Pulsed-Field Gel Electrophoresis Genotyping of *Taylorella equigenitalis* Isolates Collected in the United States from 1978 to 2010[▽]

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***Taylorella equigenitalis* is the etiologic agent of contagious equine metritis (CEM), a venereal disease of horses. A total of 82 strains of *T. equigenitalis* isolated in the United States were analyzed by pulsed-field gel electrophoresis (PFGE) after digestion of genomic DNA with restriction enzyme *Apa*I. Twenty-eight of those strains isolated from horses in the 2009 U.S. outbreak (CEM09) were further analyzed with *Not*I and *Nae*I enzymes. When *Apa*I alone was used for analysis, the 82 isolates clustered into 15 different genotypes that clearly defined groups of horses with known epidemiological connections. The PFGE profiles of the CEM09 isolates were indistinguishable after digestion with *Apa*I, *Not*I, and *Nae*I and did not match those of isolates from previous U.S. outbreaks in 1978 and 2006 or of any other isolate from the National Veterinary Services Laboratories (NVSL) culture library. Coupled with the fact that the CEM09 isolates are epidemiologically related, these results suggest a common source for the outbreak not linked to previous occurrences of CEM in the United States.**

Contagious equine metritis (CEM) is a venereal disease of horses caused by *Taylorella equigenitalis*, a Gram-negative coccobacillus. First described in England (4, 17, 18) and Ireland (22) in 1977, CEM is strictly a reproductive disease that can result in subclinical infection, endometritis, vaginal discharge, infertility, and (rarely) abortion in mares whereas stallions are typically carriers with no clinical signs. The effect on equine reproductive efficiency and international trade restrictions can create substantial economic loss in the horse industry. The gold standard for diagnosis is isolation of *T. equigenitalis* from the equine reproductive tract. Confirmation of suspect isolates relies on biochemical analysis, latex agglutination, fluorescent antibody, PCR testing (1, 24), or 16S rRNA gene sequencing. The latter two tests are also used to differentiate *T. equigenitalis* from *Taylorella asinigenitalis*, which was identified in donkeys in the United States in the late 1990s (7). *Taylorella asinigenitalis* has periodically been isolated from naturally infected horses but is not known to cause clinical signs like CEM and is not regulated in the United States (15).

The first known occurrences of CEM in the United States were diagnosed in Kentucky in 1978 (19) and Missouri in 1979 (5). Import regulations were introduced immediately, and the disease was subsequently considered to have been eradicated with respect to both instances. All sexually intact horses of reproductive age currently imported into the United States from CEM-affected countries are quarantined and tested for CEM. Officially recognized diagnostic testing for CEM is undertaken in USDA-approved laboratories, with any *Taylorella*-suspect colonies sent to the National Veterinary Services Laboratories (NVSL) for confirmation. Any stallion or mare

testing positive for *T. equigenitalis* via culture (or, for mares only, via serology) must be treated and subsequently shown to produce negative test results before being released from quarantine. Stallions that produce negative test results are further bred to mares with negative test results (referred to as “test mares”). If samples from those test mares produce positive test results via culture or serology, the stallion and mares must be treated until negative test results are obtained. Although importation of horses into the United States requires testing to certify the horses CEM free, numerous CEM-positive horses have been identified in U.S. quarantine stations since these measures were implemented.

Since the implementation of regulatory testing, there have been three incidents of CEM in the United States outside quarantine facilities. In 2006 (outbreak CEM06), postquarantine imported stallions tested positive in Wisconsin. Culture testing identified a total of three stallions with positive test results that were quickly treated until negative test results were obtained, thus returning the United States to CEM-free status. In December 2008, a stallion undergoing regulatory CEM testing for semen export was found to be culture positive for *T. equigenitalis*. The USDA immediately initiated an epidemiological investigation (CEM09) in an attempt to identify other horses with positive test results and the source of the infection. The investigation of over 1,000 exposed horses identified 23 stallions with positive test results, including one gelding, and 5 mares with positive test results, for a total of 28 culture-positive horses in a time period spanning December 2008 to March 2010 and located in 8 states. The first stallion with positive test results identified in December 2008 had never been outside the United States, but it was not known whether the stallion had become infected with a strain that had entered the United States via an unidentified positive imported horse or whether a strain previously found in the United States was perhaps still circulating. Thus, a comparison of strains was necessary to determine whether all CEM09 isolates were of the same ge-

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notype and whether this genotype matched that of any previous U.S. isolates. In May 2010 (CEM10), a stallion in California tested culture positive for *T. equigenitalis*. This horse was being tested for semen export and was not part of the CEM09 investigation. The epidemiological investigation included testing of 5 stallions and 18 mares with known contact but found no additional horses with positive test results. There was again a need to compare this isolate to U.S. isolates collected in the past.

Molecular genotyping of *T. equigenitalis* strains by the use of pulsed-field gel electrophoresis (PFGE) or crossed-field gel electrophoresis (CFGE), a variation of PFGE, has been previously performed on isolates from Europe (8), Japan (11, 16), Ireland and the United States (12), Norway (13), and Australia (10) by the use of restriction enzymes *ApaI* and *NotI*. In Europe, 46 isolates of *T. equigenitalis* from six countries were classified into 18 genotypes (8). In Japan, testing of 130 strains of *T. equigenitalis* isolated between 1980 and 1996 identified only a single genotype (11, 16). Testing of 18 strains isolated in Ireland identified three distinct genotypes, and tests of 8 U.S. strains indicated four distinct genotypes (12). In Norway, the profiles of eight strains of *T. equigenitalis* were indistinguishable (13). Testing of field strains of *T. equigenitalis* isolated between 1979 and 1980 in Australia produced three different genotypes (10). These studies demonstrated that PFGE is capable of discriminating strains of *T. equigenitalis*.

The objective of this work was to use PFGE to characterize all strains of *T. equigenitalis* isolated in the United States from 1978 to 2010. These data were further used to compare *T. equigenitalis* isolates collected within and between U.S. outbreaks, including CEM09.

MATERIALS AND METHODS

A total of 82 isolates of *T. equigenitalis* were analyzed in this study (Table 1). Of these, 46 isolates, including 14 isolates associated with CEM78, 3 isolates from CEM06, 28 isolates from CEM09, and 1 isolate from CEM10, were found in the United States outside quarantine facilities. The remaining 36 isolates, including 33 strains isolated from imported horses found culture positive in U.S. quarantine stations over the last 30 years and 3 reference isolates (ATCC 35865, ATCC 49729, and KY 188), were from the NVSL culture library. Isolates were suspended in Trypticase soy broth (TSB) with 20% glycerol and stored at -80°C . Isolates were frozen immediately after isolation, but some historical strains had been subcultured two to three times through the years prior to this study.

Culture methods for *T. equigenitalis* have changed little over the last 30 years, and isolates in this study were isolated as previously described (20). Briefly, swab samples were collected and transferred into tubes containing Amies transport medium with charcoal and plated in the laboratory within 48 h of collection. Swabs were cultured on Eugon agar medium and the more selective Timoney-Shin medium (23) and incubated at 37°C in 5 to 10% CO_2 in air. Suspect colonies were screened by Gram stain, oxidase, catalase, alkaline phosphatase, and latex agglutination tests (Monotay; Bionor Laboratories AS, Skien, Norway). Positive results for Gram-negative coccobacilli in the tests noted above were further confirmed by a fluorescent antibody test and 16S rRNA gene sequencing (MicroSeq 500; Applied Biosystems, Foster City, CA). All *T. equigenitalis* strains in the NVSL inventory were confirmed via 16S rRNA gene sequencing, since it was not available when the strains were first isolated. All *T. equigenitalis* isolates were examined for growth on Eugon agar containing 200 $\mu\text{g}/\text{ml}$ (5) streptomycin to assess sensitivity. Plates were incubated at 37°C in 5 to 10% CO_2 , and isolates were considered sensitive if no growth was observed after 96 h.

For PFGE, *T. equigenitalis* isolates were cultured for growth on Timoney-Shin medium and incubated at 37°C in 5 to 10% CO_2 for 72 h. Cell suspensions (100 mM Tris HCl, 100 mM EDTA [pH 8.0]) were prepared by removing cells from the plate surface with a sterile cotton swab and adjusting the optical density (OD) to 1.30 to 1.40 by the use of a Cole-Parmer 1200 spectrophotometer (Vernon Hills, IL) at a wavelength of 612 nm. Preparation of agarose blocks from these

cell suspensions and techniques for PFGE were performed as previously described (7, 14, 16). Genomic DNA was digested via restriction enzymes *ApaI*, *NotI*, and *NaeI*. Bionumerics software (Applied Maths Inc., Austin, Texas) was used to analyze patterns, and dendrograms were created by the use of Dice's coefficient and unweighted-pair group method using average linkages (UPGMA) settings and analyzed using 1.5% band position tolerance values. Patterns were normalized using *Salmonella enterica* subsp. *enterica* serovar Braenderup H9812, a universal size standard (6).

RESULTS

From 1978 to 2010, at least 119 horses were found culture positive for *T. equigenitalis* in the United States. Thirty-three of those horses were identified by positive test results while being held in U.S. quarantine stations and were subsequently confirmed negative by subsequent test results prior to release. However, of at least 86 horses found culture positive outside quarantine, all were linked to incidents between 1978 to 2010: from CEM78, 50 horses; from CEM79, 4 horses; from CEM06, 3 horses; from CEM09, 28 horses; and from CEM10, 1 horse. A total of 82 *T. equigenitalis* isolates were analyzed in this study, including 33 isolates from the 33 horses found positive in quarantine, 46 isolates from the 86 horses outside quarantine, and 3 isolates from reference strains (ATCC35865, ATCC49729, and KY 188) (Table 1).

Digestion of *T. equigenitalis* genomic DNA with *ApaI* typically resulted in 11 to 13 bands ranging from 15 to 450 bp in size. PFGE analysis of these 82 *T. equigenitalis* isolates with restriction enzyme *ApaI* resulted in 15 different restriction patterns, as demonstrated in Fig. 1. Each of the different restriction patterns was assigned a unique genotype designation on the basis of the *ApaI* fingerprints, as shown in Table 1. The range of homology values among the 15 different PFGE genotypes in this study was 64.59% to 95.65%.

PFGE analysis of 12 isolates from CEM78 produced two genotypes, TE003 and TE008. These two genotypes also coincided with a phenotypic difference, as TE003 was streptomycin resistant and TE008 was streptomycin susceptible. Genotype TE003 was also seen in two domestic horses testing positive in 1982 and in two reference strains, KY 188 and ATCC 35865. Analysis of 33 isolates from horses with positive test results at U.S. import stations resulted in 11 different genotypes. The countries of origin for imported horses are shown in Table 1. Genotype TE018 was isolated in 14 instances between 1989 and 2009. Twelve isolates were from horses imported from Germany, one was from a horse imported from Belgium, and one was from a horse imported from Netherlands. That was the most common genotype among the imported horses. Reference strain ATCC 49729 had the same genotypic pattern (TE009) as a strain isolated from a horse imported from Germany in 1981. All three isolates from CEM06 had the same genotype (TE020), and all were susceptible to streptomycin. This genotype was also seen in an isolate from a horse imported from Germany in 2006.

The PFGE profiles of all 28 isolates from the CEM09 outbreak were indistinguishable, resulting in one genotype, TE004. To strengthen this result, isolates from CEM09 were further analyzed with additional restriction enzymes (*NotI* and *NaeI*). The CEM09 profiles were indistinguishable when digestion was performed with all three enzymes, and all of the isolates were resistant to streptomycin. Genotype TE004 of the

TABLE 1. PFGE typing of *Taylorella equigenitalis* isolated in the United States from 1978 to 2010 by the use of ApaI

Yr of isolation or strain category	Isolate source(s) (outbreak) or designation ^a	No. of isolates	Country of import	Genotype	Streptomycin sensitivity
1978	1 stallion, 5 mares ^b (CEM78)	6	France	TE003	Resistant
1978	1 stallion, 5 mares ^b (CEM78)	6	France	TE008	Susceptible
1981	Import stallion 1	1	Germany	TE009	Susceptible
1982	Domestic stallion 1 ^c	1		TE003	Resistant
1982	Domestic mare 2 ^c	1		TE003	Resistant
1985	Import stallion 2	1	Germany	TE016	Susceptible
1989	Import stallion 3	1	Germany	TE018	Susceptible
1992	Import stallion 4	1	Austria	TE011	Susceptible
1994	Import stallion 5	1	Germany	TE018	Susceptible
1996	Import stallion 6	1	Norway	TE022	Resistant
1997	Import stallion 7	1	Germany	TE018	Susceptible
1998	Import stallion 8	1	Germany	TE018	Susceptible
1998	Import stallion 9	1	Germany	TE015	Susceptible
1998	Import stallion 10	1	Germany	TE018	Susceptible
1998	Import mare 11	1	Netherlands	TE014	Resistant
1998	Import mare 12	1	Belgium	TE017	Resistant
1999	Import stallion 13	1	Germany	TE018	Susceptible
2000	Import stallion 14	1	Germany	TE015	Resistant
2000	Import stallion 15	1	Germany	TE018	Susceptible
2000	Import stallion 16	1	United Kingdom via Germany	TE015	Susceptible
2000	Import stallion 17	1	France via Netherlands	TE018	Susceptible
2001	Import mare 18	1	Germany	TE018	Susceptible
2001	Import stallion 19	1	Germany	TE018	Susceptible
2001	Import mare 20	1	Germany	TE018	Susceptible
2001	Import stallion 21	1	Germany	TE011	Susceptible
2001	Import mare 22	1	Germany	TE015	Resistant
2002	Import stallion 23	1	Austria	TE011	Susceptible
2002	Import stallion 24	1	Belgium	TE018	Susceptible
2002	Import stallion 25	1	Germany	TE015	Resistant
2003	Import stallion 26	1	Germany	TE018	Susceptible
2003	Import stallion 27	1	Denmark	TE019	Resistant
2003	Import mare 28	1	Netherlands	TE005	Resistant
2003	Import mare 29	1	Netherlands	TE005	Resistant
2005	Import mare 30	1	Netherlands	TE016	Susceptible
2006	Import stallion 31	1	Germany	TE020	Susceptible
2006	Import stallion 32	1	Austria	TE011	Susceptible
2006	3 stallions (CEM06)	3	Slovakia	TE020	Susceptible
2009	23 stallions, 5 mares (CEM09)	28	Unknown	TE004	Resistant
2009	Import stallion 33	1	Germany	TE018	Susceptible
2010	Stallion (CEM10)	1	Belgium via United Arab Emirates	TE021	Susceptible
Reference strain	ATCC 35865	1		TE003	Resistant
Reference strain	KY 188	1		TE003	Resistant
Reference strain	ATCC 49729	1		TE009	Susceptible

^a Additional isolates from horses with positive test results in CEM78 and 4 horses with positive test results in CEM79 were not saved and thus were not available for PFGE analysis.

^b Stallions were imported, and mares were domestic (with epidemiologic links to stallions).

^c The horse had an epidemiological link to CEM78.

CEM09 strain did not match any genotype previously isolated outside U.S. quarantine when digestion with ApaI was performed. Genotype TE022 was most similar to that of the CEM09 strain. A strain of this genotype was isolated from a quarantined stallion imported from Norway in 1996 and was 95.24% similar to the CEM09 strain when digestion with ApaI was performed. The only difference was the presence of a doublet near 33 bp for TE004 and a singlet for TE022. When subjected to digestion with NotI, the isolates were 94.75% similar, differing in a doublet near 145 bp for TE022 and a singlet for TE004. When digestion with NaeI was performed, TE004 and TE022 were 100% similar. The next closest genotype of isolates tested in this study was 74.98% similar to CEM09 when digestion with ApaI was performed. The sole isolate from the CEM10 investigation did not match any strains

previously isolated in the United States. This strain was streptomycin susceptible and was designated genotype TE021.

Streptomycin sensitivity was consistent among strains with the same genotype, with the exception of strains of genotype TE015. Isolates from a total of five imported horses exhibited genotype TE015 between 1998 and 2002, with three isolates streptomycin resistant and two isolates streptomycin susceptible.

DISCUSSION

This is the first comprehensive analysis of all available strains of *T. equigenitalis* isolated in the United States from 1978 to 2010. These genotypic and phenotypic data, coupled with epidemiological information, confirmed relationships between strains isolated from horses with positive test results

FIG. 1. Dendrogram of the 15 unique genotypes of *Taylorella equigenitalis* after PFGE analysis of 82 isolates with restriction enzyme *Apa*I. Isolates from outbreaks in 1978, 2006, 2009, and 2010 were of the following genotypes: CEM78, genotypes TE003 and TE008; CEM06, genotype TE020; CEM09, genotype TE004; CEM10, genotype TE021. The PFGE pattern of *Salmonella enterica* subsp. *enterica* serovar Braenderup H9812 was used as a universal size standard.

within each CEM incident in the United States and also showed a lack of strain relationships between CEM incidents. This study and previous studies (2, 8, 9, 10, 11, 12, 13, 16) have demonstrated that PFGE is valuable in discriminating strains of *T. equigenitalis*. Isolates from horses with positive test results in U.S. outbreaks and isolates from imported horses with positive test results were tested using PFGE, resulting in 15 distinct genotypes.

Streptomycin sensitivity has also been commonly used to characterize strains of *T. equigenitalis*. Our results indicate that streptomycin sensitivity values can differ within a PFGE genotype, since, among five isolates displaying genotype TE015, three were streptomycin resistant and two were streptomycin susceptible. The epidemiological histories of these horses are not known. However, streptomycin test results were consistent for isolates with known epidemiological relationships. Since the mechanism of streptomycin resistance in *T. equigenitalis* is currently unknown, a possible explanation for variations in resistance within a PFGE genotype could be the presence or absence of a plasmid not visible in the PFGE pattern.

In the CEM78 outbreak, approximately 45 mares and 5 stallions tested culture positive for *T. equigenitalis*, and many others were suspected to be positive for *T. equigenitalis* but were treated before testing (3, 20). Twelve of those isolates were tested in this study, resulting in detection of 2 different genotypes, TE003 and TE008. All six isolates with genotype TE003 were streptomycin resistant and epidemiologically linked to a single stallion imported from France in 1977. All six isolates with genotype TE008 were streptomycin susceptible and linked to a single different stallion also imported from France in 1977. The PFGE data from this study further support the previous epidemiological and phenotypic data that indicate that the CEM78 outbreak was attributable to two different stallions and two different strains of *T. equigenitalis*.

In 1982, two domestic horses tested culture positive for *T. equigenitalis*. The source was unknown; however, it was determined that domestic stallion 1 with a positive test result in 1982 was previously bred to a culture-positive mare from CEM78 (PFGE genotype TE003) and that domestic mare 2 was subsequently bred to domestic stallion 1. Results from this study showed that the isolates from these two domestic horses were streptomycin resistant and represented PFGE genotype TE003, suggesting either a common source from outside the United States or that the strain from 1978 was still present in the United States in 1982.

In the CEM06 incident, three imported stallions tested culture positive for *T. equigenitalis* after being released from quarantine in Wisconsin. Isolates from these horses were streptomycin sensitive and had the same genotype (TE020). These PFGE data further confirm that all of the isolates from CEM06 were indistinguishable from one another and were different from the strains present in the late 1970s. A horse imported from Germany also gave genotype TE020-positive test results in 2006 while in quarantine in Maryland. There is no known epidemiologic link between the 2006 German imported horse and the CEM06 stallions that were imported from Slovakia.

Epidemiologic investigation of the CEM09 outbreak focused on the contacts with horses known to have positive test results. Additional horses with positive test results were detected through testing of both direct and indirect contacts of each known horse with a positive test result. Direct contacts were horses involved in live cover breeding with a known horse with a positive test result. Indirect contacts included mares that received semen from a known stallion with positive test results via artificial insemination and stallions that had semen collected in the same facility and within a week or less of the time that a known stallion with positive test results was collected in that facility. Findings of the investigation indicate that five

stallion breeding and collection facilities in three states were involved in the outbreak and that indirect stallion-to-stallion transmission of *T. equigenitalis* occurred at least as early as 2005. In total, 28 horses, including 23 stallions and 5 mares, were found culture positive for *T. equigenitalis* during the CEM09 outbreak. Analysis of these isolates resulted in a single PFGE profile, genotype TE004, and all isolates were streptomycin resistant. Coupled with epidemiologic evidence linking all the CEM09 horses, these genotype and phenotype data suggest that the isolates represent genetic clones and are linked to a single, common source. Evidence from the epidemiologic investigation of this outbreak indicated that one of the horses with a positive test result, a stallion imported from Denmark in late 2000, was the most likely source.

In May of 2010, a stallion in CA tested culture positive for *T. equigenitalis*. This horse had no known epidemiologic links to CEM09, and testing of stallions and mares with known contact found no additional positives. The isolate was found to be streptomycin susceptible, and PFGE indicated a unique genotype (TE021). Genotype TE021 had not previously been seen in the United States, indicating a new incident not associated with CEM09 or other outbreaks.

The CEM09 genotype TE004 isolate did not match any other isolate in the NVSL CEM culture library. Although consideration should be given to the time period between isolations (21), this suggests that the CEM09 outbreak was not a carryover from previous outbreaks in 1978 and 2006 and that it instead represents a separate introduction into the United States. The closest match (95.25% similarity when tested with *ApaI*, 94.75% similarity with *NotI*, and 100% similarity with *NaeI*) was to an isolate from a stallion imported from Norway in 1996 that had been found culture positive in quarantine. Although the strain was also streptomycin resistant, PFGE analysis indicated that this strain represented a different genotype (TE022). Even though the genotypes are highly similar, there is no known link between these horses that would suggest that the stallion imported in 1996 could have been the source of the CEM09 outbreak. At best, genotypes that are identical or that are closely related but with no known epidemiologic link may suggest a shared geographic source for the isolates prior to entry into the United States. However, more work is needed before *T. equigenitalis* PFGE genotypes can be correlated to specific geographic areas.

Comparison of bacterial strains by the use of PFGE with extended periods of time between isolations can be problematic, as was previously discussed (21). Considering the epidemiologic information from the CEM09 outbreak, some horses could have been infected for over 5 years, indicating stability of the *T. equigenitalis* PFGE profiles for at least that amount of time in that specific incident. Although the complete history of the isolates from quarantined imported horses analyzed in this study is not known, it is interesting that some genotypes are rare and that others, such as TE018, occur more frequently, spanning at least 20 years. The genotypic stability of *T. equigenitalis* might be explained by persistence in asymptomatic carrier animals as the main reservoir of infection. As an external pathogen in an apparent healthy animal, selection due to antibiotic and immune pressures might be reduced. Further work is needed to support this hypothesis.

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