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Development and application of a real-time TaqMan® qPCR assay for detection and quantification of '*Candidatus* Mycoplasma haemolamae' in South American camelids☆

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ABSTRACT

Two alpacas from a herd in southwest Switzerland died for unknown reasons. Necropsy revealed chronic weight loss and pale mucous membranes. Infection with hemotropic mycoplasmas was suspected and subsequently confirmed by molecular methods. In order to investigate the epidemiological situation in this herd, a real-time TaqMan® qPCR assay for the specific detection and quantification of hemoplasma infection in South American camelids was developed. This assay was based on the 16S rRNA gene and amplified '*Candidatus* Mycoplasma haemolamae' DNA, but not DNA from other hemoplasmas or non-hemotropic mycoplasma species. The lower detection limit was one copy/PCR, and the amplification efficiency was 97.4%. In 11 out of 24 clinically healthy herd mates of the two infected alpacas, '*Candidatus* M. haemolamae' infection was confirmed. No correlation was found between bacterial load and clinical signs or anemia. The assay described herein enables to detect and quantify '*Candidatus* M. haemolamae' and may be used in future studies to investigate the prevalence, pathogenesis and treatment follow-up of hemoplasma infections in South American camelids.

1. Introduction

Hemotropic mycoplasmas in South American camelids (SAC) are well known and are widespread in the US, where they were first described in 1990 by two independent groups (McLaughlin et al., 1990; Reagan et al., 1990). Hemoplasmas are small cell wall-free Gram-negative bacteria that parasitize red blood cells and have been described in different mammalian species (Foley et al., 1998; Messick et al., 2002; Messick, 2003; Hofmann-Lehmann et al., 2004; Sykes et al., 2005; Willi et al., 2005). The clinical spectrum of hemoplasma infections in llamas varies from asymptomatic to severe disease, depending on the host susceptibility. Clinical signs may include chronic weight loss, depression, decreased fertility, lethargy, acute collapse and, rarely, death (Reagan et al., 1990; Almy et al., 2006). In hemoplasma infections in general, co-factors, such as stress, age, immune status or co-infections, have been proposed to be involved in disease development (Grindem et al., 1990; Reagan et al., 1990; Tasker et al., 2003a; Luria et al., 2004; Messick, 2004; Willi et al., 2006; Lascola et al., 2009). Antibiotic treatment of infected SAC has been shown to improve the clinical status (McLaughlin

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et al., 1990) and reduce the bacterial burden. However, for '*Candidatus* Mycoplasma haemolamae' (CMhl), the standard tetracycline regimen seems not to completely eliminate the infection; this may promote an asymptomatic carrier state (Tornquist et al., 2009).

Species-specific real-time PCR assays have been developed for several hemoplasmas (Tasker et al., 2003b; Kenny et al., 2004; Willi et al., 2005, 2006, 2007; Hoelzle et al., 2007; Wengi et al., 2008). They permit to study the epidemiology and pathogenesis of these infections in more detail. For CMhl, most recently a conventional PCR method was described (Tornquist et al., 2009). The aims of the present study were (1) to investigate suspected hemoplasma infections in Switzerland using molecular methods and (2) to develop a specific real-time TaqMan® PCR for the detection and quantification of CMhl in llamas and alpacas for future epidemiological and experimental studies.

2. Materials and methods

2.1. Total nucleic acid isolation and PCR amplification of CMhl in Camelidae in Switzerland

Total nucleic acid (TNA) was purified from EDTA-anticoagulated blood samples from 24 alpacas and 2 llamas originating from a herd of 140 SAC with suspected hemoplasma infection in southwest Switzerland (Kaufmann et al., 2007) using a MagNA Pure LC TNA isolation kit (Roche Diagnostics, Rotkreuz, Switzerland). Two PBS controls were included in each run to monitor for cross-contamination. Part of the 16S rRNA gene sequences of CMhl was amplified by conventional PCR using a modified protocol for the amplification of feline mycoplasma species as described elsewhere (Tasker et al., 2003b). Primers (MychaeF and MychaeRW), but not the TaqMan® probes, were used to amplify 5 μl of TNA using a qPCR-based master mix (Eurogentec, Seraing, Belgium). The amplified PCR products had a predicted size of 192 bp and were analyzed on 2% or 3% agarose gels.

2.2. Sequencing of the 16S rRNA gene of the Swiss CMhl isolate

To sequence part of the 16S rRNA gene, representative amplicons (192 bp) from seven alpacas were gel purified and sequenced from both sides. Briefly, cycle sequencing was performed with approximately 20 ng of DNA and 3.3 pmol of the above-mentioned primers, MychaeF and MychaeRW, using a BigDye Terminator Cycle Sequencing Ready Reaction Kit v1.1 (Applied Biosystems, Rotkreuz, Switzerland). Cycling conditions were as follows: 25 cycles at 96 °C for 10 s, 50 °C for 5 s and 60 °C for 3 min. The products were purified with a DyeEx Spin column (Qiagen, Hombrechtikon, Switzerland) and analyzed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

To sequence the nearly complete 16S rRNA gene, specific primers were designed based on the CMhl sequence deposited in GenBank (AF306346): CMhlama.5f: 5′-GGA TTA ATG CTG GTG GTA TGC-3′ (21 bp) and CMhlama.1424r: 5′-CCA ATC AAA ATT ACC AAT CTA GAC G-3′ (25 bp). Briefly, 2.5 μl of TNA was amplified in a 25-μl reaction containing 1× Phusion HF Buffer (Finnzymes, Espoo, Finland), 500 nM of each primer, 200 nM of each dNTP (Sigma–Aldrich, Buchs, Switzerland) and 1 U Phusion™ DNA Polymerase (Finnzymes) using a T-personal 48 Thermocycler (Biometra GmbH, Goettingen, Germany). The thermal program comprised 98 °C for 3 min, 35 cycles of 98 °C for 10 s, 56 °C for 30 s and 72 °C for 1 min and finally 72 °C for 10 min. PCR products of 1420 bp were purified as described above and were cloned into the vector pCR®4-TOPO® (Invitrogen, Basel, Switzerland) according to the manufacturer's protocol. The presence of inserts was confirmed by restriction digestion with EcoRI. Purified plasmid DNA was sequenced with vector-specific M13 and two internal primers (CMhlama.375f: 5′-ACC ACG TGA ACG ATG AAG GTC-3′ (21 bp); CMhlama.975r: 5′-CGC AGT AGA TTA CAA GCC TTG GTA-3′ (24 bp)) using the BigDye® Terminator Cycle Sequencing Ready Reaction Kit v1.1 (Applied Biosystems) as described above. Sequences were analyzed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). From each animal, two clones were sequenced.

2.3. Development of a TaqMan® fluorogenic real-time qPCR assay

To develop a real-time PCR assay for CMhl a set of primers and probe were designed based on the 16S rRNA gene sequence (AF306346) using Primer Express software (Version 3.0; Applied Biosystems): forward primer CMhlama.427f: 5′-AAA AGC AGG ATA GGA AAT GAT TCT G-3′ (25 bp); reverse primer CMhlama.498r: 5′-TGC TGG CAC ATA GTT AGC TGT CA-3′ (23 bp); probe CMhlama.453p: 5′-FAM-CGT GAT TGT ACT AAT TGA AT-MGBNFQ-3′ (20 bp). The assay was designed so as not to amplify hemoplasmas other than CMhl. The 25-μl PCR reaction comprised 12.5 μl of 2× qPCR Mastermix (Eurogentec), 900 nM of each primer, 250 nM of probe and 5 μl of TNA. Assays were performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems).

2.4. Construction and production of DNA standards for absolute quantitation

The sequenced pCR®4-TOPO® plasmid (see above) containing the almost complete 16S rRNA gene of CMhl, including the target sequence encompassed by the real-time PCR assay, was linearized by restriction digestion using NotI and gel purified (QIAquick® Gel Extraction Kit, Qiagen). The plasmid DNA copy number was then calculated by spectrophotometry (NanoDrop ND-1000, Witec Ag, Littau, Switzerland) and agarose gel electrophoresis (Syngene, Gene Tools, Syngene, Cambridge, UK). 10-Fold serial dilutions (10^8–10^0 copies/5 μl) of the CMhl DNA standard were prepared and analyzed as previously described (Tandon et al., 2005).

2.5. Analytic specificity, sensitivity and amplification efficiency of the real-time PCR assay

The specificity of the assay was evaluated by amplifying DNA from different mycoplasma species: feline