ORIGINAL ARTICLE

MOLECULAR CHARACTERIZATION OF UREAPLASMA SP. ISOLATED FROM PATIENTS WITH SEXUALLY TRANSMITTED INFECTIONS IN SELECTED CENTERS NIGERIA

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ABSTRACT

Background: Urea plasma species, the main human ureolytic pathogens, usually implicated in opportunistic infections. This study was aimed at comparing the sensitivity of the culture method and PCR in identifying Urea plasma species; and to evaluate the sensitivity of PCR in distinguishing Urea plasma urealyticum and Urea plasma parvum in clinical samples using a newly designed primer from our laboratory.

Methods: Subjects included were both male and female patients attending STD reference centers (Lagos University Teaching Hospital and Jos University Teaching Hospital consisting of both male and female. A total of 119 patients (32 male, 87 female) who have been infertile for at least two years or those that present manifestations or reports of infertility, urethritis, spontaneous abortion, pelvic inflammatory diseases, preterm, low birth weight and neonatal pneumonia were included in this study. For the control, 117 asymptomatic male and female (w/o history or clinical symptoms of infertility) were recruited.

Results: The PCR assay showed more sensitivity and specificity in the identification of Urea plasma species than culture. Of the 119 patients, 26 (21.85%) were positive for U. urealyticum, while 49 (41.18%) were positive for U. parvum and 19 (15.97%) for both organisms. Of the 117 control specimens, 15 (12.82%) were positive for U. urealyticum, while 20 (17.09%) for U. parvum and 3 (2.56%) for both organisms.

Conclusion: PCR assay showed more sensitivity and specificity in the identification of Urea plasma species than culture Application of modern molecular techniques should be encouraged for the study of Urea plasma sp and other related fastidious organisms in Nigeria.

KEYWORDS: Urea plasma, culture, mycoplasma, molecular characterization, PCR

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INTRODUCTION

Urea plasma species are very important fastidious human pathogens which are implicated in some opportunistic infections.[1] A major virulence factor of these species is that they lack a cell wall; this characteristic feature confers resistance to many antibiotics against these organisms. In humans, their habitat is usually in the urogenital and respiratory tracts where they live as commensals.[2] During infections, they are mostly confined to their regions of entry however; they may invade the bloodstream and

cause systemic infections in immunocompromised subjects. The route of transmission of Urea plasma species include venereal (sexually transmitted), vertical (mother to child) or nosocomial.[3] Acute urethritis, preterm birth, bacterial vaginosis and lower respiratory tract infections have been reported as clinical manifestations of Urea plasma species.[4,5] The most frequently isolated biovar in human clinical samples is Urea plasma parvum.[6] Besides requiring special skills, the cultural method are also time-consuming, which may be dangerous in fatal cases. Due to this reason, molecular assays have been chosen as an accurate method for the detection of Urea plasma species.

Sexually transmitted infections are among the most common infections globally. Transmission of STIs is through direct contact with the infected person (genital- genital or oral- genital).[5] improperly managed sexually transmitted infections may lead to more severe complications such as infertility. Urea plasma species have been identified to cause major complications in the genitourinary tract. In pregnant females, it causes miscarriages, stillbirth and fetal defects.[7, 8] Being the most common etiological agents of human urogenital infections it causes non-gonococcal urethritis in men. Statistics have revealed that Urea plasma sp. has a possible prevalence of 40% and 67% in sexually active and non-active women, respectively.[9] According to WHO,[10] 1 million STIs globally are acquired daily; the surveillance and monitoring of STIs is therefore, highly necessary. For accurate and time effective surveillance which can help generate epidemiological data, molecular based identification and characterization is a preferable and more reliable technique. More so, in order to have clear information on the epidemiology of the different species of Urea plasma, there is a need to adopt the use of molecular techniques in the identification of this organism.[11] Real - time PCR assays have been reported to show higher specificity and sensitivity in identifying and distinguishing the biovars.

Based on this background, we compared the sensitivity of the culture method and PCR in identifying Urea plasma species. Then we evaluated the sensitivity of PCR in distinguishing Urea plasma urealyticum and Urea plasma parvum in clinical samples using a newly designed primer from our laboratory.

METHODS

Study population: Subjects used in this study include patients attending STD reference centers (Lagos University Teaching Hospital and Jos University Teaching Hospital) consisting of both male and female. Sample size was determined using Fisher's formula for cross section study. A total of 119 patients (32 male, 87 female) who have been infertile for at least two years and those that present manifestations or reports of infertility, urethritis, spontaneous abortion, pelvic inflammatory diseases, preterm, low birth weight and neonatal pneumonia were surveyed for a six month period. For the control, 117 asymptomatic male and female (i.e. no history or clinical symptoms of infertility) were used.

Urea plasma culture: Samples were inoculated in U9 broth and incubated at 35˚C for five days. During the incubation period, the broths were monitored four times daily to observe for color change. When a change of color was noticed, 100 µL of the inoculated broth was sub cultured on A7 agar. The A7 agar plates were incubated anaerobically for 48 hours at 35˚C. Using an inverted light microscope, the colonial morphology was observed. Confirmation of the species was indicated by golden-brown stained colonies with the addition of 0.167 MCO $(NH_2)_2$ and 0.04 $MnCl_2$. Urea plasma sp. positive broth suspensions (lyophilized) were used for the assay. Lyophilized U. urealyticum ATCC 27814 (serotype2) and U. parvum ATCC 700970 (serotype3) purchased from ATCC, Bethesda, MD were used as human positive culture.

DNA extraction: DNA extraction was performed using Sigma Gen Elute Genomic DNA kit. The kit contains reagents needed to isolate and purify genomic DNA from lyophilized mycoplasma cultures, including a pre-prepared 2.115 x 106 stock solution of Lysozyme (L7651) utilized to effectively lyse peptidoglycan walls and release sufficient genomic DNA required for PCR assays. Urea plasma urealyticum and Urea plasma parvum ATCC strains ordered were reconstituted inside laminar flow environment with 1.5ml of 1X PBS each. The 236 specimens from tests and controls were similarly reconstituted using 1.5ml of 1X PBS, vortexed briefly, and incubated for 10 minutes at room temperature. Then, PBS treated samples were frozen at -20 ˚C overnight.

Harvesting of cells: samples were thawed at 37˚C and centrifuged at 14,000rpm for 2 minutes. Supernatant was discarded, and then pellets were re-suspended thoroughly with 200ul of pre-prepared lysozyme. This was followed by incubation at 37 ˚C for 30 minutes. The cells were lysed by treating the sample with 20ul of the proteinase K solution, followed by 200ul of Lysis C solution (B8803), the mixture was thoroughly vortexed for 15 seconds and incubated at 55 ˚C for 10 minutes. 500 µl of the Column Preparation Solution was added to each of the pre-assembled GenElute Miniprep Binding Column seated in a 2 ml collection tube. The set up was spun at 12,000 xg for 1 minute and the eluate was discarded. The lysates were treated with 200 µl of 100% ethanol and thoroughly vortexed for 10 minutes for complete homogenous mixture. Using a wide bore pipette tip, the entire tube content was transferred into the binding column and centrifuged at 6500 xg for 1 minute. The collection tubes containing the eluate were discarded and the

column placed in a new 2ml collection tube. Initial washing was carried out to remove the contaminants by adding 500 µl of Wash solution (W0263) to the column and centrifuged at 6500 xg for 1 minute. Second washing was carried out by adding 500 µl of Wash solution (W0263) to the column and centrifuged at a maximum speed of 16,000 xg for 3 minutes.

The DNA was eluted by pipetting 200 µl of the Elution solution (B6803) directly onto the center of the column, centrifuged for 1 minute at 6,500 xg to elute the DNA. The eluate contains pure genomic DNA stored at -20 ˚C.

Polymerase Chain Reaction: Choosing of primers: Various pairs of primers and turn down cycling program (TD) were tested for their species sensitivity and specificity, prior to the actual experiments, in an attempt to establish primers and program to be used on the samples at large. Published oligonucleotide primers (Kong et al., 2000) - U8-UUA for U. urealyticum and UPS1-UPA for U. parvum were used (Table 1). We also designed and used new primers: UU16f-UU16r for U. urealyticum and UP16f-UP16r for U. parvum.

Table 1: Primers used in the assay

Oligonucleotide	Size	Length DNA Bases	Source		Nucleotide Sequence (5' -3')
U8	650	24	Uu rRNA	16S	GAAGATGTAGAAAGTCGGGTTTGC
UUA	650	22	Uu rRNA	16S	CTACAACACCGACTCGTTCGAG
UUS2	400	22	Uu gene	urease	CAGGATCATCAAATCAATTCAC
UUA2	400	21	Uu gene	urease	CATAATGTTCCCCTTCGTCTA
UPS1	650	34	Uu rRNA	16S	ATGAGAAGATGTAGAAAGTCGCTC
UPA	650	23	Uu rRNA	16S	TTAGCTACAACACCGACCCATIC
UPS2	400	22	Up gene	urease	CAGGATCATCAAGTCAATITAG
UPA2	400	23	Up gene	urease	AACATAATGTTCCCCTTTTTTATC
UU16f	350	22	Intergenic Spacer Region		AGAGATGTAGAAAGTTGCGTCGCGTTTGC
UU16r	400	24	Intergenic Spacer Region		TCACAACACCGACTCGTTCGAG
UP16f	350	24	Intergenic Spacer Region		ACGGATCATCAAATCAATTCACCA
UP16r	400	24	Intergenic spacer		ACTAATGTTCCCCTTCGTCTAGCT

PCR reactions were performed with an automated thermal cycler (Ependorff, USA). Amplification was performed in 50 µl of reaction mixture containing 10 µl of 10 x PCR buffer. PCR reaction mixture was set up in PCR tubes for each specimen as follows: The 50- µL sample to be analyzed was adjusted to a total volume of 100 µL in 1 x PCR buffer containing final concentrations of 125 µM each dATP dCTP, dTTP and dGTP; 0.2 µM each primer; and 1 U of Taq polymerase per 100 µL. Each sample was overlaid with 4 drops of mineral oil to prevent evaporation. Samples were denatured at 95 ˚C for 1 minute, and primers were annealed at 65 ˚C for 1 minute and extended at 72 ˚C for 1 minute. A total of 35 cycles were performed. In the 35th cycle, the extension time was increased to 6 minutes. 1.5% agarose in 1x TAE buffer was prepared each round. The mixture was microwaved for 2 minutes without covering until agarose melt. Two combs were inserted, gel was poured and it was allowed to set. Tank was removed with gel and placed in running tank ensuring the wells were well positioned to run from negative to positive pole. 1x TAE buffer was added to serve as the running buffer. When it was ready to be loaded, samples were inactivated at 65 C for 10minutes. 4 µL gel was taken out loading buffer into 6 spots on Para film. 15 µL of each sample was added and mixed 3 times. 4ul of QX 174 DNA Hae III Digest was loaded and visualized by Ethidium Bromide staining marker (Sigma life Science Research SYBR Grade 1 nucleic acid gel strain 2 µL/1000 µL, 1500 µL of the gel loading buffer). It was run at 80V for about 1hour 15 minutes. Polymerase Chain Reaction (PCR) products were separated on a 1.5% Ethidium Bromide-stained agarose gel electrophoresis. Deoxyribonucleic Acid (DNA) bands were first visualized with an ultraviolet illuminator, in order to check the migration patterns of the DNA bands. Consequently, photography of the DNA bands was taken using a photographic computer programme. The migration patterns of amplifiers were compared with those of the positive controls (standard) of known molecular weight.

Statistical analysis

Graphs and stables were used to compute results, while statistical analysis was carried out using Statistical Package for the Social Sciences (IBM, USA. SPSS version 21). The level of significance was set at p value <0.05.

RESULTS

The culture technique detected n= 44 (36.97%) while the PCR assay showed positive results for 55.45% (n= 66) in the patients while n= 18 (15.39%) versus n= 32 (27. 35%) of the control samples were detected by culture and PCR respectively **(Figure 1)**. The incidence of Urea plasma sp. among 119 patients attending two STD centers in Nigeria was found to be 63.03% (n= 75) **(Figure 2)**. For the control, n=15 (12.82%) and n=20 (17.09%) were detected for Urea plasma urealyticum and Urea plasma parvum (n= 20) (Figure 2). The number of patients harboring both Urea plasma parvum and Urea plasma urealyticum were 19 (15.97%) while for the control, 3 (2.56%) were detected. (Figure 3) show the PCR assay of 10 culture positive samples from Centers A and B, respectively.

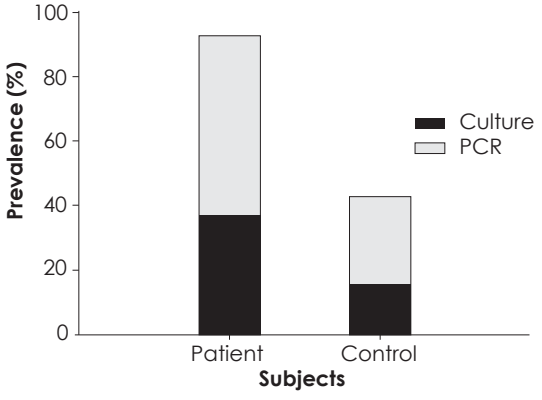

Figure 1: Frequency distribution of Urea plasma sp using cultural and molecular techniques

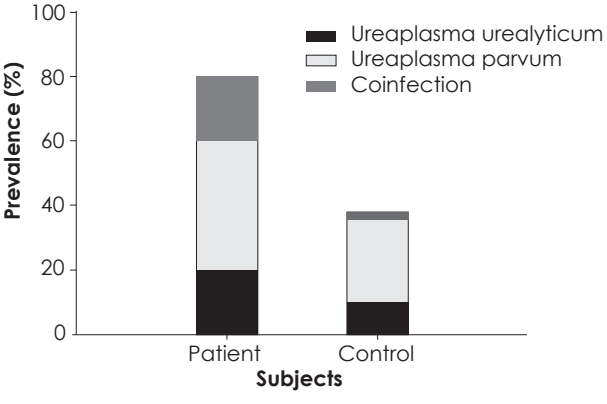

Figure 2: Percentage distribution of Urea plasma sp from the subjects

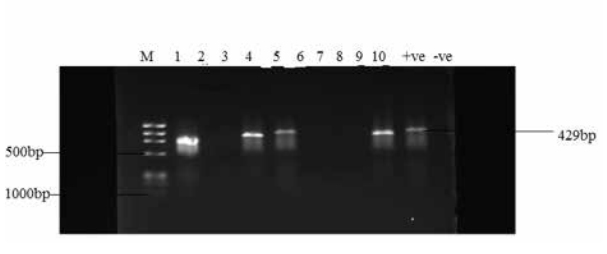

Figure 3: Test trials on 10 culture positive samples

Legend= M- marker, Lanes 1-10 is showing the migration of the PCR products post agarose gel electrophoresis, Lane 11 positive control, Lane use negative control. PCR positive control (ATCC), U. parvum PCR positive control from the host laboratory and -ve control (water)

DISCUSSION

Identifying a specific organism and quantifying its prevalence or level of pathogenicity is usually dependent on assay specificity for true positive results. Implications of false negative results could be as grave as causing disease outbreak or increased death rate in a population. Cultural method is usually the golden standard of isolation of microorganisms; however, its sensitivity and specificity in the characterization of some organisms such as Mycoplasma have been queried.[3] In this study, it was further affirmed that PCR assays are more accurate in the detection of Urea plasma sp. We also confirmed that distinguishing between the two Urea plasma sp. in clinical samples can be quantitatively done by molecular characterization. From the results of our research, we discovered that PCR was two times more sensitive than culture in the detection of Urea plasma species. In both the controls and patients, a significant difference was observed in the number of true positive results obtained through both techniques. Over time, the use of culture method for the isolation and detection of fastidious organisms have been yielding very poorly despite the amount of time it consumes. Although higher sensitivity and specificity of a PCR assay is favored by the use of the appropriate primers and protocol, the efficiency in identifying fastidious organisms is still laudable.[12]

Urea plasma parvum was the most frequently isolated biovar from the urogenital samples. This is in agreement with Kechagia et al.[13] and De Francesco et al.[9] who reported higher incidence of Urea plasma parvum in urogenital samples of women with genital diseases and clinical vaginitis. Before 2002, the level of pathogenicity of these species was not effectively quantified.[1] The speciation of Urea plasma species, have however, shown that Urea plasma parvum is often more implicated in urogenital infections especially among females. The high occurrence of Urea plasma parvum observed in this study is worrisome due to the pathology of the organism; urethritis, adverse pregnancy outcomes, neonatal meningitis, pyelonephritis and series of neonatal diseases are caused by these pathogenic mollicutes.[5, 14] From the urogenital tract, Urea plasma parvum is easily passed on to the fetus or neonate through the placenta or umbilical cord and causes complications including death in infants.

Urea plasma urealyticum also had a prevalence of 20% in the samples which is lesser compared to about 50% of the other biovar. This biovar has been linked to a high number of genital infections both in males and females. Male urethritis is a major genital infection in which 70% of the cases is being associated with Urea plasma urealyticum. Speciation has been the limitations of prior investigators in Nigeria in this field; they have not been able to utilize high molecular technique such as PCR as employed in this study. The rate of detection of U. urealyticum was higher in patients with clinical evidence of STI than in asymptomatic (control) groups. Coinfection of both biovars was also evaluated and it was observed that about 20% of the samples were positive for the occurrence of Urea plasma parvum and Urea plasma urealyticum. The concomitant infection of these biovars could be very fatal as they have been studied to cause infections with fatal complications.[15]

It has also been reported that these ureolytic pathogens show broad resistance to different classes of antibiotics,[13] thus making it difficult to effectively treat genital mycoplasma infections. We have used conventional PCR to confirm the presence of Urea plasma urealyticum and Urea plasma parvum in Nigerian patients with sexually transmitted diseases. Conclusively, Urea plasma species are frequent causes of genitourinary tract infections. Effective screening of these opportunistic pathogens is important to curb or mitigate the fatal complications that may arise from their infections. The use of PCR assay in the detection of Urea plasma sp should be encouraged. Early identification and an appropriate subsequent treatment will forestall anticipated genital complications that might arise due to Urea plasma infections.

CONCLUSION

PCR assay showed more sensitivity and specificity in the identification of Urea plasma species than culture Application of modern molecular techniques should be encouraged for the study of Urea plasma sp and other related fastidious organisms in Nigeria.

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