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Polymerase chain reaction–hybridization method using urease gene sequences for high-throughput *Ureaplasma urealyticum* and *Ureaplasma parvum* detection and differentiation

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ABSTRACT

In this article, we discuss the polymerase chain reaction (PCR)–hybridization assay that we developed for high-throughput simultaneous detection and differentiation of *Ureaplasma urealyticum* and *Ureaplasma parvum* using one set of primers and two specific DNA probes based on urease gene nucleotide sequence differences. First, *U. urealyticum* and *U. parvum* DNA samples were specifically amplified using one set of biotin-labeled primers. Furthermore, amine-modified DNA probes, which can specifically react with *U. urealyticum* or *U. parvum* DNA, were covalently immobilized to a DNA–BIND plate surface. The plate was then incubated with the PCR products to facilitate sequence-specific DNA binding. Horseradish peroxidase–streptavidin conjugation and a colorimetric assay were used. Based on the results, the PCR–hybridization assay we developed can specifically differentiate *U. urealyticum* and *U. parvum* with high sensitivity (95%) compared with cultivation (72.5%). Hence, this study demonstrates a new method for high-throughput simultaneous differentiation and detection of *U. urealyticum* and *U. parvum* with high sensitivity. Based on these observations, the PCR–hybridization assay developed in this study is ideal for detecting and discriminating *U. urealyticum* and *U. parvum* in clinical applications.

T-strain mycoplasmas were first isolated from the urethral discharge of men with nongonococcal urethritis (NGU) in 1954 [1] and ascribed to a new genus and species, *Ureaplasma urealyticum,* in 1974 [2]. *U. urealyticum* was categorized into 14 serotypes [3] and divided into 2 biotypes [4–6]. According to a phylogenetic analysis, in 1999 biovar 1 was established as a new species, *Ureaplasma parvum,* and biovar 2 retained the *U. urealyticum* designation [7]. Since *Ureaplasma spp.* were first isolated, many scientists have attempted to determine whether these bacteria cause a disease. Studies show that *U. spp.* are involved in many invasive infections such as NGU [8], prostatitis [9], arthritis [10], and meningitis [11]. However, these early studies from the 1960s to the 1990s [12–15] show conflicting results due to a lack of differentiation between *U. parvum* and *U. urealyticum.* Since the new *U. spp.* classification was established, many researchers have attempted to detect and differentiate these two species. Several studies [16–18] have reported that *U. urealyticum* is more pathogenic than *U. parvum.* For instance, NGU and an adverse pregnancy outcome are only implicated with the presence of *U. urealyticum.* Therefore, *U. urealyticum* and *U. parvum* should exhibit differences in virulence during infection.

Recently, polymerase chain reaction (PCR)-based methods have been used to distinguish the *U. spp.* However, many methods focus on NGU male patients and are based on the 16SrRNA sequence [19,20]. *U. spp.* can be isolated from approximately 40–80% of women of childbearing age [21]. Because it colonizes the lower genital tract of women, it can spread into the body and exert a pathogenic infection in females, which can be dangerous for an infant (e.g., chorioamnionitis [22], infertility [23], neonatal pneumonia [11]). The urease gene not only is used to hydrolyze urea but also is required for growth even in a highly complex medium [24].

Abbreviations used: NGU, nongonococcal urethritis; PCR, polymerase chain reaction; ATCC, American Type Culture Collection; HRP, horseradish peroxidase; SSC, standard saline citrate; SDS, sodium dodecyl sulfate.

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We developed a PCR–hybridization assay based on different urease gene sequences for high-throughput simultaneous detection and differentiation of *U. urealyticum* and *U. parvum* using random female swab samples from an infertility department.

Materials and methods

Reagents and bacteria

U. urealyticum (ATCC 26618) and *U. parvum* (ATCC 27815) were obtained from Wang Bei (Dong Nan University). *Mycoplasma genitalium* (ATCC 33530) was a generous gift from Jorgen Skov Jensen. *Hemolytic streptococcus* (ATCC 21059), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9027), and *Staphylococcus aureus* (ATCC 25923) were purchased from American Type Culture Collection (ATCC) and conserved in our laboratory.

DNA–BIND plates were purchased from Sigma–Aldrich. We purchased PPLO basic medium from Difco. Horse serum was purchased from Minhai Biotechnology. We purchased 2 × PCR Master Mix and a TIANamp Bacteria DNA Kit from Tiangen Biotech and purchased horseradish peroxidase (HRP)–streptavidin from Amresco. All other chemicals were supplied by Aldrich and used as received.

Primers and probes

The forward primer used in our study was prepared as described previously [25] (5′-CAA TCT GCT CGT GAA GTA TTA C-3′), and the reverse primer was a biotin-labeled sequence (5'-biotin-ACG ACG TCC ATA AGC AAC T-3′). The DNA probes were constructed based on amplification sequence differences between *U. urealyticum* and *U. parvum* after the first PCR step. A linker (NH_2–12C) was added to the 5′ end of each capture probe.

U. urealyticum probe: 5′ NH_2-12C-AGC AAT TAA CTT CGC TGA AGG C 3';
U. parvum probe: 5′ NH_2-12C-GGC AAT TAA TTT CGC TAG TGG T 3'.

Bacterial culture and DNA extraction

The microorganisms used in this study are listed in the “Reagents and bacteria” section above. The bacteria were cultivated in the appropriate media under growth conditions. The DNA was extracted using the TIANamp Bacteria DNA Kit in accordance with the manufacturer's instructions. The DNA was quantified by measuring the optical density at 260 nm (NanoDrop 2000, Quawell, USA).

PCR amplification

The microorganisms were amplified using PCR. The amplification experiments were performed using a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) in a total volume of 50 μl containing 25 μl of 2 × Taq PCR Master Mix (Tiangen Biotech), 0.4 μM of a set of primers, and 10 ng of DNA from the different bacteria. The PCR conditions were as follows: 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s and 56 °C for 60 s and then a final extension at 72 °C for 5 min. The PCR products were analyzed using 2.5% agarose gel electrophoresis containing ethidium bromide in 1 × TAE (Tris–acetate–EDTA) buffer for 30 min at 110 V.

PCR–hybridization assay

The PCR products were detected using a DNA–BIND plate and an HRP-based colorimetric assay. Schematic illustrations are provided in Fig. 1.

The protocol used was in accordance with the manufacturer's instructions. Briefly, the capture probes (50 pmol/well) were first pre-incubated at 37 °C for 1 h in a total volume of 50 μl. Uncoupled probes were removed by washing wells with TBS (50 mM Tris [pH 7.5] containing 150 mM sodium chloride) three times. Next, the wells were blocked by 200 μl of blocking buffer (10% BSA [bovine serum albumin] and 1 mM lysine in TBST (TBS containing 0.5% Tween 20) and incubated at 37 °C for 60 min. We added 10 μl of denatured amplification products (100 °C for 10 min before quickly transferring to the ice for 5 min) into each well with 190 μl of hybridization solution (5 × SSC [1.0% casein, 0.1% N-lauroylsarcosine, 0.02% sodium dodecyl sulfate] and 0.1% sodium dodecyl sulfate [SDS]) and incubated at 65 °C for 60 min. At the end of incubation, the wells were washed with a pre-warmed washing buffer (2 × SSC and 0.1% SDS) three times, followed by another TBST wash. HRP–streptavidin (1:1000) was added to each well with 50 μl of blocking buffer in the final volume and incubated at 37 °C for 30 min before washing two times with TBST and once more with soaking buffer (2 × SSC and 1% SDS) for 5 min. Finally, TMB (3,3,5,5'-tetramethylbenzidine) was added to each well and incubated in the dark for 20 min, followed by adding a stop solution (2 M sulfuric acid) to terminate the reaction. The absorbance was read at 450 nm using a 96-well plate reader (BioTek Synergy^2).

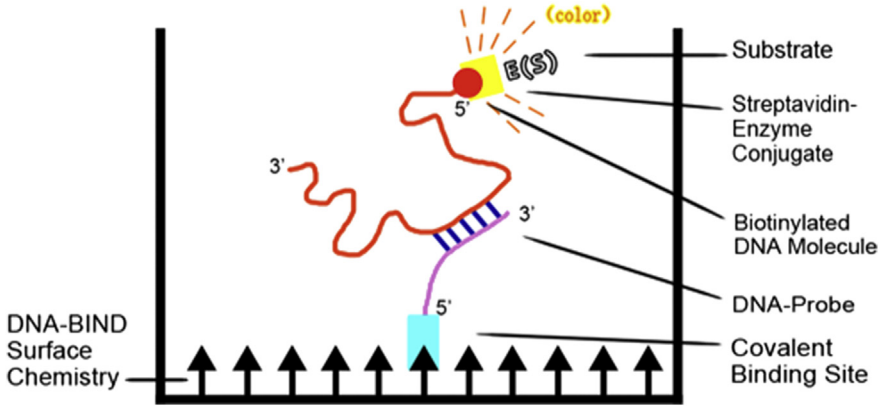

Fig.1. Schematic description of the PCR–hybridization method. A biotinylated DNA molecule was amplified from *U. urealyticum* or *U. parvum* using a normal upstream PCR primer and a biotin-labeled downstream primer. The DNA probe is a *U. urealyticum*-specific or *U. parvum*-specific nucleotide sequence for capturing the amplified biotinylated DNA molecule.