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Research Article

Rapid PCR Detection of Mycoplasma hominis, Ureaplasma urealyticum, and Ureaplasma parvum

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1. Introduction

Mycoplasma hominis, Ureaplasma urealyticum, and Ureaplasma parvum are small, fastidious bacteria belonging to the Mollicutes class. They lack a cell wall (preventing staining with Gram stain) and are not sensitively detected on routine bacterial cultures. Optimal recovery requires specialized media and growth conditions. There are several human pathogens in the genera Mycoplasma and Ureaplasma which are responsible for a variety of clinical manifestations involving multiple body systems [1]. M. hominis causes septic arthritis and postpartum fever and has been associated with pelvic inflammatory disease and bacterial vaginosis [2]. Ureaplasma species can cause acute urethritis and have been associated with bacterial vaginosis, preterm birth, and neonatal respiratory disease [1, 3].

Although M. hominis and Ureaplasma species can be cultured, this requires technical skill for interpretation of

microscopic colonies and takes two to five days. *U. ure-alyticum* was the only *Ureaplasma* species until 2002, when *U. parvum* was described [4]. The two are not distinguished based on culture characteristics alone. Real-time PCR detection of these microorganisms from clinical samples circumvents technical issues related to culture and shortens turnaround time for detection and identification.

Few real-time PCR assays and associated studies have been described for *M. hominis*. A real-time PCR assay targeting *M. hominis gap* identified two positive cervical swabs from women being evaluated for infertility [5]. 153 urogenital specimens were tested with a real-time PCR assay targeting *M. hominis yid*C, of which 45 were PCR- and culture positive and 10 PCR positive only [6]. Finally, extragenital *M. hominis* infection was diagnosed in three patients using a real-time PCR assay targeting the *M. hominis* 16S ribosomal RNA gene [7].

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	Mycoplasma hominis tuf (set 1	number 793, TIB MolBio, Aldelphia, NJ; 10X concentration) ^a
Primers	<i>tuf</i> 193F	5' AATTGATATGTTTAAAGATGATGAAAGAGA 3'
	tuf 193R	5' TGTATCAACAGCATCCATTAATTCC 3'
Probes	tuf 193fl ^b	5' GACGTAAGAAGCCTTCTATCAGAATATGGT FITC 3'
	tuf 193iLC610°	5' Red610 TGATGGTGACAATGCTCCTATTATTGCTGGTTC PO4 3'
	Ureaplasma species ure	C (set number 684, TIB MolBio; 10X concentration) ^d
Primers	ureC 158F	5' CCTGCTTCGTTTAATGTATCTG 3'
	ureC 158R	5' GAAGATCCAATCTTTGAACAAATCGTA 3'
	ureC 158R5	5' GAAGATCCAATCTTTGAACAAATTGCT 3'
Probes	ureC 158fl ^b	5' AGCAACTGTTAATGCTAAGTCAATAGCGTTTCCTG FITC 3'
	ureC 158iLC610 ^c	5' Red610 GCCCCTCAGTCTTCGTGAATCTTAAGACCACAAGC PO4 3'

TABLE 1: Primers and probes.

There has been more work on real-time PCR assays for *Ureaplasma* species, although some have described assays but have not evaluated clinical specimens or clinical isolates [8]. A real-time PCR assay that detects and distinguishes *U. urealyticum* from *parvum* was described but used to assess 87 vaginal swabs [9]. Tang et al. used a real-time PCR assay that detects and distinguishes *U. parvum* and *urealyticum* to test 346 genitourinary swabs; 120 were positive for the former and 21 for the latter, including 5 positive for both [10]. Finally, Vancutsem et al. used a real-time PCR assay for detection and differentiation of *U. urealyticum* and *parvum* to evaluate 300 lower genital tract specimens; 132 were culture positive, of which all plus an additional 19 were PCR-positive (19, *U. urealyticum*; 120, *U. parvum*; 12, *Ureaplasma* species) [11].

Herein, we present one real-time PCR assay for the detection of *M. hominis* and another for the detection and differentiation of *Ureaplasma* species and report results of these assays on 283 genitourinary specimens in comparison to culture.

2. Materials and Methods

- 2.1. Clinical Specimens. 283 genitourinary specimens (swabs, urine) submitted to the Mayo Clinic Clinical Microbiology Laboratory in transport medium (e.g., UTM, M5) for *M. hominis* and *Ureaplasma* culture were evaluated. No clinical data associated with these specimens was available. This study was approved by the Mayo Clinic Institutional Review Board.
- 2.2. Mycoplasma hominis Culture. Samples were placed into arginine broth, incubated at 35°C, and monitored four times daily for up to five days. Color change (indicating an alkaline pH shift) in the arginine broth prompted subculture of 50 μ L to an A7 agar plate. Plates were incubated anaerobically at 35°C for up to five days and examined daily with an inverted light microscope for "fried egg" morphology colonies.
- 2.3. Ureaplasma Culture. Samples were placed into U9 broth, incubated at 35°C, and monitored four times daily for up to

five days. A color change (indicating an alkaline pH shift) in the U9 broth prompted subculture of 100 μ L to an A7 agar plate. Plates were incubated anaerobically at 35°C for up to 48 hours and examined with an inverted light microscope for small, circular to irregular colonies growing into the surface of the agar, with a surrounding red zone. Confirmation of *Ureaplasma* species was indicated by golden-brown stained colonies with the addition of 0.167 M CO(NH₂)₂ and 0.04 M MnCl₂ in water.

- 2.4. Sample Processing for PCR. Samples were vortexed and 200 μ L transferred to a MagNA Pure sample cartridge (Roche Applied Science, Indianapolis, IN). DNA extraction was performed on the MagNA Pure LC 2.0 using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Applied Science) with a final elution volume of 100 μ L.
- 2.5. Polymerase Chain Reaction Assay. Primers and probes (Table 1) were designed using the LightCycler Probe Design Software, version 2.0 (Roche Diagnostics, Indianapolis, IN, USA) and DNA Workbench, version 5.7.1 (CLC Bio, Cambridge, MA, USA). Positive control plasmids were constructed for the three target-specific genes (Table 1) using the pCR 2.1 TOPO TA Cloning Kit (Invitrogen Corporation, Carlsbad, CA, USA). Sources for the inserted target sequences were M. hominis ATCC 23114, U. urealyticum ATCC 27618, and U. parvum ATCC 27815D. Plasmids were purified using the High Pure Plasmid Isolation Kit (Roche Applied Science). Sizes of the cloned inserts were confirmed by EcoR1 digestion. Plasmid inserts were sequenced using M13 forward and reverse primers included in the cloning kit, to confirm proper insert orientation. Plasmids were diluted in Tris-EDTA buffer (pH 8.0) and stored at 4°C.

The two assays were independently optimized on the LightCycler 480 II platform employing LightCycler 480 Software version 1.5 (Roche Applied Science). 15 μ L of PCR master mix, containing final concentrations of 1X Roche Genotyping Master (*Taq* DNA polymerase, PCR reaction

^atuf target corresponds to 66720-66912 of GenBank accession number FP236530.

^bLabeled with fluorescein on 3' end.

^cLabeled with LC610 on 5' end and a phosphate on 3' end.

 $^{^{\}rm d}\it{ure}\rm C$ target corresponds to 527786–527943 of GenBank accession number CP001184.

buffer, deoxyribonucleoside triphosphate with dUTP substituted for dTTP and 1 mM MgCl₂), 1 mM (additional) MgCl₂, and 1X of each of the LightCycler primer-probe sets (Table 1) were added to a 96-well LightCycler 480 plate. Extracted nucleic acid (5 μ L) was then added to each well. The cycling program was as follows: denaturation at 95°C for 10 min; amplification for 45 cycles of 10 s at 95°C, 15 s at 55°C (single acquisition), and 15 s at 72°C; melting curve analysis for 30 s at 95°C, 10 s at 59°C, 15 s at 45°C (ramp rate of 0.1°C/s), and 0 s at 80°C (ramp rate of 0.14°C/s and continuous acquisition); and cooling for 30 s at 40°C. Positive and negative controls were included in each run. The positive control consisted of the abovementioned plasmids in S.T.A.R. buffer: sterile water (1:1) at a concentration of 1,000 targets/ μ L. The negative control consisted of 1,000 colony forming units of Escherichia coli ATCC 25922 S.T.A.R. buffer: sterile water (1:1) at a concentration of 1,000 targets/µL.

2.6. Polymerase Chain Reaction Sensitivity and Specificity. Predicted amplified product, primer, and probe sequences were subjected to BLAST searches using the National Center for Biotechnology Information (NCBI) genomic database (http://www.ncbi.nlm.nih.gov/). Analytical sensitivity was assessed by spiking a series of six tenfold dilutions of quantified genomic DNA from M. hominis ATCC 23114, U. urealyticum ATCC 27816, and U. parvum ATCC 27815D into genitourinary samples. Each dilution was extracted in triplicate and each extract was assayed in duplicate. The limit of detection was the lowest dilution where all six replicates were detected. Inclusivity and cross-reactivity were assessed using a panel organisms (Table 2), including 16 members of the Mollicutes class.

Clinical sensitivity and specificity were assessed by assaying the aforementioned clinical specimens and comparing results to those of culture. Discordant samples were tested courtesy of Dr. Stellrecht, at an independent clinical laboratory (Albany Medical Center) with a previously described assay [12].

The ability of the *Ureaplasma* assay to differentiate *urealyticum* from *parvum* was assessed as follows. Cultured isolates from clinical samples were directly subjected to PCR with species differentiation based on melting curve analysis; sequence variations underlying the probed regions of *U. urealyticum* and *parvum* result in separation of the melting temperature of the two species (Figures 1 and 2). Results were compared to those of a previously described conventional PCR speciation method targeting the multiplebanded antigen using primers UMS-57 and UMA222 for *U. parvum* and UMS-170 and UMA263 for *U. urealyticum* [13].

2.7. Statistical Analysis. Assessment of the assays' sensitivity and specificity, with associated 95% confidence intervals (CI), compared to that of culture for *M. hominis* and *Ureaplasma* species was made using SAS software version 9.1 (SAS, INC, Cary, NC, USA).

3. Results

3.1. Polymerase Chain Reaction Sensitivity and Specificity. The analytical sensitivity of both assays was 100 genome copies/ μ L

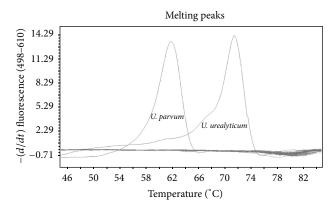


FIGURE 1: Melting curves of *Ureaplasma urealyticum* and *Ureaplasma parvum*.

genitourinary specimen. Amplified product, primer, and probe sequences were subjected to NCBI database searches using BLAST software; no significant homology was noted outside of the genera targeted by these assays. Nucleic acid material from members of the Mollicutes class, excluding *M. hominis* and the *Ureaplasma* species, was not detected (Table 2).

3.2. Clinical Sensitivity and Specificity. The M. hominis PCR assay had a clinical sensitivity and specificity of 90.7% (95% CI: 77.4%, 97.3%) and 99.2% (95% CI: 97.0%, 99.9%), respectively (Table 3). The 6 discordant results were tested at the Albany Medical Center using an assay targeting the 16S ribosomal RNA gene; [12] both PCR positive/culturenegative specimens were PCR positive, and three of four PCR negative/culture-positive specimens were PCR negative.

The *Ureaplasma* PCR assay had a clinical sensitivity and specificity of 96.5% (95% CI: 92.1%, 98.9%) and 93.8% (95% CI: 88.1%, 97.0%), respectively (Table 3). The 14 discordant results were tested at Albany Medical Center; [12] five of nine specimens that were PCR positive/culture negative were PCR positive, and all five specimens that were PCR negative/culture positive were PCR negative. Of the specimens that tested positive for *Ureaplasma* species by PCR and were culture positive, *U. urealyticum* alone was detected in 28, *U. parvum* alone in 109, and both in 2. Among the PCR positive/culture-negative specimens, *U. urealyticum* was detected in 3 and *U. parvum* in 6.

Thirty-one culture isolates of *Ureaplasma* species were tested with the *Ureaplasma* assay and a previously reported PCR method that differentiates between the two species [13]. The reference method yielded species-level identification for 20 isolates, including 4 *U. urealyticum* and 16 *U. parvum*, with identical results to the assay described herein. The remaining 11 isolates were speciated by the assay described herein but not by the reference method; they were confirmed to be *Ureaplasma* species by partial 16S ribosomal RNA gene sequencing [14]. All partial 16S ribosomal RNA gene sequences were identical to one another and were perfect matches to bases 145,365 through 145,845 of GenBank AF222894.1

 $\label{thm:constraint} \textbf{Table 2: Cross-reactivity and inclusivity panel. Only $Mycoplasma\ hominis$, $Ureaplasma\ parvum$, and $Ureaplasma\ urealyticum$ were detected (by the appropriate assays).}$

Organism	Accession no. or source	Organism	Accession no. or source
Acholeplasma laidlawii	ATCC 23206	Entamoeba histolytica	ATCC 30459
Acinetobacter baumannii	ATCC 19606	Entamoeba moshkovskii	ATCC 30042
Acinetobacter lwoffii/haemolyticus	QC Strain	Enterobacter cloacae	ATCC 13047
Actinomyces odontolyticus	ATCC 17929	Enterococcus faecalis	ATCC19433U
Aeromonas hydrophila	CAP-D-1-82	Enterococcus faecium	ATCC 19434
Arcanobacterium haemolyticum	ATCC 9345	Escherichia coli	ATCC 25922
Arcanobacterium pyogenes	ATCC 19411	Escherichia coli O142:K86(B):H6	ATCC 23985
Parabacteroides distasonis	ATCC 8503	Escherichia coli O157:H7	ATCC 35150
Bacteroides fragilis	ATCC 25285	Escherichia coli O70:K:H42	ATCC 23533
Bacteroides thetaiotaomicron	ATCC 29741	Escherichia fergusonii	ATCC 35469
Bacteroides vulgatus	ATCC 29327	Escherichia hermannii	ATCC 33650
Bifidobacterium adolescentis	ATCC 15703	Escherichia vulneris	ATCC 33821
Bifidobacterium bifidum	ATCC 29521	Eubacterium rectale	ATCC 33656
Bordetella bronchiseptica	ATCC 19395	Finegoldia magna	ATCC 29328
Bordetella holmesii	ATCC 51541	Fluoribacter bozemanae	ATCC 33217
Bordetella parapertussis	ATCC 15311	Fluoribacter gormanii	ATCC 33297
Bordetella pertussis	ATCC 9797	Fusobacterium nucleatum	ATCC 25559
Burkholderia cepacia	SCB1277	Gardnerella vaginalis	NYS 4-87
Campylobacter coli	ATCC 33559	Giardia lamblia	ATCC 30957
Campylobacter jejuni	ATCC 33560	Haemophilus influenzae	ATCC 10211
Chlamydia trachomatis	ATCC VR-348B	Human DNA	MRC-5 cells
Chlamydophila pneumoniae	ATCC 53592	Klebsiella oxytoca	ATCC 700324
Chlamydophila pneumoniae	ATCC VR-1310	Klebsiella pneumoniae	ATCC 700603
Citrobacter freundii	ATCC 8090	Lactobacillus delbrueckii ssp. lactis	ATCC 12315
Clostridium difficile	ATCC 9689	Lactobacillus rhamnosus	ATCC 7469
Clostridium perfringens	ATCC 13124	Fluoribacter dumoffii	ATCC 33279
Clostridium ramosum	ATCC 25582	Legionella jordanis	ATCC 33623
Collinsella aerofaciens	ATCC 25986	Legionella longbeachae	ATCC 33462
Corynebacterium diphtheriae	SCB-25-86	Tatlockia micdadei	ATCC 33204
Corynebacterium oseudodiphtheria	NY-4-88	Legionella pneumophila	ATCC 33152
Cryptosporidium species	feline isolate	Legionella wadsworthii	ATCC 33877
Dientamoeba fragilis	ATCC 30948	Listeria monocytogenes	ATCC 15313
Eggerthella lenta	ATCC 25559	Moraxella catarrhalis	ATCC 8176
Encephalitozoon cuniculi	JS strain	Morganella morganii	CAP-D-5-79
Encephalitozoon hellem	ATCC 50451	Mycobacterium africanum	ATCC 25420
Encephalitozoon intestinalis	ATCC 50651	Mycobacterium avium	ATCC 700398
Mycobacterium avium	ATCC 700897	Proteus mirabilis	ATCC 35659
Mycobacterium bovis	ATCC 19210	Proteus vulgaris	QC strain
Mycobacterium bovis (BCG)	ATCC 35735	Pseudomonas aeruginosa	ATCC 27853
Mycobacterium gordonae	ATCC 14470	Pseudomonas fluorescens/putida	CDC-AB4-B10-8
Mycobacterium intracellulare	ATCC 35761	Rhodococcus equi	ATCC 6939
Mycobacterium kansasii	ATCC 12478	Salmonella enterica	ATCC 35987
Mycobacterium microti	ATCC 19422	Salmonella serogroup B	CAP-D-1-69
Mycobacterium smegmatis	ATCC 19980	Shigella dysenteriae	CDC 82-002-72
Mycobacterium tuberculosis	ATCC 25177	Shigella flexneri serotype 2a	ATCC29903

Table 2: Continued.

Organism	Accession no. or source	Organism	Accession no. or source ATCC 25931	
Mycobacterium tuberculosis	ATCC 27294	Shigella sonnei		
Mycobacterium tuberculosis	ATCC 35825	Staphylococcus aureus	ATCC 25923	
Mycobacterium tuberculosis	ATCC 35837	Staphylococcus epidermidis	ATCC 14990	
Mycoplasma arginini	ATCC 23838D	Stenotrophomonas maltophilia	SCB-33-77	
Mycoplasma arthritidis	ATCC 19611D	Streptococcus bovis	CAP-D-16-83	
Mycoplasma bovis	ATCC 25523D	Streptococcus pneumoniae	ATCC 49619	
Mycoplasma buccale	ATCC 23636	Streptococcus pyogenes	ATCC 19615	
Mycoplasma faucium	ATCC 25293	Streptococcus sanguinis	ATCC 10556	
Mycoplasma fermentans	ATCC 19989	*Ureaplasma parvum	ATCC 28715	
Mycoplasma genitalium	ATTC 33530	*Ureaplasma urealyticum	ATCC 27618	
*Mycoplasma hominis	ATCC 23114	Yersinia enterocolitica	ATCC 9610	
Mycoplasma hyorhinis	ATCC 17981D	BK polyomavirus	ATCC VR-837	
Mycoplasma lipophilum	ATCC 27104	Cytomegalovirus	ATCC VR-538	
Mycoplasma orale	ATCC 23714			
Mycoplasma phocidae	ATCC 33657	Herpes simplex virus 1	Lab Control	
Mycoplasma pirum	ATCC 25960D	Herpes simplex virus 2	Lab Control	
Mycoplasma pneumoniae	ATCC 15531D	Human adenovirus 9	ATCC VR-1086	
Mycoplasma salivarium	ATCC 23064	Human coronavirus 229E	ATCC VR-740	
Neisseria gonorrhoeae	ATCC 43069	Human coxsackievirus B 1 (Enterovirus)	ATCC VR-28	
Neisseria lactamica	ATCC 23970	Human herpesvirus 6B	ATCC VR-1467	
Neisseria meningitidis	ATCC 13077	Human herpesvirus 7	ABI 08765000	
Nocardia brasiliensis	ATCC 51512	Human herpesvirus 8	ABI 08735000	
Nocardia brevicatena	ATCC 15333	Human parainfluenza virus 1	ATCC VR-94	
Nocardia carnea	ATCC 6847	Human parainfluenza virus 3	ATCC VR-93	
Nocardiopsis dassonvillei	ATCC 23218	Respiratory syncytial virus A2	ATCC VR-1540	
Nocardia farcinica	ATCC 3318	Respiratory syncytial virus B	ATCC VR-1401	
Nocardia otitidiscaviarum	ATCC 14629	Influenza A virus (H3N2)	ATCC VR-810	
Nocardia transvalensis	ATCC 6865	Influenza B virus	ATCC VR-791	
Plesiomonas shigelloides	ATCC 14029	Measles virus	ATCC VR-24	
Porphyromonas gingivalis	ATCC 33277	Mumps virus	ATCC VR-365	
Prevotella melaninogenica	ATCC 25845	Varicella-zoster virus	ATCC VR-1367	
Prevotella oralis	ATCC 33269			

	Sequence 5'-3'	
U. urealyticum	ureC 158fl ureC 158iLC610 AGCAACTGTTAATGCTAAGTCAATAGCGTTTCCTG GCCCCTCAGTCTTCGTGAATCTTAAGACCACAAGC	
U. parvum	AACAAGTT	

Figure 2: *Ureaplasma* probe design.

Table 3: Comparison of PCR with culture for *Mycoplasma hominis* and *Ureaplasma* species detection.

		M. hominis culture		
		Positive	Negative	
	Positive	38	2	40
M. hominis PCR	Negative	4	239	243
		42	241	283

Sensitivity = 90.5% (95% CI: 77.4%, 97.3%), specificity = 99.2% (95% CI: 97.0%, 99.9%)

		Ureaplasma species culture		
		Positive	Negative	
	Positive	139 ¹	9 ²	148
Ureaplasma PCR	Negative	5	130	135
		144	139	283

Sensitivity = 96.5% (95% CI: 92.1%, 98.9%), specificity = 93.5% (95% CI: 88.1%, 97.0%)

(*U. parvum*) and bases 40 through 520 of GenBank L08642.1 (*U. urealyticum*).

4. Discussion

We describe two rapid real-time PCR assays, one for detection of *M. hominis* and the other for detection of *Ureaplasma* species; they have comparable performance to culture but yield results in three hours, instead of two to five days for culture. These assays are performed on a standard platform and are adaptable to automation, a potential advantage over other described methods, especially for large reference laboratories that process large numbers of specimens.

We are not aware of other real-time PCR studies that have assessed *M. hominis* and *Ureaplasma* species using the same set of clinical samples. Overall, 14% of tested specimens were PCR positive for *M. hominis* and 52% for *Ureaplasma* species. A multiplex PCR enzyme-linked immunosorbent assay was used to detect *M. hominis* and *U. parvum* and *urealyticum* in cervical swabs from 175 Australian women with and without cervicitis; 16% tested positive for *M. hominis* and 68% for *Ureaplasma* species [15]. Multiplex PCR and autocapillary electrophoresis were used to detect *M. hominis* and *Ureaplasma* species (without differentiating *U. parvum* from *urealyticum*) in genitourinary specimens from 113 South Koreans with sexually transmitted infections; 12% were positive for *M. hominis* and 43% for *Ureaplasma* species [16]. These findings are similar to ours [15, 16].

Our PCR assay not only detects *Ureaplasma* species but also differentiates *U. parvum* from *urealyticum*. As in prior studies, *U. parvum* was more common than *U. urealyticum*, [10, 11, 15, 17] with 41% of the genitourinary specimens testing positive for the former and 12% for the latter. In one prior study, 63% of specimens were positive for *U. parvum* and 7% for *U. urealyticum* [15]. Another study showed, using

a multiplex PCR-reverse line blot assay, that 48% of first voided urine specimens from women attending sexual health clinics in Australia were positive for *U. parvum* and 25% for *U. urealyticum* [17]. In the study by Tang et al., 36% of genitourinary swabs collected from hospitalized males and females in China were positive for *U. parvum* and 8% for *U. urealyticum* [10]. Finally, in study by Vancutsem et al., 44% of lower genital tract specimens obtained from healthy women at their first prenatal visit in Belgium were positive for *U. parvum* and 10% for *U. urealyticum* [11]. Despite different geographic locales and clinical status, these numbers are strikingly similar.

In addition to the advantage of speed, the described assays overcome the challenges of detection of these organisms by culture. Although culture is considered a gold standard method (and was so considered in this study), colonial identification is challenging and subjective because it is done using the human eye and a dissecting microscope. Artifacts may be misidentified as colonies, yielding false-positive results, or colonies may be overlooked, yielding false-negative results. Although PCR may be considered more technically complex, in a laboratory where technologists are familiar with PCR, this approach is more user-friendly (and generalizable among assays for various microorganisms) than culture.

The described assays may be useful for investigating epidemiology and pathogenesis of infections with *U. parvum* and *urealyticum* [2, 18]. Although extra-genital specimens were not tested, the described *M. hominis* assay may be useful to detect extra-genital *M. hominis* infections [7].

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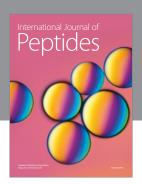
 $^{^{1}}U$. urealyticum (n = 28), U. parvum (n = 109), U. urealyticum and U. parvum (2).

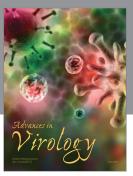
²U. urealyticum (n = 3), U. parvum (n = 6).

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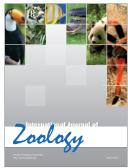








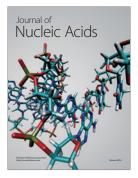




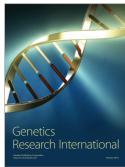


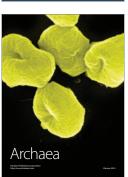


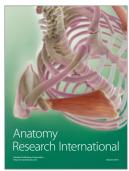
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