Isolation and identification of *Taylorella asinigenitalis* from a mare in Oklahoma, USA

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Abstract

Contagious equine metritis (CEM) is a highly contagious venereal disease of horses whose etiologic agent has been identified as *Taylorella equigenitalis*. The bacterium is a catalase and oxidase positive pleomorphic Gram-negative coccobacillus which grows best in a humidified environment of reduced oxygen (95% air containing 5% CO_2) on Eugon chocolate agar. During routine screening of an Oklahoma equine breeding population slated for export, a *T. equigenitalis*-like organism was isolated. Based on results from requisite conditions for cultivation, biochemical tests, 16S rDNA and intergenic spacer region (ISR) sequence analyses, the isolated organism was identified as *Taylorella asinigenitalis*. As required by federal regulations, this identification was confirmed at the National Veterinary Services Laboratories (NVSL) in Ames, IA. Pulsed-field gel electrophoresis (PFGE) analysis revealed that the newly identified strain has a distinct genomic fingerprint from the two previously characterized *T. asinigenitalis* isolates from the United States and represents the second strain to be recovered from a horse in the US.

Keywords: Contagious equine metritis, *Taylorella equigenitalis*, *Taylorella asinigenitalis*

Introduction

Contagious equine metritis is a bacterial infectious disease of horses caused by *Taylorella equigenitalis*. Five reported outbreaks of CEM have occurred in the United States within the past 44 years including those in Kentucky in 1978[1] and again in 1979 along with Missouri,[2] and a multi-state outbreak investigation from 2008 to 2010.[3] Incidents involving a single horse have also been reported in 2010 and 2011.[4] While symptomatic infection in mares is characterized by discharge of copious amounts of mucopurulent fluid from the vagina, infertility, and/or early abortion, stallions appear to remain asymptomatic during infection and serve as a reservoir for infection.[5] Cases of CEM are rarely, if ever, fatal to an adult animal and while no prolonged deleterious medical effects have been described, the presence of CEM in a breeding equine population has a significant economic impact.[6] To avert such financial losses, many countries require certified and validated CEM-testing of equine breeding populations prior to import or export into or from CEM-endemic countries. In the United States, testing is federally regulated and conducted exclusively by USDA-approved laboratories and diagnosticians. While direct PCR methods exist to detect *T. equigenitalis* in genital swabs,[7-9] bacteriologic examination of submitted samples is still regarded as the only definitive means of determining the presence or absence of *T. equigenitalis*.

The etiologic agent of CEM, *T. equigenitalis*, is a non-motile, Gram-negative coccobacillus with fastidious growth requirements. The bacterium is microaerophilic, and grows best on a semi-solid medium of chocolatized Eugon agar or Timoney agar in a humidified environment maintained at 5-10% CO_2, 95-90% air, and 37 °C.[5,10] While members of the genus *Taylorella* are extremely limited in the repertoire of easily detectable enzyme activities, all known isolates are biochemically positive for cytochrome C oxidase, catalase, and alkaline phosphatase. In addition, the bacterium is susceptible to most antimicrobial agents except lincosamides, sulphamethoxazole, and while streptomycin-susceptible strains have been recovered, streptomycin-resistant strains have been described.[5] Phylogenetic analysis of the 16S ribosomal DNA sequence revealed that *Taylorella*, while phenotypically similar to *Haemophilus*, occupies a unique niche in the β-subclass of the *Proteobacteria* most closely related to *Pelistega europaea*[11] and constitutes a new genus within the subclass.

In the late 1990's, three atypical *Taylorella* isolates were obtained in California and Kentucky from the genital tract of donkey jacks (*Equus asinus*). These three isolates were identified during routine regulatory testing for CEM and were found to be nearly indistinguishable from *T. equigenitalis* when assessed phenotypically. Results from 16S ribosomal DNA sequence analysis however, together with genomic DNA-DNA hybridization and G+C composition studies between these *T. equigenitalis*-like organisms and *T. equigenitalis* suggested that the former were unique and constituted a new species within the genus which was named *Taylorella asinigenitalis.*[12] Significantly, following natural breeding, disease in mares was not detected; however, clinical signs were noted in another study following experimental inoculation of mares with *T. asinigenitalis.*[13] A recent publication on the containment of the 1998 outbreak in Kentucky reported that seven mares and one stallion were infected.[14] In 2006, Båverud *et al.* reported the isolation and identification of *T. asinigenitalis* from a naturally infected stallion in Sweden and found the 16S rRNA sequence to be identical to that of the California UCD-1 Type strain, first described in the 2001 report.[15] In February 2008, Franco *et al.* reported the isolation of *Taylorella asinigenitalis* organisms, identical in 16S rDNA sequence analysis (nucleotide 505-1120) to the *T. asinigenitalis* UCD-1 Type strain (ATCC 70093) strain from two donkey jacks of the Martina Franca endangered breed in Apulia, Italy.[16] In 2011, Breuil *et al.* reported the isolation of three French *T. asinigenitalis* strains from horses, each of which could be distinguished by a combination of profiling methods.[17] Interestingly, one of these isolates was collected in 1995 and was, retrospectively, the first *T. asinigenitalis* to be recovered.

We report here the isolation and identification of *Taylorella asinigenitalis* from a mare in the state of Oklahoma. The isolate was discovered during routine testing of samples at a time concomitant with but independent of the United States 2008-2010 CEM investigation. Both phenotypic and genotypic properties were assessed in the identification of this isolate, designated *T. asinigenitalis* UMC-1, which also demonstrated that the strain is distinct from that associated with the 1998 Kentucky outbreak in horses and from the Type strain identified in a California donkey jack.

Methods

Unless noted otherwise all strains used in this study were cultivated for 48-72 hr at 37 °C in a humidified environment containing 95% (v/v) air and 5% (v/v) CO_2 on chocolatized Eugon and Timoney agars (BioMed Diagnostics, White City, OR) as previously described.[10] Additional media including tubed biochemical tests, blood and MacConkey agars were supplied by Remel® (Lenexa, KS). Initial oxidase and catalase tests were performed on colonies recovered from Timoney media after at least 48-hr incubation. The presence of catalase activity was determined by exposing selected bacterial colonies to 3% (v/v) H_2O_2 and observing the generation of oxygen. The presence of oxidase was determined using Oxoid® oxidase sticks (Remel). The specific alkaline phosphatase (AlkP) activity present in crude bacterial extracts was measured in quadruplicate using a 0.2 mL discontinuous colorimetric assay consisting of 100 mM Tris pH 10.0, 1 mM $MgCl_2$, 2 mM *p*-nitrophenylphosphate (*p*NPP), and varying amounts of suspended bacteria. The mixtures were incubated at 37 °C for 15 min with constant agitation after which time the concentration of liberated *p*-nitrophenol (*p*NP) was determined at 405 nm using a Model 680 Microplate Reader (BioRad Carlsbad, CA) and a standard curve of known concentrations of *p*-NP. The protein concentration was determined using the bicinchoninic acid technique as previously described[18] using a BSA standard. The conditions used for AlkP determination were identical to those at which significantly enhanced AlkP activity was noted for the purified recombinant *T. equigenitalis* AlkP (unpublished results).

Chromosomal DNA used for PCR and sequencing was initially isolated from 48-72 hr cultures using a Wizard Genomic DNA purification kit according to the manufacturer's protocol (Promega, Madison, WI). Primers ISR-F (5' CTGGGGTGAAGTCGTAACAAG) and ISR-R (5' TGTGATCGCCAAGGCATCCACC) were used for PCR amplification of ISR DNA as described by Tazumi *et al.*[19] Briefly, 50 ng total genomic DNA was used as template with 20 pmol of each primer in standard buffer conditions for Phusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA). Following an initial denaturation step of 98 °C for 30 s, amplification was achieved by 30 cycles of

the following parameters: 98 °C for 10 s, 57 °C for 30 s and 72 °C for 30 s. The resulting amplicons were purified (QiaQuick Spin Column, Qiagen, Valencia CA) and ligated into the positive selection vector pJET1.2 (Fermentas, Glen Burnie, MD). After transformation of *Escherichia coli* DH10B competent cells (Invitrogen, Calsbad, CA), plasmid DNA from individual colonies was prepared (Qiagen) and used as template for DNA sequencing using vector-derived primers and BigDye Terminator chemistry. Sequencing was performed at the DNA Core Facility at the University of Missouri-Columbia on a PE Biosystems 3730 capillary DNA sequencer. The resulting DNA sequences were queried against entries in GenBank available through the National Center for Biotechnology Information. The ISR sequences reported herein have been deposited in GenBank under accession numbers JQ783350 and JQ783351. Additional confirmatory tests were performed at NVSL. These tests included 16S ribosomal DNA (rDNA) sequencing, slide agglutination test (Monotayl, Bionor Laboratories AS, Skien, Norway), and direct fluorescent antibody testing using polyclonal antibody conjugate. To assess for streptomycin susceptibility, bacterial isolates were plated on chocolated Eugon agar with or without streptomycin at 200 μg/mL final concentration. Plates were incubated at 37 °C in 5% (v/v) CO_2 for at least seven days. Any growth in the presence of streptomycin indicated resistance. Pulsed-field gel electrophoresis (PFGE, CHEF-DR II system, BioRad, Carlsbad, CA) techniques to determine the relatedness of *Taylorella* strains have been described previously.[4] Briefly, cell suspensions in 100 mM Tris HCl 100 mM EDTA, pH 8.0 from 72-96 hour cultures grown on Timoney media were used to prepare agarose plugs containing genomic DNA. The DNA-containing plugs were incubated with the restriction enzyme *Not* I (Invitrogen, Carlsbad, CA) at 37 °C. Resulting PFGE patterns were standardized with a universal sized standard *Salmonella enterica* subsp. *enterica* serovar Braenderup H9812 and analyzed (Bionumerics, Applied Maths, Austin TX). A dendogram was created using Dice's coefficient and UPGMA based on band position tolerance values of 1.5%.

Results and discussion

Assessed after 48-72 hours of incubation at 37 °C in 5% (v/v) CO_2, the UMC-1 isolate along with both *T. equigenitalis* and *T. asinigenitalis* control strains were Gram-negative, nonmotile coccobacillary organisms. All three isolates were strongly oxidase and catalase positive resulting in a rapid darkening color of the oxidase stick and generation of copious amounts of bubbles (oxygen) in the presence of hydrogen peroxide, respectively. The three isolates were deemed streptomycin resistant, fastidious, and CO_2-dependent as growth of the microbes was noted only on chocolated Eugon and Timoney agars in a humidified environment composed of 95% air and 5% CO_2. The growth of the isolates was barely perceptible on blood agar incubated in CO_2 and not at all on any medium cultured in an aerobic incubator. The isolates also failed to grow anaerobically or on MacConkey agar. In addition, pre-incubation of the isolate for 24 hours on a rich medium in an air incubator prior to transfer to a CO_2 incubator failed to result in observable growth. Biochemically, all three isolates were negative in all the tests included in a TSI series consisting of various tubed media including triple sugar iron agar, lysine iron agar, motility-indole-ornithine decarboxylase agar, citrate and urea agar. The specific alkaline phosphatase activity of the UMC-1 isolate was 665 ± 64 nmole *p*NP produced/hr/mg protein; that of the *T. asinigenitalis* type strain was 637 ± 109 nmole *p*NP produced/hr/mg protein. The specific alkaline phosphatase activity of the *T. equigenitalis* type strain was 951 ± 107 nmole *p*NP produced/hr/mg protein. While certainly not definitive for identification, the alkaline phosphatase activity of UMC-1 was consistently most similar to that of the reference *T. asinigenitalis* strain than to that of the *T. equigenitalis* strain.

Amplification with ISR-flanking primers resulted in the generation of multiple amplicons in the 900-1000-bp approximate size range, as anticipated from a previously published study.[19] After cloning into a positive selection vector, plasmid DNAs corresponding to each of two differently sized amplicons were identified and subjected to DNA sequence analysis. Each ISR sequence was closely related to those determined for other *T. asinigenitalis* isolates, and each contained coding sequences for tRNA Ile and tRNA Ala. Sequence 1 was 922 bp in length and was most similar to ISR C of strains UK-1 and UK-2 (97%) and slightly less similar to ISR C of strain UCD-1 (93%) as described by Tazumi *et al* .[19] Sequence 2 (1002 bp) was 99% identical to Sequence 1 in regions of overlap between the two differently

sized ISRs, and shared 99% identical residues with ISR B of strains UK-1 and UK-2.[19] Both ISR sequences determined in this study are also distinct from those present in the complete genome of *T. asinigenitalis* strain MCE9 (isolated in 2004 from a donkey jack in France).[20] Importantly, both amplicons exhibited significantly less sequence identity to ISR regions from *T. equigenitalis*. Sequence 1 and 2 were only 72% and 70% identical, respectively, to *T. equigenitalis* EQ59, the most closely related strain of this species.

As each ISR from the mare isolate (UMC-1) had differences from the published sequences, an additional PCR/cloning was performed to eliminate the possibility of the introduction of inadvertent mutations during amplification. Sequence analysis of independent plasmid clones verified the presence of characteristic patterns of SNPs as well as insertion/deletion (indels), which may prove useful in strain discrimination as additional *T. asinigenitalis* isolates are identified. PFGE analysis of genomic DNA from the UMC-1 strain showed that it differed from both the UK isolate and the UCD-1 isolate when digested with *Not* I (Figure). The PFGE profile of UMC-1 was more similar (88%) to the UK isolate and less similar (59.4%) to the UCD-1 Type strain. Although the time between isolations of UMC-1 and UCD-1/UK strains spans approximately ten years, these results indicate that the UMC-1 isolate is not closely related to past strains of *T. asinigenitalis* found in the United States.

In conclusion, small Gram-negative coccobacilli possessing copious amounts of catalase, oxidase, and alkaline phosphatase activity were unexpectedly recovered from one horse during routine CEM screening of eight Oklahoma Quarter Horses for export. While this isolate was nearly indistinguishable from the etiologic agent of CEM, *T. equigenitalis* phenotypically, results from genomic analysis found the *T. asinigenitalis* UMC-1 isolate to be more closely related to, though distinct from the UCD-1 Type strain identified in 1997. These findings highlight the need for further epidemiological studies of the prevalence and strain profiles of *T. asinigenitalis* isolates from equid species.

Figure: PFGE analysis of *Taylorella asinigenitalis* strains UK, UMC-1, and UCD-1 after digestion of genomic DNA with *Not* I. Dendrogram was created using Dice's coefficient and UPGMA and based on 1.5% band position tolerance values.

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