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Polymerase chain reaction assay of ureaplasma strains isolated from high vaginal swabs of women in Ibadan, Nigeria

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Summary

Human ureaplasma previously had one species known as *Ureaplasma urealyticum* but was recently separated into 2 species, *U. urealyticum* and *U. parvum*. This study was carried out to separate the ureaplasma strains isolated from women attending a tertiary-care hospital in Nigeria. Thirty (30) Ureaplasma strains isolated from the vaginal tracts of 13 pregnant and 17 non-pregnant women were assayed. The polymerase chain reaction (PCR) technique was employed using two primer pairs: UMS-170/UMA-263 specific for *U. urealyticum* and UMS-57/UMA-222 specific for *U. parvum*. The positivity bands of the primer pairs were 476bp and 326bp for *U. urealyticum* and *U. parvum* respectively. All isolates were found to be *U. urealyticum* (100%). Eleven (84.6%) of the 13 *U. urealyticum* from pregnant women were from asymptomatic women while from the non-pregnant women; 6(35.3%) were from women with complaint of infertility problems; 5(29.4%) from those who complained of vaginal discharge, one (5.9%) was asymptomatic while the remaining 5 (29.4%) had various other complaints. *U. urealyticum* is thus the prevalent species of Ureaplasma among pregnant and non-pregnant women in the study population and this to the best of our knowledge is a pioneer study to speciate human ureaplasmas in this country.

Keywords: *Ureaplasma urealyticum, women, polymerase chain reaction,*

Résumé.

L'urée dans le plasma humain avait une espèce connue comme l'*urée lycitum* plasmique mais récemment séparé en deux espèces *U. Ureelycitum et U. Parvum*. Cette étude était faite pour séparer les isolats d urée plasmique aux femmes visitant les centres tertiaires universitaires au nigeria. Trente espèces d urée plasmique des voies vaginales chez 13 femmes enceintes et 17 pas enceintes étaient analysées. La technique par chaîne de réaction polymérase étaient employé utilisant deux paires de primer -170 /UMA-263 spécifiques pour *U ureeliticam* et UMS-57/UMA-222 spécifiques pour U parvum. Les bandes positives sur les paires de primes étaient à 476 bp et 326 BP pour U Ureelyticum et *U parvum* respectivement. Tous les isolats étaient *U ureelyticum* (100%). Onze (84,6%) des 13 *U Ureelyticum* des femmes enceintes n avaient que des symptômes alors que celles pas enceintes 6(35%) étaient des femmes avec des problèmes d'infertilité. 5(29,4%) avaient des problèmes de décharges vaginales, 5,9% n avaient pas des symptômes pendant que 5(29,4%) avaient des plaintes diverse. *U Ureelyticum* est l'espèce la plus prévalente parmi les femmes enceinte et pas enceinte dans la population étudiée dans ce pays.

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Introduction

Ureaplasma species are cell wall-less prokaryotes belonging to the class mollicutes. They have been implicated in a variety of urogenital infections and complications in humans some of which include non-gonococcal urethritis in men [1,2], preterm delivery and adverse pregnancy outcomes [3], neonatal infections like chronic lung disease [4] and bacterial vaginosis along with *M. hominis* and anaerobes in women [5]. Human Ureaplasmas formerly consist of one species, *Ureaplasma urealyticum*, which comprises 14 serotypes (1 to 14) [6]. This species was for a long time separated into 2 genomic clusters or biovars – the parvo biovar with 4 serotypes (1, 3, 6 and 14) and the T960 biovar with 10 serotypes (2, 4, 5, 7-13) [7]. The separation of the biovars into species was recommended [8] and few years later

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the human ureaplasma was separated into 2 distinct species – the parvo biovar as *Ureaplasma parvum* and the T960 biovar as *Ureaplasma urealyticum* [9]. Separation of these species is only possible by molecular techniques such as the PCR [10].

Human Ureaplasmas have not been previously separated into their 2 species in Nigeria. However, the prevalence of Ureaplasma spp. from the vaginal tract of women of reproductive age in Nigeria had earlier been reported [11]. Earlier investigators in the country called the Ureaplasmas various names by which they were previously known and which include T-mycoplasmas [12], *Ureaplasma urealyticum* [13, 2] or simply *Ureaplasma* species [11, 14]. Ureaplasmas were previously identified biochemically by urea hydrolysis [15] and serologically by serotyping the isolates [6]. However, the problem of obtaining all the 14 antisera needed to serotype human ureaplasmas most often posed a hindrance to serotyping the isolates. Recently, molecular study has emerged as the best method of separating the human ureaplasmas. It is fast and directly identifies the organism to its specific species. The polymerase chain reaction technique was therefore used in this investigation to separate the Ureaplasma strains earlier isolated from the vaginal tracts of women in a previous study [11] in order to know the prevalent species from women in the study population.

Materials and methods

Samples

A total of 30 Ureaplasma strains isolated in our laboratory from the high vaginal swabs of 168 women (114 pregnant and 54 non-pregnant) attending gynaecology and antenatal clinics at the University College Hospital, Ibadan were used for this study. Sample selection was case controlled and bleeding/menstruating women were excluded. The isolation and identification methods were as reported in an earlier study [11] and isolates were kept as broth cultures in a frozen state before being thawed and used for this study.

Oligonucleotide primers

Two primer pairs UMS-170/UMA-263 specific for *U. urealyticum* and UMS-57/UMA-222 specific for *U. parvum* obtained from Sigma Genosys Pty Ltd, Australia were used. These primers pairs were based on a previously published sequence [16]. The base sequences of the primers in the 5' to 3' direction are as follows;

Name	Sequence (5'– 3')
UMS-170	GTA TTT GCA ATC TTT ATA TGT TTT CG
UMA-263	AAG TGA CCT TTT RTW* GTT GCG TTT TCT G
UMS-57	GAT TRM* YAA ATC TTA GTG TTC ATA TTT TTT AC
UMA-222	TGT AAG TGC AGC ATT AAA TTC AAT G

**Key to symbols: R = A+G; Y = C+T; W = A+T; M = A+C (Sigma Genosys)*

Re-constitution of primers

The primers came in freeze-dried forms and were reconstituted by adding 1.0 ml of sterile distilled water into each of the tubes to form the stock solution. This was diluted 1 in 4 to give the working solution.

DNA preparation

DNA was extracted from the samples in a step-wise procedure using Qiagen reagents as recommended by the manufacturer (Qiagen, USA) as follows; The Qiagen protease (20ul) was added into each of the Ureaplasma broth culture (200ul) after an initial buffer (AL) was added. The tubes were incubated in a water bath for 10minutes and briefly centrifuged for 1 minute. A 200ul of ethanol was added to the sample filtrate and the mixture centrifuged for 1 minute. The filtrate was discarded and 500ul of buffer AW1 was added and again centrifuged for 1 minute after which 500ul of buffer AW2 was added to the supernatant and centrifuged at full speed for 3 minutes. Into the filtrate, 60ul of buffer AE was added and the mixture was incubated at room temperature for 1 minute and centrifuged again for 1 minute after which the supernatant was discarded and the filtrate is the extracted DNA.

PCR

The PCR mixture comprises 2.5 µl of 10X PCR buffer, 1.5 µl of 50mM $MgCl_2$, 0.5 µl of 10mM dNTPs, 0.1µl of 5m/µl of Taq platinum Polymerase (Invitrogen, Germany), 1.0µl of each primer and 6µl of sample DNA. Positive and negative controls were processed alongside the test samples. 25µl of each of the reaction mixtures were put in 1.5ml micro centrifuge tubes and transferred to a thermal cycler (Eppendorf, Germany). The thermal profile includes an initial denaturation step at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 1 minute. This was done for 35 cycles and was followed by a final extension at 72°C for 5 minutes. Five (5) µl of PCR products of each of the

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samples were analyzed by electrophoresis on 2.0% agarose gel stained with 1.5μl of ethidium bromide. The electrophoresis was run at 120mV for 20 minutes. Expected bands for positivity were 476bp (base pair) for *U. urealyticum* and 326bp for *U. parvum*.

Results

The electrophoresis analyses of the products of some of the Ureaplasma species is shown in Figure 1. All samples showed their bands at the 476bp and corresponded with the positive control used. 476bp is the expected band for *Ureaplasma urealyticum* thus showing that all the samples were *U. urealyticum*. There were no bands at 326bp in any of the samples thus ruling out the presence of *U. parvum* either alone or together with *U. urealyticum*. Apart from the band at 476bp, one or 2 non-specific bands were also seen along the lanes in some of the samples but these were all higher than the expected Ureaplasma base pair bands and were thus disregarded.

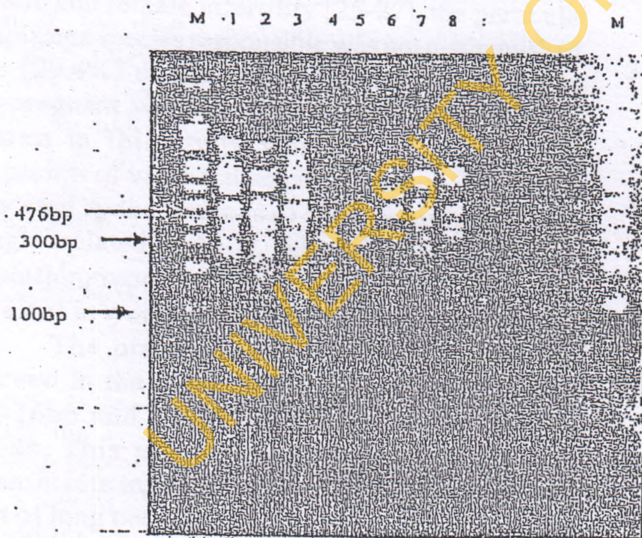

Fig. 1: Result of PCR diagnostic test for Ureaplasma strains from some of the high vaginal swabs.

Expected band for positivity is 476bp for Ureaplasma urealyticum and 326bp for ureaplasma parvum.
M =marker lanes. Samples in lanes 1,2,3 and 6 are positive for Ureaplasma urealyticum. Lane 4 contains a sterile broth, lane 5 is the negative control (N), lane 7 is the positive control and lane 8 is a mycoplasma isolate. The remaining wells were blank. There were no bands at 326bp.

The *Ureaplasma urealyticum* isolated from the pregnant women were predominantly from the asymptomatic women (84.6%) while those from the non-pregnant women were more from women with infertility problems (35.3%), followed by those from women with vaginal discharge (29.4%) and least with the asymptomatic women (5.9%) (Table 1).

Table 1: Distribution of the 30 *Ureaplasma urealyticum* according to patients' complaints

Orgnism	Source	Complaints (No. %)				Total
		Infertility	Vaginal discharge	Others	Nil	
Ureaplasma urealyticum	PW	0 (0)	1(7.7)	1(7.7)	11(84.6)	13
	NPW	6 (35.3)	5(29.4)	5(29.4)	1(5.9)	17

Key:
PW *Pregnant women*
NPW *Non pregnant women*

Discussion

Prior to the advent of molecular studies, the study of human ureaplasmas as etiologic agents of various human diseases had been marked with much controversies and even discord among researchers themselves. The reasons given for the differential pathogenicity of Ureaplasma species include; un-standardized methods; imprecise methods to distinguish among serotypes and biovars; serological reagents not widely available; antigenic variability and host factors complication [17]. Urea hydrolysis was the unique identification characteristic of the organism from culture. This was not enough as it cannot separate the organism into its 2 known species and cannot identify the species from non-viable or contaminated samples. Waites [17] stated that PCR and other molecular-based methods have now led to renewed interest in human ureaplasma research and provide more supportive evidence.

In this investigation all ureaplasma isolates were identified by PCR as *U. urealyticum*. No *U. parvum* was identified. This then gave a prevalence rate of 100% for *Ureaplasma urealyticum* and 0% for *Ureaplasma parvum*. Also using molecular techniques, investigations conducted in different parts of the world showed variable results. From Sweden, Povlsen *et al* [18] working on males with non-gonococcal urethritis reported *U. urealyticum* to be more common (60%) than *U. parvum* (37%). Similarly in Japan, Deguchi *et al.* [19] reported *U. urealyticum* to be more common (15.8%) than *U. parvum* (8.5%) from patients with non-gonococcal

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urethritis. In the United States of America, Ondondo *et al.* [20] also associated *U. urealyticum* with non-gonococcal urethritis. Contrarily though, Gupta *et al.* [21] isolated more *Ureaplasma parvum* (58.0%) than *U. urealyticum* (42.0%) from the genital discharge of male and female patients attending sexually transmitted diseases (STD) clinics.

The high prevalence rate of *U. urealyticum* obtained in this study could simply mean that it was the prevalent species among women in the environment. The prevalence of this organism from asymptomatic pregnant women was high as against it's prevalence in symptomatic women. This could be due to the fact that most of these women were routine antenatal clinic attendees and so had no complaints. This organism however had been reported as being part of the normal flora in the vaginal tracts of some women [22]. Furthermore, few pregnant women with complaints were used for this study thus necessitating the low rate of organisms recovered from them. From the non-pregnant women however, more *U. urealyticum* (35.3%) was recovered from women with infertility problems. Ureaplasma species had previously been associated with female infertility [23]. Similarly, *U. urealyticum* was isolated from the endocervical specimens of infertile women in Iran [24]. Though there had been previous studies on the roles of Ureaplasma species in male and female infertility [25-27], the particular ureaplasma species responsible was not distinguished. Five (29.4%) of the assayed *U. urealyticum* from non-pregnant women and 1 (7.7%) from pregnant women in this study were from women with complaints of vaginal discharge. This observation is supported by the findings of other investigators who though isolated more *U. parvum* from their study population, reported that *U. urealyticum* was more prevalent in women with vaginal discharge [28].

The presence of non-specific bands was observed in the PCR results. They were all above the 476bp and 326bp marks for the ureaplasma species. This probably could have been due to contaminants in the ureaplasma broth cultures as a result of long period of storage. The organisms were no longer viable at the time this PCR was done and so could not be sub-cultured for purity. It however goes to show the effectiveness of the PCR technique whereby the specific primers seek out the DNA of the specific organism (whether dead or contaminated) and multiply it to a large enough quantity to be identified. Non-specific bands were also shown by investigators who reported that non-specific nucleic-acid amplification may occur during polymerase chain reaction or other nucleic-acid amplification assays if the primers hybridize to an alternative site or bind with one another and form primer-dimers [29].

This study therefore has been able to show that *U. urealyticum* is the prevalent species of Ureaplasma among pregnant and non-pregnant women in the study population. This finding to the best of our knowledge marks the first attempt to separate human Ureaplasma strains in this country; thus leading the way for further studies which may involve the coverage of a larger population and different specimens.

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References

1. Horner P, Thomas B, Gilroy CB, Egger M and Taylor-Robinson D. Role of *Mycoplasma genitalium* and *Ureaplasma urealyticum* in Acute and Chronic Nongonococcal Urethritis. Clin. Infect. Dis. 2001; 32: 995-1003.
2. Bakare RA, Oni AA, Umar US, Kehinde AO, Fayemiwo SA and Fashina NA. *Ureaplasma urealyticum* as a cause of non-gonococcal urethritis: the Ibadan experience. Nig. Postgraduate Med J. 2002; 9: 140-145.
3. Kundsin RB, Leviton A, Alfred EN and Poulin SA. *Ureaplasma urealyticum* infection of the placenta in pregnancies that ended prematurely. Obstet. Gynaecol.1996; 87:122-127.
4. Waites KB, Crouse DT and Cassel GH. Therapeutic considerations for *Ureaplasma urealyticum* infections in neonates. Clin. Infect. Dis. 1993; 17 (supplement 1) S208 - S214.
5. Mazzuli T, Simor AE and Low DE. Reproducibility of interpretation of Gramstained vaginal smears for the diagnosis of bacterial vaginosis. J. Clin. Microbiol. 1990; 28: 1506-1508.
6. Taylor-Robinson D and Gourlay R N. The Mycoplasmas. Genus II. Ureaplasma. In: Krieg N R and Holt J G Eds, Bergeys Manual of Systematic Bacteriology. Vol. 1 William and Wilkins, Baltimore. 1984; 770 – 775.
7. Kong F, Zhu X, Wang W, Zhou X, Gordon S and Gilbert GL. Comparative analysis and serovar-specific identification of multiple-banded antigen genes of *Ureaplasma urealyticum* biovar I. J. Clin. Microbiol. 1999a; 37: 538:543.

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8. Kong F, James G, Zhenfang M, Gordon S, Wang N and Gilbert GL. Phylogenetic analysis of *Ureaplasma urealyticum* – support for the establisbment of a new species, *Ureaplasma parvum*. Int. J. Syst.Bacteriol. 1999b; 49: 1879-1889.
9. Robertson J A, Stemke G W, Davis J W Jr, Harasawa R, Thirkell D, Kong F, Shepard MC and Ford DK. Proposal of *Ureaplasma parvum* sp. nov. and emended describtion of *Ureaplasma urealyticum* (Shepard *et al.1974)* Robertson *et al.* 2001. Int. J. Syst. Evol.Microbiol 2002; 52: 587-597.
10. Waites KB.Ureaplasma infection. Retrieved January.8, 2002 from.http:spinbox.emedcine.com/
11. Agbakoba NR, Adetosoye AI and Adewole IF. The Presence of Mycoplasma and Urea plasma species in the vagina of women of reproductive age. West Afr. J. Med. 2007a; 26: 28 - 31.
12. Ladipo OM and Osoba AO. *Ureaplasma urealyticum* (T-mycoplasma) and male infertility in tropical countries. Afr. J. Med. med Sci. 1978; 7:187-190.
13. Eghafona NO, Isibor JO and Aluyi HAS. Non-gonococcal urethritis: Isolation of Mycoplasma species in adults in Benin City, Nigeria. J Med Lab Sc 1994; 4: 55-59.
14. Agbakoba NR, Adetosoye AI, Alaka OO and Adewole IF. Colonization of mouse reproductive organs following vaginal inoculation with human ureaplasma: Effect on fertility. World J. Med. Sci. 2006; 1: 140-143.
15. Purcell RH, Taylor-Robinson D, Wong D and Chanock RM. Color test for the measurement of antibody to T-strain mycoplasmas. J. Bacteriol. 1966; 92: 6-12.
16. Kong F, Ma Z, James G, Gordon S and Gilbert G. Species identification and subtyping of *Ureaplasma parvum* and *Ureaplasma urealyticum* using PCR- based assays. J. Clin. Microbiol. 2000; 38:1175-1179.
17. Waites K. B. Adventures with Urea plasmas and their search for human disease: (A Peter Hannan Award Lecture).Paper presented at the 16th International Organization for mycoplasmology Congress, . Cambridge, UK. 9th – 14th July, 2006.
18. Povlsen K, Bjornelius E, Lidbrink P and Lind I. Relationship of *Ureaplasma urealyticum* Biovar 2 to non-gonococcal urethritis. European Journal of Clinical Microbiology and Infectious Diseases 2002; 21: 97-101.
19. Deguchi T, Gilroy CB, and Taylor-Robinson D. Failure to detect *Mycoplasma fermemtans*, *Mycoplasma penetrans* or *Mycoplasma pirum* in the urethra of patients with acute non-gonococcal urethritis. Eur. J. Clin. Microbiol. Infect. Dis. 1996; 15: 169-171.
20. Ondondo RO, Whittington WLH, Astete SG. and Totten PA. *Ureaplasma urealyticum* (Biovar 2) is associated with acute non-gono coccal urethritis. Paper presented at the 16th International Organization for Mycoplasmology Congress, Cambridge, UK. 9th – 14th July, 2006.
21. Gupta V, Dhawan B, Khanna N and Chaudhry R. Detection and Biovar Discrimination of *Ureaplasma urealyticum* in Indian adults with symptoms of genital discharge. Paper presented at the 16th International Organization for Myco plasmology Congress, Cambridge, UK. 9th – 14th July, 2006.
22. Cheesbrough M. District Laboratory Practice in Tropical Countries Part 2. Cambridge University Press. 2000.
23. Horne H W, Hertig A T, Kundsin R B and Kosasa T S. Subclinical endometrial inflammation and T-mycoplasma; a possible cause of human reproductive failure. Int. J. Fert. 1973. 18: 226-231.
24. Peerayeh SN and Mirdamadi R. Comparison of culture with polymerase chain reaction for detection of *Ureaplasma urealyticum* in endocervical specimens. Medical Journal of the Islamic Republic of Iran 2005; 19: 175-179.
25. Upadhyaya M, Hibbard BM and Walker SM. The role of mycoplasmas in reproduction. Fertil. and Steril. 1983; 39: 814 - 818.
26. Levy R R, Layani-Milon M P, Giscard D S, Najioullah F, Lornage J, Aymard M, and Lina B. Screening for *Cylamydia trachomatis* and *Ureaplasma urealyticum* infection in semen from asyimptomatic male partners of infertile couples prior to in vitro fertilization. Int.J.Androl. 1999; 22:113-118.
27. Agbakoba NR, Adetosoye AI and Ikechebelu JI. Genital mycoplasmas in semen samples of males attending a tertiary care hospital in Nigeria: any role in sperm count reduction? Niger. J. Clin. Pract. 2007b (In Press).
28. Rar VA, Makimova TG, Trukhina AV, Rempel EG, Klimashevskaia NI, Riabova EN, Frolosva

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NP and Morozova OV. Level of colonization by *Ureaplasma urealyticum* of definite biovars in a group of women with different clinical symptoms. Zh. Mikrobiol. Epidermiol. Immunobiol 2004; 4: 12 – 17.

29. Winn CW, Koneman WE, Allen SD, Janda WM, Procop GW, Schreckenberger PC and Woods, GA. Konemans color Atlas and Textbook of Diagnostic Microbiology 6th Edition. Lappincott Williams & Wilkins, Philadelphia. 2006. 1023-1054.

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