 1973b; Howard and Gourlay, 1973) had reported that the metabolic inhibition test was more sensitive and less specific than the growth inhibition test.

Similarly Piot (1977) had compared the growth inhibition test with the Immunofluorescence test (IF) in serotyping clinical isolates of U. urealyticum. The study showed that the growth inhibition test, while it lacked sensitivity when compared to IF, was easy to perform and specific.

This study attempts to determine the prevalence and distribution of the various Ureaplasma serotypes in different human case presentations at the hospital and also from certain Nigerian cattle population. In addition, an evaluation of the application of growth inhibition and the metabolic inhibition test as tools for use in the sero-epidemiologic classification of the various serotypes of Ureaplasma was made.

MATERIALS AND METHODS

(a) Organisms

A total of 134 strains of U. urealyticum and 100 of bovine Ureaplasma were studied. The human

Ureaplasma strains were obtained from the following sources: 40 from the genito-urinary tract of male and female patients attending the sexually trasmitted diseases (STC) clinic, and 50 obtained from women attending the family planning (FP) clinic. These FP women were taken as the control group. Forty four (44) samples were obtained from females attending the Gynaecology clinic of the Ahmadu Bello University Teaching Hospital.

The bovine Ureaplasma strains comprised 8 from bovine semen, 20 from prepuce, 20 from cervical necks and 52 from anterior vagina.

(b) **Culture Media**

(i) **Standard Fluid Culture Media (Medium i)**

This medium was used for the general cultivation of all strains of Ureaplasma either of human or bovine origin and was also used for cloning of all isolated strains from clinical material. The medium was prepared as previously described in Chapter Three using Difco PPLO broth product.

(ii) **Standard Agar Media** was as described in Chapter Three.

(iii) Special Fluid Media for Human Antigen Production (Medium III)

Since in earlier investigation (chapter II), HEPES - buffered media gave the highest growth titre, it was selected for antigen preparation. This broth preparation was as already described. This medium was used for the propagation of human Ureaplasma antigen only.

(iv) Special Fluid Media for Bovine Ureaplasma Antigen (Medium iv)

This broth consisted of Hanks balanced salt solution (10X), 4.0ml; Hartley digest broth (Oxoid No. CM-411), 20 ml; fetal calf serum, 15ml; yeast extract (25% aqueous extract), 10ml, 1.0% phenol red, 0.2ml; 20% urea, 0.25ml; magnesium sulphate (250ug/ml), 1.0ml; benzyl penicillin (200,000u/ml), 0.5ml; double distilled deionized water as much as needed to make up total 100ml volume. The pH was adjusted to 6.0 to 6.2 with 1N HCl. The whole solution was then sterilized by passing through stacks of 0.8u, 0.45u and o.22u Millipore filter membranes. Filteration was achieved through positive pressure (Howard et al., 1978).

This medium was used for cultivation of the bovine T44, T417, D48 and 2312 typed strains for antigen production.

(c) **Cloning Procedure**

After primary isolation from clinical materials, all positive cultures were stored at -70°C. Secondary cultures were obtained by propagating these in medium (i) for all isolates and incubating at 37°C overnight.

This growth was filtered through 0.45u millipore filter. Serial 10-fold dilutions of the filtrate were made in medium (i). From each dilution, 0.1ml of the filtrate was placed on the surface of agar plates. The agar cultures were incubated for 24 to 48 hours anaerobically in a Gas-Pak anaerobic jar using oxoid (code No. BR 38) disposable generator packets.

When good well-spaced growth was obtained on the agar, a small agar block bearing a single Ureaplasma colony forming unit was excised and transfered into a tube of medium (i) using a sterile needle. This agar block was mascerated against the inner wall of the broth tube and the broth incubated at 37°C overnight. After colour change was evident,

the culture fluid material was again subjected to filteration as before and then diluted 10-fold serially and plated. This cloning process was repeated three times to obtain each strain.

(d) **Preparation of Antigens**

Standard U. urealyticum serotypes 1 to 14 were used as immunizing antigens for the human ureaplasmas, while strains D48, T44, T417 and 2312 were used as the bovine antigens. Both the human and bovine typed strains were obtained from Dr. H.L. Ruhnke of the Veterinary Services Laboratory, Guelph, Ontario, Canada, where this aspect of the investigation was actually carried out. All strains were grown in 3 litre volumes of either fluid medium (iii) or (iv). Each medium was inoculated with 30mls of an overnight starter culture of the immunizing strain grown in their respective media and incubated at 36°C for 18-24 hours. With the bovine strain D48, the medium was incubated for 36 hours because of its slow growing nature.

Good growth of the immunizing strain was ascertained before embarking on large volume cultivation.

Organisms were harvested through centrifugation using the Sorvall refrigerated centrifuge at 25,000 x g for 1 hour using the GSA rotor and at 48,000 x g for another hour using SS 34 rotor. For each serotype, the sedimented organisms were pooled and washed thrice in phosphate buffered saline, pH 7.2, and resuspended in 3.0 mls of the PBS. The suspension was sonically treated using the Sonicator MSE 100 (Watt Sonicator, O.H. Johns Scientific, Toronto) for 1 minute at maximum outburst. The sonicated suspensions were then diluted in phosphate buffered saline to yield a transmittance of 50% at 450nm (*Shepard and Lunceford*, 1978) and this was stored in the frozen state at -20^{o}C.

(e) **Preparation of Antisera**

Two rabbits were utilized for immunization with each of the respective immunizing strains of U. urealyticum and for the bovine Ureaplasma. The immunization schedule used (Table 7.1) was adapted from Shepard and Lunceford (1978) for the human Ureaplasma and from Rosendal (personal communication) for the bovine Ureaplasma (Table 7.2). Titres of raised hyperimmune sera from each rabbit were

Table 7.1 Rabbit Immunization Schedule[a] used for Preparing Hyperimmune Sera Against Human *Ureaplasma* Strains

Day	Step
0	Preimmunization bleeding
1	Prepare 5.0ml of initial immunizing antigen (2.5ml) of antigen at 50% transmittance + 2.5ml of complete Freund's adjuvant); inject 1.0ml into nape of the neck, 1.0ml into the rump, 2.0ml intraperitoneally (use half volumes bilaterally for neck, rump, and peritoneum), and 0.25ml into each of four center foot pads.
4	1.0ml antigen intravenously, no adjuvant
5	2.0ml antigen intravenously, no adjuvant
6	3.0ml antigen intravenously, on adjuvant
7	3.0ml antigen intravenously, no adjuvant
8	4.0ml antigen intravenously, no adjuvant
11	4.0ml antigen intravenously, no adjuvant
13	4.0ml antigen intravenously, no adjuvant
25	Trial bleeding. If titre was satisfactory, final bleeding by exsanguination

[a]Adapted from Shepard and Lanceford (1978)

Table 7.2: Rabbit Immunization Schedule[a] used for preparing Hyperimmune Sera Against Bovine Ureaplasma Strains

Day	Step
0	Preimmunization bleeding
1	Mix 1.5ml antigen suspension with 1.5ml Freund's complete adjuvant; inject 0.2ml subcutaneously into 10 sites along back and 0.5ml intramuscularly into each thigh
21	1.0ml antigen intravenously, no adjuvant
23	1.0ml antigen intravenously, no adjuvant
25	1.0ml antigen intravenously, no adjuvant
28	1.0ml antigen intravenously, no adjuvant
30	1.0ml antigen intravenously, no adjuvant
32	1.0ml antigen intravenously, no adjuvant
39	Test bleed. If serum unsatisfactory, repeat intravenous injections.
42	If serum satisfactory, bleed out rabbit

[a]Rosendal (Personal Communication)

determined by means of the metabolism inhibition test of Purcell et al. (1966) against homologous antigen.

The respective individual hyperimmuned sera were stored in the frozen state at -20°C without preservative.

(f) Metabolism Inhibition Test (MIT)

This test was performed using the microtitre system adopting the methods of Robertson and Stemke (1979). A 0.25ml volume of each antiserum diluted 1:10 in the medium (1) broth was placed in the first two wells of each horizontal row of a microtitre plate with U -shaped wells as the antiserum control wells.

Serial double dilutions of the antiserum in 0.025ml of medium (1) was made from wells 2 to 12 of each row. Then 0.15ml of the medium (1) to which 10% (vol/vol) pooled fresh guinea-pig sera had been added was dispensed to all test wells including the antiserum, Ureaplasma and broth control wells.

An inoculum of 0.025ml of a 10^{-3} dilution of a culture was then added to each test well and to the Ureaplasma control well. The controls received additional 0.025ml of medium (1) without added complement to make up the 0.2ml volume of the test

wells. The microtitre plates were covered with adhesive plastic tape and the wells were vented by pricking with a sterile needle. These plates were incubated in air at 35°C and examined for colour change up to at least 1 day after the inoculum control had changed colour.

A titre was recorded as the reciprocal of the highest dilution of an antiserum in which no colour change occurred.

The MIT was performed using the typed strain antigens and their homologous titre before the unknown cloned clinical isolates were subjected to this test. The test was performed on only the human Ureaplasma isolates.

All reactions for the MIT were read without knowledge of the initial results by coding the results of a previous growth inhibition test.

(g) **Agar Growth Inhibition (GI) Test**

The method of Shepard and Lunceford (1978) with some modifications was used. Standard agar medium in sterile plastic petri dishes (90mm) with about 15-20ml of agar per plate was used.

The cloned culture was incubated overnight at 37°C until colour change was evident. The actively

growing broth culture was diluted to 10^{-2} in fluid medium(i). By means of a sterile 1.0ml pipette, drops of the diluted culture were placed on the agar surface. Those drops were made to coalesce with the aid of the pipette tip to produce uniform distribution of the inoculum. The plates were left at room temperature to dry for at least 30min in a horizontal position for absorption. The bottom of the culture plates of medium were marked with numbers 1 through 14. Sterile nitrocellulose paper discs were dipped in the typed antisera and placed on the inoculated plate with the corresponding number. Each typed antiserum was again applied on the respective nitrocellulose paper disc on the plate by means of small 2ml sterile plastic syringes fitted with 21 gauge by 1.5 inch needles with extreme care to avoid mixing of antisera.

This was allowed to stand at room temperature to be air-dried for about 30 min. The inoculated antiserum-charged agar plates were then incubated right side up at 36°C in an anaerobic condition-provided by the Gas-pak generator packets. The plates were incubated overnight (18h) and were first examined the following morning for zone of growth inhibition by a specific antiserum. The serotype was

recorded, and the plate was reincubated aerobically to detect possible delayed cross-reactions. If no zones of growth inhibition were detected on the initial examination of the test (due to slow colony formation) the plate was reincubated and examined every 2 hour during the day for evidence of growth inhibition by specific antiserum. This was necessary to preclude rapid breakthrough growth of Ureaplasma colonies which initially were inhibited by a specific antiserum.

RESULTS

The growth inhibition (GI) test was easy to read and provided zones of inhibition from 1 to 5mm in diameter.

When cross-tested against homologous antisera, the GI test proved very specific with zones of inhibition between 4 and 7mm (Table 7.3), with only a few cross-reactions observed. A one-way cross reaction between serotype 4 antigen to serotype 14 antisera was seen. Similarly cross reactions were noted between serotype 1 antigen and serotype 4 antisera and between serotype 2 antigen and serotype 5 antisera.

Of the 134 clinical isolates tested 120 (89.6%) were readily typed, while 14 (10.4%) were not inhibited by any of the sera used.

A total of 40 strains isolated from male gonococcal and non gonococcal urethritis and from females attending the STC clinic were typed (Table 7.4). U. urealyticum type 8 was associated more frequently (15%) with this group of patients. The remaining serotypes isolated with some significance listed in decreasing frequency were types 2 and 4 (12.5% each) types 1 and 10 (10% each) and types 5, 9, 11 and 13 (2.5% each). Types 6 and 12 were not encountered. About 20% of the isolates could not be typed.

Of the 44 strains from gynaecology patients tested by the GI method (Table 7.4), U. urealyticum type 1 was most prevalent (11.4%) followed by types 2, 4, 8, 9 and 14, each at a rate of 9.1%. Type 7 was not isolated from these group of patients.

Ureaplasma urealyticum type 8 was associated most frequently (20%) with patients attending the family planning clinic. However types 4 (12%) followed by types 2 (10%) were the next frequently isolated organisms. Serotype 7 was also not isolated. Four strains were not typeable (Table 7.4).

The results of the MI tests of the 14 recognised serotypes of U. urealyticum tested with antisera raised against them are shown in Table 7.5.

The MI test showed titres (low dilutions) to all cross-titrations but strong reactions were, however, often observed in cases of homologous or specific type.

Mixed isolates were picked by this test method and this made interpretation of result a bit difficult in cases of weak reactions.

The degree of non-typeability was 2.3% using the MI test (Table 7.8).

The distribution of the bovine Ureaplasma serotypes as shown by the GI test is given in Table 7.9. Of the 100 bovine isolates typed, 33% belong to Type T44, 26% to type 2312, and 13% and 8% to types T417 and D48 respectively. Twenty percent of the isolates were not typeable (Table 7.9).

No serological relationship was detected between the bovine and human ureaplasmas by the GI test.

TABLE 7.3: Serological Relationship Between 14 Serotypes of Ureaplasma urealyticum determined by the Agar Growth Inhibition Test

Serotype No.	Inhibition zone[c] (mm) with antisera against homologus type													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	5	0	0	2	0	0	0	0	0	0	0	1	0	0
2	0	6	0	1	2	0	0	0	0	0	0	0	0	0
3	0	0	6	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	6	0	0	0	0	0	0	0	0	0	4
5	0	1	0	0	7	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	6	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	6	0	0	0	0	0	0	0
8	1	1	0	0	0	0	0	6	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	5	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	6	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0	5	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0	6	0	0
13	0	0	0	0	0	0	0	0	0	0	0	0	5	0
14	0	0	0	2	0	0	0	0	0	0	0	0	0	4

[c]Measurement of inhibition from edge of the well

TABLE 7.4 :Distribution of Human Serotypes of Ureaplasma urealyticum by the Growth Inhibition Test.

Source of Patients	No. of Strains Tested	Number of strains typeable by antisera: 1	2	3	4	5	6	7	8	9	10	11	12	13	14	NT
Sexually Transmitted Disease group	40	4	5	0	5	1	0	0	6	1	4	1	0	1	2	8
Gynaecology group	44	5	4	2	4	1	2	0	4	4	3	2	3	2	4	4
Family Planning group	50	4	5	1	6	0	4	0	10	2	4	3	4	4	1	2
Total	134	13 (9.7)	14 (10.5)	3 (2.3)	15 (11.2)	2 (1.5)	6 (4.5)	0 0	20 (14.9)	7 (5.)	11 (8.2)	6 (4.50)	7 (5.2)	7 (5.2)	7 (5.2)	14 (10.5)

( ) Percent of total tested

NT Non-typeable

TABLE 7.5: Serological Relationship Between 14 Serotype of Ureaplasma urealyticum Determined By the Metabolic Inhibition Test

	MIT titres of homologous and heterologous strains of U. urealyticum													
Antigen Serotype	Antisera Serotype													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	1280	40	80	80	40	40	40	40	40	40	40	40	40	40
2	40	1280	40	40	640	80	80	40	40	80	40	40	40	40
3	80	80	2560	40	40	40	40	40	40	40	40	40	40	40
4	40	40	80	1280	40	40	40	40	40	40	40	40	40	160
5	40	40	40	40	5120	80	80	40	40	40	40	40	40	40
6	80	40	40	40	40	5120	40	40	40	40	40	40	40	40
7	40	40	80	40	40	40	5120	40	40	40	40	40	40	40
8	40	80	40	40	40	80	40	2560	80	40	40	40	40	40
9	40	40	40	40	40	80	80	40	2560	40	40	40	40	40
10	40	40	40	40	40	40	40	40	80	5160	80	80	80	40
11	40	40	40	40	40	40	40	40	40	40	2560	40	40	40
12	40	40	40	40	40	40	40	40	40	80	80	5160	40	40
13	40	40	40	40	40	40	40	40	40	40	40	40	2560	40
14	40	40	40	80	40	40	40	40	40	40	40	80	40	1280

TABLE 7.6: Distribution of Human Serotypes of Ureaplasma urealyticum by the Metabolic Inhibition Test

Source of Patients	No. of Strains Tested	Number of strains typeable by antisera: 1	2	3	4	5	6	7	8	9	10	11	12	13	14	NT
Sexually Transmitted Disease group	40	4	7	0	5	2	1	0	7	1	5	1	0	1	2	2
Gynaecology group	44	5	4	3	6	1	2	0	4	4	3	2	3	2	4	1
Family Planning group	50	4	6	1	6	0	4	0	10	2	4	3	5	4	1	0
Total	134	13 (9.7)	17 (12.7)	4 (3.0)	17 (12.7)	3 (2.3)	7 (5.2)	0 (0.0)	21 (15.7)	7 (5.2)	12 (9.0)	6 (4.5)	8 (6.0)	7 (5.2)	7 (5.2)	3 (2.3)

( ) Percent of total tested

NT Non-typeable

TABLE 7.7: Result* of Metabolic Inhibition Test on Human Ureaplasma Isolates not Typeable by the Growth Inhibition Test

Source of Isolate	Isolate Number	Antiserum to strains													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	Sero-type
Gynaecology	ACC245	80	320	40	640	40	40	40	40	40	40	80	80	80	80	4, 2
" patient	ACC148	40	40	1280	40	80	40	40	80	40	40	40	80	40	40	3
" "	ACC151	40	80	80	1280	40	40	40	80	40	40	40	40	40	40	4
" "	ACC12	40	40	40	80	40	40	40	80	40	40	40	40	40	40	NT
Sexually Transmitted Diseases	STC11	40	640	40	80	40	40	80	40	40	80	80	80	40	80	2
"	STC58	40	40	40	40	40	40	80	80	40	40	40	40	40	40	NT
"	STC57	40	40	40	40	40	40	80	1280	80	640	40	40	40	40	8, 10
"	STC62	40	320	40	40	640	40	40	80	80	80	80	40	80	40	2, 5
"	STC140	40	80	40	80	40	40	40	40	40	160	80	40	40	40	10
"	STC141	40	80	40	40	40	40	40	40	80	80	80	40	40	40	NT
"	STC170	40	80	40	160	80	320	80	40	40	40	40	40	40	40	6
"	STC220	80	640	40	80	40	40	80	40	80	80	80	40	40	40	2
Family Planning	FP60	40	640	80	80	40	40	40	40	40	80	40	40	40	40	2
Family Planning	FP55	40	40	40	80	40	40	40	40	80	40	40	320	40	80	12

NT = Non-typeable

* = Expressed as reciprocal of highest antisera dilution

Table 7.8: Results of Serotyping 134 Isolates of U. urealyticum by the Growth Inhibition Test and the Metabolic Inhibition Test

Test	Tested Strains	One Serotype only	Mixed Serotype	Strains not typed
Growth Inhibition	134	120 (89.6)	0 (0.0)	14 (10.5)
Metabolic Inhibition	134	128 (95.4)	3 (2.3)	3 (2.2)

( ) Percent

TABLE 7.9 Distribution of Bovine *Ureaplasma* Isolates by Growth Inhibition Test

Sample Source	Number of Strains Tested	Number of Strains Typeable by Antisera				
		T44	T417	D48	2312	NT
Bovine Semen	8	3 (37.5)	1 (12.5)	0 (0.0)	2 (25.0)	2 (25.0)
Preputial Washing	20	7 (35.0)	2 (10.0)	1 (5.0)	5 (25.0)	5 (25.0)
Bovine Cervix	20	8 (40.0)	2 (10.0)	1 (5.0)	6 (30.0)	3 (15.0)
Bovine anterior vagina	52	15 (29.0)	8 (15.4%)	6 (11.5)	13 (25.0)	10 (19.2)
Total	100	33(33.0)	13 (13.0)	8 (8.0)	26 (26.0)	20 (20.0)

( ) Percent

NT Non-Typeable

DISCUSSION

This is the first report of Ureaplasma serotyping in Nigeria. Of a total 134 human Ureaplasma strains tested, 21 (15.7%) were of serotype 8, while 17(12.7%) were each of serotypes 2 and 4. This was followed by serotype 1 with 13(9.7%), and serotype 10 with 12(9.0%). The remaining serotypes listed in decreasing frequency of isolation in this study were type 12 (6.0%), types 6, 13 and 14 each with 5.2%, type 11 (4.5%), type 3(3.0%) and type 5 with 2.37%.

The findings in this study disagree with the report of Black (1973b) where a high incidence of serotypes 1 and 3 in ten isolates was reported in cases of non-gonococcal urethritis (NGU). In this study, serotype 8, then 2 and 4 in that order were most frequently encountered in NGU patients. Shepard and Lunceford (1978) had reported the association of genito-urinary tract infection to the presence in the tract of serotype 4. In the patients covered by this study, serotype 8 was most frequently encountered both by the MIT and GI test.

The very low frequency of isolation of some serotypes in this environment needs to be further investigated, by studying a higher number of isolates

to be able to make any definite conclusions. This study has however given an additional evidence of the presence of some serotypes of U. urealyticum in this environment and further confirmed that strains of similar types can be isolated from different countries.

Of a total of 100 bovine Ureaplasma strains tested, 33% were of T44 type, while type 2312, type T417, type D48 were 26% 13% and 8.0% respectively. It appears from this study that the four internationally known antisera were able to detect most of the bovine serotypes of Ureaplasma, as only 20% of these strains were untypeable.

It has been demonstrated that bovine strains are serologically heterogenous (Howard et al., 1978). To date no neat circumscribed serotypes exist for bovine ureaplasmas, although clusters of strains with a certain amount of antigenic similarity have been described (Howard, Gourlay and Collins, 1975). Howard et al., (1978) further confirmed this when they observed an intense cross-reaction among 6 of their 11 strains using the GI test.

A cross-reaction between 2 major groups of the bovine Ureaplasma using the MI test was also observed by Ogata et al. (1979). Probably it is this

relatedness reported between members of the bovine group and the resultant cross-reactions thereof, that have resulted in the four antisera in this study being able to type most (80%) of the isolates in this environment.

The four typing bovine antisera used in this study belong to the three main groups of bovine Ureaplasma, namely Group A - comprising strains A417 and 2312, Group B represented by strains T44, while Group C was represented by D48. The Group A and B strains were isolated with the greatest frequency from all the groups. This result agrees with the findings of Truscott (1983). To date, no evidence exists of the occurrence of any serotype which is confined to a particular anatomical site or associated with particular disease condition in cattle (Howard and Gourlay, 1973, Howard, Gourlay and Collins, 1978). However, in sheep it has been reported that one specific serotype was associated with abortion (Livingstone et al., 1978).

It was observed in this study that neither the growth inhibition (GIT) test nor the metabolic inhibition (MIT) test alone was entirely satisfactory for serotyping clinical isolates of U. urealyticum. The GI test is specific and easy to perform although

it was observed to have several disadvantages as earlier reported (Piot, 1977). Large volumes of the typing sera are needed since these have to be used undiluted. The GI test was relatively not as sensitive as the MI test. The MI test showed higher sensitivity as most isolates considered non-typeable by GI test were typed by this test.

Only 89.5% of the human isolates were serotyped by the GI test compared with 98.5% by MI test. Furthermore, the GI method is much less able to detect mixed cultures than MI test. It is singificant, though, that inspite of the rigorous cloning procedure adopted mixed colonies in 2 instances were encountered (Table 7.7). It is not known if the mixed reaction detected by the MI is a result of cross-reaction. While previous cloning has the disadvantage of masking and hindering the true detection of mixed cultures in clinical materials, both tests were not adequately sensitive to handle cases of mixed isolates. However, the distribution of the serotypes were similar in both tests with MI having slightly higher values for some types.

No common antigens were demonstrated by the GI test on the same solid media between the bovine *Ureaplasma* strains used and the 14 *U. urealyticum*

(human strains). It would appear that although serologically heterogenous, the bovine ureaplasmas seem to consist of a group of organism with many common antigens quite distinct from the human strains.

SUMMARY

This study shows the first attempt at studying human and bovine Ureaplasma serotypes in Nigeria.

There was no difference observed in the serotype distribution in the clinical cases and normal control human patients studied. Serotype 8 was most frequently encountered in the study followed by serotypes 2 and 4, while serotype 7 was not detected. However the low isolation rate of some serotypes and the non detection of serotype 7 needs to be further investigated using a larger sample size before any definite conclusions can be made.

About 80% of the bovine isolates studied were typed using four known antisera which corresponds basically to the three main groupings of the bovine Ureaplasma classification. Comparing the tests used, the metabolic inhibition test showed a greater sensitivity, but was not as specific as the growth inhibition test.

There was no common antigenic response demonstrated, using the growth inhibition test, between the bovine Ureaplasma and the human Ureaplasma confirming the distinctiveness of each of the Ureaplasma species.

CHAPTER EIGHT

EXPERIMENTAL INTRAMAMMARY INOCULATION OF BOVINE AND HUMAN STRAINS OF UREAPLASMA INTO GOATS

INTRODUCTION

The virulence for cattle of two strains of Ureaplasma from pneumonic lesions of calves has been demonstrated by the findings that calves inoculated endobronchially developed pneumonia and cows inoculated via teat canal develop experimental mastitis; however two human strains of Ureaplasma included in these tests did not cause mastitis in cows (Gourlay and Thomas, 1970; Gourlay, Howard and Brownlie, 1972).

In 1973, Gourlay and coworkers demonstrated that human Ureaplasma can infect the caprine mammary gland, thus the goat could be used as an animal model for the study of the pathogenesis of the Ureaplasma.

In Nigeria, goats are readily available at moderate prices for experimental research. It was therefore decided to use them to study the pathogenicity of selected local Ureaplasma strains from humans and cattle

MATERIALS AND METHODS

(a) Strains of Ureaplasma

Four Ureaplasma isolates obtained from bovine and human sources were used. These were strain FP81 (serotype 10) isolated from a family planning patient, strain ACC80 (serotype 1) from a gynaecology patient, strain STC 145U (serotype 4) from a male urethritis patient, and strain BS 2112 (2312) from bovine semen.

(b) Animals

Seventeen lactating Savannah Brown goats ranging in age from two to two and half years were used in this experiment.

At the time of purchase from the local market all the goats were at the last stage of pregnancy and all kidded during the conditioning period before the commencement of the experiment. During conditioning, the goats were dewormed with Panacur^{R} (Hoechst Product) and were dipped in Pfizona^{R} (Pfizer Product Ltd Nigeria) to eliminate external parasites and were ear-tagged and fed ad-libitum on wheat offal, hay,

salt lick and water. Prior to commencement of experiment, the mammary glands were checked daily for clinical evidence of mastitis. Also the California mastitis test (CMT) was performed on milk from each gland to check for evidence of sub-clinical mastitis.

Four days to the start of the experiment total aerobic plate count of milk and total somatic cell count of milk were recorded for each gland. Also rectal temperatures were recorded daily in order to establish the baseline values. Samples of milk from each half of the mammary gland, and vulval and nasal swabs were cultured for Ureaplasma. Only goats culturally free from Ureaplasma in all specimens were used.

(c) **Inoculum**

All the strains used were cloned according to the method described earlier (Chapter VII). These cloned Ureaplasma were grown in 1 litre volume each for 18 hours in Hepes buffered medium. Each growth culture was centrifuged at 30,000 x g for 45 minutes at 4°C and washed twice in PBS pH 7.2. The pellet resulting from this was suspended in fresh urea broth (UB) pH 6.0. The colony forming units (CFU/ml) was determined by standard plate count technique (Cruickshank et al., 1975).

(d) **Method of Inoculation**

The experiments were performed in two stages.

In the first stage, the goats were assigned to two pens A and B. Goats in both pens received the same dose of inoculum. The inoculation protocol are presented in Tables 8.1 and 8.2. Does in pen A were allowed to suckle their kids while those in Pen B were hand-milked twice daily. All does were left for 24 hours after inoculation before milking or suckling.

The second stage of the experiment consists of goats in Pen C. These goats all used in the first stage were infused with higher doses of inoculum 4 weeks after the termination of the first experiment. The inoculum protocol is presented in Table 8.3.

The method described by Howard (1983) was used for inoculation of the goats. All goats were milked twice daily for 4 days prior to commencement of the experiment and the milk was titrated for Ureaplasma and leucocyte counts. Also the CMT was performed.

Just before inoculation, the goats were stripped of their milk and the teat cleaned with 70% methanol. Five millilitres of the inoculum was infused into the respective half of the mammary gland

(Table 8.1 - 8.3) through the teat canal using a teat cannula with attached syringe. Infused glands were massaged to disperse the suspensions. Control glands were infused with UB, pH 6.0.

Goats were milked daily for the first 7 days and thereafter at weekly interval until day 42 post inoculation when the first experiment was terminated. The glands were however examined daily for clinical evidence of mastitis. Milk sample from each half of the mammary gland was examined for abnormal appearance, clots, and colour change. The bacterial and Ureaplasma counts were also determined on nutrient agar and UB respectively.

The CMT and somatic cell counts were performed according to the methods of Schalm et al. (1971).

A procedure similar to that described above was used on the 5 goats exposed to higher doses (stage 2)

(e) **Histopathology**

Representative portions of the mammary tissues that showed obvious change as evidenced by the CMT results and other signs of clinical mastitis in addition to persistent shedding of Ureaplasma were taken at termination of the experiment.

TABLE 8.1: Inoculation Schedule of Group A Goats with *Ureaplasma*

Goat No	Left Gland Goat			Right Gland of Goat		
	Dose[a]	Strain	Serotype	Dose[a]	Strain	Serotype
510	3.10×10^4	ACC 80	1	4.3×10^6	STC 145U	4
513	3.25×10^4	BS 2112	Bovine 2312	0	Uninoculated	Broth
514	4.50×10^3	FP 81	10	0	Uninoculated	Broth
512	3.80×10^4	STC 145U	4	3.25×10^4	BS 2112	Bovine 2312
515	0	Uninoculated	Broth	2.3×10^6	FP 81	10
188	3.16×10^4	ACC 80	1	3.25×10^4	BS 2112	Bovine 2312

[a]Dose of *Ureaplasma* inoculated in CCU/ml

TABLE 8.2: Inoculation Schedule of Group B Goats with *Ureaplasma*

Goat No	Left Gland Goat			Right Gland of Goat		
	Dose[a]	Strain	Serotype	Dose[a]	Strain	Serotype
198	3.80×10^4	STC 145U	4	0	Uninoculated	Broth
185	0	Uninoculated	Broth	1.15×10^6	ACC 80	1
181	4.50×10^3	FP 81	10	4.3×10^6	STC 145U	4
182	3.25×10^4	BS 2112	Bovine 2312	2.3×10^6	FP 81	10
184	3.10×10^4	ACC 80	1	0	Uninoculated	Broth
NN	0	Uninoculated	Broth	3.25×10^4	BS 2112	Bovine 2312

[a]Dose of *Ureaplasma* inoculated in CFU/ml

TABLE 8.3: Inoculation Schedule of Group C Goats with *Ureaplasma*

Goat No	Left Gland Goat			Right Gland of Goat		
	Dose[a]	Strain	Serotype	Dose[a]	Strain	Serotype
188	0	Uninoculated	Broth	2.1×10^7	BS 2112	Bovine 2312
198	1.2×10^9	STC 145U	4	0	Uninoculated	Broth
190	2.5×10^8	FP 81	10	1.6×10^7	ACC 80	1
181	0	Uninoculated	Broth	2.5×10^8	FP 81	10

[a]Dose of *Ureaplasma* inoculated in CFU/ml

Tissues were fixed in 10% buffered formalin and fixed sections were stained with haematoxylin and eosin stain.

RESULTS

All the does used in this experiment were negative on culture for Ureaplasma from the milk, vulval, and nasal swabs sample before onset of the experimental studies.

The various gland responses, milk changes and CMT reactions are presented in Table 8.4. A typical response to strain BS 2112 infused into the left half of the mammary gland of goat No.182 is shown in Fig. 1 as compared to response in the right half of the mammary gland of the same goat infused with strain FP81 (Fig.2). Strain BS 2112 elicited a greater somatic cell response and a higher re-isolation titre evidence of multiplication of the organism in the gland than strain FP 81 in the same goat.

The maximum titre of Ureaplasma recovery and cell count response occurred between 5 to 14 days after inoculation with this strain. All the five mammary glands in Pens A and B inoculated with strain BS 2112 developed clinical mastitis.

With increase in the dose (Pen C goats), no obvious difference in the pattern of mastitis nor that of the isolation titre was observed. The lowest and highest dose of this organism used was 10^4 cfu/ml and 10^7 cfu/ml respectively.

The response of the goats mammary gland to the other three Ureaplasma strains are shown in Fig. 2 compared to the strain BS 2112. All these strains caused some mastitis evidenced by the change in milk colour and consistency but they varied in the degree and in their persistence and multiplication in the glands. Infection with strains FP81 and Acc 80 were resolved rapidly within 7 days while with strain BS 2112 the organism was still detectable by day 42 post-exposure. With all the strains, except BS 2112 (type 2312), the infection had resolved by the 14th day of inoculation and no organisms could be isolated from the mammary gland.

The somatic cell count pattern was similar to the Ureaplasma recovery pattern.

Udder abnormalities were evident in the left gland of goat 182 inoculated with BS 2112 (Type 2312), this was manifested by induration of the gland with a drop in milk yield, while the milk became pale watery and separated.

FIG.1 Ureaplasma cell count and Somatic cell count in Milk of Goat No.182 Experimentally infected with BS 2112 and FP 81 through the left and right mammary glands respectively.

FIG.2 Ureaplasma cell count in Milk of experimentally infected Goats through Intramammary Route

*Dose used are shown on Table 8.1; 8.2; 8.3.

TABLE 8.4: Milk Changes and California Mastitis Test Reaction in Goats Experimantally Infected with *Ureaplasma* Strains.

Goat/Gland/Dose		DAYS POST INOCULATION										
		0	1	2	3	4	5	6	7	14	21	28
Left	Milk	-	**	**	***	***	***	***	***	**	**	**
Strain BS2112	Udder	-	*	*	*	**	**	***	***	**	*	*
3.25×10^4	CMT	-	*	**	**	**	**	**	**			
			*	**	**	**	**	**	**	***	***	***
182												
Right	Milk	-	*	**	**	**	*	*	*	*	-	-
Strain FP 81	Udder	-	-	-	-	*	*	*	-	-	-	-
2.3×10^6	CMT	-	*	**	**	**	**	**	*	*	-	-
Left	Milk	-	*	-	-	-	-	-	-	-	-	-
Uninoculated broth	Udder	-	-	-	-	-	-	-	-	-	-	-
	MCT	-	-	-	-	-	-	-	-	-	-	-
185												
Right	Milk	-	-	-	*	*	*	*	-	-	-	-
ACC 80												
1.15×10^6	Udder	-	-	-	-	-	-	-	-	-	-	-
	CMT	-	-	*	*	**	**	*	-	-	-	-

Goat/Gland/Dose		DAYS POST INOCULATION 0	1	2	3	4	5	6	7	14	21	28
Left												
FP 81	Milk	-	-	-	**	*	*	-	-	-	-	-
4.50 x 10^3	Udder	-	-	-	-	-	-	-	-	-	-	-
	CMT	-	-	*	**	**	**	*	-	-	-	-
181												
Right												
STC 145U	Milk	-	-	-	**	**	**	*	-	-	-	-
4.3 x 10^6	Udder	-	-	-	*	*	*	-	-	-	-	-
	CMT	-	*	*	***	***	**	**	**	-	-	-
Left												
STC 145U	Milk	-	-	*	*	*	*	-	-	-	-	-
3.80 x 10^4												
	Udder	-	-	-	-	-	-	-	-	-	-	-
	CMT	-	-	-	*	*	*	*	*	-	-	-
512												
Right												
BS 2112	Milk	-	-	-	***	***	**	**	**	**	*	**
3.25 x 10^5	Udder	-	-	-	-	*	*	*	*	*	-	-
	CMT	-	*	*	***	***	**	**	**	**	***	***
							**	**	**			

Milk Abnormalities:
*=dark yellow
**=contains clot
***=separated-clear whey and floccular material

Udder Abnormalities
* =induration
**=reduced gland size and milk secretion
***=cessation of milk secretion

CMT
*=Trace - slime forms and diseappears
**=Weak Positive - a distinct slime forms
***=distinct Positive - a gel forms with alkaline Reaction
****=strong Positive - a viscous gel with alkaline Reaction

DISCUSSION

Strains of the human Ureaplasma did cause mastitis as evident by the CMT result and the somatic cell counts, although these were transient and got resolved earlier than the bovine strain. This finding is consistent with the results of Gourlay et al. (1973) who observed subclinical mastitis with some human strains when inoculated into the caprine mammary gland. In bovine mammary glands, however, no mastitis developed (Gourlay et al., 1972; Howard et al., 1973).

In this study, Ureaplasma strains were isolated at relatively high titre for some length of time indicating the multiplication of the organism in the glands in view of the large quantity of milk produced by the goats during the course of the experiment.

The consistently high titre of the organism in milk even amongst suckling does when compared with the initial inoculum lends credence to the fact that multiplication of the organism did occur in these glands.

There were variations in the response of the goats to inoculation of Ureaplasma which was unrelated to the inoculum number with all the

strains. While it was expected that glands in the second experiment would show greater response than those in the first experiment, a reverse situation was obtained.

Earlier, Gourlay et al. (1972) had observed that cows which had high metabolic inhibiting whey titres to Ureaplasma after inoculation, had the ability to control and resolve the infection. It is therefore possible that the prior sensitization of these goats with Ureaplasma in the first experiment had produced some local immune response which prevented the second higher inoculum from causing a greater response.

Throughout the study period, Ureaplasma were not detected from the glands of goats inoculated with control broth, nor was any subclinical or clinical mastitis observed. This indicates a lack of spread of infection between gland halves which makes it convenient to use each gland as an entity or study unit.

From this study, the bovine isolate BS 2112 which had earlier been typed to be serotype 2312 by the GI test is pathogenic. Earlier report had indicated that the type 2312 is capable of causing vulvitis, endometritis and salpingitis following incubation (Doig et al., 1980a, 1980b).

Comparing the human ureaplasmas, the strains ACC 80 and FP 81 elicited milder responses compared with strain STC 145U. The pathogenic potentials of the strain STC 145U needs further investigation

When the Ureaplasma strains from the two host species were compared, the bovine strain elicited a more severe reaction in the goats than the human strains. Host specificity might be a possible explanation for the general low response of the goat gland to inoculum with human ureaplasma. However since the bovine strain could cause infection in goats it appears that this host specificity is not absolute. The finding of Taylor-Robinson et al. (1971; 1981) that only human ureaplasmas adsorbed to Hela cells but not bovine, simian or canine strains and that only simian strains adsorbed to chicken erythrocytes does indicate that some degree of specificity exists by the Ureaplasma in cell adsoption. This might possibly explain the differential response of the human and bovine strains to the caprine mammary gland.

In conclusion, caprine mammary gland can be used in this environment as a suitable model for study of the pathogenesis of Ureaplasma, especially of bovine origin.

SUMMARY

Three Ureaplasma strains of human origin (STC 145U, serotype 4; FP 81, serotype 10; ACC 80, serotype 1) and one of bovine origin BS 2112 (serotype 2312) were inoculated into various mammary glands of goat at varied determined concentrations of the organisms. The human strains multiplied and elicited a transient inflammatory response of the mammary gland which got resolved early. The bovine strain of Ureaplasma however elicited a more severe and longer lasting reaction in the gland of the goats. It is concluded that the caprine mammary gland can be used in this environment as a suitable animal model for studying ureaplasmal pathogenicity.

CHAPTER NINE

EXPERIMENTAL INTRAVENOUS AND INTRAVAGINAL INOCULATION OF GOATS WITH BOVINE UREAPLASMA STRAIN BS 2112 (LOCAL ISOLATE)

INTRODUCTION

The first isolation of Ureaplasma from cattle was from the reproductive tract (Taylor-Robinson et al., 1967), and subsequently there were other reports of isolation from the vaginal, cervical mucus, semen, and preputial washings (Taylor-Robinson et al., 1969; Onoviran et al., 1975; Panangala et al., 1975; Adegboye et al., 1980).

A report had implicated the organism in granular vulvitis in dairy herd (Ruhnke et al., 1978). Doig et al. (1980a) later reproduced this condition experimentally following vulva inoculation in cattle. To date, there are no reports on exposure of animals to Ureaplasma by the intravenous route, possibly because it is a remote portal of entry of the organism.

The purpose of this study was to determine the pathogenecity of the bovine Ureaplasma strain for the

goat reproductive system following intravaginal and intravenous inoculation.

MATERIALS AND METHODS

(a) Inoculum

Bovine Ureaplasma strain BS 2112 obtained from a bull semen and typed to be serotype 2312 by the GI test was used.

This strain was cultured in 500mls of HEPES buffered Urea broth. The culture was centrifuged at 30,000 x g for 45 minutes at 4°C. The sediment was suspended and washed twice with phosphate buffered saline (PBS), pH 7.2, and the number of CFU/ml was found to be 2.1 x 10^6 as determined by standard plate count technique (Cruickshank et al., 1975).

(b) Experimental Animals

Six pregnant Savannah Brown goats obtained from the local market were used in the study. These goats were kept in a pen and fed on hay, salt lick, wheat offal and water ad-libitum for one month during which the goats were acclamatized to the pen environment before the experiment.

(c) **Experiemental Procedure**

The goats were divided into experimental groups of three goats each. Preinoculation samples were taken for culture 4 days prior to inoculation from the vulva and from the blood.

Two goats (No.518 and 186) in group one were inoculated through the right jugular vein with 5mls each of a suspension of the Ureaplasma in PBS. The third goat (183) in this group received 5mls of the sterile PBS, and served as control.

Two goats (197 and 199) in experimental group two had Ureaplasma suspension gently deposited into the lumen of the vagina through a pipette and the vulva lips were held together for a while to keep the inoculum in position with the goat held head pointing down. The third goat (517) in this group received 5mls of sterile PBS in a similar manner.

(d) **Clinical Observation**

The appetite and rectal temperature of all the six goats were monitored. Those of group one were monitored hourly for the first 12 hours and thereafter daily as for the goats in group two.

Twice daily, a detailed examination was made of the vulval mucosa. Any external discharge was recorded and the character of the discharge noted. The discharge was also cultured for Ureaplasma spp.

(e) **Re-isolation Studies**

Blood was collected daily, post-inoculation, in tubes with EDTA from the 3 goats in group one. Approximately 0.01ml was streaked on urea agar plates and also on blood agar plates. The ureaplasma plates were incubated anaerobically in a Gaspak anaerobic jar for 2 days.

Vulval samples were taken daily, post inoculation from all the six goats for 7 days and on the 14th day. The samples were cultured routinely for Ureaplasma.

(f) **Post-Mortem**

Goat 186 aborted on day 10, and experiment in that goat was terminated to conduct gross, histopatho logical and cultural studies on the genitalia on day 10. On the 14th day post-inoculation, the study was terminated by slaughtering all the goats.

At slaughter, the reproductive tracts were removed intact and a detailed assessment of these tracts made. Swabs were taken from the entire reproductive tracts and cultured. The lungs, heart liver and brain of the fetuses were also cultured for Ureaplasma. To obtain the uterine swabs, the serosal surface of each horn was swabbed with alcohol and a 3cm incision was made into each horn and the uterine surface rubbed with sterile swabs. Thereafter the entire reproductive tract was opened and inspected.

Sections were taken from uterine wall, cotyledons, cervix, vagina and vulva of each doe and from the liver lungs and heart of the fetuses. These sections were fixed in 10% buffered formalin and processed for Haematoxylin and Eosin (H&E) stained histopathology sections routinely.

RESULTS

No obvious changes were observed in the appetite or rectal temperatures of the goats following intravenous or intravaginal inoculations (Table 9.1).

Goats inoculated intravaginally (Group 2) showed hyperaemia of the vulva. This was accompanied by mild mucopurulent vulval discharge approximately six hours post-inoculations in the experimental goats but not in the control goat (Table 9.1). The mucopurulent vulval discharge continued intermittently in goat 199 for over a week. However in goat 197 discharge was not evident after the 4th day post-inoculation.

Goat 186 aborted on the 10th day post intravenous inoculation, while Goat 199 aborted 14th day post-vaginal inoculation.

No aerobic bacteria were isolated from the blood samples of the goats.

Ureaplasma sp. was re-isolated from the blood of goats that received intravenous inoculation daily up to the 4th day post-inoculation. Thereafter the blood samples were negative. Moreover Ureaplasma was isolated from vulva cultures from goat 518 on days 3 and 4 post-intravenous inoculation. The titres were relatively low (10^2). Thereafter it was negative.

Blood samples of goats that received intravaginal inoculation were negative for Ureaplasma but vulval swabs were positive till 14 days for goat 199, but only up to 5 days for goat 197 (Table 9.2).

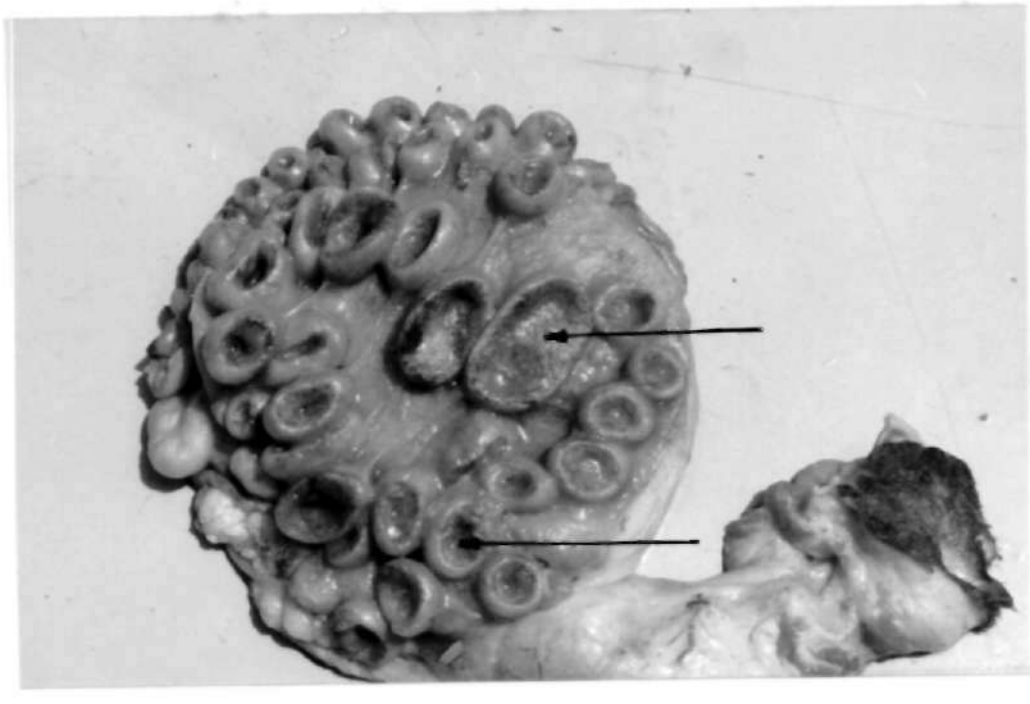


Plate 9.1a Caruncular crypts of the cotyledons in a Goat (No.199) killed 14 days following intravaginal inoculation of 10.5 x 10^6 colony forming Units Ureaplasma BS 2112. The cotyledons are filled with chessy necrotic material

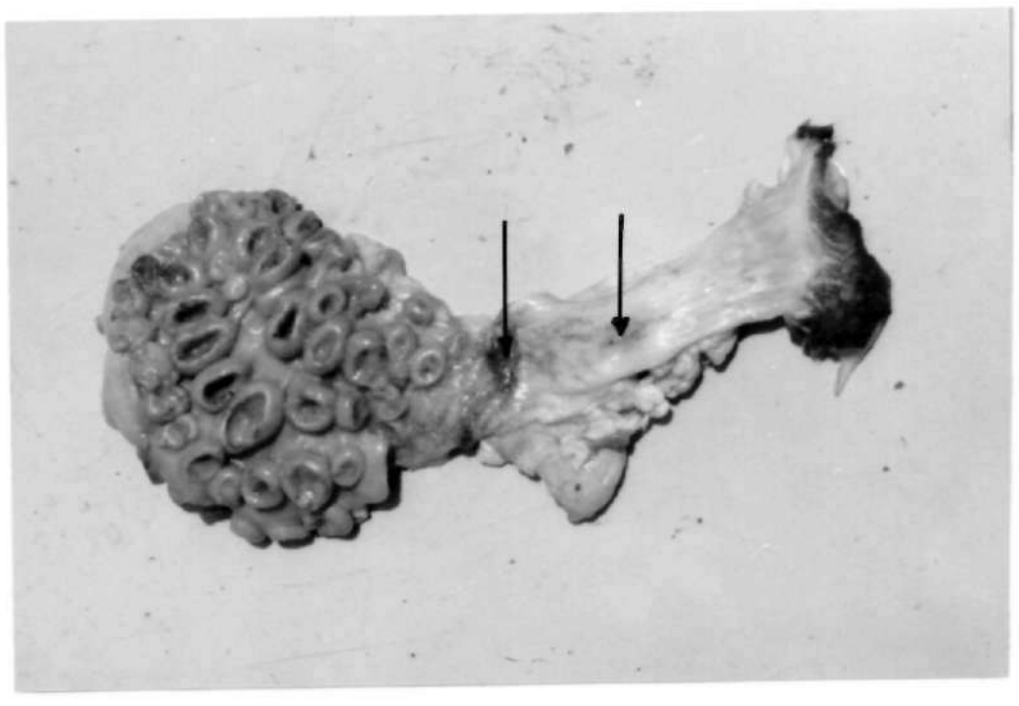


Plate 9.1b Areas of petechial haemorrhage on the vagina and cervix (arrowed) in Goat 199



Plate 9.2a Uterus with fetuses from both horns in Goat (No.197) Killed 14 days post vaginal inoculation of 10.5×10^6 Colony forming Units of Ureaplasma BS 2112.

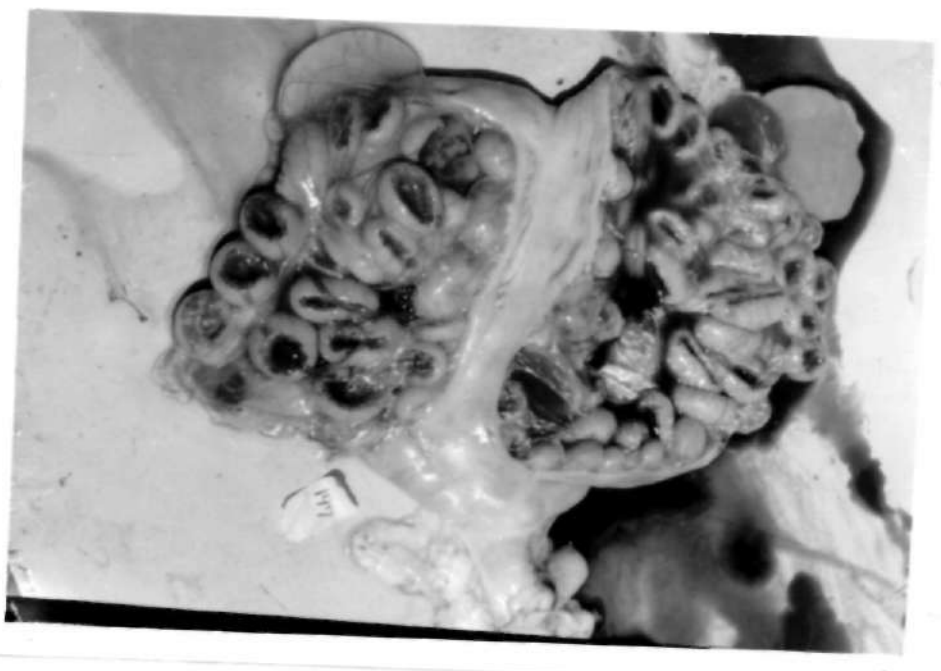


Plate 9.2b Uterus of Goat 197 with grossly normal cotyledons.

Blood cultures or vaginal swabs from the control goats were all negative for Ureaplasma throughout the study period.

Goat 199 had generalized necrosis of the uterus. The concave caruncular crypts of the cotyledons were necrotic and filled with cheesy material. The edges were lined with elevated areas of hyperaemia and petechial haemorrhages. The intercotyledonary areas were also edematous. The cervix was highly haemorrhagic and petechial haemorrhages were seen on the vagina and vulva (Plate 9.1a, 9.1b,). This goat aborted on day 14 post-inoculation just before termination of experiment.

Fetuses were observed in both horns of the uterus of goat 197 and no areas of haemorrhage or gross pathology were observed from the vulva to the horns of this goat (Plate 9.2a, 9.2b,).

Goat 518 had no gross pathology and the fetus was normal. Similarly goat 186 that aborted 10days post-intravenous inoculation had no gross pathology.

Both control does had no gross pathologic lesions. Cultures were Ureaplasma negative in both does and fetuses in all the experimental goats except in goat 199. Cultures were positive for Ureaplasma

from all specimens taken from goat 199 reproductive tract including the fetal lungs.

No histological lesions were observed in the genital tracts of goats (518 and 186) inoculated by the intravenous routes.

In goat 199, lesions observed in the cervix and vagina were characterized by massive areas of haemorrhage and by infiltrations of neutrophils beneath the cervical epithelium with some in the lumen. Endometritis characterized by massive accumulation of leucocytes around and massive exudates within the lumen of the gland were observed. The exudates contained a few lymphocytes and epithelial cells. Also areas of epithelial ulceration were observed with some fibrin strands. No significant histological lesions were however observed in the tract of goat 197.

The control goats inoculated both intravenously and intravaginally did not show any noticeable histological lesions.

TABLE 9.1 Temperature Chart of two Groups of Goats Inoculated Intravenously (Group 1) and Intravaginally (Group 2) with *Ureaplasma* strain BS 2112 (Type 2312) and the Control Animals (183, 517) inoculated with Broth only.

Route of Inoculation	Number	-2	Rectal Temperatures in °C hours post inoculation									
			0	1	2	3	4	5	6	24	48	72
Intravenous	518	39.8	39.9	39.0	39.6	39.9	40.1	40.1	39.5	39.6	39.7	39.4
	186	39.7	39.8	39.5	39.2	39.3	39.3	39.4	39.6	39.0	39.2	39.2
(Group 1)	183 (control)	39.3	39.4	39.1	39.2	39.8	39.7	39.5	39.7	39.0	39.6	39.4
Intravaginal	199	39.1	39.0	39.7	39.7	40.0	39.4	39.4	39.5*	39.0*	39.6*	39.4*
	197	39.5	39.6	39.2	39.2	39.2	39.1	39.0	39.4*	39.1*	39.4*	39.4*
(Group 2)	517 (control)	39.8	39.9	39.6	39.5	39.7	39.8	39.8	39.5	39.5	39.7	39.5

*Evidence of Pus in Vulva

Difference in temperature between inoculated and control goats in the 2 groups were not significant at $p = 0.05$ using the 'student t distribution.

TABLE 9.2 Culture of *Ureaplasma* strain BS 2112 from Goat Inoculated by Intravenous and Intravaginal Routes.

Route of Inoculation	Goat Number	Sample Source	Titres of Ureaplasma isolated days post-inoculation									
			-2	0	1	2	3	4	5	6	7	14
	518	Blood	-	-	10^4	10^3	10^2	-	-	-	-	-
Intravenous		Vaginal	-	-	-	-	10^2	10^2	-	-	-	-
	186	Blood	-	-	10^3	10^3	10^2	-	-	-	-	-
(Group I)		Vaginal	-	-	-	-	-	-	-	-	-	-
	183	Blood	-	-	-	-	-	-	-	-	-	-
		Vaginal	-	-	-	-	-	-	-	-	-	-
	199	Vaginal	-	-	10^3	10^3	10^4	10^4	10^3	10^3	10^3	+
Intravaginal	197	Vaginal	-	-	10^3	10^3	10^3	10^3	10^3	-	-	-
Group 2	517	Vaginal	-	-	-	-	-	-	-	-	-	-

+Presence or absence of *Ureaplasma* only determined
Titre not determined from the sample

-Negative for Ureaplasma

DISCUSSION

In a study by Doig et al. (1980 a and b) granular vulvitis was consistently reproduced in cattle following vulval inoculation with Ureaplasma without prior epithelial trauma. Other studies failed to recover Ureaplasma beyond the vulva when inoculated via this route (Ruhnke et al. 1978; Doig et al., 1980).

In the present study, however, Ureaplasma type 2312 was isolated from the upper reproductive tract, the uterus, and the fetus following vulvo-vaginal inoculation showing ascending dissemination to other sites. The reason for this discrepancy is not readily apparent but it is pertinent to observe that this finding was found in only one (goat 199) of the two goats exposed to Ureaplasma through the vulvo-vaginal route. The exact picture may not be known till more animals are used for the study. This study however further confirms the pathogenicity of type strain 2312 not only for the host specie cattle (Ruhnke et al. 1984) but also for the caprine reproductive tract.

The abortion in Goat 186 could not be explained as there was no attendant isolation of Ureaplasma from the upper reproductive tract of the doe nor from the aborted fetus. That there was no attendant temperature rise in both groups of goat that were inoculated by intravenous and intravaginal routes indicate that Ureaplasma infection may not be septicaemic. Although the role of Ureaplasma as a septicaemia was not established in this experiment, the haematologic routes of infection to predilection sites cannot be ruled out.

This study further confirms the suitability of the caprine as an experimental animal for the study of the pathogenicity of Ureaplasma

SUMMARY

The pathogenicity of Ureaplasma isolate BS 2112 (serotype 2312) of bovine origin was confirmed for the caprine reproductive tract. Ureaplasmal infection was not established as a septicaemia.

CHAPTER TEN

GENERAL DISCUSSION

The aim of this research was to study and determine the level of ureaplasma infection in both human and cattle population in Zaria and its environ and to characterize these isolates. This was justified by the growing interest internationally in the association of *Ureaplasma* and mycoplasmas to urogenital tract infections. There are numerous reports on the possible role of these organism in non gonococcal urethritis (Shepard, 1980), infertility (Cassel *et al.*, 1983), abortion and still birth (Kundsin *et al.*, 1981), premature birth and low birth weight of infants (Kundsin *et al.*, 1981) and pelvic inflammatory disease (Mardh *et al.*, 1970).

Several factors are known to play a role in the higher recovery rate of *Ureaplasma* on media (Yajko *et al.*, 1984). In this environment however, a higher recovery rate of *Ureaplasma* was obtained with the conventional urea broth (UB) than with other buffered media. This broth has an indicator system based on the ability of *Ureaplasma*, unique among

mycoplasmas, to degrade urea to carbon-dioxide and ammonia (Robertson, 1978). The resultant accumulation of ammonium ions increased the pH to change the indicator system. However this change is not evident until stationary growth is reached by which time the culture has lost 90% of its viability usually within 17 hours of incubation. Improved viability evident by a higher titre was however obtained with buffered media. The buffered broth contained less urea than the conventional urea broth. This is a cause of the lower final pH value accounting for prolonged viability of buffered broths. Ureaplasma possess a heat-stable, catalase resistant inactivating factor in broth that is both toxic depending on concentration. It was thus concluded that this factor may in part be responsible for the self-limiting nature of the Ureaplasma and also accounting for the poor keeping quality and resultant death of the organism during storage.

The frequency of isolation of U. urealyticum and M. hominis from the genito-urinary tract has been cited as indicating their commensal rather than pathogenic role (McCormack et al., 1973). However in this study, a statistical, and causal significance

was established between isolation of Ureaplasma and M. hominis to secondary infertility in women. In an earlier study, Braun et al. (1971), reported an association of low birth weight with isolation of these genital mycoplasmas from the maternal cervix.

In cattle, Ureaplasma was isolated from 70% of the vaginal specimens, 83% of preputial specimens and 65% of semen. There was a high degree of agreement between this study and that of an earlier one in the same environment by Adegboye et al. (1980). The finding is of important concern in that the bulls sampled were mainly those used for artificial insemination. That mycoplasmas could play a role in bovine infertility specifically causing impaired sperm motility has been well documented (Jurmanova et al., 1977; Chang et al. 1984). It is in the light of this that the significance of this finding should be viewed.

When eighteen strains of Ureaplasma obtained from isolation studies from human and also cattle and one of M. hominis were tested for their susceptibility to ten commonly used antimicrobial agent in this environment, three susceptibility patterns emerged. That tetracycline, chloramphenicol and togamycin were highly effective against both

Ureaplasma and M. hominis was in consonance with existing information. One exception to the susceptibility pattern was however observed with a Ureaplasma strain that was resistant to all dilutions of chlortetracycline used. While it is however not possible to speculate on the level of tetracycline resistance at this stage based on the sample size used, of significance is the report that about 40% of tetracycline resistant strains are now also resistant to erythromycin (Anon, 1986). This may have a bearing on the outcome of treatments of especially NGU cases where tetracyclines and erythomycin have been widely used (Bello-personal Communication).

The first attempt at serological characterization of Ureaplasma from human and cattle in this environment has yielded a broad outline of what exists. A more factual prevalence of these various types or serotypes can only be made when more isolates over a longer period of time are studied. No obvious significant differences were observed in the human serotype distribution in the clinical cases and apparently normal human. Serotype 8 was most frequently encountered in the study from all the human patients. This finding is highly significant moreso when viewed against a recent

report (Anon, 1986) that serotype 8 was associated with a fatal neonatal pneumonia in a term neonate. Predominance of serotype 8 also in the control group cannot however be explained.

It has been established from this study that human Ureaplasma multiplied and elicited a transient inflammatory response in the caprine mammary tissue. The bovine Ureaplasma strain however elicited a more severe glandular reaction resulting in mastitis thus agreeing with earlier reports (Gourlay et al., 1973). Whether all human serotypes elicite the same mild response or whether all the bovine strain cause such severe reactions is presently not known. Ureaplasma infection as a septicaemia was not established.

In conclusion, Ureaplasma occurrence in both human and cattle population was firmly established in this environment. A broad serotype scheme was established and it is hoped that this would serve as a baseline data for future studies on Ureaplasma in this environment. That the bovine strains of Ureaplasma are pathogenic for the caprine mammary glands and system was established, although the marker or reason for the pathogenicity was not ascertained. It is hoped that future studies would address itself to these problems.

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