Isolation, Characterization and Antibiotic Susceptibility of *Mycoplasma hominis* and *Ureaplasma urealyticum* from Infertile and pregnant Women in Lagos, Nigeria

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ABSTRACT

Background:

Mycoplasma hominis and *Ureaplasma urealyticum* are potentially pathogenic agents, playing aetiologic roles in both genital infections and infertility. In human in-vitro fertilization

systems, the presence of *U. urealyticum* in either semen or female genital tract results in a decline of pregnancy rate per embryo transfer as well as neonatal infections. *M. hominis* has been associated with bacterial vaginosis, pelvic inflammatory disease, postabortal fever, and a number of gynecological infections.

Aim:

The aim of this study is to isolate, characterize and determine the antibiotic susceptibility of *M. hominis* and *U. urealyticum* isolates from infertile and pregnant women in Lagos, Nigeria

Materials and Methods: The samples were collected fromObstetrics and Gynaecology clinics of Lagos University Teaching Hospital and 68 Nigerian Army reference hospital Yaba. A total of 270 specimens of urine and HVS were collected from 135 women attending the clinics for routine consultations. One hundred and eighty HVS and urine specimens were from 90 married infertile women attending the clinics as part of a work-up for fertility investigations after failing to conceive for at least one year of unprotected sexual intercourse. Ninety HVS and urine specimens were from 45 pregnant women attending the clinics for routine antenatal care. None of the subjects expressed any symptom of genitourinary tract infections.All the specimens were inoculated into *Mycoplasma* broth and subsequently Blood Agar plates, incubated appropriately and identified. Antibiotic susceptibility tests were carried out on the 52 isolates. Polymerase chain reaction (PCR) was used to detect the organisms in all the collected specimens.

Results:

Of the 90 HVS specimens collected from infertile women, 9 (10.0%) were positive for *M. hominis*, while 21 (23.3%) were positive for *U. urealyticum*. For the pregnant women using HVS specimens, 6 (13.3%) were positive for *M. hominis* while 5 (11.1%) were positive for *U. urealyticum*. The first void urine specimens gave lower values in both the infertile and pregnant women. Prevalence of U. *urealyticum* was higher in infertile women than in pregnant women ($p<0.05$). The PCR technique gave higher values of 78.5% and 71.1% using HVS specimens for the infertile and pregnant women respectively for *Mycoplasma/Urealyticum* species. The antibiotic susceptibility test showed that all the isolates of *M. hominis* (n=18) were sensitive to Tetracycline (100%) and Ciprofloxacin (100%) while all the isolates of *U. urealyticum* (n=34) were sensitive to Tetracycline (100%) and Erythromycin(100%).

Conclusion:

The significantly higher prevalence of *U. urealyticum* infection in infertile women (23.3%) compared to the lower prevalence in pregnant women (11.1%) may suggest that *U. urealyticum* can be incriminated in infertility. HVS specimen is preferred over urine specimens for the detection of *Mycoplasma and Ureaplasma.* Application of the PCR method, where affordable, is recommended for rapid and sensitive detection of *Mycoplasma and Ureaplasma* in HVS specimens. Tetracycline may be the antibiotic of choice, unless contraindicated, for the treatment of the infections, although the sample size was small.

Keywords: Prevalence, Infertility, Pregnancy, Nigeria, *Mycoplasma hominis, Ureaplasma urealyticum.*

INTRODUCTION

Infertility is considered when couples have been trying to achieve pregnancy with frequent sexual intercourse for at least a year without success[1]. Documented data revealed that approximately 72.4 million couples are infertile[1]. The causes of 25% of the cases of infertility are still unknown[2]. Majority of infertile females have inflammatory changes of the oviduct or the surrounding peritoneum and most of these alterations are caused by infections[3].

Generally, *Mycoplasma hominis and Ureaplasma urealyticum* have been isolated from genital mucosal surfaces, vagina and cervical parts of females [4, 5,6]. *U. urealyticum* is a major cause of non-chlamydial and non-gonococcal urethritis, chorioamnionitis, acute prostatitis, vaginitis, cervicitis, preterm delivery and sepsis [6, 7]. *M. hominis* is often associated with vaginitis, cervicitis, postpartum sepsis, pyelonephritis, preterm labour and premature birth[8, 9]. Bacterial vaginosis is strongly implicated in female infertility and screening and treatment of bacterial vaginosis during the course of infertility management increased the rate of pregnancy[10]. The genital mycoplasmas represent a complex and unique group of microorganisms that have been associated with a wide array of infectious diseases in adults and infants. These microorganisms particularly *U. urealyticum* are potentially pathogenic species playing etiologic roles in both genital infections and infertility. In human invitro fertilization systems, it was reported that the presence of *U. urealyticum* in either semen or female genital tract resulted in a decline of pregnancy rates per embryo transfer[11], as well as neonatal infections[12]. Similarly, *M. hominis* has been associated with bacterial vaginosis, pelvic inflammatory disease and post abortal fever as well as a number of gynaecological infections[13]. The isolation of these organisms in the diagnostic laboratories is cumbersome and takes several days to achieve. The identification from clinical specimens using Nucleic Acid Amplification Test is very expensive for routine purposes.

We therefore decided to determine the prevalence of *Mycoplasma* infections among infertile and pregnant women in Lagos Metropolis, Nigeria; and to determine the susceptibility patterns of the isolates to some of the commonly prescribed antibiotics.

MATERIALS AND METHODS

Ethical Issues: The study was approved by the Ethical Committees of Lagos University Teaching Hospital (LUTH) and the 68 Nigerian Army Reference Hospital, Yaba (68NARHY). All the participants signed informed consent form to participate in the study.

Inclusion Criteria: All the specimens (Urine and HVS) were from married infertile women attending the Gynecology Clinics as part of a work-up for fertility investigations after failing to conceive for at least one year of unprotected sexual intercourse. Also included in the study were expectant mothers who were normal antenatal clinic attendees for routine medical attention. Subjects with any clinical symptom of sexually transmitted disease were excluded from the study.

Sample Collection: From July 2012 to September 2012, a total of 270 HVS and urine specimens were collected from 135 women attending the clinics for routine consultations. One hundred and eighty of the specimens were from 90 married infertile women attending the clinics as part of a work-up for fertility investigations. Ninety specimens were from 45 pregnant women attending the clinics for routine antenatal care. The subjects were aged between 22-45 years. The HVS specimens were collected by clinicians using disposable speculum while the subjects were informed on how to collect the urine specimens devoid of contamination.

***Mycoplasma hominis and Ureaplasma urealyticum* isolation and identification:**

The specimens were inoculated into the *Mycoplasma* transport/growth medium without delay. Clearly labelled specimens were treated by inoculating 0.1ml into 5ml of prepared *Mycoplasma* broth at the site of specimen collection and transported to the laboratory. The remaining urine specimens (about 10ml each) were stored at -70°C. Both the urine and HVS specimens were incubated for up to 48h at 37°C in 5% CO_2 for *Ureaplasma* and up to 5 days for Mycoplasmas. These were examined daily for turbidity as evidence of growth. Subsequently, the broth cultures were subcultured onto solid blood agar media and incubated at 37°C in 5%CO_2 for 24 to 48 hours for *Ureaplasma* and up to 5 days for Mycoplasmas. Urea for urease activities and L-arginine for arginine utilization were used for biochemical tests. Strains of *M. hominis* ATCC 23114 and *U. urealyticum* ATCC 33175 were used as positive controls. Colonies presenting a fried egg appearance suggested the presence of *M. hominis*, while colonies that were brown and tiny indicated the presence of *U. urealyticum. U. urealyticum* also hydrolyzed urea while *M. hominis* metabolized the L. arginine.[14]

Polymerase Chain Reaction Confirmation of *U. urealyticum* and *M. hominis*

A PCR technique was used to detect *U. urealyticum* and *M. hominis*. Two millilitres of urine and HVS broth specimens was used Ref: standard strains of *U. urealyticum* (ATCC 33175); *M. hominis* (ATCC 23114) and *Clostridium difficile* (ATCC BA-2155) were used.The PCR was carried out in an Eppendiof Nexus thermal cycler with the following cycling parameters; an initial denaturation set up at 95°c for 10 minutes, followed by 30 consecutive

cycles of denaturation at 95 ^{o}c for 30 seconds, annealing at 56°c for 30 consecutive cycles. After this, a final extension at 72 ^{o}c for 5 minutes was carried out.

High vaginal swab (HVS) specimens and 2ml of first void urine from each subject were directly inoculated into 5ml of *Mycoplasma* broth, mixed and transported to the laboratory at the Nigerian Institute of Medical Research, (NIMR), Yaba, Lagos.

Sample Preparation[15]

Two milliliters of first void urine sample and HVS suspension from each of the subjects were put into a sterile 5ml tube and centrifuged at 13,000 rpm for 5 minutes. The supernatant is discarded and the cell pellets were resuspended in 50 microlitre of lysis buffer. After the homogenization, the cell pellets were incubated at 37 ^{o}c for 15 minutes so as to lyse the cells and degrade the proteins. The samples were then heated at 95 ^{o}c for 10 minutes to inactivate the protease and then centrifuged at 13,000 rpm for 5 minutes to sediment the cell debris[15]. The supernatants were transferred into new microcentrifuge tubes. Positive and negative controls were set up along side the kit controls.

The standard strains of control organisms are; *Ureaplasma urealyticum* – ATCC 33175, *Mycoplasma hominis* – ATCC 23114, strain PG 21, and *Clostridium difficile* – ATCC BA – 2155, Strain LBM 0801058. The primers were obtained from NIMR, Yaba, Lagos.

PCR Reaction Setup

Twenty microliters of each of the universal PCR mix was transferred into a tube, 2.5 microliters of the universal primers and 2.5 microliters of the test samples were added and mixed

gently using a voltex mixer. Positive and negative samples were set up alongside the controls. The tools were placed in a thermal cycler and were taken through the three steps of amplification procedure.

Gel Electrophoresis Protocol

Five microliters of the PCR product is added to 1.5 microliter of loading buffer, and mixed thoroughly. The samples and a DNA marker (1000 bp ladder) were loaded on the 3 % agarose gel. Positive and negative controls were set up alongside the kit controls. Electrophoresis was allowed to run until the tracking dye has migrated 60-70% of the gel length. The gel was then stained with ethidium bromide and viewed with ultraviolet illumination.

The kit detects 10^{-9}microgram quantities of target DNA. Samples that were positive for the presence of Mycoplasmas showed a distinct band at 434-468 bp. The positive control exhibited a 464 bp band while no visible band was noticed in the negative control lane.

Antimicrobial Susceptibility Test on the Isolates

Antimicrobial susceptibility test was carried out on the isolates using the modified Kirby-Bauer method[16] with Tetracycline (30µg), Gentamycin (10µg), Erythromycin (15µg), Streptomycin (30µg) and Ciprofloxacin (5µg).Statistical analysis was done using SPSS version 10 (computer software)

Statistical analysis

Statistical analysis was done using SPSS version 10 (computer software)

RESULTS

A total of 90 High Vaginal Swabs from infertile women and 45 HVS specimens from pregnant women were analysed using culture methods and PCR for *M. hominis* and *U. urealyticum*. Out of the 90 HVS specimens from the infertile women, 9 (10.0%) were positive for *M. hominis* while 21 (23.3%) were positive for *U. urealyticum*. The urine specimens from both the infertile and pregnant women gave lower prevalences of 3.3% for *M. hominis*, 6.7% for *U. urealyticum* and 0.0% for *M. hominis*, 4.4% for *U. urealyticum* respectively (Table 1). HVS specimens yielded more isolates than the urine specimens.

$X^2 = 72.174; P<0.05$.

Table 2 shows the distribution of *Mycoplasma/Ureaplasma* organisms in infertile and pregnant women in the study area using *Ureaplasma* using PCR. Out of the 45 HVS specimens from the pregnant women, 32 (71.1%) were *Mycoplasma/Ureaplasma*. The 78 analysed urine specimens from the infertile women gave a prevalence of 58.9% of *Mycoplasma/Ureaplasma* infections. For the pregnant women, out of the screened 45 urine specimens 12 (26.6%) were positive for *Mycoplasma/Ureaplasma*.

The antibiotic susceptibility patterns of the *M. hominis* and *U. urealyticum* is shown in Table 3. *M. hominis* showed 100% sensitivity to Tetracycline and Ciprofloxacin while *U. urealyticum* showed 100% sensitivity to Tetracycline and Erythromycin.

Table 1: Distribution of *M. hominis* and *U. urealyticum* in Infertile (n=90) and Pregnant (n=45) Women in Lagos, Nigeria.

Source	Organism	No. of Isolates	Percentage
Infertile Women			
HVS	*M. hominis*	9	10.0
	U. urealyticum	21	23.3
Urine	*M. hominis*	3	3.30
	U. urealyticum	6	6.70
Pregnant Women			
HVS	*M. hominis*	6	13.30
	U. urealyticum	5	11.10
Urine	*M. hominis*	0	0.00
	U. urealyticum	2	4.40

Table 2: Distribution of *Mycoplasma/Ureaplasma* Species in Infertile and Pregnant Women in Lagos, Nigeria using PCR

Source	Organism	No. of Isolates	Percentage
Infertile Women			
HVS (n=65)	*Mycoplasma/Ureaplasma*	51	78.5
Urine (n=78)	*Mycoplasma/Ureaplasma*	46	58.9
Pregnant Women			
HVS (n=45)	*Mycoplasma/Ureaplasma*	32	71.1
Urine (n=45)	*Mycoplasma/Ureaplasma*	12	26.6

Table 3: Antimicrobial Susceptibility Patterns of *M. hominis* and *U. urealyticum* Isolated from Infertile and Pregnant Women in Lagos, Nigeria.

Source	**Organism**	**No. of Isolates**	**Antimicrobial Agent Activities % Sensitive**				
			Te	CN	E	CPX	S
HVS	*M. hominis*	15	15(100)	0(0.0)	0(0.0)	15(100)	0(0.0)
	U. urealyticum	26	26(100)	0(0.0)	26(100)	0(0.0)	0(0.0)
Urine	*M. hominis*	3	3(100)	0(0.0)	0(0.0)	3(100)	0(0.0)
	U. urealyticum	8	8(100)	0(0.0)	8(100)	0(0.0)	0(0.0)
Total		52	52(100)	0(0.0)	34(65.4)	18(34.6)	0(0.0)

Key:

Te = Tetracycline (30µg)

CN=Gentamycin (10µg)

E=Erythromycin (15µg)

CPX=Ciprofloxacin (5µg)

S=Streptomycin (30µg)

DISCUSSION

The results of our study showed that *M. hominis* and *U. urealyticum* infections are prevalent among the infertile and pregnant women in Lagos Metropolis, Nigeria. In this study both the HVS and Urine specimens were examined using culture and PCR techniques. The culture technique gave a prevalence, using the HVS specimens, of *M. hominis* (10.0%) and 13.3% for infertile and pregnant women respectively. The urine specimens yielded lower values for *M. hominis* with a prevalence of 3.3% and 0.0% for infertile and pregnant women respectively. A prevalence of 6.7% for infertile women and 4.4% for pregnant women were recorded for *U. urealyticum* infection. The PCR technique expectedly gave higher prevalence of 78.5% and 71.1% using HVS specimens for *Mycoplasma/Ureaplasma* in infertile and pregnant women respectively. The urine specimens gave lower values of 58.9% and 26.6% for *Mycoplasma/Ureaplasma* infection in infertile and pregnant women respectively. The urine specimens gave lower values of 58.9% and 26.6% for *Mycoplasma/Ureaplasma* infection in infertile and pregnant women respectively. One possible explanation for the higher prevalences

of these infections in infertile women is hormonal disorders which can lead to reduced levels of immunity and increased bacterial colonization and survival in the vaginal epithelium[17].

In addition to the considerably high prevalence of *M. hominis* and *U. urealyticum* infections in our study, several published report revealed various prevalence rates. Agbakoba *et al*[17] reported a prevalence of 35.7% in women of reproductive age in Ibadan. The prevalence rate of *M. hominis* and *U. urealyticum* in HVS specimens ranged from 0 to 3.8%[18, 19]. The prevalence of *U. urealyticum* infection was reported from 20% in South Africa to 41.9% and 51.5% in Italy and Africa respectively[18]. In contrast, the prevalence was lower in some other studies[20, 21]. The varied prevalence rates all over the world may be due to the use of only culture methods or PCR. In addition there are many factors that will affect the culture results such as the use of transport medium, the duration between the collection of the sample and the inoculation, and the duration of incubation. The Nucleic Acid Amplification tests gave higher results due to its sensitivity and it is also a rapid test, although it is more expensive.

In our study, it was observed that *M. hominis* and *U. urealyticum* were detected more from HVS than urine specimens. This is in agreement with Taylor-Robinson[21] who reported that the numbers of isolates from urine specimens were usually 10 fold less than in swabs.

In vitro antibiotic susceptibility test showed that all the isolates of *M. hominis* and *U. urealyticum* were sensitive to Tetracycline (100%). *U. urealyticum* isolates in addition to Tetracycline were all sensitive to Erythromycin, while all *M. hominis* isolates were also sensitive to Ciprofloxacin.

In conclusion, we have indicated that *M. hominis* and *U. urealyticum* infections are prevalent in both the infertile and pregnant women in Lagos metropolis. Efforts, where feasible,

should be made to detect the presence of these organisms during the work-up of cases of infertility and in pregnancy to prevent the adverse outcomes. We therefore recommend the precise monitoring of fertile and infertile females for the presence of *M. hominis* and *U. urealyticum* and treatment of positive cases to prevent diseases and possibly infertility.

The small sample size, and the lower number of recruited pregnant women compared with the infertile womenare limiting factors in the present study. It is hoped that in future, a larger sample size will allow us to make definite conclusions.

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