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A quantitative PCR method for assessing the presence of *Pasteurella testudinis* DNA in nasal lavage samples from the desert tortoise (*Gopherus agassizii*)

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ABSTRACT

Pasteurella testudinis has been associated with upper respiratory tract disease (URTD) in the threatened desert tortoise (*Gopherus agassizii*). Our goal was to develop a sensitive and specific qPCR method for detecting DNA from *P. testudinis* in nasal lavage fluid collected from desert tortoises in the field. Probes for 16S ribosomal RNA and RNA polymerase β-subunit (rpoB) genes were designed. A standard curve generated with DNA extracted from known numbers of bacterial cells determined by flow cytometry revealed a lower detection limit of 50 fg/ml (10 bacteria/ml). The nasal lavage fluid contained no interfering substances, and the qPCR method did not recognize normal flora DNA. The nasal lavage samples from 20 desert tortoises captured in Clark County, Nevada, USA in 2007 and housed at the Desert Tortoise Conservation Center, were all positive for *P. testudinis* DNA by qPCR. Another set of 19 lavage samples collected in 2010 from wild desert tortoises in the Mojave Desert were tested and 84% were positive for *P. testudinis* DNA. Fully validated, this qPCR method will provide a means of determining colonization rate. When used in conjunction with serological methods and clinical evaluations, both infection rate and disease rate can be determined for this potential URTD pathogen. This new assay provides an important tool for managing the threatened populations of the Mojave Desert tortoise.

1. Introduction

1Populations of the desert tortoise (*Gopherus agassizii*) in the southwestern U.S. have declined in some areas by as much as 90% over the last three decades, and these reptiles are presently listed as a threatened species (U.S. Fish and Wildlife Service, 1990, 2011). Upper respiratory tract disease (URTD) has been viewed as one possible cause of this population decline (Sandmeier et al., 2009). Most of the research since 1990 has centered on two species of mycoplasma, *Mycoplasma agassizii* and *Mycoplasma testudineum*, as the major URTD pathogens (Jacobson et al., 1991; Brown et al., 1994, 2004). However, Snipes et al. (1980) identified a *Pasteurella* spp. in nasal washings collected from desert tortoises with and without URTD; this gram negative microorganism was later classified as *Pasteurella testudinis* (Snipes and Biberstein, 1982). Jacobson et al. (1991) cultured *P. testudinis* from 100% of the nasal cavities in a small cohort of desert tortoises with documented URTD. Nasal washings from 22% of 92 desert tortoises sampled in the Mojave desert between 1989 and 1993 were culture positive for *P. testudinis* (Dickinson et al., 2001), and a significant number of the positive tortoises had signs and symptoms of URTD.

It is important to determine a range-wide colonization rate for *P. testudinis* in desert tortoises, and to correlate this with serological evidence of infection with clinical signs and symptoms of URTD. Because of the logistic difficulty of culturing *P. testudinis* from samples collected in the field, we have developed a qPCR method for analyzing *P. testudinis* DNA in preserved nasal lavage samples. We tested 20 nasal lavage samples collected in 2007 from wild caught desert tortoises housed in the Desert Tortoise Conservation Center (DTCC) in Las Vegas, NV, USA. Most of these tortoises had exhibited signs of URTD, and 100% were positive for *P. testudinis* DNA by the new qPCR method reported here. In addition we tested 19 samples collected in 2010 from desert tortoises captured and sampled in the Mojave Desert and found 84% positive.

2. Materials and methods

2.1. Cultivation and flow cytometric quantification of P. testudinis

P. testudinis type strain CCUG 19802^T (=ATCC 33688^T=NCTC 12150^T) was obtained from the American Type Culture Collection (Rockville, MD). These microorganisms were cultured at 30 °C in tryptic soy broth or on 3.5% sheep red blood cell agar. The number of viable bacteria in mid log-phase broth cultures was determined

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by a modification of the flow cytometric method described by Mohammadpour et al. (2010). Briefly, 1 ml of cells from five different broth cultures was harvested by centrifugation, washed once in phosphate buffered saline (PBS, pH 7.2), then either diluted for culture on sheep blood agar plates for colony counting or labeled for 1.5 h with 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM). Viable *P. testudinis* take up the non-fluorescent CFDA-AM and intracellular esterases convert it to the fluorescent 5-carboxyfluorescein. Viable cells were counted on a Becton–Dickinson LSR-II flow cytometer (Coulter Electronics, Hialeah, FL) using counting beads to determine the number of viable bacteria/ml of culture. Colony counts were always lower, but there was a linear relationship with counts obtained by flow cytometry (data not shown). Simultaneously an equal amount of mid log-phase culture was harvested and DNA was extracted as described below.

2.2. Desert tortoises and nasal lavage

A colony of egg-reared, captive desert tortoises is housed in the reptile vivarium at the University of Nevada, Reno. These tortoises were negative for *P. testudinis* DNA in nasal washes and were used as controls. Lavage samples were obtained from desert tortoises as previously described (duPre' et al., 2011). The lavage fluid was immediately treated with 200 µl of RNALater (Qiagen, Valencia, CA) to preserve the DNA. Samples were routinely stored on ice until they could be frozen at −80 °C (usually within 48 h). Nasal lavage samples were obtained in September 2007 from 20 desert tortoises housed at the Desert Tortoise Conservation Center (DTCC, Las Vegas, NV, USA). These tortoises had demonstrated clinical signs of URTD, including nasal discharge and palpebral edema (Jacobson et al., 1991). Another 19 nasal lavage samples were collected from desert tortoises captured in Clark County, NV during the spring of 2010. All studies reported here were approved by the University of Nevada Institutional Animal Care and Use Committee, and the desert tortoises were captured under permits from the U.S. Fish & Wildlife Service and the State of Nevada Department of Wildlife.

2.3. DNA processing and quantitative PCR (qPCR)

DNA was extracted from 500 µl RNALater-treated lavage samples using the Qiagen DNeasy Blood and Tissue kit according to the manufacturer's instructions (Qiagen, Valencia, CA). Extracted DNA in a volume of 200 µl was quantified by spectrophotometry using a Nanodrop 2000 Spectrometer (Thermo Scientific, Wilmington, DE). DNA from lavage samples was aliquoted and stored at −80 °C until analyzed. The 16S ribosomal RNA and RpoB gene sequences for *P. testudinis* were obtained from GenBank (Accession nos. AY362926, EU331071.1). Primers were designed for each gene using PrimerQuest from Integrated DNA Technologies (Coralville, IA) (Table 1). Various qPCR reaction parameters were examined including temperature, cycling time, and primer concentrations, in order to obtain the optimal working conditions.

The qPCR mixture contained iQ SYBR® Green Supermix (dNTPs, 50 U/ml iTaq™ DNA polymerase, 6 mM $MgCl_2$, SYBR® Green I, 20 nM fluorescein) (Bio-Rad, Hercules, CA), 100 nmol of each primer and 1 µl of DNA template (volume of the PCR reaction: 12.5 µl). The DNA template was replaced by PCR certified nuclease-free water (Teknova, Hollister, CA) for the negative control. Amplification was performed with the MyiQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The reaction protocol consisted of denaturation at 95 °C for 3 min, and 40 cycles of denaturation at 95 °C for 15 s, annealing at 64 °C for 30 s, and extension at 72 °C for 30 s.

Standard curves for each primer set were created using a starting genomic DNA concentration of 5 ng/ml, with serial tenfold dilutions in nuclease free water. qPCR reaction analysis was performed using Bio-Rad MyiQ Optical System Software 2.0 and the threshold cycle (Ct) was set at 200 as it represented the early linear portion of the fluorescence acquisition curves. Ct values were calculated from each sample tested in triplicate. In every experiment, no-template controls were employed, and in every experiment these controls did not register a Ct value even after 40 cycles of amplification. To verify that only a single gene sequence was amplified, melting curves were generated. In addition, agarose gel electrophoresis was performed on amplified DNA using 1.5% GenePure Sieve GQA (ISC BioExpress Kaysville, UT) stained with ethidium bromide. A fifty base-pair DNA step ladder was used from Promega (Madison, WI). Finally, amplicons for both genes were sequenced at the University of Nevada Genomics Center.

Table 1
Primers used to detect DNA sequences of the 16S rRNA and rpoB genes of *P. testudinis*.

Gene	Primer Sequences	Amplicon size
16S rRNA	F 5′-TGGGAAACCGTTGCTAATACCGGA-3′ R 5′-AGGCCATTACCCTGCCAACTAACT-3′	98
rpoB	F 5′-TAGCGGCAAATGGGTGAGTAACAC-3′ R 5′-TGCGAAGTTAAGGTGTATCCGGCA-3′	156

F = forward, R = reverse.

3. Results

3.1. Generation of qPCR standard curves and specificity testing

Primer pairs were designed to amplify 16S rRNA and rpoB gene sequences for *P. testudinis* (Table 1). Representative plots of DNA concentration versus Ct value are depicted in Fig. 1A and B. Standard curves generated with both sets of primers were linear from approximately 5 ng/ml to 50 fg/ml (r^2 values, >0.97). Analysis of five standard curves generated with triplicate data points revealed a variability of less than 5% of the mean across the concentration range, and interassay variability was less than 10% coefficient of variations calculated from six repeat qPCR runs repeated at different times (data not shown).

To verify that both primer pairs amplified the expected gene sequences, samples of amplified DNA from both PCR products were electrophoresed and single bands of the expected amplicon size were observed (Fig. 1C). In addition, single sharp peaks were observed from melting curves run on DNA amplified with both primers. Amplicon identity was verified by DNA sequencing at the University of Nevada, Reno Genomics Center (data not shown).

3.2. Relationship between P. testudinis DNA and bacteria cell numbers

Having demonstrated that the qPCR standard curves for *P. testudinis* DNA using both 16S rRNA and rpoB genes could detect down to 50 fg/ml, we sought to determine the number of bacterial cells that would be represented in this amount of DNA. *P. testudinis* cells were harvested from five different mid-log phase cultures, washed once in PBS, and resuspended in 2 ml of PBS. The number of viable bacteria in each suspension was determined by both quantitative colony counts and by flow cytometry. An aliquot was frozen at −80 °C and later processed for DNA. A representative flow cytometric dot plot is shown in Fig. 2A and B. We gated on the fluorescence positive population after CFDA staining that included >90% of the total counted events. The excluded events (B) would include bacteria that failed to take up CFDA, and cell debris. DNA extracted from the same samples counted by flow cytometry was quantified by spectrophotometry to obtain the relationship between DNA concentration and cell numbers. Data averaged from these five replicates indicated that the amount of DNA/cell was 4.9 ± 1.1 fg. Since the reasonable DNA detection limit from our qPCR standard curve is 50 fg/ml, these data suggest that the method can detect the DNA from as few as 10 *P. testudinis* cells/ml of the lavage fluid.