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PREVALENCE AND ANTIMICROBIAL SUSCEPTIBILTY OF *MYCOPLASMA HOMINIS* AND *UREAPLASMA UREALYTICUM* IN PREGNANT AND NON-PREGNANT WOMEN IN PARTS OF ENUGU STATE, NIGERIA

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ABSTRACT

Mycoplasma hominis and *Ureaplasma urealyticum* are human pathogens considered as genital mycoplasmas, because infection often occurs through sexual contact. This study was conducted to determine the prevalence and antimicrobial resistance patterns of *M. hominis* and *U. urealyticum* from pregnant and non-pregnant women. A total of 614 high vaginal swab (HVS) samples collected from 300 pregnant and 314 non pregnant women in higher institutions were examined. *M. hominis* and *U. urealyticum* were isolated using mycoplasma agar, A7 agar, and urea-arginine LYO2 broth (while confirmation was done using IST2 kit). *M. hominis* and *U. urealyticum* were characterized and identified by standard methods including digitonin test, arginine, urea and glucose hydrolysis. Isolates were tested for susceptibility/resistance to different antibiotics using Mycoplasma IST2 kit. A total of 212 (35.4%) *M. hominis* and 377(61.4%) *U. urealyticum* isolates were recovered from the 614 HVS samples. Among the 300 pregnant women examined, *M. hominis* was recovered from 75(25%) samples while *U. urealyticum* was recovered from 175(58%) samples. However, actual infection, indicated by presence of $>10^4$ cells of the organism was recovered in 70(23.3%) and 170(46%) women for *M. hominis* and *U. urealyticum* respectively. For non-pregnant women, on the other, *M. hominis* and *U. urealyticum* were recovered from 142(45.2%) and 202(64.35%) of the women respectively. Concomitant infection with both organisms were recovered in 40(7%) of the pregnant women

and 70(11.4%) of non-pregnant women. Infection with both organisms was significantly ($p < 0.05$) higher in non-pregnant women than pregnant women. A higher proportion of the infected pregnant and non-pregnant women were asymptomatic. There was significant association between infection and symptoms such as offensive discharge, pruritis and pain during sex for both organisms. *M. hominis* and *U. urealyticum* isolates exhibited a high rate of sensitivity to erythromycin (96%) and ciprofloxacin (100%) while the most resisted antibiotics were josamycin (100%) and pristinamycin (100%). Some *M. hominis* and *U. urealyticum* isolates exhibited a high rate of resistance to most antibiotics tested; 14.2% of *M. hominis* were resistant to 7 antibiotics while 4.9% of *U. urealyticum* were resistant to 8 antibiotics tested. The results from this study show that there is high prevalence of *M. hominis* and *U. urealyticum* in the study area and that the two organisms are sensitive to available antibiotics.

KEYWORDS: *M. hominis* and *U. urealyticum.*

INTRODUCTION

M. hominis and *Ureaplasmas* are pathogens of humans commonly found as part of urogenital tract flora especially of women and sexually active adult males. They cause variety of infections which may lead to pelvic inflammatory disease, post-partum fever and extragenital infections for immunodepressed humans.[1] They also cause meningitis, pneumonia and abcesses in newborn children. They live parasitically and saprophytically with hosts. *M. hominis* and *Ureaplasmas* play significant roles in the microflora of men and women. They are important due to the infections they cause and growing resistant to treatment, including erythromycin.[2] Mycoplasma lack peptidoglycan and are thus resistant to all cell wall active antibiotics such as beta-lactam and glycopeptides drugs. They are also resistant to rifampin, polymyxins, nalidixic acid, sulphonamides and trimethoprim.[3] The most common antimicrobials active against mycoplasmas are included in the three major drug classes: They are; tetracyclines, macrolides-lincosamides-streptogramins-ketolides (MLSK group), and fluoroquinolones. Some differences are observed according to the species, mainly for the macrolides-lincosamides-streptogramins-ketolides group, and acquired resistance has been described.[4] These antibiotics also gave high intracellular concentrations. This is of interest because several mycoplasma species such as *M. hominis* and *M. genitalicum* localize and survive within the cells.[10] Other classes of antimicrobials such as aminoglycosides and chloramphenicol sometimes demonstrate activity *in vitro.* However, due to their toxicity, they

are normally not considered as suitable antimicrobial agents in humans except for the occasional use of choramphenicol for the treatment of systemic infections in neonates caused by *M. hominis* or *U. urealyticum* spp.[5] No study concerning the *in vitro* effect of combinations of drugs against mycoplasmas has been reported.

M. hominis and *Ureaplasma* spp. are susceptible to tetracyclines but acquired resistance to tetracyclines has been reported.[5] The resistance is associated with the acquisition of the tet(M) protein. It codes for the tet(M) protein which protects the ribosomes from the action of tetracycline. It confers high-level resistance to all tetracyclines. Glycylcyclines such as tigecycline retain activity against *M. hominis* containing tet(M) but not *Ureaplasma* spp. The prevalence of acquired tetracycline resistance among *M. hominis* and *Ureaplasma* spp. varies according to the country and the antimicrobial exposure of the population.[6, 4] *Ureaplasma* spp. is susceptible to macrolide and related antibiotics except lincosamides. *M. hominis* is resistant to erythromycin and to all 14-membered macrolides (roxithromycin, clarithromycin, dirithromycin) and 15-membered macrolides (azithromycin), but sensitive to josamycin, a 16-membered macrolide. This intrinsic resistance is associated with a guanine to adenine transition at position 2057 (*Escherichia coli* numbering) in the domain V of 23S rRNA, the molecular targets of macrolides.[5] Only a very few cases of acquired resistance to MLSK have been reported for clinical isolates of *M. hominis* and *Ureaplasma* spp. The prevalence of acquired macrolide resistance is unknown but probably very low, except perhaps in China where recent reports suggest that macrolide resistance may be frequent, presumably as a result of selective pressure because of widespread macrolide use.[8] Ureaplasmas and *M. hominis* are intrinsically susceptible to fluoroquinolones; however, newer fluoroquinolones such as levofloxacin and moxifloxacin are more active in vitro against human mycoplasmas than older ones such as ofloxacin and ciprofloxacin. Mutations in the target genes *gyrA* and *gyrB* of DNA gyrase and *parC* of topoisomerase IV are the main mechanisms conferring fluoroquinolone resistance in human mycoplasmas and ureaplasmas.[9] Resistant clinical isolates of *M. hominis* and Ureaplasma spp. show cross-resistance to all fluoroquinolones. The level of resistance depends on the number and positions of the mutations. The newest fluoroquinolones like moxifloxacin remain most effective against the mutants although they lose their bactericidal activity *in vitro.* The prevalence of fluoroquinolone resistance in genital mycoplasmas is unknown but is certainly very low, estimated at less than 1% for *Ureaplasma* spp. in France in the 2000's. However, as these drugs have been used much more extensively over the past years, cases of significant infections caused by

fluoroquinolones-resistant *M. hominis* or *Ureaplasma* spp. are being reported, especially in persons who have previously received fluoroquinolones and those who are immunosuppressed.[11]

MATERIALS AND METHODS

Media and reagents: Laboratory media used during this study were from Oxoid, Uk and Bio Merieux, Marcy-l-Etiole, France.

Specimen collection: A total of 614 high vaginal swab (HVS) samples were collected from different hospitals. A total of 300 HVS samples were gotten from pregnant women aged 20-40, attending ante-natal clinics in Bishop Shanahan Hospital, Nsukka and Enugu State University of Science and Technology, Teaching Hospital Parklane, Enugu respectively. Also, 314 HVS samples were collected from non-pregnant students aged 17-27, in higher institutions namely; School of midwifery, Bishop Shanahan hospital Nsukka and Enugu University of Science and Technology, Enugu State, Nigeria. The study was carried out during a period of 12 months (from Feb, 2016 to Feb 2017). Also, 150 samples were collected from each hospital while 157samples were collected from each school. A questionnaire was completed for each woman, recording personal data and symptoms. It was stipulated that all women participating in the study should not have taken any antimicrobial agent prior to sampling so that the growth of mycoplasma should not be affected.The approval of the Ethics Committee of Enugu State University of Science and Technology was obtained. All women gave their consent prior to enrolling. Sterile cottons swab sticks were given to each of the individual for sample collection (HVS) after which it was placed directly into RI tubes (transport medium) and subsequently, they were taken to the clinical laboratory for analysis. Isolation of organisms on Mycoplasma agar: Methods of Whithear,[29] were employed to screen for Mycoplasma. A primary inoculation was made directly on Mycoplasma agar and spread over the reminder of the agar surface with an inoculating loop. Plates were incubated at 37^0C under humidified microaerophilic atmosphere in a candle jar to provide additional carbondioxide.

Mycoplasma colonies were purified by transferring micro-colonies to mycoplasma broth and incubated at 37^0C until turbidity was observed. Mycoplasma colonies were stored in mycoplasma agar slants at 40^0C for further use.

Isolation of organisms on urea-arginine LY02 broth: Vaginal swabs were inoculated into the Mycoplasma RI vial solution and 3ml transferred to Mycoplasma R2 vial, vortexed to dissolve completely and incubated aerobically at 36^0 C for 48.

Isolation of organism on A7 agar: From the R2 positive tube, 0.1ml was inoculated onto A7 Mycoplasma agar plates and incubated at 37^0C humidified microaerophilic atmosphere with a candle jar to provide additional CO_2 checking characteristic colony morphology. Results were interpreted after 24 and 48hours of incubation. Colonies presenting with a fried egg appearance suggest the presence of *M. hominis* whereas colonies of *U. urealyticum* appear tiny granular and dark due to accumulation of manganese oxide brown. Resultant *Ureaplasma* colonies were purified by removing a block of agar containing micro-colonies and transferred to R2 vial and incubated aerobically at 37^0C for 48h, *Ureaplasma* colonies were stored in A7 slants at 4^0 C for further use.

Identification Test: Resultant colonies on each medium were aspectically isolated and characterized using established microbiological methods which hydrolysis.

Antimicrobial susceptibility Testing: The antimicrobial susceptibility pattern of the *M. hominis* and *U. urealyticum* isolates were determined using the Mycoplasma IST2 kit (Biomeriux, France). The kit contains strips that give information on the presence or absence of *M. hominis* and *U. urealyticum* and also provide additional information on antibiotic susceptibility to doxycycline, josamycin, ofloxacin, erythromycin, tetracycline, ciprofloxacin, azithromycin, clarithromycin and pristinamycin. Swabs were inoculated into R1 transport vial and 3ml of R1 was used to rehydrate the lyophilized growth medium R2. A Mycoplasma IST2 strip, consisting of 22 wells, was then inoculated with the rehydrated R2 growth medium, 55 ul per well was overlaid with drops of mineral oil and incubated aerobically at 36^o C for 48h. Positive reading showed colour change from orange to red in each cupule. Negative reading indicated yellow colour in the test cupules. Wells 1-5 provide information on the presence or absence of *M. hominis* and *U. urealyticum,* with an estimate of the density of each organism ($>10^4$ CFU), and wells 6-22 show the antimicrobial susceptibilities at different concentrations to doxycycline (4 and 8 mg/l), josamycin (2 and 8 mg/l), ofloxacin (1 and 4 mg/l), erythromycin (1,4mg/ml), tetracycline (4,8 mg/ml), ciprofloxacin (1 and 2 mg/ml), azithromycin (0.12 and 4 mg/l), clarythromycin (1 and 4 mg/ml) pristinamycin (2mg/m). *M. hominis* ATCC 15488 and *U. urealyticum* ATCC 27813 strains were used as controls.

Statistical analysis: Frequencies were obtained and percentages were calculated for study variable. Chi-square was used to calculate and determine significance. A P- value of less than or equal to 0.05 was considered to be statistically significant.

RESULTS

Identification of *M. hominis* and *U. urealyticum*

The IST2 kit gave the same result as the LYO2 broth for both organisms; with 127 (35.4%) and 377(61.4%) showing positive for *M. hominis* and *U. urealyticum* respectively and also allowed differentiation between the two organism. A comparison of the different methods of identification is shown in Table 1. The IST kit and LYO2 broth were more sensitive in detection of organisms than culture. Colonies on *Mycoplasma* agar showed typical "fried egg" appearance, characteristic of *Mycoplasma* while on A7 agar appeared as tiny granular colonies, characteristic of *U. urealyticum* on that medium.

Table1: Comparison of detective abilities of Mycoplasma agar, A7 agar, LY02 broth and IST2 Kit.

Identification Technique	Number of Samples tested	Number of sample positive(%)		
		M. hominis only	*U. urealyticum* only	*M. homins* and *U. urealyticum*
Culture on Mycoplasma Agar	614	180(29%)	0(0%)	0(0%)
Culture on A7 Agar	614	0%	209(34%)	0(0%)
Culture on Urea-Aginine LY02 Broth	614	217(35.4%)	377(61.4%)	110(18%)
Test with IST2 Kit	614	217(35.4%)	377(61.4%)	110(18%)

Microscopic appearance and biochemical characterization of *M. hominis* and *U. urealyticum*

Both *M. hominis* and *U. urealyticum* appeared as Gram-ve, pleomorphic organisms and differed in their biochemical characteristics (Table 2).

Table 2: Microscopic and biochemical characteristics of the isolates.

Characteristics	*M. hominis*	*U. urealyticum*
Digitoin sensitivity	+	_
Arginine hydrolysis	+	_
Urea hydrolysis	_	+
Glucose hydrolysis	_	+
Gram reaction	_	_

+ = positive; - = negative

Prevalence of *M. hominis* and *U. urealyticum* in samples from pregnant and non-pregnant women

Out of 300 pregnant women examined, 75(25%) were positive for *Mycoplasma*, while 175(58%) had *Ureaplasma* and 40(7%) women had both *Mycoplasma* and *Ureaplasma.* Among the 314 non-pregnant women, 142(45.2%) had *Mycoplasma,* 202 (64.3%) had while 70(11.4%) had both organisms (fig i).

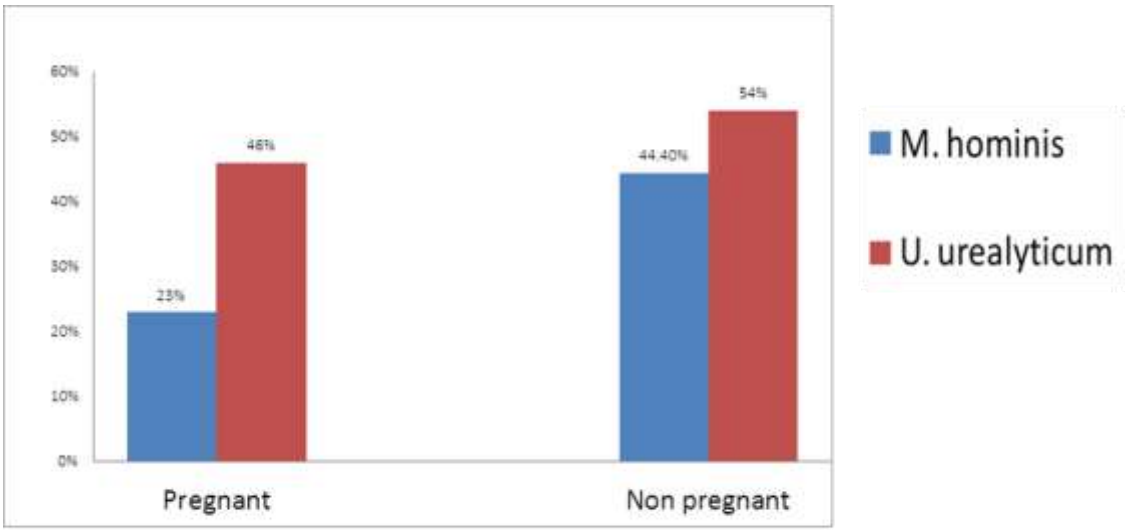

Fig i: Occurance of *M. hominis* and *U. urealyticum* in pregnant and non-pregnant women.

Prevalence of samples showing significance for infection for both organisms

For both *Mycoplasma* and *Ureaplasma*, growth > 10^4 cfu/ml is considered significant and indicative of infection. 370(60.2%) were positive for *U. urealyticum,* 210(34.2%) were positive for *M. hominis* while 110(18%) had significant growth for both organisms (Table 3).

Table 3: Number of sample showing significance for infection for both organisms.

Population	Number of Samples Examined	Number of sample positive(%) number with > 10^4CFU number > 10^4 for both				
		M.h	U.u	M.h	U.u	M.h/U.u
Pregnant women	300	75(25)	175(58)	70(23.3%)	170(46%)	40(7%)
non-pregnant women	314	412(45.2)*	202(64.3)	140(44.6%)*	200(54%)	70(11.4%
extal	614	217(35.4%)	377(61.4)	210(34.2)	370(60.2)	110(18%)

*Significant (P=0.0012) (M.h= *M. hominis*; U.u = *U. urealyticum*)

Prevalence of symptomatic and asymptomatic individuals in the two test populations.

Pre-experimental activity involved sharing of structured questionaires comprising signs and symptoms, information obtained from the respondents helped into grouping them into asymptomatic (those that did not observe symptoms) and symptomatic women (those who observed signs and symptoms). From the results a higher number of women were asymptomatic in the two test populations (fig.2).

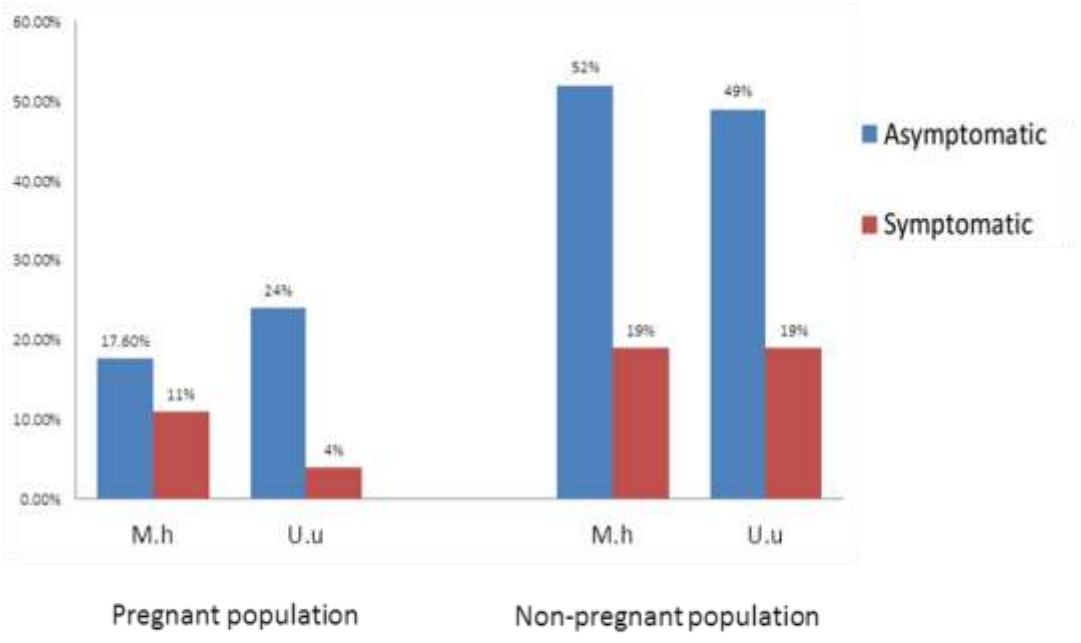

Fig ii: Occurrence of asymptomatic and symptomatic pregnant and non-pregnant population.

Prevalence of genital symptoms in women with genital mycoplasma

From the questionnaires, a total of 165 women comprising of 53 pregnant and non-pregnant women indicated having symptoms of vaginosis. Significant relationship at $p < 0.05$ (0.0012) was observed between presence of symptoms in pregnant and non-pregnant women (Table 4).

Table4: Prevalence of genital symptoms and clinical signs in women with genital mycoplasmas.

Criteria Symptoms	**No positive for symptomatic Population**	**pregnant women**	**Non-pregnant symptomatic women**	**M. hominis positive culture**	**U. urealyticum positive culture**
Offensive vaginal Discharge	110	30(57%)	80(73%)	64(30.4%)*	110(30%)*
Profuse vaginal Discharge	63	20(38%)	43(39%)	23(11%)	40(11%)
Pruritis	98	40(75%)	48(44%)	45(21.4%)*	55(15%)
Pain during sex	50	10(19%)	40(36.3%)	40(19%)*	50(14%)
Dysuria	45	12(23%)	33(30%)	20(10%)	25(7%)
Amsel's Criteria	**Total positive Population**	**Total pregnant population**	**Total non-pregnant women**	***M.hominis* positive culture**	***U. urealyticum* positive culture**
Homogeneous Discharge	480	200(67%)	280(89%)	198(94%)	282(76%)*
Positive amine test	570	270(90%)	300(96%)	200(93%)*	352(95%)*
pH> 4.5	550	245(82%)	305(97%)	198(94%)*	352(95%)*
clue cells	210	92(13%)	118(38%)	196(93.3%)	15(4%)

*Significant P<0.05 (0.0012)

Antimicromicrobial susceptibilities

The results of antibiotic susceptibilities of *U. urealyticum* and *M. hominis* from positive vaginal samples are shown in Figures 3, 4 and 5. From the results, it was observed that *M. hominis* was highly susceptible to many antibiotic tested, particularly erythromycin (96%) and ciprofloxacin (100%). Similar results were observed with *U. urealyticum.* However, resistance was observed for a few antibiotics, especially josamycin and pristinamycin; both with 100% resistance.

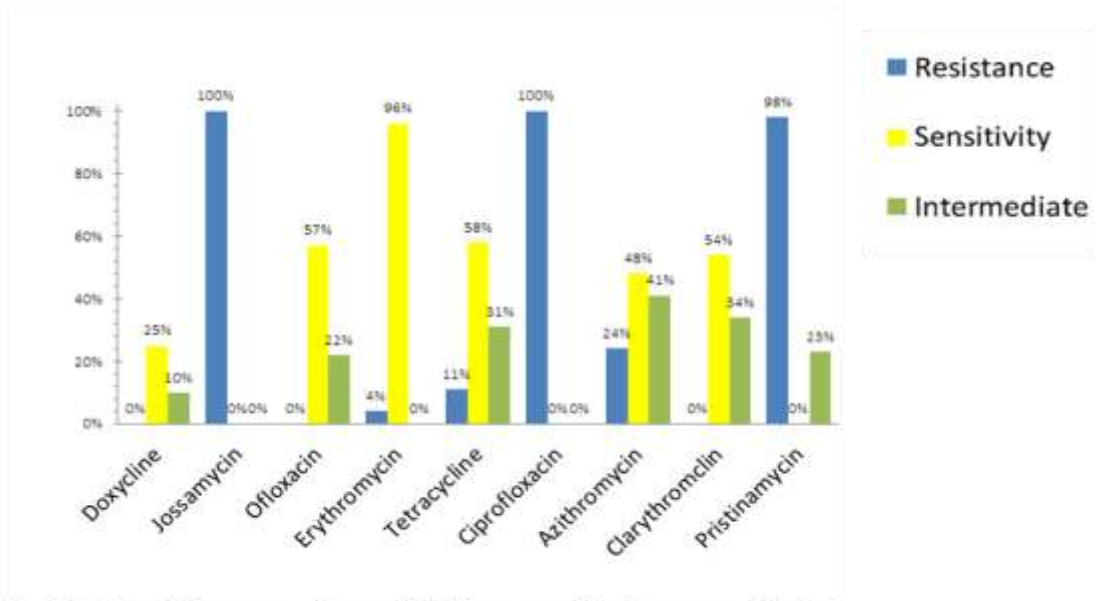

Fig iii: Resistance, Sensitivity and Intermediate reactions of *Mycoplasma hominis* to antimicrobial agents

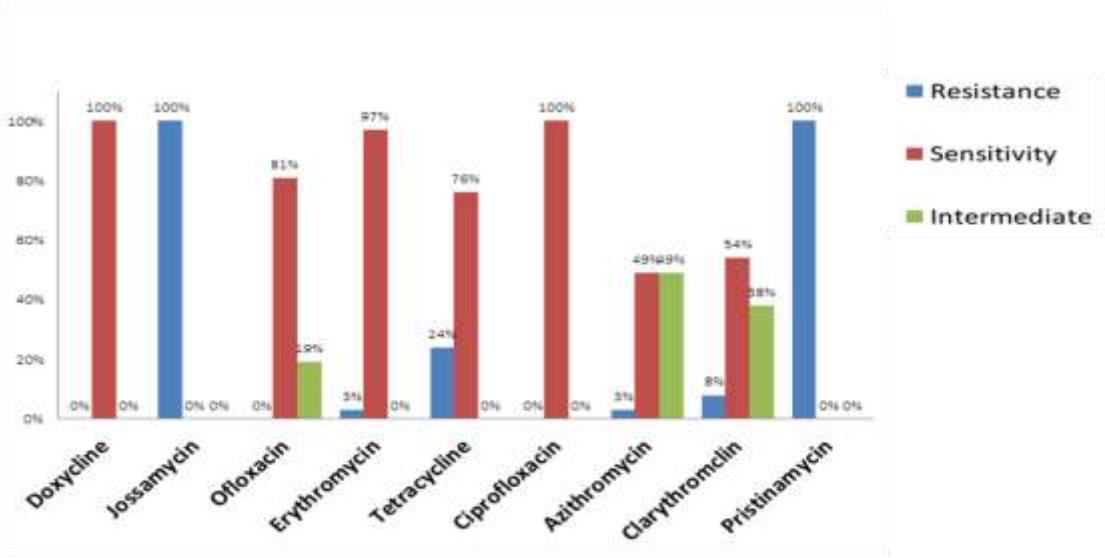

Fig iv: Resistance, Intermediate and Sensitivity reactions of *Ureaplasma urealyticum* to antimicrobial agents

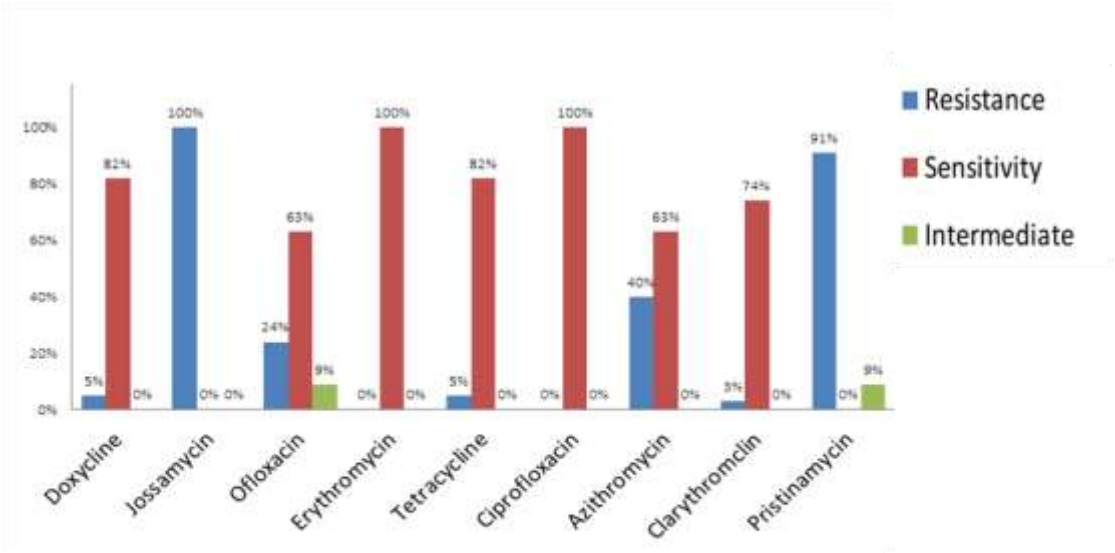

Fig v: Resistance, Intermediate and Sensitivity reactions of isolates with co-infection with *U. urealyticum* and *M. hominis* to antimicrobial agents

Prevalence of multiple-drug resistance among 201 *M. hominis* and 370 *U.urealyticum* isolates

The prevalence of multi-drug resistance in *M. hominis* and *U.urealyticum* isolates are shown in Table 5. Multi drug resistance was defined in the study as resistance to four or more of the antibiotics tested. Thus, 75 (36%) of the *M. hominis* isolates showed multidrug resistance to the antibiotics while 105(28.3%) *U. urealyticum* isolates showed multidrug resistance to the antibiotics.

Table 5: Prevalence of multidrug resistance among Mycoplasma isolates.

Types of Isolates	Resistant to 4 agents	Resistant to 5 agents	Resistant to 6 agents	Resistant to 7 agents	Resistant to 8 agents	Resistant to 9 agents
Mycoplasma hominis (n=210)	20 (9.5%)	20(9.5%	10(5%)	15(7.1%)	-	-
Ureaplasma urealyticum (n=370)	40(11%)	20 (5.4%)	15(7.1%)	12(3.2%)	18(4.9%)	-

DISCUSSION

In this study, various identification techniques were employed in the detection of these organisms. A combination of culture and commercially available diagnostic kits were used. From this study, 29% were positive with *Mycoplasma hominis* on mycoplasma agar. Culture

on urea-arginine LYO2 broth and IST2 kits had more sensitive results at 34% for *M. hominis* and 61.4% for *U. urealyticum* (Table 1). This result is in line with the works of Naher *et al*.,[30] who had more positive results in commercial kits than in culture. *Mycoplasma hominis* grew by binary fission and produced "fried egg" colonies on Mycoplasma agar plates. The present study revealed that 29% of the isolates showed "fried egg" colonies typical of *Mycoplasma hominis* and 34% isolates of *U. urealyticum* were granular, with extremely small colonies (T-strains) on A7 agar and are extracellular Gram-negative pleomorphic bacterium. This supports the work of Baczynska *et al*.[1] Strains of Mycoplasma and Ureaplasma required cholesterol for growth and membrane synthesis while Mycoplasma and Ureaplasma were differentiated by their requirement of arginine and urea for growth.[13] All the *M. hominis* isolates in the study hydrolysed arginine while U. urealyticum hydrolysed urea (Table 2). Among the 300 pregnant women examined, *M. hominis* was recovered from 75(25%) samples while *U. urealyticum* was recovered from 175(58%). However, $>10^4$ cells of the organisms were recovered from 70(23.3%) and 170(46%) women for *M. hominis* and *U. urealyticum*, respectively. Also, *M. hominis* and *U. urealyticum* were recovered from 142(45.2%) and 202(64.3%) of non pregnant women respectively. Concomitant infection with both organisms were recorded in 40(7%) of the pregnant women and 70(11.4%) (Table2). This is in agreement with the study of Bayraktar *et al*.,[26] who found genital mycoplasmas present in the vaginal mileu of pregnant and non-pregnant women. This study also is in agreement with the study of Zdrodowska *et al*,[14]; Kechagia *et al*,[15] who found 50% of women colonized with *U. urealyticum* and 30% with *M. hominis.* Prevalence of genitourinary infections due to *U. urealyticum* was considerably higher as compared to *M. hominis* infection in both pregnant and non-pregnant women. Infection with both organisms was significantly ($p<0.05$) higher in non-pregnant than pregnant women (Figure 1). Studies have shown that *U. urealyticum* are more commonly detected than *M. hominis* in both pregnant and non-pregnant women.[16, 17, 18] The results of the study showed prevalence of asymptomatic pregnant and non-pregnant women with *M. hominis* at 17.6% and 52% respectively and asymptomatic pregnant and non-pregnant women with *U. urealyticum* at 24% and 49% respectively. This is line with the study of Lila et al., (2000) who found *U. urealyticum* on the mucosal surfaces of the cervix or vagina of sexually active asymptomatic women at 30 to 45%, whereas *M. hominis* may occur in 21 to 53% (Fig 2). There was significant relationship between *U. urealyticum* and presence of signs and symptoms in pregnant and non-pregnant women at $p< 0.05(0.0012)$ (Table 4). This is in agreement with Lilia et al.,[19] who indicated that some of these signs and symptoms are indicators of non

specific vaginitis. This study also matched the results obtained by Thulas,[20]; Donder et al.,[21] who found that *M. hominis* and *U. urealyticum* are associated with a number of genital signs and symptoms. *U. urealyticum* strains were susceptible to ciprofloxacin and erythromycin (100% and 97%) and *M. hominis* were susceptible to ciprofloxacin and erythromycin (100% and 96%). This is in contrast with the study of Eunha *et al*.,[22] and Bayraktar *et al*.,[26] who reported that *M. hominis* and *U. urealyticum* strains were in-active to ciprofloxacin and erythromycin. In the present study *M. hominis* strains were susceptible to tetracycline and ofloxacin at (58% and 57%) and *U. urealyticum* strains were more susceptible to ofloxacin and tetracycline at 76% and 81% and this is in line with the works of Eunha *et al*.,[22] who reported similar susceptibility results at 81% and 88.6% respectively. *M. hominis* and *U. urealyticum* strains showed complete resistance to josamycin and pristinamycin at 100% and 98%; 100% and 100% respectively. This is not in line with the studies of Waites *et al*.,[24]; Eunha[22]; Bayraktar *et al*.,[26] who reported that *M. hominis* and *U. urealyticum* strains were completely susceptible to josamycin and pristinamycin (Fig 3,4 and 5). It is important to note that there are differences in susceptibility patterns among studies reported from different countries. In Enugu state, Nigeria this study shows that the level of resistance to josamycin, pristinamycin, azithromycin and clarithromycin are generally high, while the rate of susceptibility to fluoroquinolones (ciprofloxacin), erythromycin and tetracycline were high. In the study, mixed isolates of *M. hominis* and *U. urealyticum,* showed similar pattern of antimicrobial susceptibility to that observed in *U. urealyticum.* It was alarming to observe that 11% of *U. urealyticum* and 9.5% of *M. hominis* strains were completely resistant to eight of the antimicrobial agents tested (Table 5). The increasing resistance of *M. hominis* and *U. urealyticum* strains to antibiotics makes guidance of therapy by invitro susceptibility test of paramount importance. *M. hominis* and *U. urealyticum* strains resistance to multiple drugs can be attributed to in-appropriate prescription, self medication and indiscriminate use of antibiotics. The empirical treatment can be ineffective for these reasons. It is difficult to establish common guidelines for the empirical treatment of genital mycoplasma infection. In the present study, we determined the antimicrobial susceptibilities of genital mycoplasma in Enugu which is a specific geographic region, rather than Nigeria as a whole. Treatment of genital mycoplasma infections in Nigeria may require a nationwide survey to help establish new guidelines for treatment.

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