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Genotyping of German and Austrian *Taylorella equigenitalis* isolates using repetitive extragenic palindromic (REP) PCR and pulsed-field gel electrophoresis (PFGE)

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ABSTRACT

A total of 124 *Taylorella* (*T.*) *equigenitalis* and five *T. asinigenitalis* field isolates collected between 2002 and 2014 were available for genotyping using REP- (repetitive extragenic palindromic) PCR and PFGE (pulsed-field gel electrophoresis). The study comprised 79 *T. equigenitalis* field isolates originating from ten defined breeds of German horses and revealed a spectrum of five REP (rep-E1-E4, rep-E3a) and 15 PFGE (TE-A1-A9, TE-B1-B3, TE-C, TE-E1, and TE-E2) genotypes. *T. equigenitalis* field isolates ($n = 40$) obtained from Austrian Lipizzaner horses were differentiated into three REP (rep-E1, rep-E3a, and rep-E4) and three PFGE genotypes (TE-A2, TE-A5, and TE-D); those isolated from four Austrian Trotters belonged to the REP/PFGE genotype rep-E2/TE-A1. Interestingly, a *T. equigenitalis* isolate recovered from a Holsteiner stallion living in South Africa revealed the REP/PFGE genotype rep-E1/TE-A5 which was otherwise exclusively present in the majority of Austrian Lipizzaner horses in our study. The type strain included in this study revealed the genotype REP/PFGE rep-E1/TE-F. Six strains of *T. asinigenitalis* including the type strain were separated into three REP (rep-A1-A3) and six PFGE genotypes (TA-A1, TA-A2, TA-A3, TA-B, TA-C, TA-D). Overall, the generated REP and PFGE genotypes showed a good correlation, whereas REP-PCR proved to be a suitable method for molecular epidemiological screening of *T. equigenitalis* and *T. asinigenitalis* isolates that should be differentiated in detail by genotyping using PFGE.

1. Introduction

Taylorella (*T.*) *equigenitalis* is the etiologic agent of contagious equine metritis (CEM), the most relevant venereal disease in horses worldwide with the greatest impact on horse breeding. After the first appearance of CEM in the UK and in Ireland in 1977 (Crowhurst, 1977), *T. equigenitalis* has spread globally (Matsuda and Moore, 2003; Timoney, 1996) supported by its highly contagious nature and long-term survival in asymptomatic animals, predominantly stallions, but also in mares (Schulman et al., 2013). These attributes allow *T. equigenitalis* to survive over a long period of time, especially in countries where CEM is endemic and poses a risk of introduction and spread to countries free of CEM (Erdman et al., 2011). This situation has been aggravated by the introduction of artificial insemination by certain breed societies (Matsuda and Moore, 2003; Schulman et al., 2013). Consequently, international trade of equine animals and germplasm is closely regulated and routine testing of breeding stallions has been implemented to prevent CEM outbreaks (Council Directive 92/65/EEC, 1992; Dennis et al., 2014).

Genotyping of *T. equigenitalis* has been performed following CEM outbreaks in the USA, Japan, Australia, UK, Norway and a few other European countries (Aalsburg and Erdman, 2011; Erdman et al., 2011; Luddy and Kutzler, 2010; Matsuda and Moore, 2003; Kagawa et al., 2001; Matsuda et al., 1997, 1998, 1999, 2000; Thoresen et al., 1995;

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Bleumink-Pluym et al., 1990; Miyazawa et al., 1995; Ozgur et al., 2001). However, no current data are available on the epidemiology of CEM in Germany and Austria. Genotyping of *T. equigenitalis* may assist in tracing routes and sources of global *T. equigenitalis* infections (Erdman et al., 2011). This is especially true for *T. equigenitalis* strains originating from Germany, since a considerable number of German isolates are suspected to be responsible for CEM outbreaks in the USA (Aalsburg and Erdman, 2011) and recently in the United Arab Emirates (Dwyer et al., 2013). Furthermore, a CEM outbreak in a Lipizzaner herd in South Africa in 2011 was presumed to have originated from Europe (May et al., 2012, 2015; Schulman et al., 2013).

In the present study REP- (repetitive extragenic palindromic) PCR was used for genotyping of *T. equigenitalis* isolates and the results were compared for the first time with datasets obtained by PFGE (pulsed-field gel electrophoresis). REP-PCR is an easy to perform PCR-based technique that has already been used to genotype a variety of diverse groups of pathogens (Bricker, 2011). PFGE analyses were performed as the gold standard method (Olive and Bean, 1999) to provide data comparable with previous studies.

The aim of the present study was to gain insight into the spectrum of *T. equigenitalis* genotypes found in Germany and Austria between 2002 and 2014 and to allow comparisons on an international basis.

2. Material and methods

2.1. Bacterial isolates

In the present study, a total of 124 *T. equigenitalis* field isolates recovered from horses (79 stallions, 34 mares, 11 without information on gender) and *T. asinigenitalis* field isolates recovered from five donkeys between 2002 and 2014 were included. The isolates were provided by the Chemical and Veterinary Investigations Office Stuttgart, the Friedrich-Loeffler-Institute in Jena, the Institute of Microbiology, University of Veterinary Medicine Vienna, the Aulendorf State Veterinary Diagnostic Centre, and the IDEXX Vet·Med·Laboratory. In addition, type strains of *T. equigenitalis* (ATCC 35865/DSM 10668) and *T. asinigenitalis* (ATCC 700933), respectively, were included in the study. All strains (listed in Table 1) were stored at −70 °C in a commercially available preservation system (Cryobank, Mast Diagnostica, Reinfeld, Germany) until use.

2.2. REP-PCR analysis

REP-PCR was performed as described by Versalovic et al. (1991) with some modifications. In brief, *T. equigenitalis* and *T. asinigenitalis* isolates and the type strains were cultivated for 72 h at 37 °C under microaerophilic conditions (GasPak EZ, CO_2 Container system, BD, Heidelberg, Germany) on lysed horse blood agar (Mast Diagnostica, Reinfeld, Germany) and harvested with a 1 μl inoculation loop (Sarstedt, Nümbrecht, Germany), suspended in 100 μl sterile deionized water and incubated for 15 min at 99 °C. After centrifugation of the bacterial suspension for 1 min at 20,000 rcf, the supernatant was used as template or stored at −20 °C until use.

For PCR, 1.5 μl of the supernatant was added as a template to 25 μl (total) PCR mixture containing 1 μM of each primer REP2-I (5′-ICGICTTATCIGGCCTAC-3′) and REP1R–I (5′-IIIICGICGICATCIGGC-3′), 200 μM of each dNTP, 1.25 U Taq DNA polymerase (Roche, Mannheim, Germany), 1× reaction buffer, and 2.5 mM (total) $MgCl_2$. In some cases the template had to be diluted 20- to 50-fold. An automated thermal cycler (Mastercycler, Eppendorf, Hamburg, Germany) provided the following amplification conditions: an initial denaturation at 95 °C for three minutes followed by 30 cycles of denaturation at 90 °C for 30 s, annealing at 40 °C for 60 s and extension at 72 °C for 90 s. The reaction ended with a final extension at 72 °C for eight minutes and subsequent cooling to 8 °C. Ten μl of each PCR reaction were electrophoresed in a 2.5% agarose gel with 1× TBE containing 0.3 μg/ml ethidium bromide at 5 V/cm for 2.5 h. DNA products were made visible with an UV transilluminator and documented with a gel imaging system (Herolab, Wiesloch, Germany).

Table 1
Genotyping of *T. equigenitalis* and *T. asinigenitalis* isolates and type strains using REP-PCR and PFGE.

REP genotype	PFGE genotype	Number of isolates/strains	Isolates
T. equigenitalis			
rep-E1	TE-A5[a]	46	2374, 9009, 9010, 9011, 9012, 9015, 9016, 9017, 9201, 9202, 9203, 9204, 9205, 9206, 9207, 9208, 9209, 9210, 9212, 9213, 9214, 9215, 9216, 9221, 9223, 9224, 9225, 9226, 9227, 9228, 9229, 9230, 9231, 9232, 9233, 9234, 9236, 9237, 9238, 9239, 9240, 9241, 9242, 9243, 9244, 9245
	TE-C	3	2362, 2955, 5061
	TE-F[a]	1 (2)	365/3139 (ATCC 35865/DSM 10668)
rep-E2	TE-A1	4	9217, 9218, 9219, 9220
	TE-B1[a]	3	2364, 9018, 9020
	TE-B2	1	4972
	TE-B3	2	4408, 5567
	TE-E1[a]	1	9022
	TE-E2[a]	1	9029
rep-E3	TE-A1	15	1639, 7299, 9039, 9050, 9051, 9054, 9056, 9211, 9401, 9402, 9403, 9404, 9405, 9406, 9443
	TE-A3	2	2370, 2371
	TE-A4	3	2144, 2363, 5060
	TE-A7	1	2368
	TE-A8	3	7994, 9021, 9025
	TE-A9	17	2353, 2356, 2359, 2360, 2361, 2365, 2954, 3023, 3024, 3026, 3027, 9027, 9456, 9457, 9458, 9459, 9462
rep-E3a	TE-A2	20	2372, 4226, 5033, 5768, 8373, 8374, 8375, 8376, 8377, 8378, 8379, 8380, 8381, 8382, 8383, 8384, 8497, 8498, 9053, 9246
rep-4	TE-A6	1	9055
	TE-D	1	2953
Total		125	
T. asinigenitalis			
REP	PFGE	Number of isolates/strains	Isolates
rep-A1	TA-A1	1	9222
	TA-AB	1	9004
	TA-AC	1	5785
	TA-AD	1	5307 (ATCC 700933)
rep-A2	TA-A3	1	2475
rep-A3	TA-A2	1	9001
Total		6	

[a] profiles identical to those described by Aalsburg and Erdman (2011), namely TE-A5 = TE011, TE-B1 = TE018, TE-E1 = TE014, TE-E2 = TE015, and TE-F = TE003.

2.3. PFGE analysis

T. equigenitalis and *T. asinigenitalis* isolates and type strains were cultured on Eugon agar (Becton Dickinson, Schwechat, Austria) supplemented with 10% defibrinated sheep blood (*v*/v) and 1% IsoVitalex Enrichment (v/v, Becton Dickinson, Schwechat, Austria) and incubated at 37 °C in 6% CO_2 atmosphere. Agarose block preparation from *T. equigenitalis* and *T. asinigenitalis* cell suspensions, digestion with *Apa*I, and PFGE analysis techniques were performed as previously described (Aalsburg and Erdman, 2011; Matsuda et al., 1994; Miyazawa et al., 1995). Bionumerics software (Applied Maths Inc., Sint Martens Latem, Belgium) was used to analyse PFGE patterns and a dendrogram was constructed using Dice's coefficient and UPGMA settings based on 1% band position tolerance. Patterns were normalized employing *Salmonella enterica* subsp. *enterica* serovar Braenderup H9812 size standard. PFGE profiles generated were compared to those described by Aalsburg and Erdman (2011).