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Isolation and characterization of Ureaplasma parvum from the semen of infertile men and detection of virulence factors genes

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***Abstract*---**The bacterial infection of seminal fluid is one of the most important factors behind infertility in many cases. The biological diversity of the *Ureaplasma* and *Mycoplasma* have widely been reported to be associated with the infertility grade in many cases. The purpose of this research was to identify and characterize and the phylogenetic positioning of twenty-one isolates of *Ureaplasma* in seminal fluids. Two genetic loci were included in this study *ureB/ureA* and *upV* for *Ureaplasma*. Each amplified locus was investigated in both bacterial infections to identify the identity of the possible bacterial infection of those patients and to assess the pattern of the genetic variation for each genetic locus in investigated patients. The results of the DNA sequence analysis of the *urease* and *upv* gene extracted from these bacteria showed that *Ureaplasma parvum serovar* 3 was the cause of infections in the studied samples. The studied isolates were also matched with the global isolates registered in the gene bank. After NCBI matching for these gene segments, polymerase chain experiments confirmed the diagnosis. The NCBI BLASTn The algorithm discovered up to 99.5 percent sequence similarity. between the studied samples and the reference samples for these gene. The substitution mutation in sample S19 led to a change in the amino acid resulting from the translation process by changing the amino acids from 14 V > I and 9 S > N respectively, while the substitution mutation in sample S16 did not show any change in amino acids.

***Keywords*---**ureaplasma, infertile, genotype, urease, upv gene.

Introduction

Mycoplasma is a one-of-a-kind bacterial species, the tiniest creature on the planet known living entity capable of reproducing on its own (1). Because *Mycoplasma* lacks a cell wall, it can take on a variety of shapes and sizes, making identification challenging. When it was cultured in the laboratory from Urethritis in males with non-clinical urethritis, Shepard was the first to describe a small mycoplasma strain (strain T)(2), which was later identified as *Ureaplasma*. It attaches to host cells through its protein-coated apical tip, which adheres to the host cell's protein layer, which is why antibiotics categorized as "protein synthesis inhibitors," such as Tetracycline, are commonly used to treating Mycoplasma infection (3).(4) The reason for the differences in the appearance of bacteria in the medium used in the development of mycoplasma and the method of transmission and presentation according to different diagnostic methods is attributed to the nature of the study population . The mechanism of damage to *Mycoplasma* when present in the semen comes through its adhesion with the sperm, further increase of toxins and H_2S_2, as well as its release of some enzymes such as phospholipase (5).Ureas is a nickel-based mineral enzyme that catalyzes the hydrolysis of urea to produce ammonia and carbamate, and the latter compound spontaneously decomposes to form carbonic acid and ammonia . The first sequence for this *ureas gene* was recorded in 1989 and it was for *Proteus mirabilis*. The Ureas consists of three different polypeptides in *P. mirabilis*, three structural subunits were found :three structural genes, *ureA*, *ureB*, and *ureC*, respectively, encode this polypeptide. While it was found that the Urease enzyme in Helicobacter Pylori consists of only two subunits: The *ureA* and *ureB* genes encode 26.5 kDa ureA (subunit) and 61.7 kDa ureB (subunit) (6). (7) The *upv gene* is a special gene for the detection of *Ureaplasma parvum* (8) that *upv* is a methylation-inducible gene that encodes for the enzyme DNA methylation (cytosine-5)-methyltransferase, and it also encodes for the CATG-cutting enzyme, as the enzyme Upv showed (This enzyme belongs to the family of glycosyltransferases, It is specifically pentosyltransferase expressed by endonuclease activity and recognizes the CATG sequence, resulting in a sharp cut between A and T This was observed in a study (8) involving Vsa (variable surface proteins) that is classified as a microbial pathogen in *Mycoplasma pulmonis*, which is closely related to *Ureaplasma spp. It* plays an important role in virulence and escape from the host immune system.

Material and Method

150 semen samples were collected in sterile special containers, inoculated in PPLO-modified medium (MDCS) (9), and incubated anaerobically for 24-72 hours at 37°C. On the sloped surface of the solid media, colonies appeared. After being stained with Denise dye, the developing colonies were microscopically diagnosed using a dissection microscope. The carbohydrate fermentation test, the arginine group depletion test, and the urease detection test were all used to diagnose genital bacteria.A prepared DNA extraction kit (Geneaid, Taiwan) was used to extract DNA from each sample, then the samples were kept at -20°C until PCR was used for DNA extraction. The bacterial genotypes were determined based on the DNA sequence of the *urease gene* after amplifying the gene using the PCR method, and the *upv gene* was also detected using the PCR method. The primers

used in this study were designed by the Korean company Macrogen and were based on the database site NCBI-Gene Bank for the purpose of obtaining the nucleotide sequence of the gene used in the study .Inside this study, amplified by pcr was ramped up through using additions.20µl plus 5 µl of dna templet , 1 µl (10 pmol) of each forward : 5' –CAA TCT GCT CGT GAA GTA TTAC- 3' and reverse : 5' –ACG ACG TCC ATA AGC AACT- 3' primers for *Ureas gene* , and 13µl of Water containing free nuclease to the PCR tube ,PCR premix kit (bioneer , Korea) which contain other pcr reaction requirements (dNTPs , KCl , MgCl2 , Taq DNA polymerase ,tris HCl pH :9.0 , tracking dye and stabilizer) , The *urease gene* 424bp fragment underwent for amplification in following conditions: initial denaturation at 94 °C for 3 min. , subsequently 30 cycles of denaturation at 94 °C for 1min. , annealing at 52°C for 1min. , elongation at 72°C for 1min. after final extension at 72°C for 10 min. and holding at 4°C . Using agarose gel electrophoresis, the PCR product of the *urease gene* (424bp) was analyzed.The same materials were also added to amplify the *upv gene* except for the primer each forward :5'–TGC GGT GTT TGT GAA CT- 3' and reverse :5'–TGA TCA AAC TGA TAT CGC AAT TAT AGA- 3' primers for *upv gene* . Then the primers were added together to make multiple PCR for the two genes in the following way: add 20 µl including 5 µl of DNA templet, 1 µl (10 pmol.) of each primer (*urease* and *upv*), and 11µl of free nuclease water to the PCR tube, The multiplex PCR It was completed under the following conditions: initial denaturation at 95 °C for 3 minutes, 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 52 °C for 1 minute, elongation at 72 °C for 1 minute, final extension at 72 °C for 5 minutes, and holding at 4 °C. Using agarose gel electrophoresis, the PCR product of the multiplex PCR was analyzed.

Result and Discussion

The Results of Isolating Colonies on Culture Media

The results of this study showed that *Ureaplasma parvum* bacteria were isolated from the semen of infertile men. 150 samples were collected isolates positive for the examination were 68 isolates. The percentage of *U. parvum* bacteria was 45%. Ureaplasma colonies appeared after 48-24 hours of incubation in dark golden or brown color. As shown in the (fig 1) .

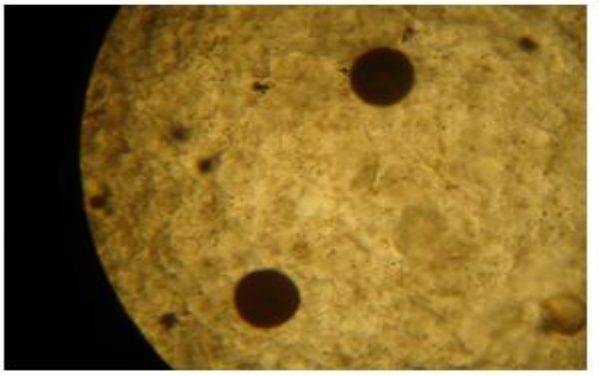


Figure (1) Under the microscope,Ureaplasma spp. on power 10x , stained with Denise.

Sequencing Methods

Nucleic acids sequencing of PCR amplicons

The resolved PCR amplicons were sequenced commercially in the forward direction, following the sequencing company's instructions (Macrogen Inc. Geumchen, Seoul, South Korea). Only clear chromatographs from ABI (Applied Biosystem) sequence files were analyzed further, ensuring that the annotation and variations were not caused to PCR or sequencing artifacts. Virtual positions and other details of the retrieved PCR fragments were identified by comparing the observed nucleic acid sequences of investigated bacterial samples with the retrieved nucleic acid sequences.

Interpretation of sequencing data

Using BioEdit Sequence Alignment Editor Software Version 7.1, the sequencing results of the PCR products of the targeted samples were edited, aligned, and assessed as long as they matched the appropriate sequences in the reference database (DNASTAR, Madison, WI, USA). Each sequenced sample's detected differences were numbered in PCR amplicons and their matching locations within the referencing genome. The nucleic acids found inside the PCR amplicons were numbered, as were their respective locations in the reference genome. The Snap Gene Viewer (version 4.0.4) (https://www.snapg-ene.com) was used to annotate each discovered variant among the bacterial sequences.

Nucleic acid variations were converted into amino acid residues

The amino acid sequences of the targeted proteins were obtained from the protein data bank (http://www.ncbi.nlm.nih.gov) on the internet. Using the Expasy online program (http://web.expasy.org/translate/), the observed nucleic acid variants in the coding portions of the analyzed genetic loci were translated into a reading frame corresponding to the corresponding amino acid residues in the encoded protein. Using the BioEdit server's "align" script, multiple amino acid sequence alignment was conducted between the referring amino acid sequences and their observed mutated counterpart.

Construction of even a comprehensive phylogenetic tree

A specific comprehensive tree was constructed in this study according to the neighbor-joining protocol described by (10) The observed variants were compared with their neighbor homologous reference sequences using the NCBI-BLASTn server (11). Then, a full inclusive tree, including the observed variant, was built by the neighbor-joining method and visualized as a circular cladogram using the iTOL suit (12). The sequences of each incorporated species in the comprehensive tree were colored in an appropriate color to be differentiated from the other species. Whereas the sequences color of each species was taken as one unified color.

PCR Technique

The success of the process of amplifying DNA extracted from cells for the Ureas gene, *upV* gene, and multiplex PCR for two genes simultaneously was shown by the results of this technique. The positive findings of DNA samples amplified for the Ureas gene, *upV* gene, and multiplex retrieved from the cell after being placed onto an agarose gel are shown in (Fig 2,3,4).

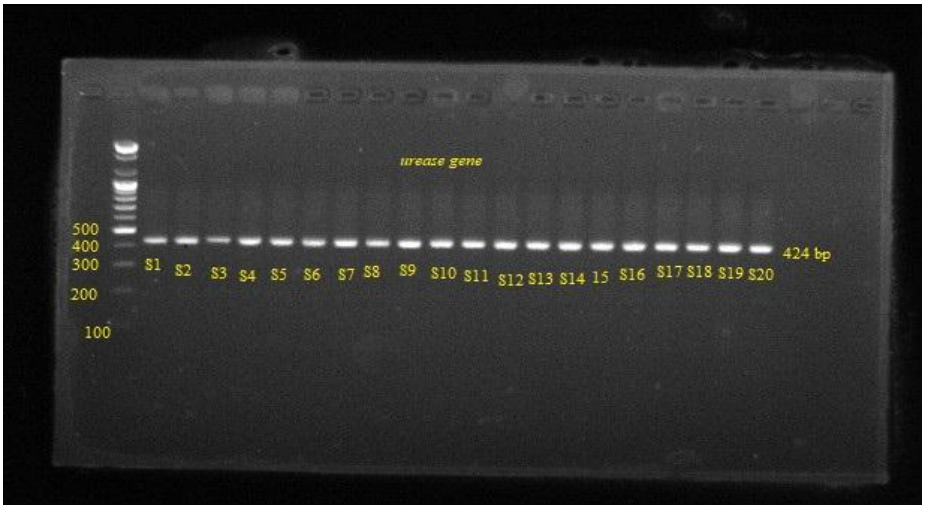


Figure (2) representing electrophoresis on agarose gel to amplify the urease gene extracted from the DNA.

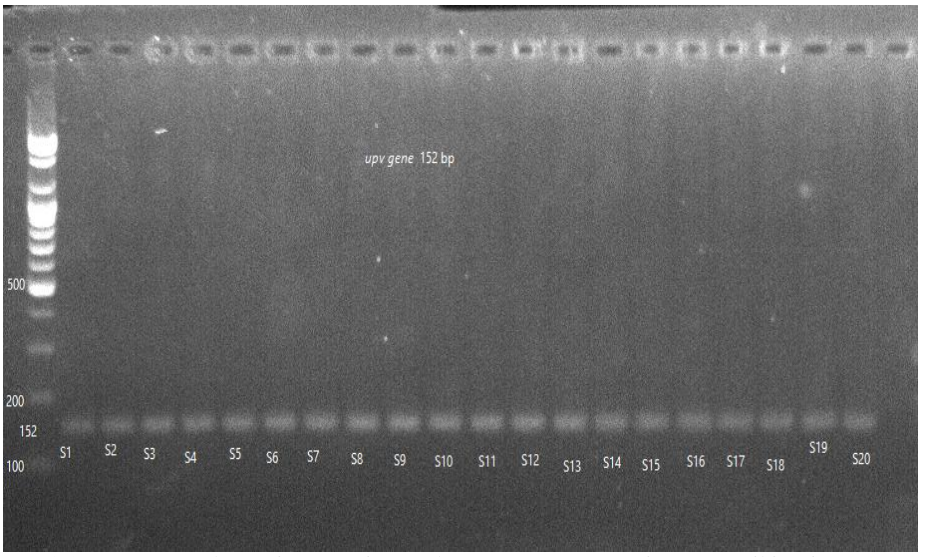


Figure (3) representing electrophoresis on agarose gel to amplify the *upv gene* extracted from the DNA.

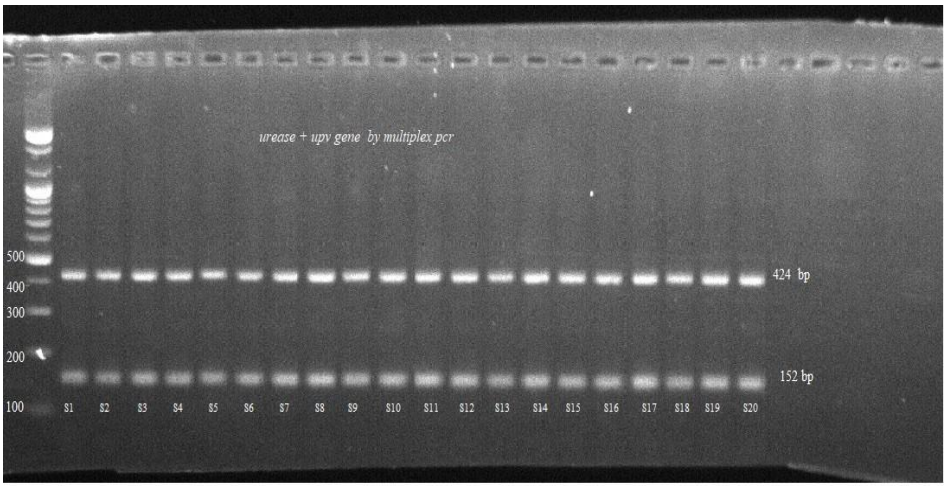


Figure (4) representing electrophoresis on agarose gel to amplify,the *urease* & *upv* by multiplex pcr gene extracted from the DNA.

Ureas gene sequences

Within this locus, twenty samples (assigned S1 to S20) were considered in this research. These samples were examined to see if they were partially amplify the DNA *ureB* gene and *ureA gene* that code for urease subunit beta and urease subunit gamma in the *Ureaplasma* sequences respectively. As a result of its possible ability to adapt to variable genetic variety as seen in different bacterial types, the variance of the DNA ureB/ureA genes can be used for genotyping of this bacteria. After completing NCBI blasts for these PCR amplicons, the sequencing assays revealed their precise identification (11). concerning the 424 bp amplicons, the NCBI Blasting engine showed up to 99.5% sequences similarities between the sequenced samples and *Ureaplasma parvum* reference target sequences. By comparing the observed nucleic acid sequences of these investigated samples with the retrieved nucleic acid sequences (GenBank acc. CP041199.1) accurate positions and other details of the retrieved PCR fragments were identified. In addition to the partial amplification of the *ureB* locus, the currently utilized amplicons were also partially covered the *ureA* locus, which is concerned with the production of urease subunit gamma. The NCBI server was used to determine the total length of the targeted loci, and the start and end positions of the targeted locus within the most homologous bacterial target. The alignment results of the 424 bp samples indicated three nucleic acid variants in the S16 sample and two nucleic acid variations in the S19 sample, which was interesting in comparison with the most similar referring reference nucleic acid sequences (GenBank acc. no. CP041199.1). our results indicated the presence of three nucleic acid variants observed in the investigated S16 sample, namely 288T>A, 289G>A, and 305A>G, and two nucleic acid variants, namely 204C>T and 218C>T, observed in the investigated S19 sample. These differences observed in the currently observed nucleic acid sequences in the analyzed samples were not found in the corresponding reference sequences. To confirm these variations, The investigated samples' sequencing chromatograms, as well as their detailed annotations, were verified and documented, and the sequences' chromatograms were shown according to their positions in the PCR amplicons. Each of these

variants' presence was confirmed in its original chromatogram, and also the absence of any possible technical error.

The investigated nucleic acid sequences were however converted to their corresponding positions in the urease subunit beta and urease subunit gamma. The Expasy translate suite was being used to translate all nucleic acid sequences in the studied samples to their matching amino acid sequences. Amino acid alignment of amino acid sequences with their references showed that the investigated 424 bp consisted of 81 amino acid sequences in the urease subunit beta and 44 amino acid sequences in the urease subunit gamma. The identified nucleic acid alterations were further investigated to see if they caused a change in their corresponding positions in the urease subunit beta and subunit gamma, respectively. Due to the observed nucleic acid variations, Using the Expasy translate suite, all nucleic acid sequences of S16 to S19 were translated to their corresponding amino acid sequences. Amino acid alignment of amino acid sequences with their references showed that two of these variants exhibited a missense effect on the urease subunit beta as it was found in the S19 sample, while S16 variations were found to be located in the urease subunit gamma region and were not exhibit any missense (non-synonymous) mutation in this protein. Then, these non-synonymous variants were exemplified in the entire urease protein sequences depends on their relative positions in the whole protein, namely p.14V>I and p.9S>N . However, the bacterial sequences usually alter their sequences to adapt to the host environment in which they are living (13).

To give a phylogenetic understanding of the actual distances of the investigated S1 – S20 samples, The nucleic acid sequences detected in the amplified 424 bp of the *ureB*/*ureA* gene amplicons were used to create a thorough phylogenetic tree in this study. This phylogenetic tree was created using these twenty samples, and other related nucleic acid sequences from human-infecting bacteria. Sequences of *Ureaplasma parvum*. In addition to *Ureaplasma* sequences, To analyze patterns of biological intra-species and inter-species variability within the incorporated sequences of the tree, two more bacterial genomes were incorporated inside the same tree outgroup sequences. Streptococcus thermophiles (outgroup-1) and Staphylococcus hominis (outgroup-2) are the outgroup sequences (outgroup-2).

Within this tree, all our investigated samples were aligned alongside other relative sequences to constitute the currently incorporated sequences within the cladogram. In this comprehensive tree, there were 61 aligned nucleic acid sequences. Within the human-infecting *Ureaplasma* sequences, the integrated samples were clustered into five phylogenetic clades in the cladogram. The most interesting finding in our bacterial isolates is a link between their positioning and nearby *Ureaplasma parvum* clade sequences. This clade consisted of the majority of incorporated samples of *Ureaplasma* sequences as 27 sequences of variable strains of *Ureaplasma parvum* sequences were incorporated within variable phylogenetic distances within this clade (Fig. 5). Within this major clade, it seems that both S16 and S19 samples were slightly tiled from the other investigated samples and suited beside the GenBank acc. no. of AF222894.1 that belonged to *Ureaplasma parvum* serovar 3 (ATCC 700970) deposited from the USA. This style of positioning was due to the presence of three genetic variations in S16 and two genetic variations in S19 samples. However, there is no remarkable deviation with

respect to the original positioning of these bacterial sequences occupied within the major clade of this cladogram. Furthermore, the clustering of all integrated bacterial samples within the clade of *Ureaplasma parvum* may confirm the presence of phylogenetic distributions of these sequences within this clade that are reasonably close. The current observation of this tree has confirmed sequencing reactions because it explained the actual neighbor-joining-based positioning in such investigated sequences. However, the positioning of the other non-variant samples in the immediate vicinity to other strains of *Ureaplasma parvum* has been determined. The determining positioning of all studied (S1 – S20) samples inside the *Ureaplasma parvum* serovar 3 was the most important concept in this context. This observation indicated that all investigated samples shared the same serovar. In addition to the American origins of these samples, the Asian origin was also observed in the same clade, such as the Chinese origin. The different origins of our investigated samples could not be excluded as a factor in this.

In the vicinity of the *Ureaplasma parvum* clade, in which the S1 – S20 samples were positioned, it was found that the *ureB/ureA* sequences have relatively close phylogenetic positions to the clade of *Ureaplasma urealyticum*. Thus, both types of *Ureaplasma* sequences share a high ratio of homology in these sequences. The second-largest clade within this tree is the clade of *Ureaplasma urealyticum*. This clade consists of 16 strains, three of these strains (GenBank acc. no. CP001184.1, CP041200.1, and CP039963.1) have close connections with the clade of *Ureaplasma parvum*. Whereas part of this clade has a close connection with the clade of *Ureaplasma diversum* in which only one strain was incorporated. However, the *Ureaplasma diversum* clade was found to be the closest clade to the outgroup-1 clade of *Streptococcus thermophiles*.

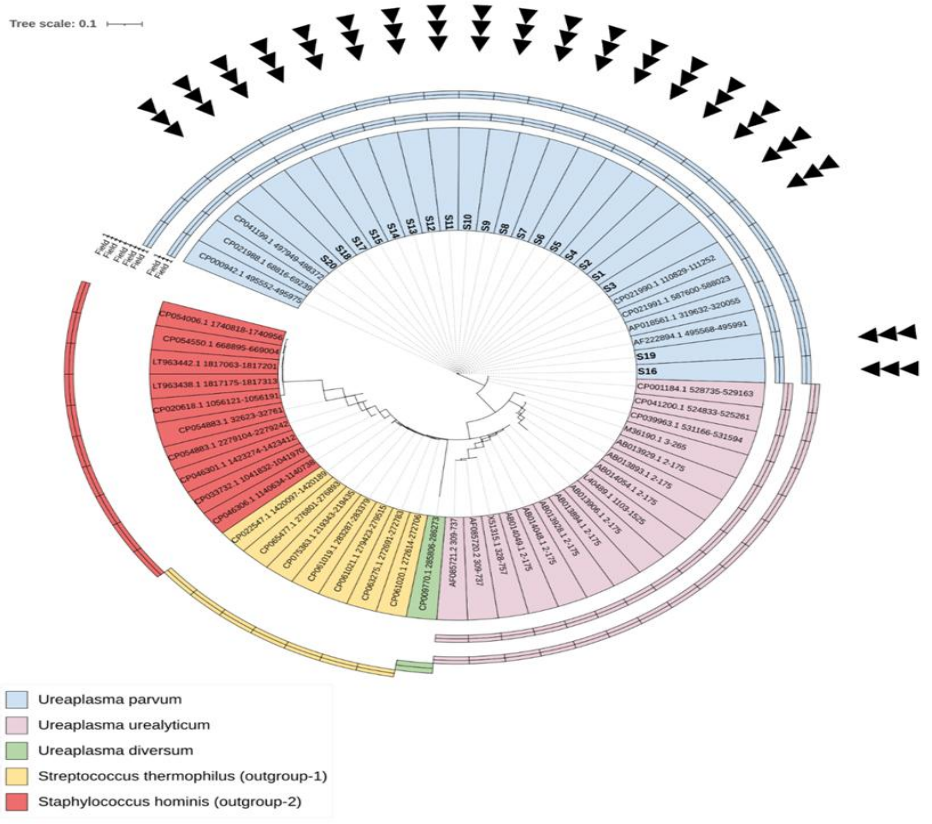


Figure (5). The comprehensive cladogram phylogenetic tree of genetic variants of the DNA *ureB/ureA gene* fragment of twenty samples of human-infecting *Ureaplasma parvum*. The black-colored triangle refers to the analyzed bacterial sequences. All the mentioned numbers referred to the GenBank accession number of each referring species. The number "0.1" at the top portion of the tree refers to the degree of scale range among the comprehensive tree categorized organisms. The letter "S#" refers to the code of the investigated sample.

Importantly, use of DNA *ureB* gene sequences in this study has provided additional evidence for the presence of exact serotype identification of this bacterial organism. This comprehensive tree based on the urease subunit beta has provided the tool for the higher ability of such fragments to effectively distinguish among the recently researched bacterial isolates using the *ureB*-based genetic fragment. This, in turn, demonstrates the superiority of the *ureB* gene over the *ureA* gene in the identification of the currently researched human-infecting *Ureaplasma parvum* and its precise phylogenetic locations.

***upV* gene in *Ureaplasma parvum* sequences**

Within this locus, only one sample (assigned *upV*) was included in the study. The *upV* gene, which codes for DNA (cytosine-5)-methyltransferase in *Ureaplasma parvum* sequences, was partially amplified in this sample. Because of its potential ability to adapt to diverse genetic diversity, as observed in different bacterial

kinds, the variance of the DNA *upV* gene can be used for genotyping of this bacteria. After executing the NCBI blast for these PCR amplicons, the sequencing reactions revealed the precise identity. The NCBI BLASTn engine found complete sequence similarities between the sequenced samples and *Ureaplasma parvum* reference target sequences for the 152 bp amplicons. The exact positions and other characteristics of the retrieved PCR fragment were identified by comparing the observed nucleic acid sequences of this sample with the retrieved nucleic acid sequences (GenBank acc. AP018561.1). The *upV* gene, which is involved in the production of DNA (cytosine-5)-methyltransferase, is partially covered by the currently employed amplicons in addition to the partial amplification of the *upV* locus. The entire length of the targeted loci was determined using the NCBI server, and the start and end positions of the targeted loci were confirmed using the most homologous bacterial target.

Our results indicated the absence of any nucleic acid variants observed in the investigated *upV gene*. To confirm these results, The chromatograms of the sequences of the samples analyzed were confirmed and documented, and the chromatograms of their sequences were shown according to their positions in the PCR amplicons, and the chromatograms of their sequences were directly observed. The absence of any variants in the original chromatogram was confirmed, as was the absence of any probable technical error in the visible chromatogram.

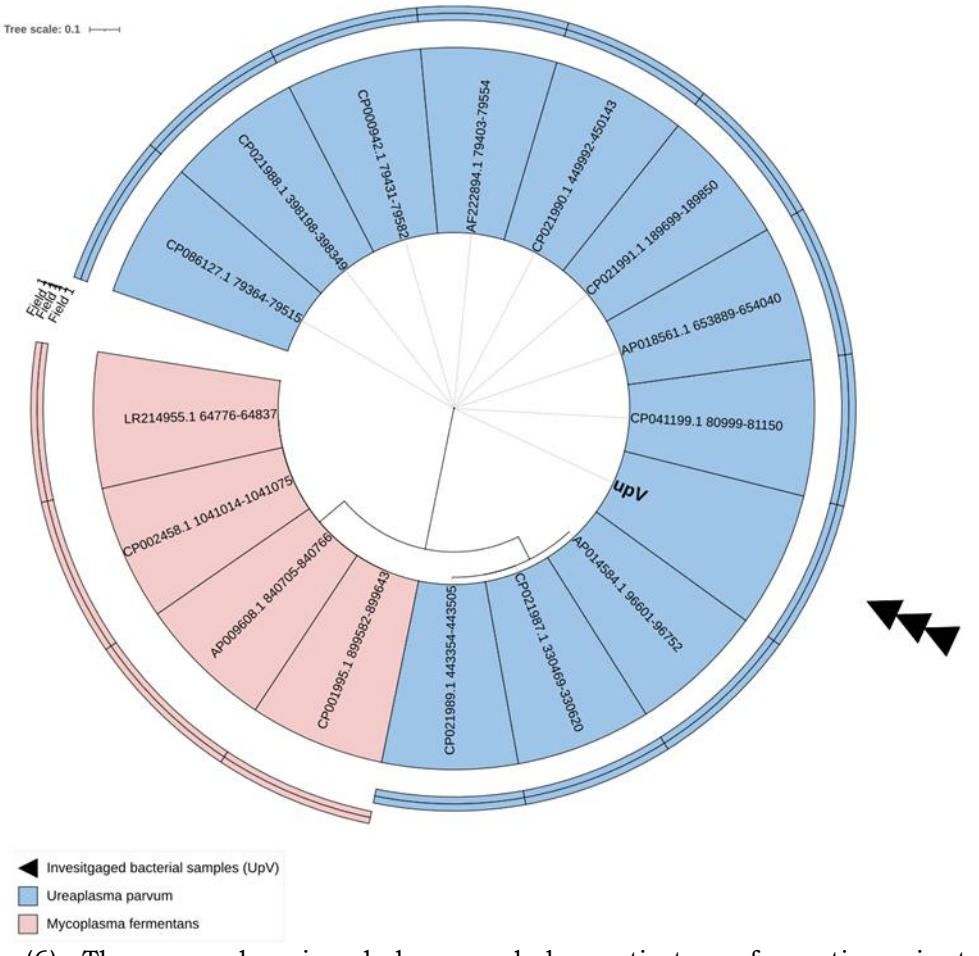
Investigated nucleic acid sequences were however converted to their corresponding positions in the DNA (cytosine-5)-methyltransferase. The Expasy translate suite was used to translate all nucleic acid sequences in the samples analyzed to their corresponding amino acid sequences. Amino acid alignment of these amino acid sequences with their references showed that the investigated 152 bp consisted of 50 amino acid sequences in DNA (cytosine-5)-methyltransferase. The amplified nucleic acid sequences were further analyzed to identify their accurate positions in the entire enzyme.

To give a phylogenetic understanding of the actual distances of the investigated *upV gene*, nucleic acid sequences observed in the amplified 152 bp of the *upV* gene amplicons were used to create a comprehensive phylogenetic tree in this study. This phylogenetic tree was generated to incorporate this investigated sample alongside other relative nucleic acid sequences of human-infecting *Ureaplasma parvum* sequences. In addition to *Ureaplasma parvum* sequences, other bacterial sequences were incorporated as outgroup sequences in same tree to assess patterns of biological inter-species variations within the tree's incorporated sequences. These outgroup sequences are *Mycoplasma fermentans*, which represented the only outgroup incorporated in the tree.

Within this tree, all our investigated sample was aligned alongside other relative sequences to constitute the currently incorporated sequences within the cladogram. The total number of the aligned nucleic acid sequences in this comprehensive tree was only 16. In the constructed cladogram, the incorporated samples were clustered into two phylogenetic clades within the human-infecting *Ureaplasma parvum* sequences. Our investigated bacterial isolate was incorporated in the *Ureaplasma parvum* clade. This clade represented the major

clade, which was made of 12 incorporated samples of *Ureaplasma parvum* (Fig. 6). Within this major clade, the Upv sample was not tilted from the other investigated samples due to the absence of any detectable variation in this sample. It was inferred that this sample was suited beside several Asian, European, and American strains of the same species. The Upv sample was placed near four Chinese strains (GenBank acc. no. CP041199.1, CP021991.1, CP021990.1, and CP021988.1), one Japanese strain (GenBank acc. no. AP018561.1), one English strain (GenBank acc. no. CP086127.1), and two American strains (GenBank acc. no. AF222894.1 and CP000942.1), of the same species. This style of positioning was due to the absence of any genetic variations in the investigated sample. Furthermore, the clustering of these bacterial samples inside the primary clade could indicate the presence of relatively similar phylogenetic distribution patterns across these sequences within this clade. As a result, the numerous Asian, American, and European origins of our examined samples could not be ruled out. The most essential concept in this context, as with the ureB/ureA fragment, was represented by the determined location of the integrated samples within the *Ureaplasma parvum* serovar 3.

In the vicinity of the *Ureaplasma parvum* clade, in which the Upv sample was positioned, it was found that the *upV* sequences have relatively close phylogenetic positions to the clade of *Mycoplasma fermentans* with only four strains. Thus, it was indicated that *Ureaplasma parvum* sequences share a relatively high ratio of homology with *Mycoplasma fermentans* sequences. However, it was noted that this fragment has the lowest biological diversity among all other investigated loci in this study. This is due to the lower numbers of the observed similar sequences of the Upv fragment than that found in *ureA/ureB* sequences. So, it is rational to say that the currently utilized Upv fragment has exerted a lower biological power in terms of providing further in-depth details of this fragment. The reason behind this reduced ability has resulted from the low number of deposited nucleic acid sequences of the Upv fragment in the NCBI database. This reduced number of the Upv fragments that were deposited in NCBI did not detect any biological reason to go further in the exploration of this locus. Alternatively, the amplified fragments of *ureA/ureB* have provided better results, whether in terms of identification or discrimination of the currently investigated samples.



Figure(6) The comprehensive cladogram phylogenetic tree of genetic variants of the DNA *Upv* gene fragment of one sample of human-infecting *Ureaplasma parvum*. The black-colored triangle refers to the analyzed bacterial sequences. All the mentioned numbers referred to the GenBank accession number of each referring species. The number "0.1" at the top portion of the tree refers to the degree of scale range among the comprehensive tree categorized organisms. The letter "*Upv*" refers to the code of the investigated sample.

Conclusions

Ureaplasma parvum serovar 3 is the strain that causes human infection in Iraq, according to the current study, and the *urease gene* is useful for both diagnosing and detecting polymorphisms within the species. The *upV gene* also exhibited the potential to molecularly diagnose this type of bacteria. Understanding the genetic differences between bacterial strains may help in the development of future bacterial infection diagnostic and treatment methods.

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