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## A two-step species-specific 16S rRNA PCR assay for the detection of *Taylorella equigenitalis* in horses

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A two-step PCR assay was developed for the molecular detection of *Taylorella equigenitalis*, a Gram-negative genital bacterial pathogen in horses. Two specific oligonucleotide primers (TE16SrRNABChf [25mer] and TE16SrRNABChr [29mer]) were designed from multiple alignments of the 16S rRNA gene loci of several closely related taxa, including *T. asinigenitalis*. Subsequent enhanced surveillance of 250 Thoroughbred animals failed to detect the presence of this organism directly from clinical swabs taken from the genital tract of mares and stallions. Such a molecular approach offers a sensitive and specific alternative to conventional culture techniques, and has the potential to lead to improved diagnosis and subsequent management of horses involved in breeding programmes.

### Key words

Horse,  
*Taylorella equigenitalis*,  
16S rRNA,  
Contagious equine  
metritis,  
Polymerase chain reaction.

### Introduction

Contagious equine metritis (CEM) caused by *Taylorella equigenitalis* is a sexually transmitted disease in horses, generally leading to a loss of fertility in the mare. Since CEM was first reported in England (Crowhurst, 1977), this disease and its causative agent have been detected in many countries and in various breeds of horses (Timoney, 1996). Diagnosis of CEM has been based mainly on the isolation of *T. equigenitalis* by culture, from swabs of the central *clitoral sinus* in mares and from the *urethral fossa* in stallions. Previously, a three-step PCR assay based on the amplification of the 16S rRNA of *Taylorella equigenitalis* was described by Bleumink-Pluym *et al.* (1994), yielding a 585bp amplicon specific to *T. equigenitalis*. However, through careful re-design of primers, it is possible to modify the assay conditions, by eliminating the annealing time during the PCR reaction. Therefore, it was the aim of this study to

develop a PCR diagnostic assay specific for *T. equigenitalis*, based on 16S rRNA, in a two-step amplification procedure, thereby reducing assay time and hence allowing for more rapid laboratory turnaround times to aid with the management of animals being screened for this organism. The two-step assay was used to survey 250 Thoroughbred horses for the presence of this organism.

### Materials and methods

#### Primer design

Alignments of the 16S rRNA gene sequence of *Taylorella equigenitalis* [GenBank Accession no: X68645] was performed against 23 bacterial species of close phylogenetic relatedness, as previously described (Bleumink-Pluym *et al.*, 1994), employing MegAlign software (Dnastar Inc, Wisconsin, USA), as well as with *Alcaligenes xylosoxidans*, *Bordetella bronchiseptica*, *Haemophilus parainfluenzae*, *H. influenzae*, *H. ducreyi*, *H. parasuis*, *H. aphrophilus*, *H. parahaemolyticus*, *H. paraphrophilus*, *H. segnis*, *H. haemoglobinophilus*, *H. paracuniculus*, *H. paragallinarum*, *H. taxon C.*, *H. haemolyticus*, *H. somnus*, *H. aegyptius*, *H. haemophilus paraphrohaemolyticus* and *Taylorella asinigenitalis*. Primers were designed to allow for a two-step PCR amplification with a high melting temperature ( $T_m$ ), as described in Figure 1.

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### DNA extraction from bacterial organisms

All DNA isolation procedures were carried out in a Class II Biological Safety Cabinet (MicroFlow, England) in a room physically separated from that used to set up nucleic acid amplification reaction mixes and also from the “post-PCR” room in accordance with the Good Molecular Diagnostic Procedures (GMDP) guidelines of Millar *et al.* (2002), in order to minimise contamination and, hence, the possibility of false positive results. Genomic DNA from *T. equigenitalis* (Type strain NCTC 11184 and two wild-type strains isolated previously from horses with CEM), *Pseudomonas aeruginosa*, *Alcaligenes xylosoxidans*, *Haemophilus influenzae*, *Stenotrophomonas maltophilia*, *Burkholderia cepacia*, CDC Group IV organisms, *Streptococcus pneumoniae* and *Staphylococcus aureus* was extracted from pure culture employing the Roche High Purity PCR DNA extraction kit (Roche Ltd., England), as per the manufacturer’s instructions.

### PCR amplification

Amplification reactions were set-up in accordance with Good Molecular Diagnostic Procedures, as detailed in the guidelines of Millar *et al.* (2002). In order to minimise contamination, all reaction mixes were set up in a PCR hood in a room separate from that used to extract DNA and from the amplification and post-PCR room. Initially, PCR amplification conditions were optimised by separately varying magnesium chloride concentration, annealing temperature, primer concentration and DNA template concentration. Following optimisation, reaction mixes (25µl) were set up as follows: -10 mM Tris-HCl, pH 8.3, 50mM KCl, 2.5mM MgCl<sub>2</sub>, 200µM (each) dATP, dCTP, dGTP and dTTP; 1.25U of

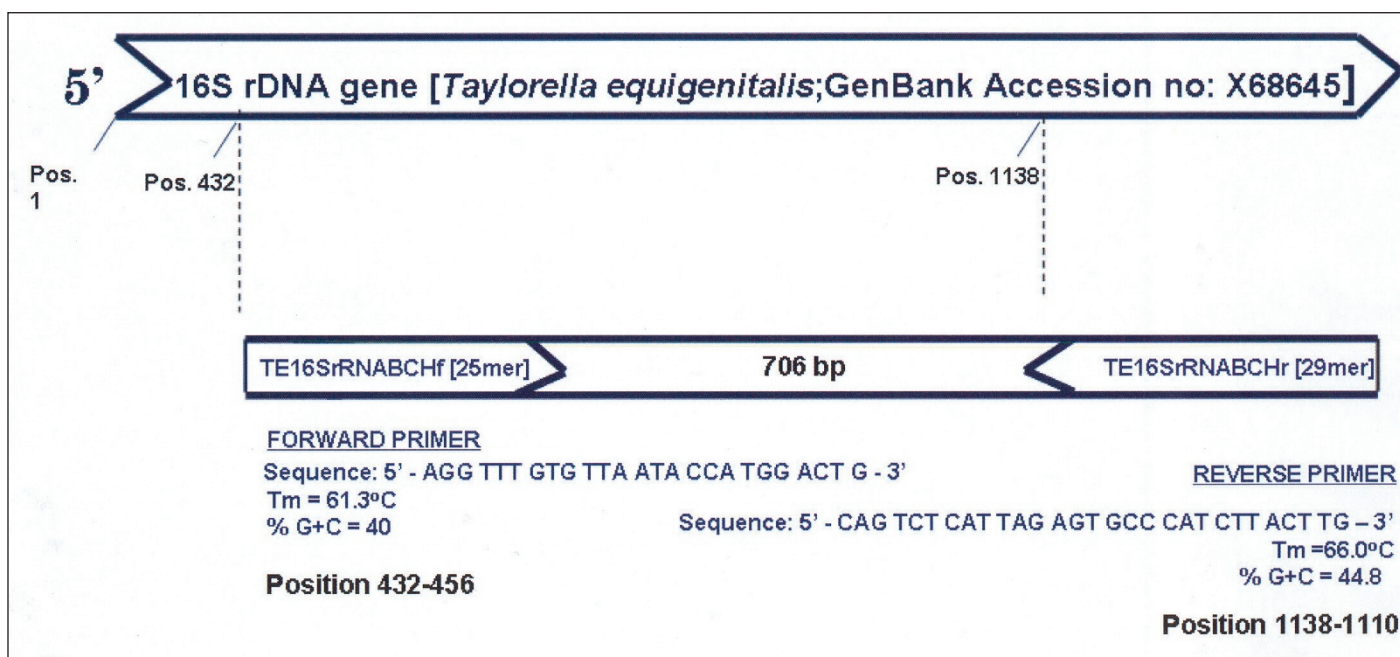
*Taq* DNA polymerase (Amplitaq; Perkin Elmer), 0.1µM (each) of each primers (TE16SrRNABChf and TE16SrRNABChr; **Figure 1**) and 2µl of DNA template. Following a “hot start”, the reaction mixtures were subjected to the following empirically optimised thermal cycling parameters in a Perkin Elmer 2400 thermocycler: 96°C for five minutes followed by 40 cycles of two-step PCR; 96°C for one minute, 72°C for one minute, followed by a final extension at 72°C for 10 minutes. During each run, molecular grade water was included randomly as negative controls and appropriate DNA templates from *T. equigenitalis* DNA were included as positive controls, as appropriate.

### Detection of PCR products

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

### Surveillance study of Thoroughbred horses

Two hundred and fifty Thoroughbred horses were examined in this study for the presence of *T. equigenitalis* by PCR and cultural techniques. One swab was taken from each animal by a veterinary surgeon. In mares, the swab was taken from either the *clitoral fossa* or the *clitoral sinus* and in stallions, from the *urethral orifice*, the



**FIGURE 1:** Description and location of novel PCR primers employed in the PCR assay.

*urethral fossa or the penile sheath*. Swabs were transported to the laboratory in Amies charcoal transport media and were examined within 24h. For the conventional detection of *T. equigenitalis*, swabs were plated on to four different media:

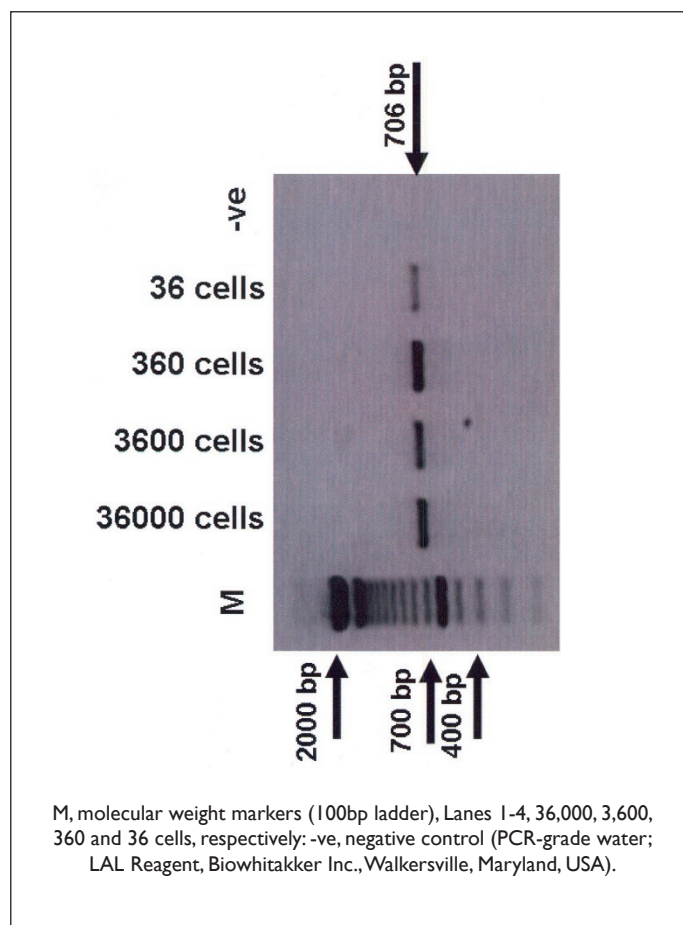
- (i) CEMO agar (MAST DM470, MAST Diagnostics Ltd, Merseyside, England) containing streptomycin (250µg/ml) and fungizone (5µg/ml);
- (ii) CEMO agar supplemented with 5% chocolate and fungizone (5µg/ml);
- (iii) Columbia agar base supplemented with 5% defibrinated horse blood; and,
- (iv) McConkey agar. The plates were incubated for six days (37°C; 5% [v/v] CO<sub>2</sub>).

Swabs from animals were placed in 1.5ml micro-centrifugation tubes containing phosphate-buffered saline (PBS)(200µl) and sonicated in a sonicating bath for five minutes. The resulting suspension was boiled for 10 minutes in a heating block (Stuart Scientific Ltd., England) and subsequently centrifuged at 13,000g for five minutes. A 100µl volume of supernatant was transferred to a tube and stored at -80°C until amplification. Following this, the pellet was re-suspended in sterile distilled water (50ml) and boiled for 15 minutes.

A sterile non-inoculated swab and a swab spiked with *T. equigenitalis* (Type strain NCTC 11184) underwent the above extraction procedure to serve, respectively, as a negative and a positive extraction control. The sensitivity of the assay was calculated by serially diluting a known number of bacterial cells in PBS and carrying out an extraction and PCR amplification, as described above. The highest serial dilution that yielded a positive PCR reaction was considered to be the threshold of sensitivity for the assay.

## Results

Two primers [TE16SrRNABCH<sub>forward</sub> and TE16SrRNABCH<sub>reverse</sub>] were selected from the 16S rRNA gene of *T. equigenitalis* as outlined in **Figure 1**. We tested the specificity of the primers both by BLAST searching at <http://www.blast.genome.ad.jp>, as well as by challenging the primers to DNA from non-*T. equigenitalis* organisms. BLAST searching demonstrated a score of 50 [E value 6x10<sup>-5</sup>] and 49 [E value 2x10<sup>-4</sup>] for the forward and reverse primers, respectively. There was no shared homology with any other bacterial or eukaryotic DNA with either primer, nor with the other species member within the genus *Taylorella* (i.e., *T. asinigenitalis*) where there were eight and four nonaligning base pairs in the forward and reverse primers, respectively, between these two species. An amplified product of approximately 706bp was generated with all tested *T. equigenitalis* isolates (**Figure 2**) and no other bacterial genomic DNA was amplified, demonstrating the specificity of the primers for *T. equigenitalis*. Of the 250 animals screened for *T. equigenitalis*, none was positive for this organism by either culture or PCR methods, where the molecular sensitivity of the assay was shown to be no lower than 36 cells, which compares favourably to the three-step PCR assay, previously described by Bleumink-Pluym et al. (1994), who calculated the sensitivity of their assay as 15 bacteria.



**FIGURE 2:** Sensitivity of the two-step PCR assay; PCR amplification of a 706bp region of the 16S rDNA of *Taylorella equigenitalis*

## Discussion

The traditional basis for the identification of pathogenic and commensal bacteria has been their isolation or propagation in the laboratory. Biochemical, morphological and serological tests usually require growth of the organism. Reliance on these parameters may have significantly limited the awareness of true bacterial diversity and is impractical in many situations. The rapidly expanding use of 16S rRNA gene sequences for phylogenetic, evolutionary and diagnostic studies offers an opportunity for alternative approaches: 16S rRNA genes are found in all bacteria and accumulate mutations at a slow, constant rate over time, hence they may be used as “molecular clocks” (Woese, 1987). Highly variable portions of the 16S rRNA sequence provide unique signatures to any bacterium and useful information about relationships between them. Alternatively, since 16S rRNA molecules have crucial structural constraints, certain conserved regions of sequence are found in all known bacteria (i.e., the eubacteria) and in all known organisms. Species-specific primers may then be designed to recognise these conserved bacterial 16S rRNA gene sequences and used to amplify



intervening, variable or diagnostic regions (Weisburg *et al.*, 1991; Wilson *et al.*, 1990). This procedure avoids the need to grow the bacterium and does not require pre-existing phylogenetic information. Employment of conventional culture techniques for the detection of *T. equigenitalis* can be both slow and insensitive, potentially leading to the generation of false-negative results. In the case of *T. equigenitalis*, normally six days are required for the detection by culture (Swaney and Sahu, 1978). Phenotypic identification can be further compromised due to the presence of unusual colony morphology variants (Sahu *et al.*, 1982) and streptomycinsensitivity variants (Kamada *et al.*, 1986). Recently, there is an increasing tendency to employ PCR-based detection assays for a number of clinically relevant pathogens. Although such a molecular approach may have certain disadvantages, overall the employment of PCR-based detection assays has numerous advantages, which outweigh those offered by the conventional approach: in particular, improvements in the speed and specificity of the detection of *T. equigenitalis*, as well as the ability to detect this organism in animals undergoing antimicrobial chemotherapy. Disease prevention strategies rely heavily on the results of swabs taken from mares and stallions involved in breeding programmes. Sole reliance on problematical culture techniques may translate into complications in the management of horses at stud, e.g., where stallion owners should not accept mares unless they have an up-to-date negative laboratory certificate (Anon, 2000). Conventionally, most PCR assays involve a three-step process, i.e., denaturation, annealing and extension. However, this method sought to develop a novel two-step process, i.e., denaturation and annealing/extension, to gain on time of phenotypic identification. Consequently, large 25+mers were designed to be able to tolerate such a two-step PCR assay. Overall, it was estimated that the two-step assay would gain approximately one hour in comparison with the three-step assay, allowing for 40 cycles of one minute annealing at approximately 50°C to 60°C, in addition to the ramp times in lowering the temperature from denaturation to annealing and from annealing to extension. This advantage in saving time may be beneficial in busy service diagnostic laboratories, to help facilitate several PCR runs within the working day. Although the ribosomal primers that were designed in this study are specific for *T. equigenitalis*, it must be remembered that the 6S rRNA gene is highly conserved throughout the eubacteria. Consequently, if a positive swab is detected by PCR, it must be regarded as being presumptively positive, until confirmed by sequencing of the 706bp PCR amplicon. As positive results are extremely rare, it is indeed feasible to sequence all presumptive positives to avoid any molecular misidentifications. In conclusion, we describe a novel two-step PCR assay for the detection of *T. equigenitalis* based on specific primers within the 16S rRNA genes. Such a molecular approach offers a sensitive and specific alternative to conventional culture techniques, which could potentially lead to improved diagnosis and subsequent management of horses involved in breeding programmes.

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