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Short communication

A quantitative TaqMan PCR assay for the detection of *Ureaplasma diversum*

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ABSTRACT

Ureaplasma diversum in veterinary studies is an undesirable microbe, which may cause infection in bulls and may result in seminal vesiculitis, balanopostitis, and alterations in spermatozoids, whereas in cows, it may cause placentitis, fetal alveolitis, abortion, and birth of weak calves. *U. diversum* is released through organic secretions, especially semen, preputial and vaginal mucus, conjunctival secretion, and milk. The aim of the present study was to develop a TaqMan probe, highly sensitive and specific quantitative PCR (qPCR) assay for the detection and quantification of *U. diversum* from genital swabs of bovines. Primers and probes specific to *U. diversum* 16S rRNA gene were designed. The specificity, detection limit, intra- and inter-assay variability of qPCR to detect this ureaplasma was compared with the results of the conventional PCR assay (cPCR). Swabs of vaginal mucus from 169 cows were tested. The qPCR assay detected as few as 10 copies of *U. diversum* and was 100-fold more sensitive than the cPCR. No cross-reactivity with other Mollicutes or eubacteria was observed. *U. diversum* was detected in 79 swabs (46.42%) by qPCR, while using cPCR it was detected in 42 (25%) samples. The difference in cPCR and qPCR ureaplasma detection between healthy and sick animals was not statistically significant. But the *U. diversum* load in samples from animals with genital disorders was higher than in healthy animals. The qPCR assay developed herein is highly sensitive and specific for the detection and quantification of *U. diversum* in vaginal bovine samples.

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1. Introduction

Ureaplasmas are pleomorphic organisms, microaerophilic and mesophilic that hydrolyze urea, release ammonia and thus increase pH *in vitro*. Their genome size is approximately 750 kbp, with a G–C% content of 26.9–28.0% in organisms isolated from humans and 29.7–30.2% in organisms isolated from bovine. Ureaplasmas colonize the respiratory and urogenital tract of animals and humans. *Ureaplasma diversum* is a bovine origin mollicute, which was previously considered a non-pathogenic species. These organisms were initially isolated from the reproductive tract of a bovine by Taylor-Robinson et al. (1967); 11% of clinically healthy cows had ureaplasmas in vaginal mucus. Several subsequent studies detected ureaplasma in the genital tract of bovines without reproductive problems. Therefore, it was initially of interest that these organisms were not pathogenic.

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Currently *U. diversum* is considered a pathogenic species due to its strong association with cattle diseases such as placentitis, fetal alveolitis, abortion and birth of weak calves (Miller et al., 1983; Doig et al., 1980a,b). Doig et al. (1980a,b) performed experimental infections in cows and observed granular vulvovaginitis. Experimental infection with *U. diversum* strains inoculated in the mammary gland of sheep and cattle indicated variable virulence among the studied strains. This justified the belief that the ureaplasma found in healthy animals does not cause disease (Ball and Mackie, 1985; Howard et al., 1973). Nevertheless, little is known about the virulence and pathogenic mechanisms of this mollicute, including its capsule, toxins and adherence. In the present study we developed and standardized a quantitative TaqMan PCR to detect *U. diversum* in bovine vulvovaginal swabs.

2. Material and methods

2.1. Bacterial strains and culture conditions

The *U. diversum* ATCC 449782 was used as positive control. The following microorganisms were also used as controls: *Mycoplasma bovis* Donetta, *Mycoplasma dispar* M29/79, *Mycoplasma mycoides* subsp. *mycoides* SC PG1, *Mycoplasma mycoides* subsp. *mycoides* LC Y-goat, *Mycoplasma bovirhinis* PG43, *Mycoplasma bovoculi* M165/69, *Mycoplasma bovigenitalium* PG11, *Mycoplasma alkalescens* PG51, *Mycoplasma canadense* 275C, *Mycoplasma arginini* G230, *Mycoplasma verecundum* GM893, *Mycoplasma cynos* H831, *Mycoplasma edwardii* PG24, *Mycoplasma caprine* SM12, *Mycoplasma felis* CO, *Mycoplasma ovipneumoniae* Y-98, *Mycoplasma pulmonis* PG34, *Mycoplasma mycoides capri* PG3, *Mycoplasma salivarium* PG20, *Mycoplasma fermentans* PG18, *Mycoplasma gallisepticum* PG31, *Mycoplasma synoviae* WUU1853, *Mycoplasma conjuntivae* HRC581, *Mycoplasma flocculare* Ms42, *Mycoplasma capricolum* Calif kid, *Mycoplasma hyopneumoniae* J, *Acholeplasma laidlawii* PG38, *Ureaplasma urealyticum* T960, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 10145, *Staphylococcus aureus* ATCC 25293, *Streptococcus pyogenes* ATCC 12345, *Klebsiella pneumoniae* ATCC 13883, *Salmonella typhimurium* C5 CIP, *Bacteroides fragilis* C45A AUS 00306, *Bacteroides vulgatus* C18E AUS 00293, and *Actinomyces viscosus* ATCC 910144. The strains were obtained from the Universidade de São Paulo and Universidade Federal Fluminense, Brazil. The species were cultured on modified Friis (FF) medium and the ureaplasma medium with 5% CMRL-1066 (with glutamine and without bicarbonate). The walled bacteria included as controls were cultured in brain heart infusion medium. DNA was extracted from log phase of cultures by a boiling method described elsewhere (Fan et al., 1995).

2.2. Field samples

A total of 169 vulvovaginal swabs were collected from cows from 7 different ranches in the state of Bahia. The samples were collected from 47 (27.8%) animals with vulvovaginitis and 122 (42.2%) from clinically healthy animals. The vulvovaginal mucus samples were transported in 3 ml of modified Ureaplasma medium (Ruhnke and Rosendal, 1994) at 4 °C. Samples were initially stored at −20 °C. After thawing the samples, the DNA was extracted using PureLink™ Genomic DNA Mini Kit. The DNA was analyzed using spectrophotometry (NanoDrop ND-1000, Witec Ag, Littau, Switzerland) to verify the success of the extraction.

2.3. Conventional PCR assays

A PCR for detecting Mollicutes was performed using primers described by (Van Kuppeveld et al., 1992), and positive samples were then submitted to a specific PCR to detect *U. diversum* based on a 16S rRNA gene sequence (Cardoso et al., 2000). Nuclease-free water was used as negative control in both assays. The amplified products were analyzed on a 1.5% agarose gel containing 0.5 mm/ml ethidium bromide in TAE buffer (40 mM Trisacetate, 2 mM EDTA, pH 8.0). The products were visualized and photodocumented under ultraviolet light using a photodocumentation system (Vilber Lourmat, Marne-la-Vallée, France).

2.4. Design of primers and probe for quantitative PCR

Primers and probes were designed based on the 16S rRNA gene of the *U. diversum*. Briefly, 16S rRNA gene sequences from other mycoplasma were aligned using the ClustalW algorithm of the MEGA4 Software. Variable regions were manually selected for the design of primers and probes used in this assay. The selected region is the same used in the cPCR described by Cardoso et al. (2000). Oligonucleotide properties were further analyzed using the Primer Express Software ver. 3.0 (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA). Primers and probe specificity were tested *in silico* using BLAST algorithms.

The following forward and reverse primer sequences were selected to amplify a 61-bp fragment of the *U. diversum* 16S rRNA gene: UDF 5′-CAT TAA ATG ATG TGC CTG GGT AGT AC-3′ and UDR 5′-CCC CGT CAA TTC CGT TTG-3′. The TaqMan probe specific to the amplicon was designed to contain the fluorescent dye 2′-chloro-7′-phenyl-1,4-dichloro-6-carboxyfluorescein (VIC) at the 5′-end and a minor groove binding (MGB) at the 3′-end (ProbeUD: 5′-TTC GCA AGA ATG AAA C-MGB 3′). The probe was custom synthesized at a commercial source (Applied Biosystems, Life Technologies Corporation).

2.5. Quantitative PCR

Absolute quantification assays were performed using a StepOne Real-Time PCR System (Applied Biosystems, Life Technologies Corporation). The real-time PCR mixture was prepared as follows: 12.5 μl of Universal PCR Master Mix (Applied Biosystems), 10 μM each primer, 10 μM VIC-labeled TaqMan MGB probes, 20 ng of or 1 μl of extracted DNA, and RNase-free water to a final volume of 25 μl. Cycling conditions were as follows: 50 °C for 10 min; 95 °C for 10 min; and 45 cycles of denaturation at 95 °C for 15 s, annealing at 59 °C for 1 min. All reactions were performed

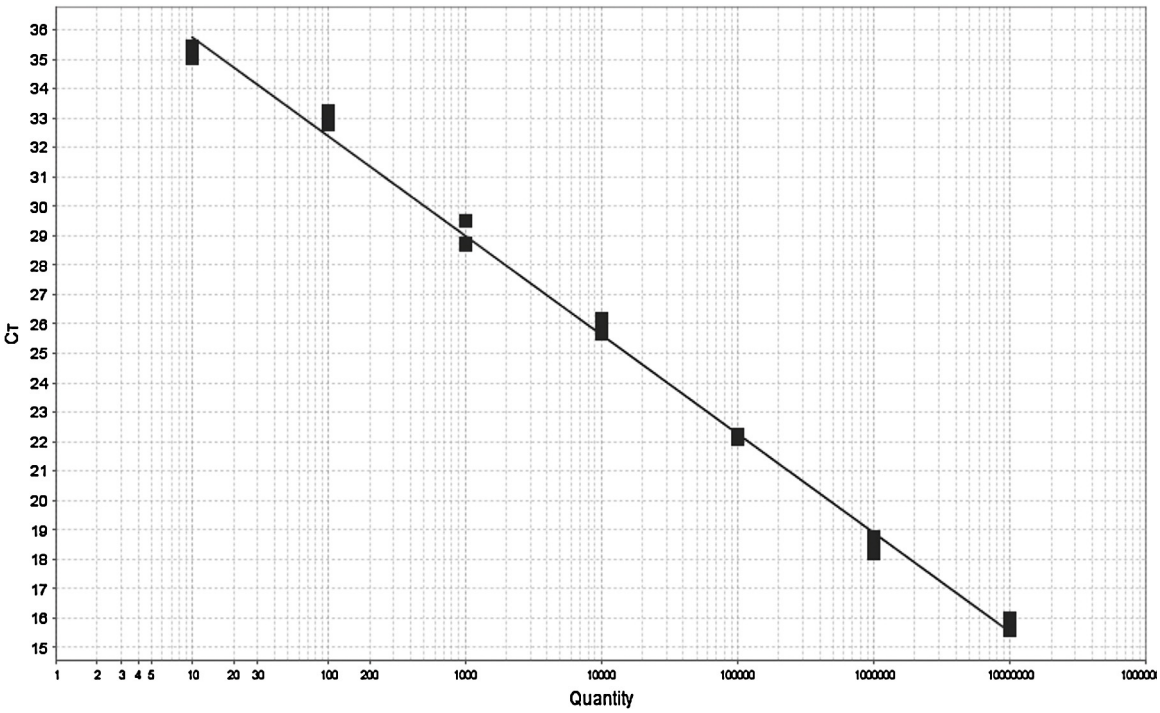

Fig. 1. Standard curves of serial dilutions of *Ureaplasma diversum* positive control calculated by StepOne Software 2.1 software (Applied Biosystems, Life Technologies Corporation).

in duplicate in a StepOnePlus real-time PCR instrument (Applied Biosystems).

For constructing DNA standards for absolute quantitation, the ureaplasmas were first cultured in 2 ml of ureaplasma medium (UB) at 37 °C and increased to 100 ml in the same broth. In a logarithmic growth phase (based in colorimetric changes), the culture was centrifuged at 20,600 × *g* for 30 min at 25 °C. The DNA was extracted using a PureLink™ Genomic DNA Mini Kit. The genomic DNA copy number was then calculated by spectrophotometry (NanoDrop ND-1000, Witec Ag, Littau, Switzerland). Ten-fold serial dilutions (10^7–10 copies/ml) of the *U. diversum* DNA standard were prepared and analyzed.

The data were acquired during the annealing step and analyzed using the StepOne Software 2.1 (Applied Biosystems, Life Technologies Corporation). The threshold was manually adjusted within the logarithmic curve, above the background level and below the linear and plateau phase. The threshold cycle (C_t) of the genome dilutions was plotted against the log number of genome copies and used as input to create the standard curve. Linear regression analyses were then applied to calculate the r^2 and slope values. Assuming 100% efficiency if the DNA template is doubled in each cycle, the PCR efficiency was calculated as $E = 10(-1/\text{slope}) - 1$, where E is PCR efficiency.

2.6. qPCR specificity and intra- and inter-assay repeatability

The DNA of the microorganisms used here as controls was tested for the specificity of the qPCR assays (above-bacterial strains). The intra-assay repeatability was determined by running five replicates of the genome ureaplasmal dilutions (10^8–1 copy of genome-bacteria/reaction) in the same run. The standard curve was then generated using the StepOne Software 2.1 (Applied Biosystems, Life Technologies Corporation). The inter-assay variability was determined by running triplicates of the same genome dilution in three different runs, on separate days. These replicates were used to determine the mean, standard deviation and coefficient of variation in C_t values for each genome dilution.

3. Results and discussion

The qPCR assay had a reaction efficiency of $E = 98.20\%$ ($r^2 = 0.996$) and consistently detected as few as ten copies of genome/reaction when using the genomic DNA copy number standards diluted in TE (Fig. 1). The intra-assay and inter-assay repeatability of the qPCR is shown in Table 1. The assay was specific for *U. diversum* because no amplification was observed with the microbial controls used. Furthermore, the qPCR technique is extremely beneficial for high-throughput laboratories, such as those serving industry, as the elapsed time for obtaining results is shorter and the technique is much less labor intensive than cPCR (Guimaraes et al., 2011). The TaqMan MGB probes have the advantage of being more specific and sensitive for detecting targeted DNA (Kutyavin et al., 2000). The MGB moiety increases the probe melting temperature (T_m) and allows for the design of a shorter and more specific probe compared with regular TaqMan probes (Kutyavin et al., 2000). The MGB probes also bind more

Table 1
Intra- and inter-assay repeatability of the qPCR assay.

Genome copies	Intra-assay repeatability Genome bacteria diluted in 1× TE		Inter-assay repeatability Genome bacteria diluted in 1× TE	
	Mean-crossing point ($C_t \pm$ SD)	CV (%)	Mean-crossing point ($C_t \pm$ SD)	CV (%)
10	35.97 ± .51	1.62	35.72 ± 0.64	1.80
10^2	33.52 ± 0.49	1.47	33.37 ± 0.46	1.37
10^3	29.97 ± 0.41	1.37	30.16 ± 0.39	1.28
10^4	26.60 ± 0.35	1.32	26.86 ± 0.29	1.10
10^5	22.29 ± 0.19	0.86	22.62 ± 0.32	1.43
10^6	19.40 ± 0.17	0.90	19.32 ± 0.41	2.10
10^7	16.03 ± 0.40	2.49	16.53 ± 0.57	3.44

CV, coefficient of variation.

Table 2
PCR for Mollicutes, cPCR and qPCR for *U. diversum* frequency in samples from healthy and sick animals and the odds ratio, confidence interval and *p*-value.

PCR	Positive clinical signs $N = 47$ *n* (%)	Negative clinical signs $N = 122$ *n* (%)	Odds ratio	IC 95%	*p*
General PCR					
Positive	34 (27.6)	89 (72.4)	0.94	[0.44; 2.00]	0.873
Negative	13 (28.9)	32 (71.1)	1		
cPCR *U. diversum*					
Positive	16 (38.1)	26 (61.9)	1.89	[0.90; 3.96]	0.092
Negative	31 (24.6)	95 (75.4)	1		
qPCR *U. diversum*					
Positive	18 (24.7)	55 (75.3)	0.74	[0.37; 1.48]	0.401
Negative	29 (30.5)	66 (69.5)	1		

efficiently to the target DNA, which increases sensitivity by decreasing background noise (Kutyavin et al., 2000; Marois et al., 2010).

The PCR for Mollicutes detected these bacteria in 123 (72.8%) clinical samples. In the samples collected, 42 (24.9%) were positive by cPCR. Surprisingly, a total of 73 (43.2%) of the 169 samples were positive by the qPCR. All samples positives in PCR for Mollicutes were positive in cPCR and qPCR. Six (8.2%) samples were positive in cPCR and negative in qPCR and 37 (50.7%) samples were positive only in qPCR. Positive samples in qPCR were sequenced and all of the sequenced PCR products showed a similarity of 96–97% with *U. diversum* ATCC 449782. The frequency of detection to PCR for Mollicutes, cPCR and qPCR to *U. diversum* in samples from healthy and sick animals and the odds ratio, confidence interval and *p*-value are summarized in Table 2. The difference in cPCR and qPCR ureaplasma detection between healthy and sick animals was not statistically significant. But the *U. diversum* load in sick animals (mean load: 2.844×10^4 CFU/μl – DNA concentration) was higher than in healthy animals (mean load: 2.469×10^3 CFU/μl – DNA concentration) (Mann–Whitney test, $p < 0.001$) (Fig. 2). As sick and healthy animals could be infected by *U. diversum*, the enhanced sensitivity of qPCR is particularly important for gaining a better understanding of the extent and impact of such infections. Probably the bacterial CFU/μl – DNA concentration could be closely related to the development of the disease. Sachse et al. (2010) also observed that the *M. bovis* load in herds with clinical disease was significantly higher than in disease-free herds.

Quantitative PCR using probes can increase the specificity of the assay, and the T_m of the probe and amplicon hybrid can be measured for further verification of PCR specificity (Cai et al., 2008). It has been reported that different sample salt concentrations due to the difference in sample type and DNA extraction may cause variation in T_m, which may be overcome using internal T_m control and high resolution melting techniques (Seipp et al., 2007). In addition, the real-time PCR methodology is more accurate and is easier to use in quantifying the target DNA.

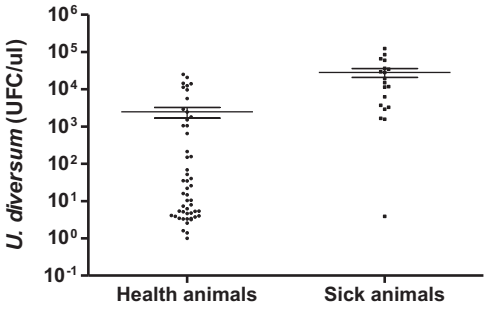

Fig. 2. *Ureaplasma diversum* load between healthy and sick animals. The *U. diversum* CFU/μl – DNA concentration in sick animals was higher than in healthy animals (Mann–Whitney test, $p < 0.001$).

Accordingly, the MGB detection system employed herein proved to be specific for *U. diversum* based on the specificity testing against other Mollicutes and *in silico* analyses using the BLAST algorithm.

In conclusion, we developed a specific, sensitive, reproducible and quantitative real-time PCR assay for the detection of *U. diversum*. This quantitative PCR showed a higher sensitivity than conventional PCR. Furthermore, it may also be a useful tool for the detecting *U. diversum* in specimens for which the culture lacks sensitivity (Férandon et al., 2011).

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