

Short Note

**Possible misidentification of *Bacteroides* sp.,
probably *B. ureolyticus* as *Taylorella equigenitalis*:
Implications for the laboratory diagnosis of CEM**

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(Received 9 February 2001; accepted 21 June 2001)

Abstract – A wild-type isolate with similar morphological and phenotypic properties to *Taylorella equigenitalis*, the causative bacterial agent of contagious equine metritis (CEM), was referred for molecular identification by PCR amplification of the 16S rRNA gene. A species-specific PCR failed to yield a product compatible with that of *T. equigenitalis*. The direct sequencing of the universal 16S rRNA PCR amplicon suggested the presence of a *Bacteroides* sp., probably *Bacteroides ureolyticus*, with no consequent effects on the movement and transportation of the animal. Adoption of such a molecular means of identification through sequencing may aid in the identification of the atypical forms of *Taylorella equigenitalis*, as recently described, as well as differentiating this species from *Taylorella asinigenitalis*.

horse / CEM / PCR / *Taylorella equigenitalis* / *Bacteroides*

Résumé – Possibilité d'erreur dans l'identification d'un *Bacteroides* sp., probablement *B. ureolyticus*, détecté comme *Taylorella equigenitalis* : conséquences pour le diagnostic en laboratoire de la métrite contagieuse équine. Un isolat de type sauvage ayant des caractéristiques morphologiques et phénotypiques similaires à celles de *Taylorella equigenitalis*, l'agent bactérien responsable de la métrite contagieuse équine, a été soumis pour identification moléculaire par la technique d'amplification en chaîne par la polymérase (PCR) du gène de l'ARNr 16S. Une PCR spécifique à l'espèce n'a pas permis d'obtenir un produit compatible avec celui de *T. equigenitalis*. Le séquençage direct de l'amplicon de PCR de l'ARNr 16S universel suggère la présence d'un *Bacteroides* sp., probablement *Bacteroides ureolyticus*, n'ayant aucune conséquence sur le mouvement et le transport de l'animal. L'adoption d'une telle technique moléculaire d'identification par le séquençage pourrait aider à l'identification de formes atypiques de *Taylorella equigenitalis* qui ont été récemment décrites, ainsi que pour différencier cette espèce de *Taylorella asinigenitalis*.

cheval / amplification en chaîne par polymérase / métrite contagieuse équine / *Bacteroides*

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1. INTRODUCTION

Contagious equine metritis (CEM) is a sexually transmitted disease of horses caused by the bacterium, *Taylorella equigenitalis*. Originally CEM was first reported in England in 1977 [4]. Since then, CEM and its causative agent have been detected in many countries and in various breeds of horses. CEM continues to be a cause for concern in veterinary microbiology, especially due to its economic implications. Diagnosis of CEM has been mainly based on the isolation of *T. equigenitalis* by bacteriological culture, with the central clitoral sinus in mares and the urethral fossa in stallions being the most likely locations, where this organism may be found. Usually this organism is cultured from swabs, by means of conventional selective bacteriological techniques. Growth requires between two to six days and is often difficult to observe due to competition from other commensal organisms in the equine genital tract.

We report a laboratory case of potential misidentification of *Bacteroides* sp., probably *B. ureolyticus*, as *T. equigenitalis* during routine screening of horses for CEM.

2. MATERIALS AND METHODS

2.1. Bacteriological culture

Routine swabs taken from the genital area of an animal were cultured on *T. equigenitalis* selective agar. For the conventional detection of *T. equigenitalis*, swabs were plated onto different media namely (i) CEMO agar (MAST DM470, MAST Diagnostics Ltd, Merseyside, England) containing streptomycin (250 µg/mL) and fungizone (5 µg/mL) (ii) CEMO agar supplemented with 5%, v/v, chocolate and fungizone (5 µg/mL) and (iii) Columbia agar base supplemented with 5%, v/v, defibrinated horse blood. All plates were incubated for 4-6 days (37 °C; 5%, v/v, CO₂).

A bacteriological culture, yielding a few small convex colonies, which grew on CEMO Selective Agar, with atypical colonial characteristics to *Taylorella equigenitalis*, i.e. greyish yellow, glistening with an entire edge, approximately 2-2.5 mm in diameter, but which shared several phenotypic characteristics, including Gram stain and morphology (Gram -ve rods), oxidase (+ve), microaerophilic growth (+ve in 5%, v/v, CO₂) and aerobic growth (-ve), was referred for molecular identification, as possibly being that of *T. equigenitalis*.

2.2. DNA extraction

Genomic bacterial DNA from the culture was obtained by employing the High Purity PCR template DNA extraction Kit (Roche Ltd., England) in accordance with the manufacturer's instructions.

2.3. Ribosomal PCR amplification

All reaction mixes were set up in a PCR hood in a room separate from that used to extract DNA and the amplification and post-PCR room in order to minimise contamination. Reaction mixes (50 µL) were set up as follows: -10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 200 µM (each) dATP, dCTP, dGTP and dTTP; 1.25 U of *Taq* DNA polymerase (Amplitaq; Perkin Elmer), 0.2 µM (each) of the universal 16S rRNA primers [12], i.e. forward primer: P11P, 5' GAG GAA GGT GGG GAT GAC GT 3' and reverse primer: P13P 5' AGG CCC GGG AAC GTA TTC AC 3' and 4 µL of DNA template, containing approximately 50 ng DNA/mL DNA. After a "hot start", the reaction mixtures were subjected to the following thermal cycling sequence in a Perkin Elmer 2400 thermocycler: 96 °C for 3 min followed by 40 cycles of 96 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. During each run, molecular grade

water was included randomly as negative controls and appropriate DNA templates from *T. equigenitalis* were included as positive controls when appropriate.

2.4. PCR confirmation by specific PCR

Genomic DNA was amplified by means of a species-specific PCR for *T. equigenitalis*, as previously described [2]. A positive control of *T. equigenitalis* and a negative control of molecular grade water (Biowhittaker Inc., USA) were included as appropriate controls.

2.5. Detection of PCR products

Following amplification, aliquots (15 µL) were removed from each reaction mixture and examined by electrophoresis (80 V, 45 min) in gels composed of 2% (w/v) agarose (Gibco, UK) in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3), stained with ethidium bromide (5 µg/100 mL). Gels were visualised under UV illumination by means of a gel image analysis system (UVP Products, England) and all images archived as digital graphic files (*.bmp).

2.6. Sequencing of amplicons and analysis of sequence data

Amplicons chosen for sequencing were purified by means of a QIAquick PCR purification kit (Qiagen Ltd., UK) eluted in Tris-HCl (10 mM, pH 8.5) before sequencing, particularly to remove dNTPS, polymerases, salts and primers. The forward universal primer [5' GAG GAA GGT GGG GAT GAC GT 3'] was used for sequencing with the ABI PRISM™ Dye Terminator Cycle Sequencing Reaction with AmpliTaq DNA Polymerase®, FS (PE Biosystems, Foster City, CA, USA) (96 °C for 1 min, followed by 25 cycles of 96 °C for 10 s,

50 °C for 5 s, 60 °C for 4 min, then held at 4 °C). The products were ethanol-precipitated and analysed on an ABI 373 Automatic Sequencer (PE Biosystems, Foster City, CA, USA). *Campylobacter jejuni* NCTC11168 and *T. equigenitalis* were included as positive sequencing controls. The resulting sequences obtained were compared with those stored in the Genbank Data system by means of BLAST alignment software [1] (<http://www.blast.genome.ad.jp/>).

3. RESULTS

The species-specific PCR failed to yield a product compatible with *T. equigenitalis* (Fig. 1). The isolate produced an amplicon of the correct size with the universal 16S rRNA primers P11P/P13P (Fig. 2) and the sequencing of the amplified 216bp PCR amplicon derived from the query isolate suggested the presence of a *Bacteroides* sp., possibly *B. ureolyticus*.

4. DISCUSSION

This study describes a laboratory case of uncertainty over the identification of an organism growing on CEMO selective agar under microaerophilic conditions. Although the colonial characteristics of the colonies did not closely resemble *T. equigenitalis* and hence were atypical in this respect, they shared several other phenotypic properties with *T. equigenitalis*. As atypical variants of *T. equigenitalis* have been recently described in mares [8] and taking into consideration the economic importance of CEM, it was decided to delay transportation of the animal from where the swabs originated pending a detailed molecular work-up of the identification of the suspected colonies.

The genus *Taylorella* was first proposed in 1983 by Sugimoto et al. [10]. It was the strain first isolated and described by Taylor et al. [11] and accepted by the UK

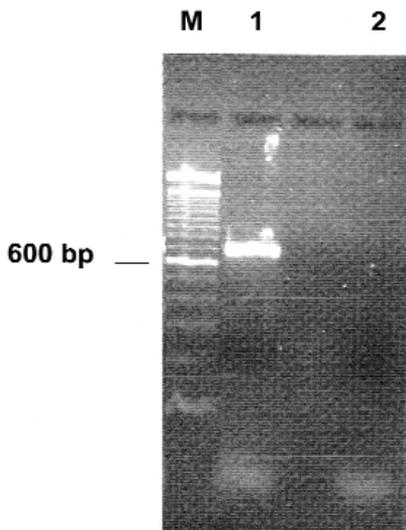


Figure 1. Species-specific PCR for *Taylorella equigenitalis*. Lane M: molecular weight marker (100 bp ladder; Gibco Ltd., Scotland, UK). Lane 1: positive control *Taylorella equigenitalis*. Lane 2: query isolate.

National Collection of Type Cultures (NCTC), as the Type Strain NCTC 11184 which was originally included as a species *incertae sedis* in the genus *Haemophilus* in Bergey's Manual of Determinative Bacteriology [7].

Phenotypically, the species shares characteristics of organisms in Group 4B (Gram-negative aerobic/microaerophilic rods and cocci) within Major Category I (Gram-negative Eubacteria that have cell walls) according to the 9th Edition of Bergey's Manual of Determinative Bacteriology, along with species in the genera *Wolinella* and *Bacteroides*. Phenotypically, species in the genera *Taylorella* and *Bacteroides* are very similar, being non-motile, microaerophilic and oxidase positive (*B. ureolyticus*), as described (Tab. I), although the latter genus has little clinical significance in CEM, whereas *Taylorella* is highly significant in this disease state. In addition, although phenotypically similar, *T. equigenitalis* is

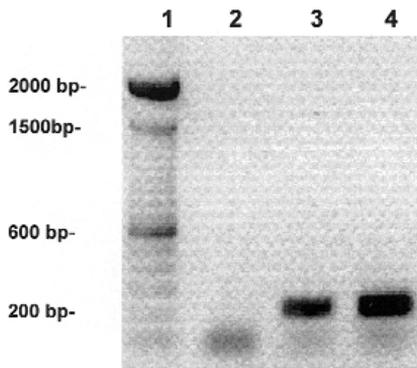


Figure 2. Gel electrophoresis of PCR products which resulted following amplification of a 216 bp region of the 16S rRNA gene from prepared genomic DNA. Lane 1: 100-bp DNA molecular weight ladder (Gibco, UK). Lane 2: negative control (molecular grade water). Lane 3: positive control (*Taylorella equigenitalis*). Lane 4: query isolate.

markedly different phylogenetically from *Bacteroides ureolyticus*. Analysis of the 16S rRNA nucleotide sequence of *T. equigenitalis* (Genbank Accession No. X68545) and *B. ureolyticus* (Genbank Accession No. L04321) using alignment software (DNASTAR Inc., Wisconsin, USA) showed 63.7% homology between these two organisms.

Initially, it was surprising to detect a *Bacteroides* sp. on CEMO agar grown microaerophilically, as species within this genus are regarded as being obligate anaerobes. This is largely true for the genus with the exception of *B. ureolyticus*, which is microaerophilic and hence could grow in 5% CO₂ conditions, which are normally achieved in common CO₂ laboratory incubators. Indeed, this species of *Bacteroides* will not grow in anaerobic conditions. Furthermore this organism has been previously cultured from vaginal discharges from mares on three previous occasions [3, 5, 6]. In the

Table I. Differential characteristics of *Taylorella equigenitalis* and *Bacteroides ureolyticus*.

Phenotypic characteristic	<i>Taylorella equigenitalis</i>	<i>Bacteroides ureolyticus</i>
Bergey's Classification	Major category I Subgroup 4B	Major category I Subgroup 4B
Morphology	Rods	Rods
Gram reaction	Gram-negative	Gram-negative
Motility	Non-motile	Non-motile
Temperature growth range	30-42 °C	25-37 °C
Oxygen requirements	microaerophilic	microaerophilic
Oxidase	positive	positive
Carbohydrates utilised	none	none
Natural habitat	Genital tract	Urogenital tract

Fodor et al. study [5], seven cultures resembling *T. equigenitalis* were grown in microaerophilic conditions, which were confirmed as *B. ureolyticus*. All isolates were catalase positive, oxidase positive and alkaline phosphatase positive, being similar to *T. equigenitalis*. This study concluded that the *Bacteroides ureolyticus* organisms isolated closely resembled *T. equigenitalis*. Overall, this study demonstrated that both species are very similar in terms of their phenotypic characteristics, however differences included urease production by *B. ureolyticus*, leucine arylamidase and alanine arylamidase production by *T. equigenitalis*. For those laboratories which do not have access to molecular techniques, these differential phenotypic characteristics may be a useful aid in helping with laboratory identification.

The isolation of *T. equigenitalis* may prove difficult since the species is fastidious, requiring nutritionally-supplemented media and specialised reduced oxygen conditions. Furthermore, it is easily outgrown by other non-pathogenic organisms in the microflora found including commensal organisms in the genital tract of horses and hence may be misidentified in routine practice.

We report here on a case of possible misidentification of *Bacteroides* sp., as *T. equigenitalis*.

Direct sequencing of the 16S rRNA locus from the query isolate allows to identify a *Bacteroides* sp., possibly *B. ureolyticus*. As the PCR amplicon was 216 bp, only a relatively short region of the 16S rRNA locus was amplified and sequenced. Hence it was not possible to differentiate the sequence identification lower than the genus level, without the need to sequence a larger stretch of this gene. However, the subsequent BLAST search led to the identification of *Bacteroides* sp.

Adoption of such a molecular means of identification through sequencing may aid in the identification of the atypical forms of *Taylorella equigenitalis*, as recently described [8], as well as differentiating this species from *Taylorella asinigenitalis* (GenBank Accession number AF067729) [9].

The correct identification of *T. equigenitalis* as the aetiological agent responsible for CEM is very important, as CEM requires special control measures and restrictions have to be introduced to prevent the transmission of disease locally, nationally and internationally, whereas colonisation/infection of the genital tract with *Bacteroides* spp. do not necessitate such measures.

With the adoption of highly discriminatory molecular methods such as PCR and direct automated sequencing into routine clinical microbiology, in cases of difficulty in identifying atypical colonies, we

therefore recommend molecular identification by species-specific PCR amplification of a locus specific for *T. equigenitalis* [2], as well as by sequencing the 16S rRNA locus after attempts to identify suspect colonies with a combination of biochemical (e.g. urease production, API Identification System) and serological testing have proved unsuccessful.

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