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PERFORMANCE OF CONVENTIONAL PCR-BASED ASSAYS FOR THE SPECIFIC DETECTION OF UREAPLASMA UREALYTICUM AND UREAPLASMA PARVUM IN ASYMPTOMATIC WOMEN USING ENDOCERVICAL SWAB SAMPLES

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ABSTRACT

Background & Objectives: Ureaplasma infection in asymptomatic women shows a significance of women's reproductive health. Culture-based methods are slow, labor-intensive, and can miss non-culturable or fastidious strains. Targeting 16SrRNA gene, a highly conserved genetic marker found in the bacterial genome, has been widely utilized for microbial identification and phylogenetic analysis. The main aim of this study is to identify the prevalent genotypes of Ureaplasma species by the application of specific 16SrRNA target in asymptomatic women.

Methodology: This cross-sectional study was performed with 100 asymptomatic women aged from 18 to 45 years with infertility. Conventional PCR for 16SrRNA was taken to determine the presence of U. urealyticum and U. parvum using endocervical samples. Amplified PCR products of were analyzed and positive products were sent for sequencing and the results were compared with GenBank and phylogenetic analysis was constructed.

Results & Discussion: The overall result was found that 24.0% of asymptomatic women were positive for Ureaplasma species. One of our study sequences were closely clustered with common ancestors of reference strains from Austria, Ireland, Canada and China. Remaining five sequences forms a separate ancestor lineage for the USA strain JQ901478.1.

Conclusion: The study concludes that the major identification of Ureaplasma species in asymptomatic women is Ureaplasma parvum which represents a multifaceted challenge with significant clinical implications.

Keywords: Ureaplasma urealyticum, Ureaplasma parvum, sexually transmitted diseases (STDs), Conventional PCR, 16SrRNA, Asymptomatic women

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1. INTRODUCTION

Ureaplasma infection in asymptomatic women shows a significance of women's reproductive health [1-2]. Ureaplasma urealyticum and Ureaplasma parvum is belonging to the class Mollicutes, a small, self-replicating, fastidious agent to colonize in the female genital tract [3]. While Ureaplasma species are often considered commensal organisms, recent evidence suggests that their presence has been associated with adverse clinical outcomes such as pelvic

inflammatory disease (PID), preterm birth and infertility [4]. In pregnant women, Ureaplasma species have been linked to preterm birth, chorioamnionitis, and neonatal complications [5-6]. Conservative methods for the screening of Ureaplasma were microscopy and culture-based techniques, but it had several limitations. Culture-based methods are slow, labor-intensive, and can miss non-culturable or fastidious strains [7]. Microscopy, on the other hand, lacks specificity and cannot differentiate among Ureaplasma species. Hence, the sensitivity and specificity has been increased for an early detection of Ureaplasma species by the molecular diagnostic assays [8-10]. Targeting 16SrRNA gene, a highly conserved genetic marker found in the bacterial genome, has been widely utilized for microbial identification and phylogenetic analysis [11]. Its ability to provide species – level resolution makes an attractive target for the detection of Ureaplasma species in a endocervical sample. On accurate detection of Ureaplasma species is helps for the appropriate treatment and prevention of potential complications like infertility, bacterial vaginosis etc. [11]. Clinical significance of Ureaplasma species often associated with urogenital infections in both symptomatic and asymptomatic individuals [12-16] Moreover, their presence in asymptomatic women has raised questions about their infectious role in women's reproductive health. At least 14 serovars have been identified in human ureaplasmas; U. parvum contains serovars 1, 3, 6, and 14, while U. urealyticum contains the remaining 10 serovars, 2, 4, 5, 7, 8, 9, 10, 11, 12, and 13 [17]. This research paper delves into a prospective application of the 16srRNA gene target for the identification of Ureaplasma species, particularly in asymptomatic women. The 16srRNA gene, with its variable regions, offers a robust molecular tool that can provide insights into their diversity, and potential pathogenicity of Ureaplasma species in asymptomatic individuals [8, 17-18]. To address these diagnostic challenges, the present study sets a stage for our initial research and this introduction will explore the significance of Ureaplasma species in clinical contexts and their association with reproductive health. The main purpose of this study is to identify the prevalent genotypes of Ureaplasma species by the application of specific 16SrRNA target for asymptomatic women.

2. MATERIALS & METHODS

This cross sectional study was performed with 100 asymptomatic women aged from 18 to 45 years with infertility. The study was carried out for the period of six months from November 2023 to April 2024 who attended for regular checkup due to infertility as an outpatient department (OPD) of Obstetrics & Gynecology in a tertiary care hospital. After getting a written consent from the asymptomatic women were recruited for the collection of endocervical swabs and transferred to the sterile phosphate buffered saline (PBS) solution. Specimens were immediately transported to the research facility for molecular testing. After testing, the vials will be autoclaved and disposed as per biomedical waste management rules, 2016 [19].

Molecular Diagnosis of Ureaplasma Species:

Genomic DNA was extracted from the endocervical swab using the commercial extraction kit "QIAGEN Blood Mini DNA, Germany". The genomic DNA was extracted according to their manufacturer's instructions, and the concentration of the extracted DNA was measured by spectrophotometry (NanoDrop 2000 Spectrophotometer, Thermo Scientific). The purity of the extracted DNA was determined by absorbance (A) using Eppendorf Spectrophotometer. A260/A280 ratios were in the range of 1.7-1.8 for the samples. The extracted DNA was preserved at -20℃ for molecular testing. About 200 µl PBS containing the patient samples were used for the genomic DNA extractions as per the procedure of DNA Blood Mini Kit

(Qiagen, Germany). QIAamp DNA Blood Kits simplify DNA purification from blood and other body fluids with fast spin-column, vacuum, centrifugation or automated procedures [4]. Conventional PCR Reaction for 16SrRNA gene target was performed for 100 samples. The reaction mix was constitutes of 25µl which includes 12.5 µl of 2X Taq DNA polymerase PCR (Ampliqon, 2X Master Mix RED, Denmark), 1.5 mM MgCl2 final concentration followed by 1.0µl of each 0.2µm forward and reverse primer are mentioned in Table - 1, 8.5µl of Molecular grade distilled water and 2.0µl of genomic DNA is used as a template for this reaction setup. Amplification protocol for conventional PCR was carried out with initial denaturation at 95ºC for 8 mins followed by denaturation 95ºC for 30 secs, annealing 58ºC for 60secs for 35 cycles, extension at 72ºC for 30 secs along with the final extension 72ºC for 5 mins. The conventional PCR was carried out using Applied Bios-system, VeritiDx 96-wells Thermal cycler (Thermo Fisher Scientific, Singapore).

Table – 1: Details of Conventional PCR primers for 16SrRNA specific Ureaplasma gene targets

Primer Name	5' Sequence 3'	Amplicon size
16SUu-S	TACCCTTAAGTTGGGGATAA	898bp
16SUu-AS	ACTATATTTCTATAGCGTCGCAA	

Analysis of Amplified Products:

The gel was prepared and immersed in 1x TAE buffer (HiMedia, Biosciences, India). After amplification, 8µl PCR products were loaded onto a 2.0 %agarose gel electrophoresis stained with Ethidium bromide solution. About 2µl of 100-bp DNA ladder (HiMedia, BioSciences, India) was added to the well for determination of amplicon size for the specific target. The voltage 90ºC for 40 mins was applied by using a Power Pac 300 (Bio-Rad, USA) power supply for separation of the different size of DNA bands. The gel was visualized using Gel+ Documentation system (Bio-Rad, USA). The sample was considered as positive for 16SrRNA Ureaplasma species, when amplified product size at 898-bp.

Purification of PCR Products and Gene Sequencing:

In the present study, 16SrRNA Conventional PCR positive samples were subjected for purification using QIAquick PCR purification kit (Qiagen, Germany) as per the procedure outlined by the manufacturers. The purified PCR products are sent for sequencing. Briefly, one volume of amplified PCR product was added to five volume of PB buffer with pH Indicator I to check if the color of the solution remains yellow (pH- 5.0). The sample was added to QIAquick spin column placed inside 2ml collection tube and centrifuged at 8000 rpm for 30-60secs. After centrifugation the filtered waste was discarded and the column was replaced in the same collection tube. About 750µl PE buffer was added to the column and centrifuged for 30-60 secs to wash the residuals from the spin column. An additional brief centrifugation was also made as per the recommendation of the kit to avoid impurities. Spin column was then placed in a clean1.5 ml micro-centrifuge tube and 30 µl of EB (Elution Buffer) buffer (10mM TrisHCl, pH 8.5) was added to the center of the column and centrifuged for one min. For confirmation, one volume of loading dye was added to five volumes of purified products in a 2.0% agarose gel electrophoresis stained with Ethidium bromide solution. This purification kit contains three different marker dyes (bromophenol blue, xylene cyanol, and orange G) to estimate the migration of DNA and standardization of agarose gel running time. PCR purified amplicons were sent to Mediomix, Bangalore and they were sequenced by using Applied Biosystems ABI3730XL. PCR amplified products will be sent for sequencing to Mediomix Pvt. Ltd., Bengaluru.

Phylogenetic Analysis of Ureaplasma Species:
For phylogenetic investigations, the sequenced PCR products was utilized for further determination using Nucleotide BLAST (Basic Local Alignment Search Tool) program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (National Centre for Biotechnology Information - NCBI database). These sequenced data were aligned with corresponding nucleotides from GenBank by using ClustalW (https://www.ebi.ac.uk/Tools/msa/clustalo/) and Maximum Likelihood (ML). Phylogenetic tree generated using IQ-TREE tool (default parameters) based on multiple sequence alignment. The maximum likelihood bootstrap support values are denoted as blue circles as described in the legend.

3. RESULTS

Endocervical swabs were collected from 100 asymptomatic patients who visited our Outpatient Department (OPD) of Obstetrics & Gynecology without any symptoms. Figure – 1 shows that 100 asymptomatic patients were tested for specific species of 16SrRNA and yielded 24 (24.0%) and remaining 76% were negative for the specific target. Figure – 2 shows the positive samples for 16SrRNA target amplified in 898bp. Figure – 3: showed that the study isolate PP195967 were closely clustered with common ancestors of reference strains like T735169.1, OR879215.1, MK615035.1, MK615038.1, KR514490.1, MH428101.1 and JF975749.1 from Austria, Ireland, Canada and China respectively. Study sequences such as PP195970, PP195966, PP195969, PP195971 and PP195968 forms a separate ancestor lineage for the USA strain JQ901478.1.

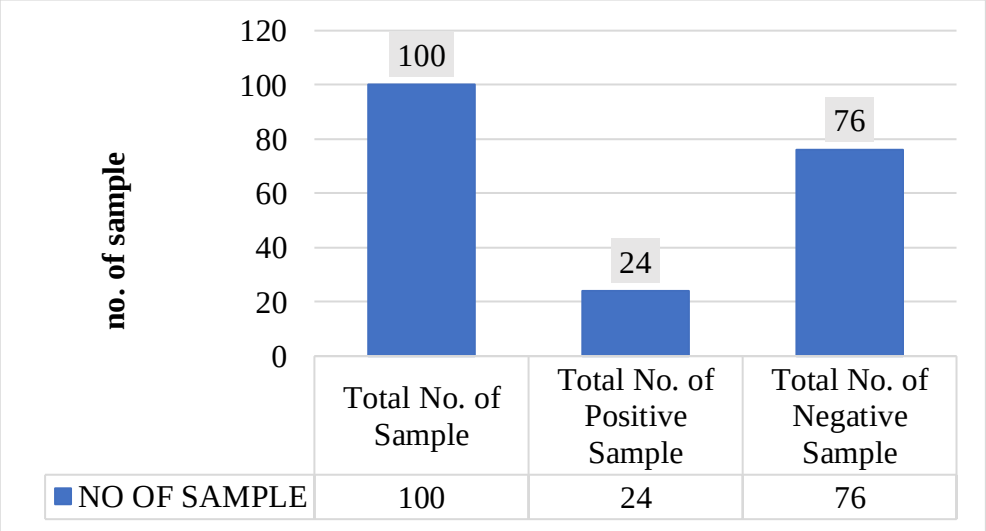

Figure – 1: Positivity of 16SrRNA Ureaplasma species in asymptomatic patients (n=100)

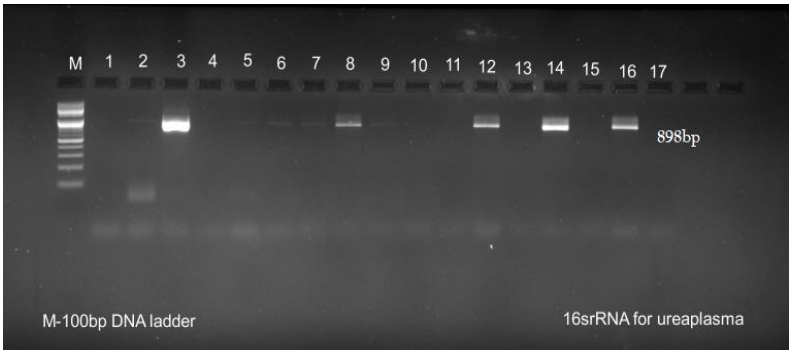

Figure – 2: Amplified PCR products of 16SrRNA Ureaplasma species in asymptomatic

women. M – 100bp DNA Ladder; Samples 1, 2, 3, 10, 13, 15 – Negative and Samples 3, 5, 6, 7, 8, 9, 12, 14 – Positive shows at band 898bp and well no: 16 – Sample positive control and 17 – Negative control.

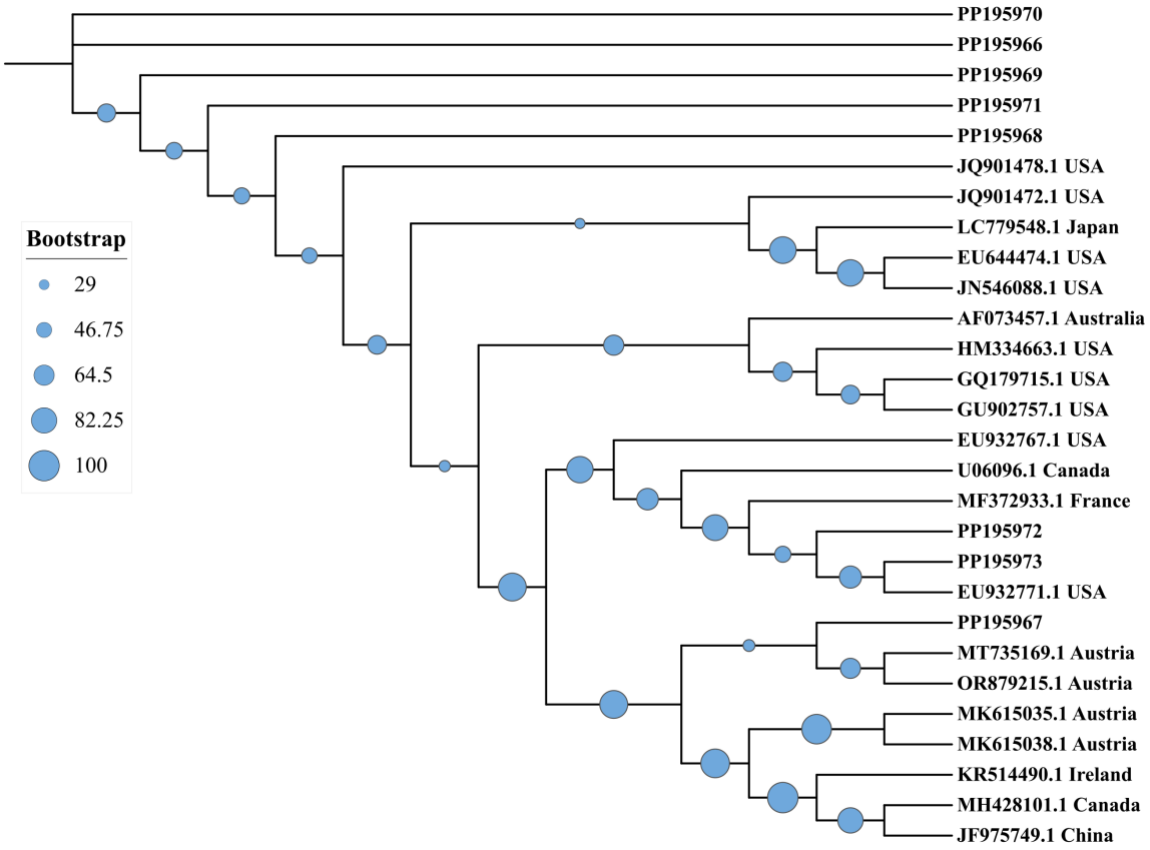

Figure – 3: Maximum Likelihood (ML) Phylogenetic tree generated using IQ-TREE tool (default parameters) based on multiple sequence alignment of 16S ribosomal RNA genes of study isolates.

4. DISCUSSION:

The 16srRNA gene is our specific target which is underpinned by its well-established utility in phylogenetic as well as species identification in diverse microbial taxa [8]. The 16srRNA gene, widely recognized a high degree of conservation across the microbial kingdom, serves as a promising molecular target to unravel the diversity within the genus [17]. The 16srRNA gene, found in all prokaryotes, offers a promising target for molecular identification. Its conserved regions provide a stable scaffold for primer design, while the variable regions contain unique sequences that can discriminate between species [18]. Moreover, its ability to differentiate between closely related species at the molecular level makes it an attractive candidate for studying the diversity within the Ureaplasma genus. Our research aims to leverage the power of the target to improve the accuracy for identification of Ureaplasma species and shed the light in the reproductive health of asymptomatic women. Similarly, from Australia and Brazil Kong et al., 1999 reported that U. parvum is the predominant pathogen than U. urealyticum by using 16SrRNA and multiplex PCR [4, 8]. In India, New Delhi has conducted a prospective study using Multiplex PCR and culture for the detection of Ureaplasma species in 38 of 147 (25.7%) by Dhawan et al., 2012 [3]. Another study from the

same research in the year 2006 found that the performance of culture and polymerase chain reaction U. urealyticum 32% and 45% respectively [20]. In Puducherry, Multiplex Real Time PCR was performed and 17 were detected both U. parvum and U. urealyticum using cervical swabs by Stephen et al., 2019 [4]. Globally, many researchers have found that U. parvum is the predominant isolates in cervico-vaginal samples in both fertility as well as infertility groups [2]. U. urealyticum infection affected majority of the Morocco population [13]. Kacerovsky et al., 2022 Paired amniotic and cervical fluid samples using PCR found Ureaplasma species 61% in Czech Republic population [5]. Incidence of Ureaplasma positivity was observed 30% and 14.3% in asymptomatic women among Nigerian population [1]. Kasprzykowska et al., recorded that U. urealyticum infection was observed 3.6% in asymptomatic women [21]. The present study, finds that 24.0% of asymptomatic women was positive for U. parvum. Since its quite higher when compared to the other studies from Nigeria and Poland. Most of the studies have shown that these microorganisms are present up to 70% of sexually active women, and making them one of the most common genital flora. The study isolate PP195967 were closely clustered with common ancestors of reference strains from Austria, Ireland, Canada and China. Remaining study sequences such as PP195970, PP195966, PP195969, PP195971 and PP195968 forms a separate ancestor lineage for the USA strain JQ901478.1.

The impact of these microorganisms is not limited to women; they have also been associated with male infertility and prostatitis. However, the exact role of Ureaplasma species in these clinical conditions remains a subject of ongoing research. The study of Ureaplasma species has been hampered by the limitations of traditional culture-based methods, which often fail to distinguish between species and are insensitive to low bacterial loads. Asymptomatic women, represent a unique challenge, as the absence of clinical symptoms can lead to under-diagnosis and underestimated prevalence. Finally, the present study we will elucidate the rationale behind the selection of the 16srRNA gene as a target for this study, and the potential implications of our research on understanding the role of Ureaplasma species in the reproductive health of asymptomatic women.

Ureaplasma parvum and Ureaplasma urealyticum have gained attention in the field of women's reproductive health due to their association with genitourinary infections. Notably, these infections can remain asymptomatic in many cases and making them challenging to diagnose and manage effectively. In the current research, we focus on the potential target of specific 16srRNA as a robust for identifying Ureaplasma species, especially in the context of asymptomatic carriers.

5. CONCLUSION

In conclusion, the identification of Ureaplasma species in asymptomatic women presents a multifaceted challenge with significant clinical implications. This paper sets the stage for our research, emphasizing the importance of precise Ureaplasma species identification and the limitations of existing diagnostic methods. We proposed 16srRNA gene as a promising molecular target, and in the subsequent sections, will delve into the methods, results, and implications of our prospective study, which aiming to contribute to a better understanding on the role of Ureaplasma species in the reproductive health of asymptomatic women.

Conflict of Interest: None to declare

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