Molecular epidemiological investigation of contagious equine metritis in South Africa

By

Catherine Edith May

Submitted in partial fulfillment of the requirements for the degree

Philosophiae Doctor (Veterinary Science)

In the

Department of Production Animal Studies
Faculty of Veterinary Science, University of Pretoria

October 2020

Declaration of originality

I, Catherine Edith May, do hereby declare that I understand what plagiarism entails and am aware of the University's policy in this regard. I declare that this these is my own, original work. Where someone else's work was used (whether from a printed source, the internet or any other source) due acknowledgement is given and reference is made according to departmental requirements. I have not made use of another student's previous work and submitted it as my own. I have not allowed and will not allow anyone to copy my work with the intention of presenting it as his or her own work.

Catherine Edith May

26 October 2020
Date

Ethics statement

The author, whose name appears on the title page of this thesis, has obtained, for the research described in this work, the applicable research ethics approval. The author declares that she has observed the ethical standards required in terms of the University of Pretoria's Code of ethics for researchers and the Policy guidelines for responsible research.

Summary of thesis

Molecular epidemiological investigation of contagious equine metritis in South Africa

Catherine Edith May

Supervisor: **Prof Martin Schulman**

Co-supervisor: **Prof Alan Guthrie**

Degree: **Philosphiae Doctor (PhD)**

Department: **Production Animal Studies**

Contagious equine metritis (CEM) is a non-systemic, venereally-transmitted disease of horses caused by the bacterium, *Taylorella equigenitalis.* The second member of the genus, *Taylorella asinigenitalis,* is found in donkeys and is considered non-pathogenic. Contagious equine metritis was initially identified during the 1977 Thoroughbred breeding season in Newmarket, United Kingdom (UK) where, due to its virulence and contagious nature, CEM was estimated to have caused a markedly decreased foaling rate. The disease was speculated to have originated from France and is now considered endemic in non-Thoroughbred populations in mainland Europe. Since then, sporadic outbreaks of seemingly less virulent strains *of T. equigenitalis* have occurred worldwide in many non-endemic countries, often associated with assisted reproductive techniques (ART).

Contagious equine metritis is a World Organisation for Animal Health (OIE) notifiable disease and is regarded internationally as one of the most highly regulated equine diseases of global importance to equine health and international trade. Stallions affected with *T. equigenitalis* are unapparent carriers of the organism and the main source of infection with this carrier status persisting for months or even years. Infected mares typically develop a transient endometritis or cervicitis resulting in irregular interoestrus periods and temporary infertility. Most mares develop a short-lived humoral immunological response and rid themselves of infection, however a small population of asymptomatically infected mares may attain carrier status and pose a potential source of infection to stallions.

South Africa was considered free of *T. equigenitalis* until May 2011 when an outbreak was confirmed. Initially the outbreak was thought to be confined to one property, affecting the index case, a stallion and a mare bred to him via fresh semen artificial insemination. However, institution of a national stallion screening programme and further epidemiological traceback of in-contact animals during the period July 2011 to May 2020, revealed a total of 42 horses on 15 properties that were affected. All affected animals were traced back to the index property.

In the first chapter, the problem statement, hypotheses and objectives that lead to the publication of the work presented in this thesis are considered.

In the second chapter, the broader context of the risk factors contributing to outbreaks of equine infectious diseases are considered and the pertinent literature on both *T. equigenitalis* and *T. asinigenitalis,* including the diagnosis, pathogenesis, clinical signs, transmission, treatment, control and prevention are reviewed.

In the third chapter, the materials and diagnostic methods used during the South African *T. equigenitalis* outbreak and subsequent epidemiological surveillance are described. These include the diagnostic tools used to identify the first incursion of *T. asinigenitalis* into South Africa. Later in the chapter, the epidemiological investigations of the *T. equigenitalis* outbreak and *T. asinigenitalis* incursion are described and the progressive development of an adapted treatment protocol for the South African context is explained.

In the fourth chapter, the results obtained from outbreak investigation and subsequent epidemiological surveillance are presented. The successive findings from the development of the adapted treatment protocol are also presented.

Finally, in the fifth chapter, the findings of these studies are discussed, in particular how the use of newer molecular technologies and the application of comparative phylogenetic analysis definitely traced the origin of the outbreak. These findings are aligned with future directions and in the final chapter, the overall conclusions from these studies are presented.

Overall, this work reported on the use of more current molecular technologies for epidemiological investigation of the origin and circumstances of both the *T. equigenitalis* outbreak and *T. asinigenitalis* incursion into South Africa. Experience gained during the investigation and control of these outbreaks informed subsequent legislation and knowledge regarding the diagnosis and treatment of both *T. equigenitalis* and *T. asinigenitalis.*

Dedication

To Martin Schulman – mentor, colleague and friend. Long live Barbara Cartland!

Acknowledgements

I would like to acknowledge the following people for their support and kindness:

My supervisors, Prof Martin Schulman and Prof Alan Guthrie. Thank you for starting me down this road in 2011, for your endless faith in me and for all the amazing opportunities that you've pushed my way. I definitely wouldn't be here if it weren't for you.

To all the staff at the Equine Research Centre for their continuous help and support over the years. Chris Joone and Mpho Monyai, thank you for all the help with samples and Jenny du Toit for your fantastic filing system and always managing to find those lab reports!

To my colleagues at the Section of Reproduction, thank you for your support and friendship throughout this time.

To my family, John, Mandy, Liam and Sean, for bearing with me through the endless studies. Thank you for always being there for me.

And to my husband, Christopher. Thank you for your endless patience and the numerous cups of tea! Thank you for understanding how important my work is to me and for the constant support. A very big thank you for doing those 2am feeds for little Isabella so that I was able to function properly the next day.

And finally, to my daughter, Isabella May Woodrow. The plan was to have this done before you arrived, but that didn't quite work out! Thank you for your patience, my little one, while I had to work and for always brightening up my day.

Table of contents

Declaration of originality ... **ii**
Ethics statement ... **iii**
Summary of thesis ... **iv**
Dedication ... **vi**
Acknowledgements ... **vii**
Table of contents ... **viii**
List of tables ... **x**
List of figures ... **xii**
List of abbreviations ... **xiii**
Chapter 1 General introduction ... **1**
1.1 Introduction ... 1
1.2 Problem statement ... 2
1.3 Hypotheses ... 3
1.4 Objectives ... 4
Chapter 2 Literature review ... **5**
2.1 Equine infectious diseases ... 5
2.2 Historical perspective ... 11
2.3 The genus *Taylorella* ... 12
2.4 Transmission ... 15
2.5 Pathogenesis and clinical signs ... 15
2.6 Diagnosis ... 17
2.6.1 Sampling sites ... 17
2.6.2 Sampling interval ... 17
2.6.3 Sampling procedures for imported animals ... 17
2.6.4 Sampling procedures for detection of infection ... 18
2.6.5 Polymerase chain reaction ... 19
2.6.6 Bacterial culture ... 20
2.6.7 Serology ... 20
2.6.8 Loop-mediated isothermal amplification (LAMP) ... 21
2.7 Treatment ... 21
2.7.1 Treatment of *T. equigenitalis*-positive horses ... 21
2.7.2 Treatment of *T. asinigenitalis*-positive equids ... 22
2.8 Analysis using restriction enzymes ... 22
2.9 Multilocus sequence typing (MLST) ... 23
2.10 Whole Genome Sequencing ... 25

2.11 Comparative phylogenetic analysis using the PathoSystems Resource Integration Centre 26
Chapter 3 Materials and methods **28**
3.1 The diagnostic methods used in the South African investigation 28
3.1.1 Real-time PCR 28
3.1.2 Bacterial culture 29
3.1.3 MLST analysis 30
3.1.4 Whole genome sequencing 32
3.1.5 Comparative phylogenetic analysis 32
3.2 Epidemiological investigation of the South African outbreak of *Taylorella equigenitalis* and the *Taylorella asinigenitalis* incursion 34
3.3 Development and evolution of treatment protocols in South Africa 35
Chapter 4 Results **38**
4.1 The results from the South African outbreak 38
4.1.1 Real-time PCR 38
4.1.2 Bacterial culture 43
4.1.3 MLST analysis 43
4.1.4 Whole genome sequencing 43
4.1.5 Comparative phylogenetic analysis of *Taylorella equigenitalis* sequence type 4 44
4.2 The epidemiological sequence of the South African outbreak of *Taylorella equigenitalis* 48
4.3 The results of treatment regimens used during the course of the South African outbreak (2011-2017) 49
Chapter 5 Discussion **53**
Chapter 6 Conclusions **63**
References **64**
Appendix A: Draft Genome sequence of *Taylorella equigenitalis* strain ERC_G2224 isolated from the semen of a Lipizzaner stallion in South Africa **76**
Appendix B: Direct culture-independent sequence typing of *Taylorella equigenitalis* obtained from genital swabs and frozen semen samples from South African horses **78**
Appendix C: In-treatment monitoring and use of a novel topical therapy shortens the duration to eliminate *Taylorella asinigenitalis* in donkey stallions **81**

List of tables

Table 2-1 The International Federation of Horseracing Authorities' (IFHA) international flat and jump racing statistics and the global events hosted by the International Federation for Equestrian Sports (FEI) from 2007-2018 7

Table 2-2 The cumulative number of stallions and semen and total foal crop for 2019 registered by studbooks (n=75) belonging to the World Breeding Federation for Sport Horses (WBSFH) 8

Table 2-3 A summary of the comparison between *Taylorella equigenitalis* and *Taylorella asinigenitalis* in terms of genomics, disease caused, clinical signs, carrier status, transmission and diagnosis.. 14

Table 3-1 The bioproject and biosample numbers and sequence reads archive (SRA) numbers with hyperlinks of all ST4 (n=32) and one ST1 genomes available on the National Centre for Biotechnology Information database 33

Table 4-1 Summary of qPCR assay and confirmatory bacterial culture results from swabs obtained from the predilection sites on the external genitalia of three male horses identified in 2011 during the initial outbreak investigation of the South African *Taylorella equigenitalis* outbreak..................................... 39

Table 4-2 Summary of cycle threshold (Ct) results from the qPCR assay and confirmatory bacterial culture results from swabs obtained from the predilection sites on the external genitalia of 36 male horses identified during the epidemiological traceback and national stallion screening programme during 2011-2017 of the South African *Taylorella equigenitalis* outbreak... 40

Table 4-3 The distribution of *Taylorella equigenitalis* by sample predilection sites of acutely (n=1) and chronically (n=2) infected mares during the 2011 South African outbreak 41

Table 4-4 Summary of results from a duplex qPCR assay for *Taylorella equigenitalis/asinigenitalis* and bacterial culture from swabs obtained from the predilection sites on the external genitalia of two miniature donkey stallions 42

Table 4-5 Genomic characteristics of all ST4 *Taylorella equigenitalis* strains (n=32) found on GenBank plus a reference ST1 outgroup assembled on PATRIC... 45

Table 4-6 Summary of treatment periods for 10 Lipizzaners from the South African Lipizzaner Centre positive for *Taylorella equigenitalis* on qPCR alone, including the treatment product and total number of days required to clear infection 49

Table 4-7 Summary of treatment periods for 13 Lipizzaners from the South African Lipizzaner Centre positive for *Taylorella equigenitalis* on qPCR and bacterial culture, including the treatment product and total number of days required to clear infection 50

Table 4-8 Summary of all courses of treatment for the horses (n=5) treated by University of Pretoria personnel including two treated with the additional inclusion of an intramammary antimicrobial formulation 51

Table 4-9 Sequential incidence and distribution by anatomic site of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* in Lipizzaner stallions following topical treatment for *Taylorella equigenitalis* by treatment group 52

List of figures

Figure 2-1 Factors affecting the risk of international disease transmission 10

Figure 4-1 Phylogenetic tree based on the SRA data of 32 ST4 genomes using the maximum likelihood method, rooted with *Taylorella equigenitalis* ST1 as the outgroup 47

Figure 4-2 Schematic temporal representation of *Taylorella equigenitalis*-positive horses identified during the South African outbreak showing the proposed transmission between animals involved in the outbreak. Date represents the date of sample collection. 48

List of abbreviations

AI	Artificial insemination
AHSV	African horse sickness virus
APHA	Animal and Plant Health Agency
ART	Assisted reproductive techniques
BRC	Bioinformatics Resource Centre
CEM	Contagious equine metritis
CEMO	Contagious equine metritis organism
CC	Clonal complex
CF	Complement fixation
CFGE	Crossed field gel electrophoresis
CRISPR	Clustered regularly interspaced short palindromic repeat
DEFRA	Department for Environment, Food and Rural Affairs
DNA	Deoxyribonuclease acid
EAV	Equine arteritis virus
EHV	Equine herpesvirus
EU	European Union
EVA	Equine viral arteritis
FEI	International Federation for Equestrian Sports
Fic	Filamentation induced by cyclic AMP (*Fic*)
FIGE	Field inversion gel electrophoresis
IFA	Immunofluorescent antibody
IFHA	International Federation of Horseracing Authorities
HBLB	Horserace Betting Levy Board
HHP	High health, high performance
MLST	Multilocus sequence typing
NCBI	National Centre for Biotechnology Information
OIE	World Organisation for Animal Health
PATRIC	Pathosystems Resource Integration Centre
PFGE	Pulsed field gel electrophoresis
PubMLST	Public databases for molecular typing and microbial genome diversity
qPCR	Real-time polymerase chain reaction
RAST	Rapid Annotation using Subsystem Technology
RT-PCR	Reverse transcriptase polymerase chain reaction

SALC	South African Lipizzaner Centre
SRA	Sequence Read Archive
ST	Sequence type
T2SS	Type II secretory system
T3SS	Type III secretory system
T4SS	Type IV secretory system
T6SS	Type IV secretory system
T. equiperdum	*Trypanosoma equiperdum*
T. equigenitalis	*Taylorella equigenitalis*
T. asinigenitalis	*Taylorella asinigenitalis*
TRACES	Trade Control and Expert System
UAE	United Arab Emirates
UK	United Kingdom
US	United States
USDA	United States Department of Agriculture
WBFSH	World Breeding Federation for Sport Horses

Chapter 1
General introduction

1.1 Introduction

A number of questions remained unanswered at the conclusion of the initial investigation following the 2011 South African contagious equine metritis (CEM) outbreak. South Africa was hitherto considered free of CEM until May 2011 when the first case of *T. equigenitalis* [1] was confirmed. Initially, it was presumed that the index case, an imported stallion was the source of the outbreak, however, further epidemiological investigation disproved this.

A comprehensive literature review of both *T. equigenitalis* and *T. asinigenitalis* covering their diagnosis, pathogenesis, transmission, treatment, control and prevention is presented in Chapter 2. Contagious equine metritis is also considered within the broader context of equine infectious diseases and the risks posed by amongst other things, the international trade and movement of horses.

In Chapter 3, the methods used for diagnosis, epidemiological surveillance and treatment of *T. equigenitalis*- and *T. asinigenitalis*-positive animals identified in South Africa are described. A duplex PCR assay able to distinguish *T. equigenitalis* from *T. asinigenitalis* was developed as part of the outbreak investigation [2] and was incorporated into the epidemiological surveillance scheme and for post-arrival quarantine testing of imported equids. This duplex PCR assay has shown no incursions of *T. equigenitalis* since its introduction and additionally identified a *T. asinigenitalis*-positive miniature donkey stallion in 2015 on post-importation quarantine testing. Since outbreak identification, newer molecular sequencing tools such as whole genome sequencing [3] and an open-access online multilocus sequencing typing (MLST) scheme [4] became more accessible for veterinary diagnostics. These led to the reporting of the draft genome sequence of a strain isolated from a South African Lipizzaner stallion in 1996 as well as the MLST sequence type (ST) from 36 horses identified over the course of outbreak investigation. Using genomic data from open-source online databases, the origin and evolution of the South African *T. equigenitalis* strains were further characterized.

Internationally recognized treatment protocols [5; 6] were applied during the initial response to the 2011 South African outbreak. However, experience of prolonged treatment periods with frequent re-treatments and a reliance on bacterial culture with an associated delay for detecting treatment endpoint prompted subsequent modifications to these protocols [7]. The serendipitous but rare occasion of identifying a relatively large number of concurrent cases allowed an opportunity to compare various diagnostic and treatment approaches and to make cogitated changes where necessary. The large cohort of cases also presented the opportunity to monitor the effects of prolonged antimicrobial therapy on the genital microbial flora. These changes informed several modifications to the national diagnostic [8] and treatment [9] protocols. The modified treatment protocol for *T. equigenitalis* was subsequently adapted to treat the *T. asinigenitalis*-positive donkey stallion identified in 2015.

Chapter 4 sequentially describes the results obtained from these studies.

In Chapter 5, the important findings from these studies are discussed and aligned to proposed future research directions and in the final chapter, the pertinent conclusions from these studies are presented.

1.2 Problem statement

Initially, it was suspected that *T. equigenitalis* had been imported into South Africa with the index case. Subsequent epidemiological investigation however, revealed that the index case was not the source of the outbreak and that *T. equigenitalis* had been circulating in the South African equine population for a number of years prior to outbreak identification [2]. Since the South African outbreak, newer molecular technologies such as MLST [4; 10] and whole genome sequencing [3; 11; 12] have become more accessible and have been used to gain an overview and characterize the genetic diversity of outbreaks of *Taylorella* spp. worldwide. Further investigation of the wider disease context of the South African strain or strains was indicated, including defining the origin, evolution and global epidemiological links to other *T. equigenitalis* outbreaks.

Invaluable experience was gained in targeted outbreak control of CEM during the South African outbreak in 2011. In particular, the occurrence and subsequent management of a large number of cases on a single property [2] indicated that the current internationally recognized therapeutic protocols [5; 6] were inadequate for the purposes of effective and practical disease eradication and required reconsideration.

1.3 Hypotheses

The hypotheses for this study were developed sequentially with experience gained during treatment and epidemiological investigation of cases linked to the South African *T. equigenitalis* outbreak, in particular, how the application of molecular diagnostic technologies could further elucidate the origin, evolution and global context of *T. equigenitalis* in South Africa.

- The index case during the 2011 South African CEM outbreak was not the source of infection
- A point introduction of *T. equigenitalis* into South Africa had occurred prior to CEM outbreak recognition in 2011 and there had been no further incursions of *T. equigenitalis* into South Africa
- There would be similarity amongst all strains of *T. equigenitalis* isolated during the South African epidemiological investigation
- The topical application of a bovine intramammary preparation to the bacterial predilection sites of *T. equigenitalis*-positive horses would shorten the duration of treatment

1.4 Objectives

The objectives of this study were:

1. To use newer molecular technologies to facilitate identification of the origin and evolution of the South African *T. equigenitalis* outbreak and its global context.
2. To use molecular methodologies to facilitate the development of an efficacious treatment protocol.

-----

*text that is **bold** and <u>underlined</u> is hyperlinked

Chapter 2
Literature review

2.1 Equine infectious diseases

Until recently, there had been exponential growth in the global horse industry due to a favorable economic climate with a concomitant increase in the international movement of both horses and their germplasm [13-15]. Horses, second only to man, are the most travelled species in the world. The increasing economic significance of the horse industry is clear. A 2017 report by the American Horse Council Federation stated that the US horse industry contributed 122 billion dollars towards the US economy [16]. While in the United Kingdom (UK), the Thoroughbred Breeders' Association estimated that the total economic contribution of horseracing to the UK economy in 2017 was approximately £3.5 billion, providing employment for more than 3 500 people in the industry and supported positions for an additional 15 500 persons [17]. A 2019 national equestrian survey by the British Equestrian Trade Association placed the economic value of the equine sector at £4.7 billion [18] and according to UK trade data, the total value of UK exports of pure-bred breeding horses exceeded £300 million and that of other live horses exceeding £50 million in 2019 [19].

The international movement of horses occurs for a variety of reasons. Some horses are intended for permanent entry into the importing country for either commercial or recreational purposes, while others are intended only for temporary entry, usually for the purposes of competition either in racing or performance events [15]. The number of flat and jump races hosted in 2018 by 63-member countries of the International Federation of Horseracing Authorities' (IFHA) was 141 723 and 8 225, respectively which included 219 236 and 20 131 horses in flat races and jump races, respectively (see Table 2-1). Elite horses are shipped around the world to compete in these prestigious and lucrative events; for example the winner's purse for the 2020 Saudi Cup was $10 million, the Dubai World Cup, $7.2 million and the Everest horse race, $4.5 million [20]. Similarly, the number of major international events hosted by the International Federation for Equestrian Sports (FEI), including show jumping, dressage, eventing, driving, pole vaulting, reining and endurance riding, has increased by 115% from 2 072 in 2007 to 4 464 in 2018 (See Table 2-1) [21]. These high-level equine athletes pose a unique challenge as they temporarily travel across

borders for sports events posing potential health risks to both the local horse population and themselves [13; 22]. Due to the unique management of these horses, international movement of this specific subpopulation is administered separately under the auspices of the World Organisation for Animal Health's (OIE) 'High health, high performance (HHP) horse' strategy [23] as these horses are considered a low risk for transmitting infectious diseases [24; 25]. Under HHP management, specific measures are applied to ensure a 'functional separation between horses in the 'high health equine population' and other equids, at all times', and allows for temporary international movement of horses competing in worldwide FEI and IFHA competitions or races [23]. A key component in the success of the HHP concept is the identification and traceability of qualifying horses [23] which has been facilitated in the European Union by the TRACES (Trade control and Expert System) platform [26; 27].

Table 2-1 The International Federation of Horseracing Authorities' (IFHA) international flat and jump racing statistics and the global events hosted by the International Federation for Equestrian Sports (FEI) from 2007-2018

	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018
Flat races	146 710	151 980	150 820	154 340	150 586	151 759	148 473	146 646	142 549	142 045	140 869	141 723
Number of horses	213 401	223 075	224 632	234 468	228 507	225 471	216 083	221 291	223 118	218 937	216 546	219 236
Jump races	7 788	8 126	8 139	7 919	8 610	8 196	8 418	8 413	8 399	8 309	8 383	8 225
Number of horses	19 760	21 132	23 254	23 705	23 580	22 285	22 002	21 119	21 395	20 709	19 694	20 131
FEI events hosted	2 072	2 401	2 660	2 945	3 216	3 318	3 541	3 785	3 989	4 206	4 428	4 464

Source: IFHA annual reports 2007-2018 [28]; FEI [21]

Over the past 20 years, there has been an increasing trend in the practice of dual-hemisphere breeding of stallions due to the restriction on assisted reproductive techniques such as artificial insemination (AI) in the Thoroughbred industry [29]. These so called, 'shuttle stallions' are shipped mainly from the Northern to the Southern hemisphere allowing them to service mares in both hemispheres in one calendar year [15; 29]. Apart from obvious commercial gain, these movements also help improve genetic diversity within the closed population of Thoroughbreds [30]. In Ireland, the third largest producer of Thoroughbred horses worldwide, 26% (1076/4141) of all coverings in 2016 were by stallions that had been shuttled [31]. The international disease risk posed by shuttle stallions is well known, with the 2007 Australian equine influenza outbreak being traced back to imported shuttle stallions and mares from Japan [32]. The movement of mares for breeding is done on a far greater scale, albeit usually over shorter distances. National movement of mares follows a seasonal pattern, with a spike in movement during the commercial breeding season of thoroughbreds and standardbreds [29]. Following the annual sales, Thoroughbred yearlings are often shipped globally for example New Zealand exports ~40% of their foal crop (n ~ 1 500) annually [33].

In contrast to the Thoroughbred industry, where the movement of live animals is still *de rigueur*, the use of assisted reproductive technologies such as AI by other breed registries has transformed the global equine landscape [15]. The 2019 statistics from the international federation of studbooks for sport horses, the World Breeding Federation for Sport Horses (WBFSH), shows a total of 10 440 registered stallions, with an additional 7 139 either foreign stallions or semen used (See Table 2-2).

Table 2-2 The cumulative number of stallions and semen and total foal crop for 2019 registered by studbooks (n=75) belonging to the World Breeding Federation for Sport Horses (WBSFH)

Own studbook-approved stallions	Other stallions or semen used	Total foal crop
10 440	7 139	92 022

A final category, which does not pose a major disease risk, is the shipment of horses to Europe for processing and human consumption [15].

The advent of jet transportation has also increased the risk of disease transmission exponentially, posing a threat to both equine and public health [15; 34]. There are a number of factors that influence the risk of disease transmission between countries, importantly, these include presence of national disease surveillance structures and transparency of disease-reporting channels. The Terrestrial Animal Health Code [35] published by the OIE documents the principal control standards required to prevent the spread of specific diseases. It is mandatory for all member countries of the World Trade Organisation to report the occurrence of any OIE-listed disease, facilitating risk assessment analysis between countries. An essential component of any disease-monitoring programme is the availability of reliable diagnostic laboratory capability [15; 34] and of permanent individual identification to ensure the integrity of sampling, transportation procedures and traceability [35]. In order to mitigate the risk of disease transmission, animals are required to spend a variable amount of time in dedicated pre-export and post-import quarantine where they may undergo further testing. In order to facilitate trade, multinational trade agreements such as the European Union Free Trade Agreement [37] reduces pre- and post-import health requirements, including quarantine, among participating countries. Whether or not the importation is permanent or temporary as well as the mode of transmission of specific diseases play a role in the likelihood of disease introduction. For instance, respiratory diseases such as equine influenza, equine herpesvirus (EHV) type-1 and -4, equine arteritis virus (EAV), strangles and glanders can occur irrespective of whether importation is permanent or temporary. Permanent importation of persistently infected animals, specifically of those with vector-borne diseases, pose a risk if their specific vector or a new potential vector is available in the country of import. These diseases included African horse sickness virus (AHSV), alphaviruses such as Western and Venezuelan equine encephalitis viruses, equine infectious anaemia virus, *Trypanosoma equiperdum, Theilleria equi* and *Babesia caballi.* The carrier state is also characteristic of many venereal diseases, such as *T. equigenitalis, T. equiperdum,* and EAV, which may result in the transmission of disease via natural mating or artificial insemination with contaminated semen. Pregnant mares can also be a source of *T. equigenitalis* or EHV-1. A less common means of disease introduction is through the importation of an animal infested with the intermediate state of a parasite, such as the botfly *Cochliomyia hominivorax*, one of the causes of screwworm myiasis [15; 34] (See Figure 2-1).

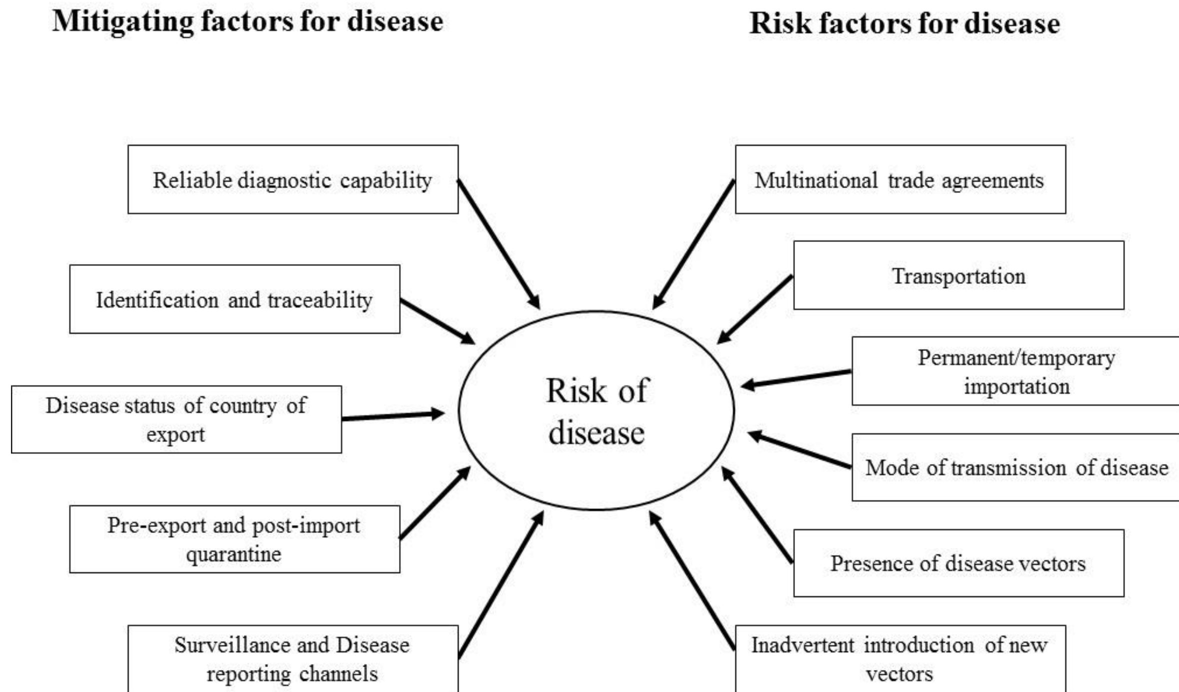


Figure 2-1 Factors affecting the risk of international disease transmission

From 1995 when the OIE was first recognized under the Sanitary and Phytosanitary agreement of the World Trade Organisation, until 2015, there have been 54 international occurrences of 10 OIE-listed equine infectious diseases associated with the international movement of live horses [38]. Of the 10 OIE-listed diseases, three are considered venereal pathogens; contagious equine metritis (CEM), dourine and equine viral arteritis (EVA). Of these 54 disease events, CEM (12/54, 22%) was surpassed only by equine influenza (13/54, 24%) in the total number of occurrences and the number of introductions that led to disease transmission to the local equine population (5 versus 9). Most equine influenza disease events were associated with the vaccination status of the imported animal, with horses either being unvaccinated or the import requirements not being in-line with OIE requirements for unvaccinated horses while the majority of CEM disease events were due to the importation of horses for breeding where the import requirements were not in-line with OIE requirements [38]. CEM is therefore one of the most highly regulated equine diseases in the world [39].

Due to the potentially serious economic implications of a disease outbreak particularly to the Thoroughbred racing industry, the most common equine venereal diseases; CEM, coital exanthema (EHV-3), equine viral arteritis and dourine [40], in addition to international

controls [41] and country-specific importation requirements, are often governed by various local regulations. The United Kingdom (UK), France, Germany, Ireland and Italy have instituted voluntary codes of practice [42] overseen by the respective horseracing authorities for the surveillance and control of these pathogens amongst others. The United States Department of Agriculture (USDA) also offers some guidance on screening for these and other pathogens [43].

2.2 Historical perspective

Contagious equine metritis (CEM) is a non-systemic, venereally-transmitted disease of horses caused by the bacterium *Taylorella equigenitalis.* The organism was first described as *Haemophilis equigenitalis* [44] but was later renamed *Taylorella equigenitalis* [45]. It was first described in the United Kingdom (UK) during the 1977 Thoroughbred breeding season [46-48], where it was quickly given the colloquial name, 'The Jubilee Clap' as it was also the year of HM Queen Elizabeth II's Silver Jubilee [49]. The infection was thought to have been introduced to the UK via mares from Ireland brought over to breed with National Stud stallions. There was early evidence that this unknown organism was spread by horizontal fomite transmission, initiating changes in breeding shed management and hygiene [49]. Following reports that the newly identified contagious equine metritis organism (CEMO) had been identified in Australia in 1976 in mares covered by a recently imported Irish stallion [49], the potential origin of the disease was traced to France after Thoroughbred mares were shipped to Ireland in early 1976 [5; 50]. By the end of the 1977 breeding season, *T. equigenitalis* had been diagnosed on 29 Thoroughbred studs in the Newmarket area, with 23 stallions and approximately 200 mares affected [5; 49; 50]. This resulted in closure of the National Stud and other stud farms in the Newmarket area, resulting in significant losses both economically in stud fees and foal sales and in foaling rates, which reportedly decreased to 70% from 86% the year before [49].

Despite the rapid imposition of a ban on the importation of horses into the United States (US) and Canada from the UK and Europe, *T. equigenitalis* was confirmed in Kentucky (US) in 1978 [51; 52], Missouri (US) and Canada in 1979 [50]. The 1978 outbreak was estimated to have cost the Kentucky Thoroughbred breeding industry one million dollars a day and the estimated cost to eradicate *T. equigenitalis* from the US was 13.5 million dollars [6].

The disease attained worldwide significance with the regulatory restrictions associated with CEM continuing to limit the movement and trade of horses internationally. Since the first reported cases of CEM in the UK [46], the disease has been confirmed in various countries worldwide including several in Europe [53-61], the US [5; 51], Australia [62], Japan [63], South Africa [64], Iran [65] and South Korea [66]. The costs associated with CEM are due not only to direct economic loss as a result of infertility, but also to significant indirect economic costs in terms of quarantine and surveillance measures and those accrued by outbreak management and treatment protocols in those countries where CEM is regulated [39].

In the US following routine regulatory testing, an atypical strain of *T. equigenitalis* was identified in donkey stallions in California (1987) and Kentucky (1988) [67]. The organism was further characterised and shown to be a new (second) member of the genus *Taylorella* and based on taxonomic studies was classified *T. asinigenitalis* [67; 68]. Since then, *T. asinigenitalis* has been reported from other states in the US [69-71], Sweden (2004) [72], Italy (2008) [73], France (2011) [74] and the UK (2018) [75].

2.3 The genus *Taylorella*

Within the genus *Taylorella* are two known species; *T. equigenitalis*, which causes CEM and *T. asinigenitalis*, a closely-related species found mainly in donkeys which is considered non-pathogenic. *Taylorella equigenitalis* was initially classified as *Haemophilus equigenitalis* [44], however, further analysis of its phenotypic characteristics, DNA base composition and DNA-DNA hybridization revealed a different taxonomic position and a novel genus, *Taylorella* was proposed [45]. Analysis of the DNA (rDNA) encoding the 16S ribosomal (rRNA) sequence clarified the phylogenetic position of *T. equigenitalis* in the ß subclass of the class *Proteobacteria* in the Alcaligenaceae family which supported its exclusion from the genus *Haemophilus* [76]. Further work demonstrated that the 16S rDNA sequences of geographically diverse *T. equigenitalis* isolates (n=23) gave high sequence similarity (> 99.5%) but were not identical [77]. Analysis of the rRNA sequence of *T. asinigenitalis* revealed that it belonged to the same cluster as *T. equigenitalis* [67], but that the rRNA sequence provided a reliable means of distinguishing *T. equigenitalis* from *T. asinigenitalis* [72]. *Taylorella* spp. are non-motile, microaerophilic Gram-negative,

frequently pleiomorphic bacteria [47; 48; 78-80] that are fastidious, slow growing and rapidly overgrown by other bacteria present in the reproductive tract of horses [81-83]. They are sensitive to a broad range of antimicrobials and disinfectants [6; 39; 79].

Historically, two biotypes of *T. equigenitalis* were differentiated by either sensitivity or resistance to streptomycin [44; 82].

Taylorella asinigenitalis was first discovered in 1997 when the National Veterinary Services Laboratory in California identified a bacterial isolate (UCD-1^T) from a donkey stallion that showed similar colony morphology, growth rate and immunofluorescence characteristics to *T. equigenitalis* [67]. Interestingly, serum from this donkey stallion reacted with antibodies in the complement fixation (CF) test for *T. equigenitalis*, a feature absent in the sera of *T. equigenitalis*-positive stallions [67]. In early 1998, atypical isolates were additionally confirmed from two donkey stallions in Kentucky (UK-1 and UK-2), which also reacted positively to the CF test for *T. equigenitalis* antibodies [68]. Analysis of the genomic DNA of *T. equigenitalis* and these three donkey isolates using sequence analysis of the 16S rDNA (97.6% similarity), DNA-DNA hybridization studies (23% similarity) and G+C composition analysis confirmed that the donkey isolates were closely related but not identical to *T. equigenitalis* strains and a new species of the genus *Taylorella*¸ *T. asinigenitalis* was proposed [67]. This new species had slower growth rates and a weaker positive reaction in the immunofluorescent antibody (IFA) test than *T. equigenitalis* [67]. Interestingly, *T. asinigenitalis* isolates, unlike *T. equigenitalis,* are resistant to a greater number of antimicrobials including streptomycin, trimethoprim/sulphamethoxazole, oxytetracycline, oxacillin [71; 72], ampicillin and cephalothin [74].

Further work carried out on the 16S ribosomal gene diversity of 43 French strains of *T. asinigenitalis* showed a similarity of 99.3-100% within the species with a difference in only two polymorphic nucleotide sites and 97-97.6% similarity with a reference strain of *T. equigenitalis*. Three clusters (Clusters 1, 2 and 3) were distinguishable and correlated with distinct geographical sites in France [74]. It also appeared that *T. asinigenitalis* was present in France prior to its first official isolation in California in 1997 (UCD-1) [67] and the presence of strain UCD-1 in Cluster 2 suggests a common European origin [74]. Analysis of the rRNA sequence of *T. asinigenitalis* isolated from a stallion in Sweden revealed it to be identical to the UCD-1 strain which also suggested a common origin [72]. Analysis of the

23S rRNA genes of *T. asinigenitalis* strains from the United States (n=3) and France (n=32) found multiple intervening sequences (IVS) of unknown function [84-86]. These findings were deposited in the DDBJ/EMBL/Gen-Bank with accession number, AB259168 [85]. In contrast, only one identical 70 bp IVS (TeIVS2) was found in the central region of the 23S rRNA gene of 19 strains of *T. equigenitalis* including the type strains [NCTC11184 (UK), Kentucky188 (US) and EQ59 (Japan)] [87]. No IVSs were found in the 16S rRNA gene sequences of either *T. equigenitalis* (n=22) or *T. asinigenitalis* (n-35) [88]. The significance of these findings is currently undefined.

A table comparing *T. equigenitalis* and *T. asinigenitalis* is shown in Table 2-3.

Table 2-3 A summary of the comparison between *Taylorella equigenitalis* and *Taylorella asinigenitalis* in terms of genomics, disease caused, clinical signs, carrier status, transmission and diagnosis

	Taylorella equigenitalis		***Taylorella asinigenitalis***			
	Male horses	**Mares**	**Male horses**	**Donkey jacks**	**Mares**	**Donkey jennies**
Class	Betaproteobacteria					
Order	Burkholderiales					
Family	Alcaligenaceae					
23S rRNA genes	70 bp IVS (TEIVS2)		Multiple IVS			
Genome length	1,695,860 bp		1,638,559 bp			
G+C content	37.42%		38.3%			
Virulence factors	Secretion systems Types II (T2SS), III (T3SS), VI (T6SS)					
	Secretion system type IV (T4SS) Lactoferrin Transferring receptors					
Invasion factors	Hsp60 homolog					
Disease caused	Contagious equine metritis		Considered non-pathogenic			
Clinical signs	Asymptomatic	Vaginitis, cervicitis, endometritis	Uncommon; asymptomatic	Asymptomatic	Cervicitis, metritis	Asymptomatic
Carrier status	Uretha, urethral fossa, lamina interna	Clitoral sinus & fossa	Not recorded	Uretha, urethral fossa, lamina interna	Uterus & cervix	
Transmission (method)	Venereal, ART, fomite, transplacental (rare)					
Diagnosis (method)	Bacterial culture, qPCR					
International control	Yes		No			

2.4 Transmission

Taylorella equigenitalis is a venereally-transmitted organism spread directly during natural mating or indirectly via AI with contaminated fresh, cooled, extended or cryopreserved semen from a carrier stallion [79; 89].

An increasingly relevant trend in recent outbreaks [2; 66; 90; 91] has been indirect transmission by fomites, including: (i) during assisted reproductive procedures e.g. breeding phantoms, artificial vaginas; (ii) equipment and procedures employed during examination of the reproductive tract of the mare, e.g. examination gloves, tail bandages, specula; and (iii) housing and routine management e.g. grooming equipment, buckets, bedding [39; 54; 58; 59; 79; 92; 93]. Survival of the organism outside the body is reportedly brief, with the organism susceptible to many disinfectants, ultraviolet light, high temperatures and reduced humidity [39; 79].

Cases of apparent transplacental transmission have been reported with *T. equigenitalis* isolated from the placenta and genital tracts of young foals [5; 49; 94]. Young equids may also become directly infected through contact with bedding or pasture contaminated by discharges from an infected mare [94].

Transmission of *T. asinigenitalis* is similar with cases of direct and indirect venereal [69] and fomite [70; 72] transmission reported. There have also been two proposed cases of transplacental transmission [69].

2.5 Pathogenesis and clinical signs

The incubation period of *T. equigenitalis* varies from two to 12 days [94; 95]. Mares with the acute form of the disease suffer from temporary infertility, typically showing a muco-purulent vaginal discharge and irregular interoestrus periods because of clinical endometritis, cervicitis or vaginitis. Strain virulence appears to have decreased over time, with mild or inapparent clinical signs observed in the majority of mares in recent outbreaks [5; 54; 92; 94]. Most mares rid themselves of infection and develop a short-lived humoral immune response [5; 79; 94; 96], however, up to 20-25% of asymptomatically infected

mares attain a carrier status which may persist for months or even years [5; 79; 82; 97]. Chronically infected carrier mares most commonly harbor the organism in the clitoral fossa and sinuses [98]. Occasionally, the organism may be carried in the endometrium, where is has been reported to persist throughout pregnancy [94]. Abortion reports are rare [54; 79].

Affected stallions are unapparent carriers and the principal source of infection. This smegma-associated commensal merely colonises the predilection sites of the external genitalia without eliciting clinical signs or an immune response. The organism shows a tropism for the urethra, the urethral sinus and the *lamina interna* [5; 6; 91; 94; 97; 99] with the carrier state persisting for months or years [39; 94].

Taylorella asinigenitalis is considered non-pathogenic [67; 70; 100] but has been cultured from the reproductive tract of mares following natural service with infected stallions and donkey stallions [67; 70] and following intrauterine infusion [68] has been shown to cause a transient metritis and cervicitis. In that study, all experimentally infected mares (n=4) cleared themselves of clinical signs by day 35 post inoculation, although *T. asinigenitalis* was still recovered from one mare throughout the 111 day observation period. In another study [70], *T. asinigenitalis* persisted in 2/7 nurse mares despite two rounds of treatment, with one mare that remained culture-positive > 300 days. Although a small reported sample size (n=5), a chronic carrier state does appear to develop in some mares, with the organism showing a tropism for the proximal (uterus, cervix) rather than the distal (clitoral fossa and sinuses) reproductive tract [68; 70] as seen in chronic *T. equigenitalis*-positive mares. A long-term carrier state [69; 70] exists in male equids with the organism showing a similar tropism as *T. equigenitalis* for the urethral fossa, urethral sinus and urethra [69; 75]. Aside from the far-reaching implications of misidentification of *T. asinigenitalis* as *T. equigenitalis* using bacteriology, the risk posed to horses by *T. asinigenitalis* is currently undefined. Although *T. asinigenitalis* has been shown to cause transient metritis and cervicitis [68] there have been no reports on the number of breeding's per pregnancy in affected mares which would give an indication of the impact of *T. asinigenitalis* on reproductive performance.

2.6 Diagnosis

2.6.1 Sampling sites

Swabs are obtained from the genital predilection sites for diagnosis of both *T. equigenitalis* and *T. asinigenitalis;* in stallions from the urethra, urethral fossa and *lamina interna* and in mares from the non-pregnant endometrium, clitoral sinus and clitoral fossa.

For bacterial culture, swabs are transferred in Amies charcoal transport medium and must be transported to an accredited laboratory within 48 hours of collection [101]. For qPCR testing, dry swabs without transport media are also appropriate [2; 102].

2.6.2 Sampling interval

Although not currently mandated by the OIE [101], international regulations [103; 104] prescribe multiple sampling opportunities to increase the sensitivity of culture and qPCR assays for both *Taylorelleae* spp.

2.6.3 Sampling procedures for imported animals

The USDA has differing pre-export testing protocols subject to the CEM disease status of the country of origin [103]. Horses from countries considered free of CEM are released without any additional tests immediately following initial Federal quarantine. If the country of origin is considered to be affected by CEM then the following regulations apply: subsequent to Federal quarantine, all stallions and mares > 731 days of age and stallions and mares < 731 days of age if ever used for breeding, must undergo mandatory CEM isolation and testing at a State-approved CEM quarantine centre. Mares require an initial complement fixation (CF) test followed by three sets of swabs taken for culture over a 12-day period. This is followed by a five-day treatment regimen consisting of daily scrubbing and coating of the external genitalia with an antibacterial ointment. Testing of stallions requires one set of swabs taken from the external genitalia for culture, followed by live-cover breeding of two test mares. Following test breeding, the stallion's external genitalia are scrubbed and coated with an antibacterial ointment daily for five days. Beginning on day three after test breeding, three sets of swabs are collected from the test mares on separate occasions over a 12-day period. The test mares are also tested using CF between days 21-28 after test

breeding. Following negative tests, the animals may be released from quarantine. Geldings and non-domesticated zoo equine species are exempt from testing. Despite these stringent regulations, there have still been sporadic outbreaks of *T. equigenitalis* in the US that have gone undetected for a number of years [5]. Many carrier animals require treatment intervals longer than five days to ensure elimination of the organism [105] and therefore, tailoring treatment duration to an individual animal warrants consideration. A carrier state has also been proven to exist in both geldings [106] and non-domesticated zoo equine species [75] with substantial evidence that fomite transmission played a significant role in most recent outbreaks [39; 66; 91; 106] therefore, exempting geldings and non-domesticated zoo equid species from testing is a conceivable risk.

2.6.4 Sampling procedures for detection of infection

The UK Horserace Betting Levy Board's (HBLB) 2020 International Codes of Practice [104] recommends annual swabbing of all stallions used for natural mating, semen collection or teasing. They recommend obtaining two sets of swabs and submission for culture and, or PCR at an interval of no less than seven days. Additionally, 'High risk' stallions are recommended to be swabbed after the first few matings of the season and again in mid-season. A 'High risk' stallion is defined as one that had not previously been used for breeding purposes, or from which the contagious equine metritis organism (CEMO), *Klebsiella pneumoniae* (capsule types 1, 2 or 5) or *Pseudomonas aeruginosa* has been isolated, or which in the last 12 months, has been on a property where these diseases have been isolated or which has mated a mare that has not been swabbed negative in accordance with the HBLB Code of Practice. 'High risk' mares require duplicate clitoral swabs taken at an interval of at least seven days and sent for culture and, or PCR. A 'High risk' mare is defined as one from which CEMO, *K. pneumoniae* (types 1, 2 or 5) or *P. aeruginosa* has been isolated, or which in the last 12 months, has visited a property where these diseases have been isolated, or one arriving from France, Germany, Italy, Ireland and the UK that has been mated in the last breeding season by a stallion resident outside these countries, or has been in countries other than France, Germany, Italy, Ireland or the UK within the previous 12 months.

Taylorella equigenitalis has been eradicated from Japan in 2010 since the inception in 2001 of an annual qPCR testing programme of all Thoroughbred breeding stock prior to the breeding season. Any positive cases were either culled or treated. Treated positive animals

were classified as high risk for a period of three years and had to undergo three additional PCR tests prior to mating [107].

2.6.5 Polymerase chain reaction

Real-time PCR has revolutionized the utility of high throughput laboratories for routine diagnosis, offering sensitive and specific results with a rapid turnaround time [108]. Real-time PCR also increases the ability to detect and differentiate micro-organisms that are difficult to culture or confirm by analysis of their products [109]. Since real-time PCR visualises the reaction as it is taking place, it allows for immediate quantification and analysis of the reaction, reducing the risk of false negatives due to PCR inhibition, unlike in traditional systems that rely on endpoint analysis. Real-time PCR is also performed in a closed system, thereby reducing the risk of contamination and false positives due to operator errors [108-110].

Therefore, due to the limitations associated with isolating *T. equigenitalis,* various PCR tests have been developed to overcome these challenges and, following the discovery of the second member of the genus, to distinguish it from *T. asinigenitalis.* Initially, a real-time quantitative PCR test (RT-qPCR) was developed using genus-specific primer-probes for the 16S ribosomal DNA sequence [81]. Due to concerns regarding specificity due to conservation of short DNA segments within the 16S rRNA genes, original oligonucleotide primers were developed and evaluated in both a single- and two-step PCR [111]. Following the discovery of *T. asinigenitalis* in 1997 [67], a PCR-based method was developed to distinguish between these two organisms based on four unique DNA sequences coding for the 16S rRNA [100]. Other researchers have continued to make modifications and improvements [112], with development of rapid, direct, real-time PCR assays that do not require prior DNA extraction or bacterial isolation [83; 102; 113-116]. Pooling of samples for qPCR does not affect the ability to detect a positive animal [117].

Real-time PCR shows enhanced sensitivity and specificity compared with traditional bacteriology and is associated with greater practicality, quicker turnaround times and lower costs [2; 81; 115; 116; 118].

2.6.6 Bacterial culture

Bacterial culture is currently the gold standard test for *T. equigenitalis* recognized by the OIE [101]. Swabs are accordingly transferred in charcoal Amies medium and on ice to reach the laboratory within 48 hours. According to the OIE manual, the samples are inoculated onto chocolate agar with the addition of trimethoprim (1 μg/ml), clindamycin (5 μg/ml), and amphotericin B (5-15 μg/ml). Plates are incubated at 35-37 °C in 5-10% (v/v) CO_2 in air or by use of a candle jar. At least 72 hours is normally required before colonies of *T. equigenitalis* become visible, after which daily inspection is needed. A standard incubation time of at least seven days is advisable before certifying cultures negative for *T. equigenitalis*. If *T. equigenitalis* colonies are suspected, a single colony is stained with Gram stain and a catalase and oxidase reaction is performed. Thereafter, the organism should be tested for reactivity with *T. equigenitalis*-specific antiserum [101].

Taylorella asinigenitalis is analogously cultured with similar colony morphology to *T. equigenitalis* but is slower growing and has a weaker reaction to the indirect fluorescent antibody (IFA) test. Bacterial culture runs the risk of potential misidentification of *T. asinigenitalis* as *T. equigenitalis* [4; 119] with important repercussions for national and international trade.

2.6.7 Serology

Seroconversion is reported in mares as a transient feature of the acute phase of endometritis associated with *T. equigenitalis* and is absent in stallions [5; 97] thus markedly limiting the application of serology. Antibody titres rise from seven days post exposure to reach a peak at three weeks before declining at around six to 10 weeks. Serology is therefore infrequently used as a routine diagnostic method, due to the often-protracted interval between suspected exposure and testing for *T. equigenitalis*. Due to their practicality and increased sensitivity, molecular diagnostic methods such as qPCR has replaced serology for epidemiological investigation of recent outbreaks [2; 55; 66; 90]. However, in the US, serology is currently still applied to test mares as part of the post-importation test-breeding protocol of stallions with the complement fixation (CF) test being performed between Days 21-28 post breeding [103]. In certain countries, the CF test for CEM is occasionally listed as a pre-export testing requirement for mares and male horses. This seems totally inappropriate.

Interestingly, initial reports of experimentally-infected mares (n=6) [68] and the three original *T. asinigenitalis*-positive donkey stallions [67] indicated positive results on the *T. equigentitalis* CF test. Subsequent reports of serological testing of *T. asinigenitalis*-positive stallions, mares and donkey stallions have been either negative or anti-complementary [69; 70].

2.6.8 Loop-mediated isothermal amplification (LAMP)

In an effort to expedite the detection of *Taylorella* spp. in less well-equipped laboratories, loop-mediated isothermal amplification (LAMP) methods, Te-LAMP and Ta-LAMP, for detecting *T. equigenitalis* and *T. asinigenitalis* respectively were developed [120]. There are a number of concerns regarding the reliability and ability to obtain valid results using this method if it is not performed in a laboratory with appropriately trained people [121].

2.7 Treatment

2.7.1 Treatment of *T. equigenitalis*-positive horses

As *T. equigenitalis* is sensitive to most antibiotics, various protocols have been described for the treatment of positive horses. Most regimens prescribe daily topical treatment of the external genitalia for five consecutive days in stallions. With the penis fully extruded, all smegma is removed and then the urethral fossa, urethral sinus and penis are either rinsed with 2% chlorhexidine [5; 97; 122] or cleaned with 4% chlorhexidine gluconate [6; 123]. The penis is dried and an antimicrobial ointment containing either 0.2% nitrofurazone [5; 97; 122; 123] or 1% silver sulphadiazine [6; 122; 123] is applied to the external genitalia. One protocol mixed 1% silver sulphadiazine with an intramammary preparation containing procaine penicillin and dihydrostreptomycin for topical application to the external genitalia [6]. Certain regimens also prescribe additional oral treatment with trimethroprim-sulfamethoxazole at 30 mg/kg twice daily for 10 days [6; 123].

In mares, the recommended treatment regimen prescribes that on the first day of treatment, the clitoral sinuses are flushed with a ceruminolytic agent and 0.2% nitrofuracin solution and packed with 0.2% nitrofurazone ointment. Thereafter, the clitoral sinuses and fossa are cleaned with 4% chlorhexidine gluconate and packed with 0.2% nitrofurazone ointment or

another efficacious agent for an additional four days [123]. Several courses of treatment may be required to eliminate the organism in mares [5; 97]. Surgical ablation of the clitoral sinuses has been described in the event that repeated treatments prove unsuccessful [97].

2.7.2 Treatment of *T. asinigenitalis*-positive equids

Despite the recognized significance of the disease, there is a surprising paucity of prescribed treatment protocols for *T. asinigenitalis* with most protocols apparently loosely based on those recommended for treatment of *T. equigenitalis*-positive horses. In one study [72], a stallion was treated for five consecutive days with a combination of washing of the external genitalia with 2% chlorhexidine and topical application of gentamicin based on antimicrobial-sensitivity testing. In another [73], two positive donkey stallions were treated for 10 days with a combination of twice daily intramuscular injections of trimethoprim-sulfadiazine at 30 mg/kg and cleaning of the external genitalia with 4% chlorhexidine.

One study reported on the treatment of nurse mares [70], and recommended daily washing of the external genitalia with 4% chlorhexidine gluconate followed by application of topical 0.2% nitrofurazone ointment for five consecutive days.

2.8 Analysis using restriction enzymes

Historically, the genomic DNA profile of the *Taylorella* spp. have been elucidated using electrophoresis following cleavage by restriction endonucleases. The choice of restriction endonucleases based on the low G+C content (36.5%) of the *Taylorella* genome [44] included only those with recognition sequences containing G and C, namely *Apa*I, *Not*I and *Nae*I [124]. These enzymes cut the genome into a limited number of restriction fragments (< 15) which are then used to generate distinct profiles using electrophoresis. Most researchers used pulsed-field gel electrophoresis (PFGE) [59; 93; 125] or variations thereof including crossed field gel electrophoresis (CFGE) [124; 126-131], a variation of PFGE where the gel is rotated and field inversion gel electrophoresis (FIGE) [132] where the electrical field is periodically inverted [133].

Various researchers have employed gel electrophoresis to study the epidemiology of *T. equigenitalis*. Field inversion gel electrophoresis was used to compare 20 Dutch with 12 other isolates and determined that all strains could be designated into one of five different restriction patterns, designated A-E [132]. Strains from Thoroughbred horses from all countries belonged to a single group (A) which also included the original English type strain, NCTC 11184.

Both CFGE and PFGE were used to describe various isolates from around the world and found a common strain, designated Kentucky 188, in 28/82 isolates from England, Ireland, France, the US and Australia, which suggested a common source [125; 127; 130]. CFGE was also used on 109 isolates obtained from Thoroughbred broodmares in Japan from 1980-1993 and showed a common source and genotype, designated 'Genotype J' [128; 131].

Application of PFGE to analyse strains isolated from eight Norwegian horses showed one distinct strain, designated 'Genotype N' [129]. More recently, PFGE analysis of 82 strains of *T. equigenitalis* isolated in the US identified 15 different epidemiologically linked genotypes. These findings suggested that a novel introduction of *T. equigenitalis* not linked to previous incursions was responsible for the 2009 US outbreak [93]. Austrian and German isolates (n=124) collected between 2002-2014, analysed using repetitive extragenic palindromic PCR (REP-PCR) and PFGE, yielded five REP and 15 PFGE genotypes [59]. Five of the PFGE genotypes in this study had identical profiles to those found in the recent US outbreak [93] which proved common sources. Analysis of six *T. asinigenitalis* isolates from the same study [59] revealed three REP and six PFGE genotypes.

Chromosomal DNA fingerprinting of 14 *T. equigenitalis* strains isolated from Norwegian horses showed genetic homogeneity amongst all the Norwegian strains tested and which were identical to four Swedish strains [134].

2.9 Multilocus sequence typing (MLST)

The whole genome sequencing of *T. equigenitalis* MCE9 [3] and *T. asinigenitalis* MCE3 [135] allowed for the development of a multilocus sequence typing (MLST) scheme for the *Taylorella* genus using seven conserved housekeeper genes [4]. Housekeeper genes are

chosen based on their stable allelic profile and relatively slow accumulation of nucleotide changes [136]. The housekeeper genes chosen for the *Taylorella* spp. were: citrate synthase (*gltA*); gyrase subunit B (*gyrB*); putative fumarate hydratase (*fh*); serine hydroxymethyltransferase (*shmt*); tyrosine aminotransferase (*tyrB*); adenylate kinase (*adk*) and thioredoxine (*txn*). The *Taylorella* MLST scheme is publicly available at http://pubmlst.org/taylorella/ [4]. An MLST analysis is highly discriminatory and allows direct, unambiguous inter-laboratory comparison of results [137]. The MLST assays have enormous application during bacterial outbreak investigation due both to their practicality and ability to elucidate epidemiological relationships between separate points and as such, have been used for a myriad of different applications, such as the 2017-2018 listeriosis outbreak in South Africa [138].

Multilocus sequence typing performed on 163 strains (*T. equigenitalis*, n=113 and *T. asinigenitalis,* n=50) collected in several countries over 35 years (1977-2012) revealed 39 sequence types (ST), 27 *T. equigenitalis* and 12 *T. asinigenitalis*. Interestingly, Japanese strains, originally all classified as one genotype using CFGE (Genotype J) [128; 131], were shown to have two distinct sequence types (ST3 and ST9) on MLST analysis [4]. An eBURST analysis grouped the *T. equigenitalis* STs into four clonal complexes (CC1-4) and five unlinked STs. The *T. asinigenitalis* STs were grouped into three clonal complexes (CC5-7) and five unlinked STs. A more recent study by the same group [10], compared 367 *T. equigenitalis* strains using MLST typing according to geographical location, year of isolation and breed. The strains could be divided into 49 ST, with three major and three minor CCs and 11 singletons. Strain distribution over time was heterogenous, with 32 strains isolated from 1977-1997 and 310 during 1998-2018. However, ST diversity was greater during the initial (0.34 STs/strain) compared to the latter interval (0.12 STs/strain). Geographically, the first CEM outbreaks in 1977-1978 in the United Kingdom, Australia and the United States were associated with the founding complex, CC1. According to this dataset, CC1 was circulating internationally in the 1970s-80s, but does not appear to have spread throughout Europe. European and non-European countries could be linked through ST1, ST4, ST17 and ST30. Six singletons [ST3 & ST9 (Japan), ST31 and ST42 (United Arab Emirates (UAE))], ST55 (South Korea) and ST58 (US) were only associated with non-European strains and CC3, CC8, CC9, CC10 and singletons ST5 (France) and ST50 (Belgium and Poland) were only associated with European strains. The STs from Asia (ST3 & ST9 – Japan, ST55 – South Korea) had no connection to other countries, whereas CC1

linked Australia, the US and Europe, CC2 and ST30 linked the UAE with Europe and ST4 linked South Africa, Europe and the US. The fact that over time, distinct genotypes have emerged in different countries and continue to emerge [139; 140] supports the presence of an as yet, unidentified natural worldwide reservoir [4].

Currently, MLST analysis has superseded gel electrophoresis as the method of choice for epidemiological investigations of *Taylorellae* in outbreaks being highly discriminatory and allows direct, unequivocal comparison of results from different laboratories [137] using a dedicated online tool.

2.10 Whole Genome Sequencing

In 2011, the genome of *T. equigenitalis* MCE9 (GenBank accession number CP002456), a strain isolated in 2005 from a French stallion and maintained at the French National Reference Laboratory for CEM (Anses, Dozulé Laboratory for Equine Diseases, Dozulé, France) was sequenced [3] and compared to the genome of *T. asinigenitalis*, MCE3 (GenBank accession number CP003059) the following year [11]. The *T. equigenitalis* genome is 1 695 860 bp long, has a total G+C content of 37.42% without evidence of plasmids and one restriction/modification (R/M) system. The *T. asinigenitalis* genome is 1 638 559 bp long with a 38.3% G + C content and one prophage, despite the fact that *T. asinigenitalis* appears to have more systems in place to defend itself against horizontal gene transfer, such as clustered regularly interspaced short palindromic repeat (CRISPR) and a R/M system. Although no CRISPRs were shown in *T. equigenitalis* strain MCE9, they were recently identified in 14/17 *T. equigenitalis* and 4/14 *T. asinigenitalis* strains [141; 142] and five diverse *T. equigenitalis* isolates from the US, Germany, Austria and the Netherlands collected in the US from 1978-2009 [12]. Notwithstanding the presence of antiphage factors, the majority of strain-specific genes were associated with atypical GC content suggesting that these genes were only recently incorporated during horizontal gene transfer, most likely from the genital microbiome of host Equidae. *Taylorella* spp. lack the catabolic pathways to survive in many environments and it is unlikely that they are able to proliferate outside of the host. Therefore, the suggested worldwide reservoir [4] is unlikely to be environmental, but rather from clinically unapparent carrier animals. In terms of virulence factors, these were mainly associated with attachment to the host rather than causing damage to host

tissues. Numerous secretion systems were identified in both species such as Type II secretory system (T2SS), Type III (T3SS) and Type VI (T6SS). Type IV (T4SS) was only found in *T. equigenitalis* and may party explain the difference in virulence capacity of this species. Other differences in virulence in *T. equigenitalis* can be ascribed to the presence of lactoferrin and transferrin receptors allowing for the acquisition of ferric iron from the host and an Hsp60 homolog which acts as an invasion factor [141; 142]. Filamentation induced by cyclic AMP (*Fic*) proteins, known in bacteria to be toxins secreted by Type III or IV secretion systems were also identified [12] in *T. equigenitalis* isolates. A single point mutation previously shown to confer streptomycin resistance [143; 144] was identified in three *T. equigenitalis* isolates known to be streptomycin-resistant and was absent in two streptomycin-susceptible isolates [12].

Subsequently, whole genome sequencing was performed on the type strain of *T. equigenitalis* (NCTC11184, NCBI accession numbers CP003264) and draft genome sequences were produced for both a *T. equigenitalis* strain isolated from a stallion in Dubai in 2009 (NCBI accession number HE681423) and a *T. asinigenitalis* strain isolated in Sweden in 2004 from an Ardennes stallion (NCBI accession number HE681424) [145]. These were compared to MCE9 with similar findings.

2.11 Comparative phylogenetic analysis using the PathoSystems Resource Integration Centre

The PathoSystems Resource Integration Centre (PATRIC) (https://www.patricbrc.org) is the bacterial bioinformatics resource funded by the National Institute of Allergy and Infectious Diseases (NIAID) and provides an easy-to-use, publicly available online platform for bioinformatic analysis of bacteria. The PathoSystems Resource Integration Centre was one of the original centres within the Bioinformatics Resource Centre (BRC) programme that was established by the NIAID in 2004. The primary mandate of PATRIC was to support comparative analysis of bacterial pathogens. In 2009, PATRIC merged with the National Microbial Pathogen Database Resource BRC which had developed the successful SEED database and developed the RAST (Rapid Annotation using Subsystem Technology) annotation system. The SEED database was the first annotation environment to ‘support the creation, curation, population and exchange of subsystems’ where a subsystem is a ‘set of

functional roles that together implement a specific biological process or structural complex' [146]. A populated subsystem is one where the exact genes the implement the functional roles in that subsystem have been annotated by a subsystems expert [146]. The RAST annotation system allows for uniform genome annotations across microbial species, enabling comparative analysis and alleviating the need for users to develop custom annotation pipelines. In 2014, PATRIC began providing various bioinformatic services using RAST as a template [147].

One of these bioinformatic services was the Comprehensive Genome Analysis Service which assembles and annotates sequencing reads into user-friendly descriptions of the genome. 'The output includes a genome quality assessment, antimicrobial resistance genes and phenotype predictions, specialty genes, subsystem overview, identification of the closest genome sequences, a phylogenetic tree and a list of features that distinguish the genome from its nearest neighbors' [147].

Another service, the Phylogenetic Tree Service, allows the evolutionary relationships between bacterial genomes to be reconstructed and visualized [147].

Chapter 3
Materials and methods

3.1 The diagnostic methods used in the South African investigation

3.1.1 Real-time PCR

Following confirmation of *T. equigenitalis* in 2011, we were looking for a rapid and sensitive diagnostic method capable of distinguishing *T. equigenitalis* from *T. asinigenitalis* and that additionally overcame the inherent difficulties associated with bacterial culture. After a review of available laboratory resources and the impracticality of transporting a large number of samples from more remote collection points to the laboratory within the stipulated 48 hour window [101] it was decided to implement a qPCR assay for practical surveillance. Over time and as experience was gained, several modifications were made to the national diagnostic [8] and treatment [9] protocols, to include the use of a modified duplex PCR assay [2; 102] capable of distinguishing *T. equigenitalis* from *T. asinigenitalis* for initial screening and for in-treatment monitoring. This duplex PCR assay was also incorporated into routine post-arrival quarantine testing of equids entering South Africa.

The method developed and validated by Wakeley et al. [102] fulfilled the appropriate criteria and was real-time, negating the need for agarose gel electrophoresis [81; 100; 111]. These researchers [102] designed discriminatory TaqMan® probes conjugated with different fluorophores, FAM™ and HEX™, to distinguish *T. equigenitalis* from *T. asinigenitalis* respectively, based on an 8bp divergence area (positions 434-454 bp inclusive) between the two species. The sequences of the primers and probes were as follows:

- Forward primer, Tay377for – CCGCGTGTGCGATTGA;
- Reverse primer, Tay488rev – TTTGCCGGTGCTTATTCTTCA;
- *T.equigenitalis*probe (TequiPERC)
  – TequiFAM, AAAGGTTTGTGTTAATACCATGGACTGCTGACGG; and
- *T. asinigenitalis* probe (TasiniPERC)
  – TasiniHEX, AAAGTTTTAGGATAATACCCTAGGATGCTGACGG.

Wakeley et al [102] also designed 16S rDNA TaqMan® control designed to detect the 16S rDNA of many commensal bacteria found in the genital tract of the horse. The assay described by Wakeley et al [102] was used during the initial South African outbreak investigation with primers and probes supplied by the Animal Health Trust, Newmarket, UK.

Minor modifications were made subsequently to both primers and probes for optimizing efficiency on the real-time PCR machine in our laboratory. In short, the fluorescent dye on the *T. asinigenitalis* probe was changed from HEX™ to NED™, Black Hole quencher was changed to an MGB (Applied Biosystems, Cheshire, UK) probe which allowed shortening of the probes and the mastermix (Kapabiosystems, Cape Town, South Africa) was changed. The sequences of the primers and probes were as follows:

- Forward primer – T CGGGTTGTAAAGCACTTTTGTC;
- Reverse primer – GCCGGTGCTTATTCTTCAGGTA;
- *T. equigenitalis* probe – FAM-TGTGTTAATACCATGGACTGC-MGB; and
- *T. asinigenitalis* probe – NED-TAGGATAATACCCTAGGATGC-MGB.

The 17 µl PCR mix consisted of 7 µl of a diluted primer/probe mix and 10 µl of Kapa Probe Fast ABI Prism 2 x PCR mastermix, which was added to each well of a PCR plate to which 3 µl of the extracted template was added. Positive and negative template controls were included on each plate The PCR was performed on a StepOnePlus Real-Time PCR System (Life Technologies, Carlsbad, California, US) according to the manufacturer's protocol and the cycle threshold (Ct) was calculated as the point when the normalised fluorescence exceeded a 0.1 threshold within 40 PCR cycles [2]. This modified assay was used for the epidemiological traceback exercise and the national stallion screening programme as described in May et al [64].

3.1.2 Bacterial culture

The OIE gold standard for diagnosis of *T. equigenitalis* is bacterial culture. Swabs for bacterial culture must be placed in a transport medium containing activated charcoal, for example, Amies charcoal medium, must be kept cool during transportation to arrive and be plated out at the laboratory within 48 hours of collection.

In short, the preferred culture media for isolating *T. equigenitalis* is produced by adding trimethoprim (1 µg/ml), clindamycin (5 µg/ml) and amphotericin B (5-15 µg/ml) to ‘chocolate’ blood agar. After inoculation, plates must be incubated at 35-37 °C in 5-10% (v/v) CO_2 in air. Plates are examined for contaminants after 24 hours incubation. At least 72 hours are required before *T. equigenitalis* colonies become visible, after which time daily inspection is needed. *Taylorella equigenitalis* colonies may be up to 2-3 mm in diameter, smooth with an entire edge, glossy and yellowish grey in colour [101].

3.1.3 MLST analysis

The development of the *Taylorella* genus MLST scheme and the establishment of the online database [4] allowed for further epidemiological investigation of the South African *T. equigenitalis* outbreak and *T. asinigenitalis* incursion. *Taylorella equigenitalis*-positive genital swab samples were collected from mares and male horses in South Africa from 2011-2017 (n=33) during both a nationwide stallion screening program, legislated subsequent to outbreak recognition, and an epidemiological traceback [2]. Heightened awareness of the CEM-associated risk in South Africa prompted additional voluntary submissions of cryopreserved semen straws derived from ejaculates collected and processed from stallions prior to outbreak identification in 2011. In 2015, this submission of cryopreserved semen identified an additional *T. equigenitalis*-positive case linked to a stallion that had semen collected and cryopreserved in 2008. The stallion was subsequently gelded and on follow-up seven years later was found still to be positive.

The traceback exercise had furthermore identified a subpopulation focus at the South African Lipizzaner Centre and, based on this finding, archived semen samples (n=7) were accessed in August 2014. These samples had been collected from Lipizzaner stallion residents of this Centre during an equine viral arteritis investigation [148] in 1996 and were included in the MLST analysis.

Unfortunately, few isolates had been stored necessitating the experimental use of crude extract to obtain samples.

In 2015, two *T. asinigenitalis*-positive miniature donkey stallions were identified on post-arrival quarantine. Multilocus sequence typing analysis was performed on the isolate obtained from one of the donkey stallions.

In brief, swabs were agitated for 5 s in 0.4 mL of 0.1 M phosphate-buffered saline (PBS; pH 7.4) in separate 1.5 mL microfuge tubes. In the case of semen samples, after thawing, 0.1 mL was added to 0.4 mL of 0.1 M PBS as described above. All samples were then pelleted by centrifugation (18 000 × *g*, 60 s), and the supernatant was aspirated. The resultant pellet was resuspended in 0.1 mL of nuclease-free water (MilliporeSigma, St. Louis, MO) and then heated at 95 °C for 15 min. Insoluble material was pelleted by centrifugation (18 000 × *g*, 60 s) and the supernatant stored at -20 °C.

Lysates were amplified using specific primers for seven loci (*gltA*, citrate synthase; *gyrB*, gyrase subunit B; *fh*, putative hydratase; *shmt*, serine hydroxymethyltransferase; *tyrB*, tyrosine aminotransferase; *adk*, adenylate kinase; *txn*, thioredoxin) as described previously [4]. Primers for the PCR [149] were standardized with universal tail C and tail D sequences (Integrated DNA Technology, Whitehead Scientific, Cape Town, South Africa). A PCR amplification (KAPA SYBR FAST qPCR master mix, ABI Prism; Kapa Biosystems, Cape Town, South Africa) following the manufacturer's instructions on a PCR machine (StepOnePlus real-time; Applied Biosystems, Thermo Fisher Scientific, Johannesburg, South Africa) was performed as follows: 95 °C for 5 min followed by 35 cycles of 95 C for 30 s and 60 C for 60 s. We visualized the PCR outcome using commercial software (StepOne software v.2.3; Thermo Fisher Scientific). The PCR products with a cycle threshold value < 25 were purified (illustra ExoProStar 1-step; GE Healthcare, Little Chalfort, Buckinghamshire, UK) and diluted in half with Tris-EDTA buffer (MilliporeSigma) before sequencing. A commercial sequencing kit (ABI Prism BigDye terminator v.3.1 cycle; Applied Biosystems) was used for the sequencing reactions with C and D tail primers using a quarter of the recommended concentration of BigDye terminator v.3.1. Sequencing products were purified with an ethanol precipitation and analyzed (3130*xl* genetic analyzer; Applied Biosystems).

Geneious v.8.0.4 (https://www.geneious.com) was used to assemble forward and reverse sequences, and the resulting consensus sequences were uploaded to the relevant Public databases for molecular typing and microbial genome diversity (PubMLST) database

(http://pubmlst.org/perl/bigsdb/bigsdb.pl?db=pubmlst_taylorella_seqdef&page=batchSequenceQuery) for strain type identification. No ambiguous sequencing results were observed.

3.1.4 Whole genome sequencing

To better characterize the South African outbreak, the genome of *T. equigenitalis* strain ERC_G2224 was sequenced using the Ion Torrent (Life Technologies) platform. This isolate was obtained from archived semen samples collected from seven Lipizzaner stallions during an equine arteritisvirus investigation in 1996 [148]. High-molecular-weight DNA was extracted, and the size, quantity, and quality were checked using previously described methods [150]. The library was constructed from 1 µg of genomic DNA using the Ion Xpress Plus fragment library kit (Life Technologies). The size selection was performed on a 2% E-Gel SizeSelect gel (Invitrogen) using the 400 bp selection criterion. Fragments were not amplified during the library-building process. Template amplification was performed using the Ion OneTouch 2 system (OT2) with the Ion PGM Hi-Q OT2 kit, and the templated particles were enriched on the Ion OneTouch ES system (Life Technologies). The samples were loaded on an Ion 316 Chip version 2 and sequenced on the Ion PGM system (Life Technologies) using the Ion PGM Hi-Q sequencing kit (Life Technologies) for 400 bp chemistry.

3.1.5 Comparative phylogenetic analysis

In order to characterize the origin and evolution of the South African *T. equigenitalis* strains, we used the Pathosystems Resource Integration Centre (PATRIC, https://www.patricbrc.org) as a novel method to compare all 32 ST4 genomes available on the National Centre for Biotechnology Information (NCBI – www.ncbi.nlm.nih.gov) genetic sequence database, GenBank with the type strain of *T. equigenitalis.* Sequence Read Archive (SRA) data for 32 ST4 and one ST1. *Taylorella equigenitalis* genomes available publicly on the NCBI website under the BioProjects 384636, 436694, 294225 and 385665 were uploaded into the Comprehensive Genome Analysis service available on the PATRIC website to facilitate genome assembly and analysis (see Table 3-1).

The Phylogenetic Tree Building service in PATRIC was used to generate a phylogenetic tree based on 1 000 proteins from each of the isolates. The data was downloaded in Newark format and used to generate a phylogenetic tree in GENEIOUS Prime® (Version 2020.2.2).

Table 3-1 The bioproject and biosample numbers and sequence reads archive (SRA) numbers with hyperlinks of all ST4 (n=32) and one ST1 genomes available on the National Centre for Biotechnology Information database

Strain	Bioproject number	Biosample number	SRA
USDA_92_0920[c]	PRJNA384636	SAMN06843504	SRR5484568
NVSL_14_0141[a]	PRJNA436694	SAMN08630307	SRR6799980
NVSL_15_0094[a]	PRJNA436694	SAMN08630301	SRR6799972
NVSL_14_0144[a]	PRJNA436694	SAMN08630309	SRR6799899
NVSL_14_0135[a]	PRJNA436694	SAMN08630302	SRR6799971
UPERC_96_G2224[b]	PRJNA294225	SAMN04017970	SRR2221504
NVSL_14_0140[a]	PRJNA436694	SAMN08630306	SRR6799975
NVSL_14_0136[a]	PRJNA436694	SAMN08630303	SRR6799978
NVSL_14_0146[a]	PRJNA436694	SAMN08630310	SRR6799900
NVSL_14_0139[a]	PRJNA436694	SAMN08630311	SRR6799897
NVSL_01_0619[a]	PRJNA436694	SAMN08630161	SRR6799906
NVSL_15_0085[a]	PRJNA436694	SAMN08630292	SRR6799948
NVSL_15_0087[a]	PRJNA436694	SAMN08630294	SRR6799942
NVSL_15_0086[a]	PRJNA436694	SAMN08630293	SRR6799941
NVSL_06_0158[a]	PRJNA436694	SAMN08630171	SRR6799985
NVSL_15_0090[a]	PRJNA436694	SAMN08630297	SRR6799949
NVSL_02_0320[a]	PRJNA436694	SAMN08630163	SRR6799908
NVSL_02_0316[a]	PRJNA436694	SAMN08630238	SRR6799940
NVSL_15_0088[a]	PRJNA436694	SAMN08630295	SRR6799943
NVSL_15_0092[a]	PRJNA436694	SAMN08630299	SRR6799974
NVSL_15_0089[a]	PRJNA436694	SAMN08630296	SRR6799944
NVSL_15_0091[a]	PRJNA436694	SAMN08630298	SRR6799950
NVSL_15_0093[a]	PRJNA436694	SAMN08630300	SRR6799973
NVSL_13_0154[a]	PRJNA436694	SAMN08630208	SRR6800009
NVSL_13_0161[a]	PRJNA436694	SAMN08630209	SRR6799881
NVSL_13_0165[a]	PRJNA436694	SAMN08630228	SRR6799847
NVSL_13_0239[a]	PRJNA436694	SAMN08630212	SRR6799884
NVSL_13_0252[a]	PRJNA436694	SAMN08630225	SRR6799854
NVSL_13_0424[a]	PRJNA436694	SAMN08630267	SRR6799920
NVSL_13_0264[a]	PRJNA436694	SAMN08630227	SRR6799848
NVSL_13_0253[a]	PRJNA436694	SAMN08630226	SRR6799853
NVSL_13_0238[a]	PRJNA436694	SAMN08630213	SRR6799877
USDA_79_1587[c]	PRJNA385665	SAMN06899366	SRR5515305

Key: Sequencing platform: [a] = Illumina MiSeq; [b] = Ion Torrent; [c] = PacBio; Country of origin: Aus = Austria; SA = South Africa; US = United States of America; NC = No data collected; Green = South African isolates; Red = Austrian isolates; Blue = United States of America isolates

3.2 Epidemiological investigation of the South African outbreak of *Taylorella equigenitalis* and the *Taylorella asinigenitalis* incursion

The first two cases of *T. equigenitalis* identified in South Africa were confirmed in an imported Warmblood stallion (index) and a mare bred to him using fresh semen AI [1; 64]. Immediately following post-importation quarantine, the stallion had been transferred to an equine breeding centre for assisted reproductive procedures. Traceback of all in-contact horses present at the breeding centre (index property) during the period when the index case was resident, initially identified an additional two *T. equigenitalis*-positive stallions [151].

The epidemiological traceback of all in-contact horses and exposed mares and offspring identified an additional two stallions and two mares [64]. Concomitantly, effective from 31 August 2011, the national stallion screening programme which required all stallions used for breeding to have obtained a CEM clearance certificate was legislated [8]. A CEM clearance certificate was issued following two negative qPCR tests for *T. equigenitalis* performed on genital swabs obtained ≥ 7 days apart according to the prescribed methods outlined in the legislation [8]. To facilitate compliance, only offspring from stallions with a valid CEM clearance certificate were eligible for registration by their relevant breed society.

The requirements for the qPCR-based national stallion screening programme were based on the known pathogenesis of *T. equigenitalis* and supported by evidence from the 2008-2010 US outbreak [91]. Erdman and others' [91] report of an increased incidence in male horses and highlighted the importance of sampling all three predilection sites in male and female horses corroborated our findings.

A subpopulation focus was identified at the South African Lipizzaner Centre (SALC). Further epidemiological scrutiny of the South African Lipizzaner population resulted in archived semen samples, collected as part of a 1996 equine arteritisvirus investigation from seven Lipizzaner stallions, being accessed and submitted for qPCR and bacterial culture.

In 2015, the voluntary submission of cryopreserved semen straws derived prior to outbreak identification led to the detection of an additional *T. equigenitalis*-positive animal. This stallion had visited the index property for semen collection and cryopreservation in 2008 and had been subsequently gelded. Follow-up testing seven years later found that the gelding

remained positive for *T. equigenitalis.*

There have been various iterations of this programme in keeping with the changing disease situation. Current South African legislation [8] requires all stallions used for natural mating to be tested once every five years and stallions used as semen donors for assisted reproduction techniques to be tested on an annual basis. No foals may be registered with their relevant registering authorities if their sire had not been issued with a CEM clearance certificate prior to breeding. Semen is not allowed to be collected from stallions without a valid CEM clearance certificate, and any semen for which a valid CEM clearance certificate is not available, will be required to undergo additional testing [8].

In 2015, two imported miniature donkey stallions were identified *T. asinigenitalis*-positive on duplex qPCR during routine post-quarantine testing. The initial diagnosis of *T. asinigenitalis* was made only on the third (and final) sampling attempt, which strongly supported multiple sampling opportunities for effective screening. Interestingly, the second mature donkey stallion was initially negative and only became positive following their housing within the same stable during treatment. The donkey stallion initially diagnosed positive was a yearling and was probably prepubertal at the time of his diagnosis. The two donkeys were sent to an approved quarantine centre for follow-up testing and treatment.

3.3 Development and evolution of treatment protocols in South Africa

Due to a lack of local experience at the time of outbreak recognition, internationally recognized protocols [5; 6] were consulted and then adapted for the South African context. Treatment endpoint was defined when an animal was confirmed negative on qPCR testing of all sampling sites for two consecutive days after which they were treated for an additional three days to confirm negative status. For the initial modified treatment regimen [105], male horses were sedated once daily using an intravenous combination of detomidine hydrochloride [Domosedan, Novartis SA (Pty) Ltd, Animal Health, Isando] at 0.01 mg/kg and butorphanol tartrate (Torbugesic, Fort Dodge Animal Health, Iowa, US) at 0.01-0.02 mg/kg to facilitate extrusion of the penis. In order to monitor treatment efficacy and determine treatment endpoint, prior to commencement of daily treatment in all cases, a set of dry swabs was taken from the predilection sites and sent for qPCR testing. This was

followed by two days of cleaning of the penis and prepuce with a 5% docusate sodium (Docusol® Kyron Laboratories, Benrose, RSA) surfactant solution to remove smegma and external debris. From the third day, cleaning with the surfactant solution was followed by irrigation with a disinfectant solution containing 4% chlorhexidine gluconate (Dismed Bioscrub®, Dismed Pharma, Halfway House, RSA). Dry swabs were dipped in the 4% chlorhexidine gluconate solution and used to thoroughly clean the urethral fossa and sinus. The penis was thoroughly dried using paper towel. Thereafter, one of two topical antimicrobial therapies, either 0.2% nitrofurazone (Furex®, Aspen Pharmacare, Woodmead, South Africa) or 1% silver sulphadiazine (Silbecor, Biotech Laboratories, Midrand, RSA) ointment, were liberally applied to the prepuce and surface of the penis, including the urethral fossa and sinus.

In the first mare treated, a similar regimen was used to clean the clitoral fossa and sinuses. The clitoral fossa and sinuses were cleaned, using normal swabs and dedicated paediatric swabs (Copan Innovation, Brescia, Italy) respectively, dipped in 4% chlorhexidine gluconate. Thereafter, 1% silver sulphadiazine ointment was applied liberally to the external genitalia.

To facilitate treatment of the largest cohort of *T. equigenitalis*-positive horses (n=23) at the SALC, the animals were divided into smaller groups. The entire stallion population was removed and housed at separate isolation facilities under strict quarantine measures to facilitate a depopulation-repopulation exercise at the SALC. During the depopulation phase, the stables at the SALC were thoroughly cleaned, decontaminated and disinfected and only certified negative horses were allowed to return to the facility [105]. A five-day antimicrobial treatment regimen was instituted for the initial treatment group (Group 1, n=5), as previously described. Follow-up qPCR testing after 10 days showed that all stallions remained positive for *T. equigenitalis.* The second group of stallions (Group 2), comprised of seven previously untreated stallions and an additional three stallions from the initial treatment group (Group 1), were treated using the same protocol for nine consecutive days. Due to the inconsistent response to the prescribed limited treatment period, daily in-treatment qPCR monitoring was instituted to track treatment efficacy and to determine treatment endpoint for the final treatment group. This group was comprised of all remaining untreated animals (n=11) and all animals that had remained *T. equigenitalis*-positive despite prior treatments (n=7).

All negative stallions (n=9) were tested repeatedly using qPCR to confirm negative status before being allowed to return to the SALC. All *T. equigenitalis*-positive stallions were treated in manageable batches at an isolation facility where they remained until treatment endpoint. Thereafter, negative status was confirmed ≥ 21 days post-treatment by both bacterial culture and qPCR testing before the stallions were allowed to return to the SALC. This was followed by a monthly qPCR testing protocol of all stallions resident at the SALC for a period of 12 months [106].

In order to monitor potential recrudescence of *T. equigenitalis* and the consequences of prolonged topical antimicrobial treatment on the microflora population of the resident horses at the SALC, a monthly swabbing regimen was instituted approximately four weeks after the last administered treatment and concluding approximately 12 months later. On five separate occasions during that period, duplicate swabs for bacterial culture and qPCR were obtained as previously described to monitor the effects of prolonged antimicrobial treatment. On the first occasion, four weeks post final treatment, all 32 horses at the SALC were swabbed. These included the 23 horses (22 stallions and 1 gelding) that had been treated for *T. equigenitalis* and the nine untreated, *T.* equigenitalis-negative stallions. Based on their treatment history, the horses could be subdivided into three groups; Groups A (0.2% nitrofurozone; n=12) and Group B (1% silver sulphadiazone; n=11) were post-treatment *T. equigenitalis*-positive horses and Group C (n=9) were untreated *T. equigenitalis*-negative stallions. Thereafter, any stallion showing at least one positive culture for either *Klebsiella pneumoniae* or *Pseudomonas aeruginosa* was reswabbed for bacterial culture on three subsequent occasions with an interval of approximately one month. Finally, approximately 12 months after the initial swabbing date, all horses (n=32) were resampled for culture.

Based on these experiences of protracted treatment periods and the known susceptibility of *T. equigentialis* to most antimicrobials [5], a topical bovine dry cow intramammary preparation containing 200 mg sodium cloxacillin and 75 mg sodium ampicillin (Curaclox DC intrammary, Norbrook Laboratories) for application to the urethral or clitoral fossae and sinuses was introduced to the treatment protocol for the second *T. equigenitalis*-positive mare identified in 2013 and later for the treatment of a *T. equigenitalis*-positive gelding identified in 2015 [7] and two stallions identified in 2017 [152]. Similarly, this protocol was used to treat the two *T. asinigenitalis*-positive miniature donkey stallions identified in 2015 [9].

Chapter 4
Results

4.1 The results from the South African outbreak

4.1.1 Real-time PCR

Since first reporting of the index case during the South African outbreak [1], a total of 42 *T. equigenitalis*-positive horses and two *T. asinigenitalis*-positive donkeys have been identified using qPCR over the nine-year period May 2011 to May 2020.

A subpopulation focus was identified at the SALC where 24/33 (72.7%) resident stallions tested positive for *T. equigenitalis* on qPCR. Five out of seven archived semen samples that had been collected in 1996 as part of an equine arteritisvirus investigation [148] at the SALC were accessed and tested positive for *T. equigenitalis* on qPCR.

The distribution of *T. equigenitalis* across the sampled predilection sites in all 39 identified *T.* equigenitalis-positive male horses is shown in Table 4-1 and Table 4-2. These results included both screening qPCR tests and confirmatory bacterial culture. All male horses identified during the South African outbreak tested positive on qPCR assay. Across the predilection sites the most commonly affected site was the urethral fossa (36/39; 92.3%), followed by the urethra (33/39; 84.6%) and the lamina interna (25/39; 64.1%). In the 36 horses identified using the modified qPCR assay, the copy number from the urethral fossa was at least 50-fold more than from the urethra and at least 500-fold more than from the lamina interna (see Table 4-2).

The breed distribution in the 39 identified male horses was Lipizzaner (n=25; 64%), Warmblood (n=11; 28%), Selle Francais (n=1; 2.6%), Andalusian (n=1; 2.6%) and Connemara (n=1; 2.6%).

Table 4-1 Summary of qPCR assay and confirmatory bacterial culture results from swabs obtained from the predilection sites on the external genitalia of three male horses identified in 2011 during the initial outbreak investigation of the South African *Taylorella equigenitalis* outbreak

Animal identification	Breed	qPCR results from screening tests			Confirmatory bacterial culture result
		Urethra	Urethral fossa	Lamina interna	
CEM_2011_01_01	WB	+	+	+	+
CEM_2011_02_03	WB	-	+	+	+
CEM_2011_02_04	WB	+	+	+	+

Key: qPCR results reported as + (positive) or – (negative), assay as per Wakeley et al [102]; bacterial culture results reported as + (positive) or – (negative); WB=Warmblood

Table 4-2 Summary of cycle threshold (Ct) results from the qPCR assay and confirmatory bacterial culture results from swabs obtained from the predilection sites on the external genitalia of 36 male horses identified during the epidemiological traceback and national stallion screening programme during 2011-2017 of the South African *Taylorella equigenitalis* outbreak

Animal identification	Breed	qPCR results from screening tests (Ct value)			Confirmatory bacterial culture result
		Urethra	Urethral fossa	Lamina interna	
CEM_2011_03_05	WB	28.5	17.6	24.4	+
CEM_2011_04_06	Con	33.8	32.6	≥ 40	+
CEM_2011_05_07	WB	39.0	23.5	≥ 40	+
CEM_2011_06_08	Lip	24.9	17.2	≥ 40	+
CEM_2011_07_09	WB	≥ 40	23.8	≥ 40	+
CEM_2011_08_10	Selle	29.6	21.8	36.0	+
CEM_2011_09_11	Lip	34.2	29.6	35.3	-
CEM_2011_09_12	Lip	28.8	23.9	36.5	+
CEM_2011_09_13[#]	Lip	28.5	22.7	≥ 40	+
CEM_2011_09_14	Lip	≥ 40	33.0	35.7	+
CEM_2011_09_15	Lip	26.0	28.9	31.1	-
CEM_2011_09_16	Lip	≥ 40	≥ 40	29.6	+
CEM_2011_09_17	Lip	36.3	≥ 40	34.8	-
CEM_2011_09_18	Lip	33.3	≥ 40	34.1	+
CEM_2011_09_19	Lip	27.2	24.7	≥ 40	+
CEM_2011_09_20	Lip	22.4	17.6	28.6	-
CEM_2011_09_21	Lip	30.9	25.7	31.6	+
CEM_2011_09_22	Lip	26.3	25.7	35.6	-
CEM_2011_09_23	Lip	31.6	24.8	32.9	+
CEM_2011_09_24	Lip	23.1	18.7	35.4	-
CEM_2011_09_25	Lip	21.4	38.8	37.5	-
CEM_2011_09_26	Lip	28.7	20.9	36.8	+
CEM_2011_09_27	Lip	29.5	30.2	≥ 40	+
CEM_2011_09_28	Lip	30.9	22.8	≥ 40	-
CEM_2011_09_29	Lip	30.9	22.2	34.3	-
CEM_2011_09_30	Lip	30.0	21.5	≥ 40	+
CEM_2011_09_31	Lip	16.9	15.8	25.2	+
CEM_2011_09_32	Lip	27.7	21.8	≥ 40	+
CEM_2011_09_33	Lip	23.2	17.6	30.3	+
CEM_2011_09_34	Lip	≥ 40	19.8	29.1	-
CEM_2011_10_35	And	29.1	25.6	≥ 40	+
CEM_2011_11_36	WB	38.0	29.2	≥ 40	+
CEM_2011_12_37	WB	31.0	18.0	≥ 40	+
CEM_2015_41[#]	WB	≥ 40	21.5	≥ 40	+
CEM_2015_42	WB	28.1	19.1	34.4	+
CEM_2015_43	WB	29.9	21.6	28.3	+
Mean of positive results (Ct ≤ 40)		**29.0**	**23.6**	**32.6**	
Standard deviation of positive results (Ct ≤ 40)		**4.8**	**5.3**	**3.8**	

Key: Culture results reported as + (positive) or – (negative); [#]Gelding; And = Andalusian; Selle = Selle Francais; Con = Connemara; Lip = Lipizzaner; WB = Warmblood

The distribution of *T. equigenitalis* across the predilection sites of acutely (n=1) and chronically (n=2) affected mares during the South African outbreak is shown in Table 4-3. These results include both qPCR and bacterial culture results. The organism was present in the endometrium during acute infection (n=1) and in the clitoral sinus and fossae in the chronic carrier state (n=2).

Table 4-3 The distribution of *Taylorella equigenitalis* by sample predilection sites of acutely (n=1) and chronically (n=2) infected mares during the 2011 South African outbreak

Mare ID	Breed	*T. equigenitalis* infection	Endometrium		Clitoral fossa		Clitoral sinus	
			Culture	qPCR	Culture	qPCR	Culture	qPCR
CEM_2011_01_02	TB	Acute	+	+	Not done		Not done	
CEM_2011_13_38	TB	Chronic	-	-	-	29.9	-	37.2
CEM_2011_08_39*	WB	Chronic	n/a	n/a	-	39.7	+	-

Key: TB = Thoroughbred; WB = Warmblood; + = positive; - = negative; * pre-pubertal filly therefore endometrial swabs not taken

The qPCR assay and bacterial culture results from swabs obtained from the predilection sites on the external genitalia of the two miniature donkey stallions identified positive for *T. asinigenitalis* during post-importation quarantine in South Africa are shown in Table 4-4. Both miniature donkey stallions tested positive for *T. asinigenitalis* on qPCR.

Of the qPCR positive results, the most commonly affected sites were the urethral fossa and sinus: Jack 1 = 4/6 (66.67%) and Jack 2 = 6/8 (75%), followed by the urethra in both donkey stallions: Jack 1 = 2/6 (33.34%) and Jack 2 = 2/8 (25%). All results from follow-up testing at five- and six-weeks post-treatment were negative on qPCR. All qPCR results are shown in Table 4.4.

Table 4-4 Summary of results from a duplex qPCR assay for *Taylorella equigenitalis/asinigenitalis* and bacterial culture from swabs obtained from the predilection sites on the external genitalia of two miniature donkey stallions

		Timeline (2015)																													
		Post arrival quarantine				In-treatment testing																								Follow-up testing	
Animal	**Assay**	**16 Sept**	**23 Sept**	**30 Sept**	**09 Oct**	**15 Oct**			**16 Oct**			**17 Oct**			**18 Oct**			**19 Oct**			**20 Oct**			**21 Oct**			**22-26 Oct**			**1 Dec**	**8 Dec**
						a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c		
Jack 1	Culture				+																									-	-
	qPCR	-	-	30.8		-	24.3	-	31.6	28.5	-	35.3	32.0	-	-	34.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Jack 2	Culture				-																									-	-
	qPCR	-	-	-		-	37.5	-	-	30.6	-	-	32.3	-	35.9	32.3	-	32.5	-	-	-	33.8	-	-	39.3	-	-	-	-	-	-

Key: a = urethra; b = urethral fossa and sinus; c = *lamina interna*; + = positive culture; - = negative culture/qPCR, qPCR expressed in cycle threshold (Ct) values

4.1.2 Bacterial culture

In male horses, 29/39 (74%) tested positive on bacterial culture (see Tables 4-1 and 4.2).

In female horses, 2/3 (66.7%) tested positive on bacterial culture (see Table 4-3).

Five out of seven (71.4%) archived semen samples collected in 1996 as part of an equine arteritisvirus investigation [148] at the SALC were positive for *T. equigenitalis* on bacterial culture.

Only one of the two *T. asinigenitalis*-positive donkeys tested positive on bacterial culture. All results from follow-up testing at five- and six-weeks post-treatment were negative on culture (see Table 4-4).

4.1.3 MLST analysis

A single sequence type (ST4) was identified from all South African *T. equigenitalis* samples.

In the two donkey stallions, the following allelic profile was identified for *T. asinigenitalis*: new allele-unable to type-17-10-8-10-15.

4.1.4 Whole genome sequencing

In total, 2.25 million reads (mean length, 314 bp) generated 706 Mb of data, of which 1,123,016 reads were assembled (estimated coverage, < 80 x into 18 large contigs (> 500 bp), giving a consensus length of 1 670 247 bp; the contigs were ordered and compared with the genome of *T. equigenitalis* strain MCE9 (accession no. CP002456) using IonGAP: integrative bacterial genome analysis for Ion Torrent sequence data, released in 2015 (http://iongap.hpc.iter.es/iongap/) [153]. Annotation was added by the NCBI Prokaryotic Genome Annotation Pipeline, released in 2013 (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/), yielding 1,540 genes, 38 tRNAs, and 12 rRNAs. One clustered regularly interspaced short palindromic repeat (CRISPR)/Cas loci was detected. The average G+C content of the draft genome sequences is 37.4%.

This whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession no. LIYJ00000000 (https://www.ncbi.nlm.nih.gov/nuccore/LIYJ00000000).

4.1.5 Comparative phylogenetic analysis of *Taylorella equigenitalis* sequence type 4

A summary of the genomic characteristics from all 32 ST4 and one ST1 type strain of *T. equigenitalis* found on GenBank is shown in Table 4-5. A phylogenetic tree based on 1 000 proteins from each isolate and showing the epidemiological links between strains is shown in Figure 4-1.

Table 4-5 Genomic characteristics of all ST4 *Taylorella equigenitalis* strains (n=32) found on GenBank plus a reference ST1 outgroup assembled on PATRIC

Strain	MLST	Contigs	Genome Length	GC Content	Coarse Consistency	Fine Consistency	Genome Quality	Isolation Source	Isolation Country	Comments
USDA_92_0920[c]	ST4	1	1666291	37.52	99.9	99.9	Good	Swab	US	Imported into US from Austria in 1992
NVSL_14_0141[a]	ST4	20	1646045	37.43	99.9	99.9	Good	Swab	SA	
NVSL_15_0094[a]	ST4	28	1642330	37.44	99.9	99.9	Good	Swab	SA	Index case for South Africa outbreak
NVSL_14_0144[a]	ST4	20	1645023	37.43	99.7	99.7	Good	Swab	SA	
NVSL_14_0135[a]	ST4	21	1645323	37.43	99.9	99.9	Good	Swab	SA	
UPERC_96_G2224[b]	ST4	18	1670247	37.52	98.6	97.7	Good	Semen sample	SA	Isolated from semen sample collected in 1996
NVSL_14_0140[a]	ST4	22	1646241	37.44	99.7	99.7	Good	Swab	SA	
NVSL_14_0136[a]	ST4	21	1645752	37.43	99.9	99.9	Good	Swab	SA	
NVSL_14_0146[a]	ST4	19	1645536	37.44	99.9	99.9	Good	Swab	SA	
NVSL_14_0139[a]	ST4	20	1645913	37.44	99.9	99.9	Good	Swab	SA	
NVSL_01_0619[a]	ST4	25	1637477	37.41	98.7	98.6	Good	Swab	US	Imported into US from Germany in 2001
NVSL_15_0085[a]	ST4	17	1649299	37.45	99.9	99.9	Good	Swab	Aus	
NVSL_15_0087[a]	ST4	19	1649216	37.45	99.9	99.9	Good	Swab	Aus	
NVSL_15_0086[a]	ST4	20	1648698	37.45	99.9	99.9	Good	Swab	Aus	
NVSL_06_0158[a]	ST4	18	1640225	37.42	98.7	98.6	Good		US	Imported into US from Austria in 2006
NVSL_15_0090[a]	ST4	20	1649003	37.45	99.9	99.9	Good	Swab	Aus	
NVSL_02_0320[a]	ST4	23	1639338	37.42	98.7	98.6	Good	Swab	US	Imported into US from Austria in 2002
NVSL_02_0316[a]	ST4	17	1639662	37.42	98.7	98.6	Good	Swab	US	Imported into US from Austria in 2002
NVSL_15_0088[a]	ST4	17	1645006	37.44	99.9	99.9	Good	Swab	Aus	
NVSL_15_0092[a]	ST4	18	1644467	37.44	99.9	99.9	Good	Swab	Aus	
NVSL_15_0089[a]	ST4	20	1644004	37.43	99.9	99.9	Good	Swab	Aus	
NVSL_15_0091[a]	ST4	17	1644321	37.44	99.7	99.7	Good	Swab	Aus	
NVSL_15_0093[a]	ST4	17	1644574	37.45	99.7	99.7	Good	Swab	Aus	
NVSL_13_0154[a]	ST4	19	1636646	37.42	98.7	98.6	Good	Swab	NC	
NVSL_13_0161[a]	ST4	22	1635532	37.41	98.7	98.6	Good	Swab	NC	
NVSL_13_0165[a]	ST4	17	1636792	37.42	98.7	98.6	Good	Swab	NC	
NVSL_13_0239[a]	ST4	16	1633296	37.41	98.4	98.3	Good	Swab	NC	
NVSL_13_0252[a]	ST4	19	1637280	37.42	98.7	98.6	Good	Swab	NC	
NVSL_13_0424[a]	ST4	19	1637280	37.42	98.4	98.4	Good		NC	
NVSL_13_0264[a]	ST4	19	1636931	37.42	98.7	98.6	Good		NC	
NVSL_13_0253[a]	ST4	35	1633395	37.42	98.7	98.3	Good	Swab	NC	
NVSL_13_0238[a]	ST4	17	1636232	37.41	98.7	98.6	Good	Swab	NC	
USDA_79_1587[c]	ST1	1	1739054	37.31	99.9	99.9	Good	Swab	US	Original *T. equigenitalis* type strain in US

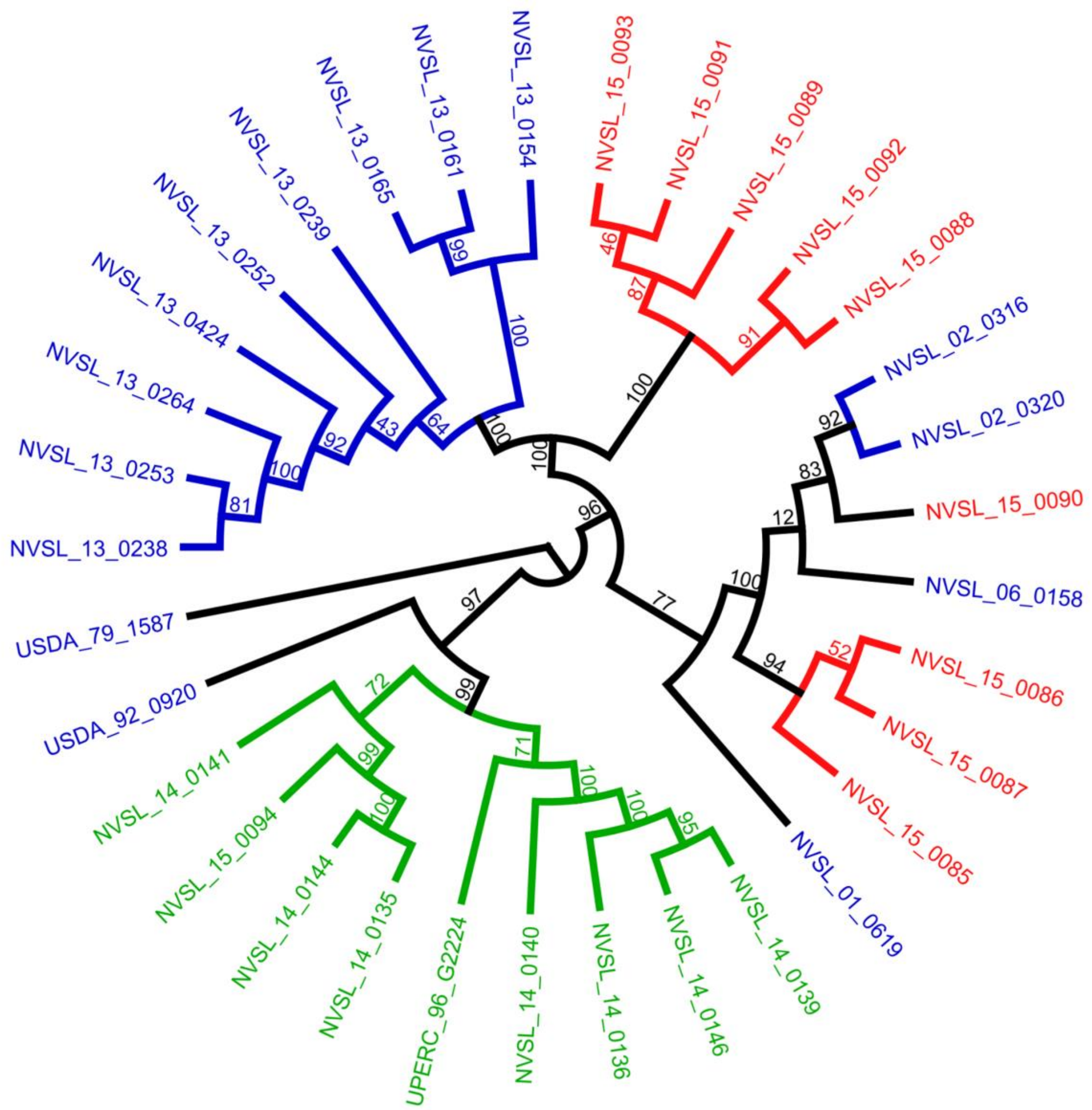
Key: Sequencing platform: [a] = Illumina MiSeq; [b] = Ion Torrent; [c] = PacBio; Country of origin: Aus = Austria; SA = South Africa; US = United States of America; NC = No data collected; Green = South African isolates; Red = Austrian isolates; Blue = United States of America isolates

The first branch of the dendrogram in Figure 4-1 shows that all South African *T. equigenitalis* isolates (n=9) shared a common ancestor with an Austrian horse (USDA_92_0920) imported into the US in 1992. Lipizzaners were exported from Austria into the US in 1992, 2002 and 2006 (Hubinger, personal communication) strongly suggesting that these four Austrian imports (USDA_92_0920, NVSL_06_0158, NVSL_02_0320 and NVSL_02_0316) were Lipizzaners.

The second branch links horses imported into the US from Germany in 2001 (NVSL_01_0619) with three horses imported from Austria in 2002 (NVSL_02_0320 and NVSL_02_0316) and 2006 (NVSL_06_0158) and four *T. equigenitalis*-positive strains isolated in Austria in 2015 (NVSL_15_0085, NLSL_15_0086, NVSL_15_0087 and NVSL_15_0090). This branch shows that multiple incursions of ST4 from Europe were detected at post-arrival quarantine in the US over a number of years.

The third branch links a clade of Austrian strains (n=5) with nine strains isolated in the US from an unknown source in 2013 (see Figure 4-1).

The majority of isolates were sequenced with Illumina MiSeq, apart from two older isolates (USDA_92_0920 and USDA_79_1587) which were sequenced with PacBio and one isolate (UPERC_96_G224) sequenced with IonTorrent technology. These sequencing technologies show a minor difference in their ability to generate usable data [154].



Green = South African isolates; Red = Austrian isolates; Blue = United States of America isolates

Figure 4-1 Phylogenetic tree based on the SRA data of 32 ST4 genomes using the maximum likelihood method, rooted with *Taylorella equigenitalis* ST1 as the outgroup

4.2 The epidemiological sequence of the South African outbreak of *Taylorella equigenitalis*

A diagram of the epidemiological events and the sequence of the South Africa outbreak of *T. equigenitalis* was obtained from the results of molecular diagnostics as shown in Figure 4-2.

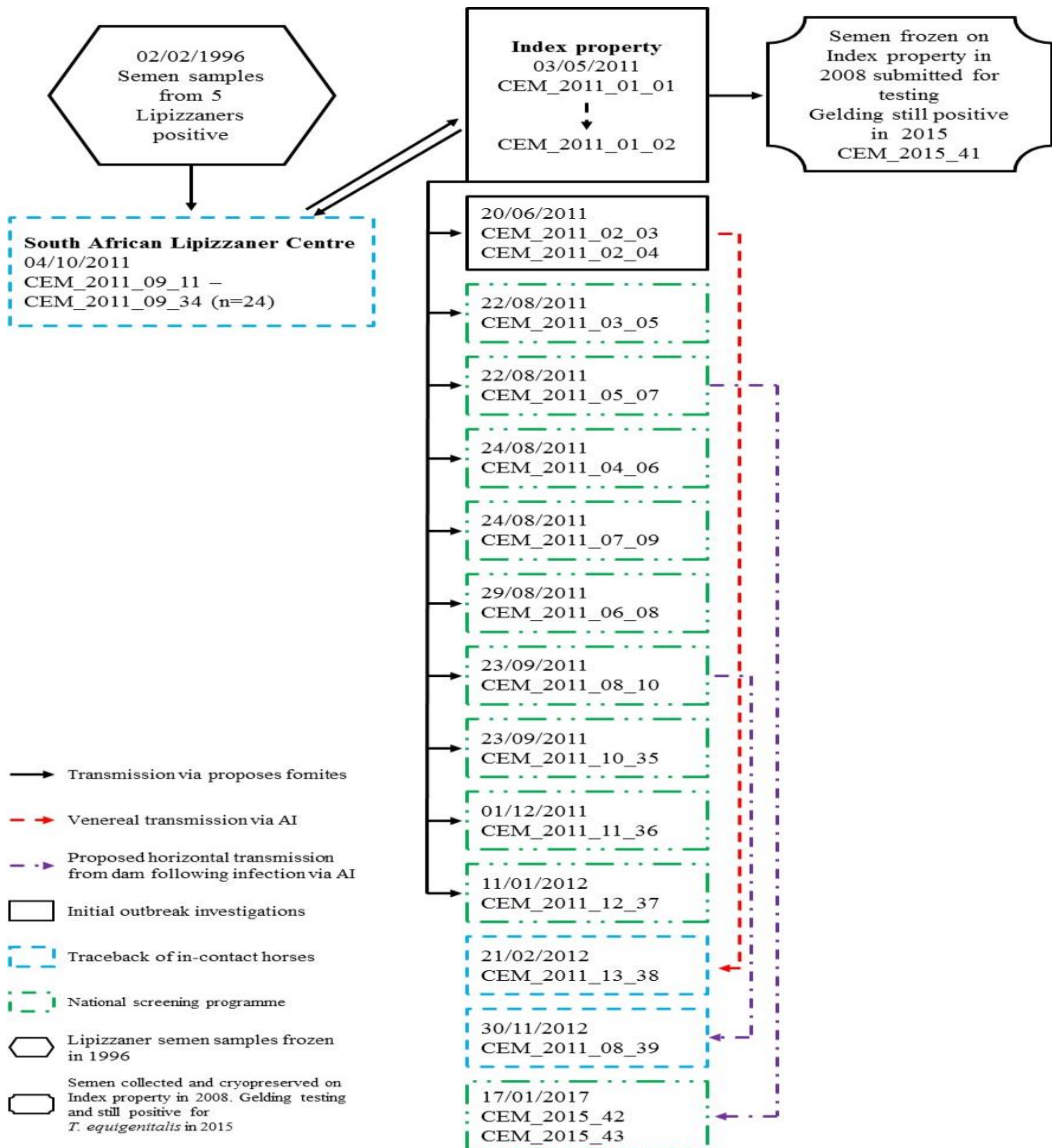


Figure 4-2 Schematic temporal representation of *Taylorella equigenitalis*-positive horses identified during the South African outbreak showing the proposed transmission between animals involved in the outbreak. Date represents the date of sample collection.

4.3 The results of treatment regimens used during the course of the South African outbreak (2011-2017)

A summary of the treatment regimens for 23 Lipizzaners from the SALC is shown in Table 4-6 and Table 4-7. All horses in Group 1 (n=5), initially treated for five days, remained positive. During the second nine-day treatment programme (Group 2), 4/7 (57%) previously untreated horses and 1/3 (33.3%) of the previously treated horses from Group 1 cleared themselves of infection. The final treatment protocol, which incorporated daily in-treatment qPCR monitoring, resulted in 11 previously untreated horses and seven previously treated horses clearing themselves of infection with the range of treatment periods spanning 8-51 days.

In horses that were *T. equigenitalis*-positive on qPCR alone (n=10), the range of treatment periods was 8-34 days, with a mean of 17.6 days and a sample standard deviation of 9.58. In horses that were *T. equigenitalis*-positive on both qPCR and bacterial culture (n=13), the range of treatment periods was 9-65 days, with a mean of 21.2 days and a sample standard deviation of 16.2. There was no difference between the two topical antimicrobial agents in the interval to elimination of *T. equigenitalis* [106].

Table 4-6 Summary of treatment periods for 10 Lipizzaners from the South African Lipizzaner Centre positive for *Taylorella equigenitalis* on qPCR alone, including the treatment product and total number of days required to clear infection

OIE number	DOB	Lowest initial Ct values	Final treatment group	Total (days)	Product used
CEM_2011_09_11#§	2000	29.6	07-16/02/2012 (10 d)	19	S
CEM_2011_09_15#	2004	26.0	Cleared during previous treatment	9	S
CEM_2011_09_17	1995	34.8	07-16/02/2012 (10 d)	10	N
CEM_2011_09_20§	1993	17.6	28/02-07/03/2012 (8 d)	8	S
CEM_2011_09_22§	2008	25.7	30/01-24/02/2012 (26 d)	26	S
CEM_2011_09_24#	2002	18.7	11/02-07/03/2012 (25 d)	34	S
CEM_2011_09_25§	2003	21.4	30/01-10/02/2012 (12 d)	12	N
CEM_2011_09_28§	2007	22.8	30/01-17/02/2012 (19 d)	19	N
CEM_2011_09_29§	2008	22.2	30/01-20/02/2012 (22 d) + 13/03-20/03/2012 (8 d)	30	S
CEM_2011_09_34#	2005	19.8	Cleared during previous treatment	9	N

Key: #Group 2 treated for 9 d (16-24/01/2012); § used for breeding previously; $ gelding; S = silver sulphadiazine; N = Nitrofurazone

Table 4-7 Summary of treatment periods for 13 Lipizzaners from the South African Lipizzaner Centre positive for *Taylorella equigenitalis* on qPCR and bacterial culture, including the treatment product and total number of days required to clear infection

OIE number	DOB	Lowest initial Ct values	Final treatment group	Total (days)	Product used
CEM_2011_09_12	2000	23.9	30/01-14/02/2012 (16 d)	16	N
CEM_2011_09_13[$]	2003	22.7	30/01-08/02/2012 (10 d)	10	S
CEM_2011_09_14[#]	2006	33.0	Cleared during previous treatment	9	S
CEM_2011_09_16	1993	29.6	30/01-10/02/2012 (12 d)	12	N
CEM_2011_09_18[#]	1995	33.3	Cleared during previous treatment	9	N
CEM_2011_09_19[*#§]	2006	24.7	07/02-29/03/2012 (51 d)	65	S
CEM_2011_09_21[*#]	1995	25.7	Cleared during previous treatment	14	N
CEM_2011_09_23*	1997	24.8	28/02-07/03/2012 (8 d)	13	N
CEM_2011_09_27*	2003	29.5	30/01-20/02/2012 (22 d)	27	N
CEM_2011_09_30[§]	2005	21.5	30/01-10/02/2012 (12 d)	12	N
CEM_2011_09_31[*#§]	2000	15.8	07/02-05/03/2012 (27 d)	41	N
CEM_2011_09_32	2002	21.8	30/01-27/02/2012 (29 d)	29	S
CEM_2011_09_33[#]	2006	17.6	07-16/02/2012 (10 d)	19	N

Key: *Group 1 initially treated for 5 d (n=5, 05-09/12/2011); [#]Group 2 treated for 9 d (16-24/01/2012); [§] used for breeding previously; [$] gelding; S = silver sulphadiazine; N = Nitrofurazone

The total treatment periods required for three non-Lipizzaner horses treated by University of Pretoria personnel are shown in Table 4-8. The range of these treatment periods was 11-26 days. The first horse (CEM_2011_01_01) was treated using the original treatment protocol. Subsequent horses were treated with a modified protocol which included the use of in-treatment qPCR monitoring. The male and female horse were negative on qPCR assay after 8 and 18 days respectively. Table 4-8 also shows the total treatment periods required to treat an additional two non-Lipizzaner horses using a modified treatment protocol which included the use in-treatment monitoring and the application of a topical intramammary antimicrobial formulation. The range of treatment in these horses was 7-8 days, with the female and male horse being negative on qPCR after 2 and 1 day respectively.

Table 4-8 Summary of all courses of treatment for the horses (n=5) treated by University of Pretoria personnel including two treated with the additional inclusion of an intramammary antimicrobial formulation

OIE number	Sex	Treatment periods (Dates)		Total treatment period (days)
		Period 1	Period 2	
CEM_2011_01_01	M	23-25/08/2011	08-15/07/2011	11
CEM_2011_10_35	M	26/02-09/03/2012		12 (qPCR negative after 8 days of treatment)
CEM_2011_13_38	F	05-30/07/2011		26 (qPCR negative after 18 days of treatment)
CEM_2011_08_39[#]	F	24-30/04/2013		7 (qPCR negative after 2 days of treatment)
CEM_2015_41[#]	M	06-14/08/2015		8 (qPCR negative after 1 day of treatment)

Key: [#]treated using protocol including an intramammary antimicrobial formulation

In the two donkey stallions, the interval to elimination or treatment endpoint was four days (Jack 1: 15-18 October) and seven days (Jack 2: 15-21 October), respectively (see Table 4-4).

Table 4-9 shows the five occasions when duplicate swabs for bacterial culture were taken from the predilection sites of the Lipizzaner population at the SALC to monitor the consequences of prolonged topical antimicrobial treatment on the microflora population of the external genitalia. The sequential incidence and distribution of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* in Lipizzaner stallions by treatment group (A = 0.2% nitrofurazone; B = 1% silver-sulfadiazine; C = untreated) and anatomic site are shown. *Pseudomonas aeruginosa* or *K. pneumoniae* was isolated from 10/23 (43.5%) treated stallions during the four months post-treatment with the isolation of *P. aeruginosa* (6/10) having a stronger treatment-association than *K. pneumoniae* (4/10). At one year post-treatment, *K. pneumoniae* was isolated from nearly half the untreated (4/9), but none of the treated stallions. During the course of the observation period, five (15.6%) of the stallions were occasionally used for breeding purposes.

There appeared to be no influence of site on the incidence of either *P. aeruginosa* or *K. pneumoniae*.

Table 4-9 Sequential incidence and distribution by anatomic site of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* in Lipizzaner stallions following topical treatment for *Taylorella equigenitalis* by treatment group

Stallion ID	Treatment Group	Total treatment period (days)	Date of sampling									
			12/04/2012		03/05/2012		23/05/2012		26/06/2012		02/04/2013	
			Pseudo	***Kleb***	***Pseudo***	***Kleb***	***Pseudo***	***Kleb***	***Pseudo***	***Kleb***	***Pseudo***	***Kleb***
1	C		-	+	-	-	-	-	-	-	-	+
2*	C		-	-	ns		ns		ns		-	+
3	C		-	-	ns		ns		ns		-	+
4	C		-	-	ns		ns		ns		-	+
CEM_2011_09_16	A	12	+	-	+	-	+	-	-	+	-	-
CEM_2011_09_27	A	27	-	-	ns		ns		ns		+	-
CEM_2011_09_30[§]	A	12	+	-	-	-	-	-	-	-	-	-
CEM_2011_09_31[§]	A	41	+	+	+	-	-	-	-	-	-	-
CEM_2011_09_11[§]	B	19	+	-	-	-	-	-	-	-	-	-
CEM_2011_09_13[#]	B	10	-	+	-	-	-	-	-	-	-	-
CEM_2011_09_14	B	9	-	+	-	-	-	-	-	-	-	-
CEM_2011_09_22[§]	B	26	-	+	-	-		-	-	-	-	-
CEM_2011_09_24	B	34	+	-	-	-	-	-	-	-	-	-
CEM_2011_09_29[§]	B	30	+	-	-	-	-	-	-	-	-	-
Total positive animals			**6**	**5**	**2**	**0**	**1**	**0**	**0**	**1**	**1**	**4**

Key: A = 0.2% nitrofurazone; B = 1% silver-sulfadiazine; C = *T. equigenitalis*-negative and untreated;
Pseudo = Pseudomonas aeruginosa;, Kleb = Klebsiella pneumoniae;
ns = not sampled; + = positive culture; - = negative culture;
* bred 2 mares by natural mating during the 12 month periods (08 and 19/01/2013, 07 and 09/02/2013); [§]had been used for breeding previously; [#]gelding

Chapter 5
Discussion

The international movement of equids can be traced back most notably to Christopher Columbus' voyage to the New World where the introduction of equids, amongst other things, changed the geopolitical landscape [155]. Since then, the frequency and ease with which horses are transported around the globe has increased exponentially and with that, so has the risk of disease transmission. Despite various safeguards in place to prevent the spread of disease, the vagaries of the aetiological agent and in particular, the asymptomatic carrier state, requires additional vigilance on the part of the veterinary profession. The international movement of horses is well regulated but requires a level of trust in the diagnostic capabilities, the veterinary infrastructure and the disease-reporting networks of both the exporting and importing countries. International trade agreements, such as the European Union's Free Trade Agreement [37], have encouraged economic expansion of the equine industry but place the onus for disease-reporting on an often unwary public and rely on the integrity of individual horse owners who may be poorly informed of the greater disease risks. Similarly, the international shipment of stallion semen has the potential to disseminate various venereal pathogens, including equine arteritisvirus [148; 156; 157], *T. equigenitalis* [89], *K. pneumoniae*, *P. aeruginosa* and *Trypanosoma equiperdum* [15; 158]. However, the actual disease risk posed by shipped semen is largely undefined [39].

It is not only the international movement of horses that poses a risk. The extensive local movement of horses for competitions and breeding purposes within countries are forerunners to the potential dissemination of pathogens. Again, the disease risk is largely undefined, relying on the requirements of local competition bodies and individual on-farm biosecurity measures. A 2009 survey of 27 Thoroughbred and Standardbred stud farms in New Zealand, reported a median of 127 movement events from each stud including movement of mares, stallions and yearlings, and an annual total of 5 449 movement events [29]. In terms of the risks for disease transmission, the majority (96%) of these stud farms had biosecurity measures in place for arriving mares, although very few included the isolation of new arrivals. Only 15% had on-farm policies in place for working with different groups of horses and visitor protocols were absent on the majority of farms (93%) leaving them potentially

vulnerable to outbreaks of infectious disease [29]. In sport horses, the lack of an centralized, integrated registry provides a barrier to the accurate management and recording of data for this cohort [159; 160].

The initial objective of this study was to use newer molecular technologies to identify the origin and evolution of the South African *T. equigenitalis* outbreak. As described in Chapter 4 (section 4.1.3), MLST analysis of all South African *T. equigenitalis* strains identified during the period 1996-2017 identified a single sequence type (ST4) that strongly suggested a point introduction [161]. Unfortunately, isolates from all horses were not kept, necessitating the ultimately successful use of crude extract to obtain samples and highlighting the need for stringent sample and record keeping during outbreaks in order to facilitate epidemiological tracebacks. A study reporting genotyping of German and Austrian *T. equigenitalis* isolates using repetitive extragenic palindromic (REP) PCR and pulsed-field gel electrophoresis (PGFE) included a South African Holsteiner stallion isolate, which was the index case in our study and which was allocated the REP/PGFE genotype rep-E1/TE-A5 [59]. In that report [59], rep-E1/TE-A5 was present exclusively in the majority of Austrian Lipizzaners (n=38) and additionally was identical to the TE011 genotype reported as part of the US outbreak in 1978-2010 [93] from four stallions imported from Austria and Germany. These data show that ST4, rep-E1/TE-A5, and TE011 represent a single *T. equigenitalis* type. Using MLST, we were therefore able to demonstrate that the index case in the South African outbreak was not the source of infection [161] and that Lipizzaner horses [59] were the common breed and Austria the common country of origin implicated in both the South African [2] and US outbreaks (1978-2010) [93]. Although a small sample size (n=2), the same novel *T. asinigenitalis* ST was identified from both miniature donkey stallions diagnosed during post-arrival quarantine and most closely matched a strain isolated from a male horse in Sweden [72]. The donkey stallions were imported from the US in 2015 and a link has previously been established between *T. asinigenitalis* strains from mainland Europe and the US [74]. Application of MLST has standardized epidemiological analysis of *Taylorellae* species isolates and eliminated taxonomic discord due to multiple analyses using differing techniques [59; 93; 125; 127-132; 134]. Multilocus sequence typing has also provided an unambiguous and robust tool to investigate the genetic relationships between samples from different laboratories [137; 162].

Decreasing costs, optimising reproducibility and the portability of results have allowed next-generation sequencing technologies to standardize the assembly of bacterial genomes [163] and enhance straightforward inter-laboratory comparison of results [164]. In the past, various molecular typing tools including PFGE [59; 93; 125], FIGE [132], CIGE [124; 126-130] and chromosomal DNA fingerprinting were used to genotype *Taylorella* isolates. However, comparison of inter-laboratory results using these methods is complicated, making them unsuitable for global epidemiological studies [10]. Recently, MLST has been used to determine the spatio-temporal distribution of specific *T. equigenitalis* genotypes from internationally isolated strains [10] enabling an overview of the reported CEM outbreaks worldwide. Whole genome sequencing has also been used to characterize the genetic diversity of *T. equigenitalis* isolates within specific countries [12]. However, the use of whole genome sequencing to determine epidemiological links and wider disease context, as frequently applied to various other agents [165], has not yet been applied to *Taylorella* spp. In order to elucidate the relationship between the South African isolates and global strains, publicly available SRA data on the NCBI database from the genomes of 32 ST4 and an original type strain (ST1), were accessed and submitted to the Comprehensive Genome Analysis service in PATRIC, as described in Chapter 3 (section 3.1.5). The phylogenetic analysis of these isolates demonstrated a distinct separation between the ST1 and the ST4 clades, with ST4 strains having a limited distribution, being reported in only four countries; Germany, Austria, the US and South Africa as shown in Table 4-5. These data also supported Austrian Lipizzaners as the common breed and country of origin for the outbreaks in both the US [93] and South Africa (shown in Figure 4-1).

The application of newer molecular technologies such as qPCR, MLST and online software for genomic analysis facilitated the epidemiological investigation into the origin and circumstances of the South African outbreak of *T. equigenitalis*. Publicly available repositories of genetic data such as the NCBI platform can be used for genome assembly and phylogenetic analysis similar to that performed on the ST4 isolates in this study and the phylogenetic analysis applied to epidemiological investigations in order to determine global links between isolates. The presence of a single sequence type, ST4 in South Africa documented over a 24-year interval (1996-2020) warrants additional phylogenetic and genomic analysis to investigate potential evolutionary changes in the genome of *T. equigenitalis* that may have occurred over this period. Reportedly, albeit anecdotally, there appears to have been a change in the severity of the disease from when it was first

described, with more recent outbreaks showing milder clinical signs. Further elucidation of the genetic basis for virulence factors may explain whether these differences are due to attenuation of the organism itself or whether the severity of the disease process depends on distinct sequence types.

The majority of *T. equigenitalis*-positive horses (n=42) identified during the South African CEM outbreak were Lipizzaners (n=25). These were introduced into South Africa via two main importation events in 1948 and 1956, respectively. South Africa remains an important breed nucleus and is home to the only population of performing Lipizzaner horses outside Austria. Uniquely, all training and performances take place at the SALC where the Lipizzaner stallions are resident while mares and young stock are kept at a separate satellite facility approximately 40 km away where all breeding-related procedures occur. A limited number of selected stallions are used for the SALC's breeding programme, mainly by means of natural cover. The Lipizzaners had previously been maintained a closed herd following their introduction into South Africa in 1948 until 2001. From 2001-2006, several SALC-resident stallions had visited the index property for purposes of semen collection for breeding mares by AI. It was only during the national stallion screening programme of resident stallions (n=35) at the SALC that 23 stallions and one gelding were identified as positive for *T. equigenitalis.* None of the Lipizzaner mares or young stock resident on a separate property were tested positive for *T. equigenitalis.* We proposed that the organism had persisted and circulated undetected for a number of years at the SALC by means of horizontal transmission via fomites. The index property was contaminated with the organism subsequent to the Lipizzaner stallions' visits for semen collection procedures at some timepoint from 2001-2006. Candidate fomites included the breeding phantom, equipment or personnel used during semen collection and handling or tack and grooming equipment [2; 39; 91; 94; 97]. As a consequence, various other stallions that visited the index property became colonized by horizontal fomite transmission and *T. equigenitalis* was established for the first time outside the Lipizzaner population (see Figure 4-2). Interestingly, a similar situation had occurred in 2006 in Austria at the Spanish Riding School in Vienna where a similar proportion of 48/68 (71%) of Lipizzaner stallions tested positive for *T. equigenitalis* by proposed horizontal fomite transmission. At the Federal Stud in Piber, 22/50 colts, five geldings, eight non-breeding mares and 2/50 brood mares also tested positive using a combination of PCR and culture. Most mares in Piber are bred by AI and no clinical signs of disease were observed in these breeding mares [61].

The precise date that *T. equigenitalis* was introduced into South Africa is currently unknown. Pre-export and post-arrival quarantine testing for *T. equigenitalis* was instituted in South Africa in 1978, shortly after the first CEM outbreak was reported in the United Kingdom [48]. As part of the 2011 South African epidemiological traceback following CEM outbreak identification, *T. equigenitalis* was isolated from semen samples collected from five Lipizzaner stallions in 1996 during the investigation of an equine viral arteritis outbreak [148]. The whole genome sequencing and subsequent MLST strain typing of one of these archived samples [2], strongly suggested a point introduction into South Africa prior to 1996, possibly even before importation testing for *T. equigenitalis* was legislated. Lipizzaner stallions have been imported into South Africa from Europe on several occasions between 1948-1996, which raised the possibility that *T. equigenitalis* may even have entered prior to global disease recognition in 1977 [46-48].

A highlight of both the South African *T. equigenitalis* outbreak and the *T. asinigenitalis* incursion was the importance of fomite transmission [39]. Most recent outbreaks reported in countries considered non-endemic for *T. equigenitalis* have been due to a combination of natural and assisted breeding and fomite transmission [39]. In the US: natural mating, AI and fomites [91]; UK: AI and fomites [90] and South Korea: natural mating and fomites [66].

During the South African outbreak, there were only two confirmed cases of venereal transmission associated with AI of contaminated semen from known positive stallions (See Figure 4-2). The total number of affected mares (n=3) identified during the 2011 South African outbreak may arguably have been significantly higher [2], however the actual numbers will remain undefined due to both the mare's known ability to clear herself of infection [5; 79; 96; 166] and the time interval between suspect exposure and commencement of the epidemiological traceback. Sampling in mares is complicated by difficulty in accessing the clitoral sinuses which require paediatric swabs in order to take a representative sample. Sampling technique may therefore indirectly affect the sensitivity of the assay. The third mare, a filly, was the offspring of a *T. equigenitalis*-positive stallion. Her dam was negative for *T. equigenitalis* at the time of testing, but it was presumed that she was initially acutely infected and the filly was subsequently infected by either vertical or horizontal transmission prior to the dam clearing herself of infection. The distribution of *T. equigenitalis* across the predilection sites of acutely (n=1) and chronically (n=2) affected

mares during the 2011 South African outbreak is shown in Table 4-3. Although the sample population was small (n=3), our findings substantiated reports of the organism being present in the endometrium during acute infections and in the clitoral fossa and sinuses in the chronic carrier state [98] and support sampling of all three predilection sites in mares.

From all 42 *T.* equigenitalis-positive horses, a single predilection site was positive on qPCR in only four cases (three males and one female). In these horses, these were the urethral fossa (n=2), the lamina interna (n=1) and the clitoral fossa (n=1). This finding strongly supported sampling of all the predilection sites [2; 91] in both females and males. Our findings showed that the most commonly affected site in male horses was the urethral fossa, followed by the urethra and then the lamina interna. Real-time PCR results in male horses showed that the amount of nucleic acid in the urethral fossa was significantly higher than in other sites [167], followed by the urethra and then the lamina interna.

Since its inception in 2011, the national stallion screening programme has proved invaluable in providing surveillance of the national herd and has significantly aided the efforts to re-establish South Africa's CEM-free status. Compliance with the national screening programme has been enhanced by the practicality and low costs associated with the qPCR assay [2; 102]. To the author's knowledge, this is the first comprehensive and nationally legislated programme instituted globally to monitor CEM. It is interesting to note the absence of regulated mandatory venereal disease screening and biosecurity protocols by some leading Thoroughbred-producing nations including the US, Australia and Argentina. The reasons for this are unclear given the devastating detrimental impact a *T. equigenitalis* outbreak would have in this sector. Although instrumental in eradicating CEM from the British Thoroughbred sector, the comprehensive HBLB international code of practice for breeders only provides voluntary recommendations for the prevention and control of specific diseases, relying on the integrity and honesty of responsible livestock keepers. A novel approach to addressing a treatable notifiable disease was implemented in 2018 by the UK Department for Environment, Food and Rural Affairs (DEFRA) which resulted in shared responsibility for the control of CEM between the government and the equine industry [168].

Our experience to date has informed both national legislation and international equine stud health practices [104] and continues to support the targeted surveillance of stallions and exposed mares. The decision to use qPCR as a screening test was based, amongst others, on

experience of prolonged turnaround times and lower sensitivity of bacterial culture when compared to qPCR. Revised legislation to include a duplex qPCR into the South African post-quarantine testing regimen for imported equids led to the identification of two *T. asinigenitalis*-positive miniature donkey stallions. To date, 42 equines have been identified positive for *T. equigenitalis* by qPCR screening with 31 being confirmed positive using bacterial culture (see Table 4-1 and Table 4-2) [2]. The main discrepancy occurred amongst Lipizzaners at the SALC, where 10/24 (41.6%) tested positive on qPCR alone. Possible reasons for this discrepancy were twofold; based on the history, the risk of exposure to these horses was considered high and to expedite resolution of the outbreak, treatment was initiated shortly after the animals were shown to be positive on qPCR and prior to the completion of the bacterial cultures. It has been shown that multiple sampling attempts are often required in order to identify *T. equigenitalis* [39]. Furthermore, initiation of treatment precluded the collection of further samples for bacterial culture. While these practical limitations could be partially responsible for the difference in detection rate between qPCR and bacterial culture, it has been reported that the sensitivity of qPCR exceeds that of bacterial culture [56; 115; 118; 169].

Interestingly at the SALC, there was no statistically significant difference in treatment periods between horses that were *T. equigenitalis*-positive on qPCR alone (n=10) and horses that were *T. equigenitalis*-positive on both qPCR and bacterial culture (n=13).

Regulatory reliance on bacterial culture therefore requires additional safeguards such as test-mating and subsequent culture of samples obtained from test-mares to supplement diagnosis of *T. equigenitalis* [6]. This reliance is arguably questionable with the advent of significantly more sensitive and practical molecular methods [4; 56; 115; 118; 169]. Quantitative PCR provided a robust, highly sensitive assay with a rapid turnaround time that could be used on large numbers of samples at a reasonable cost, making it uniquely suited for use in both the epidemiological traceback during the South African outbreak and the subsequent national stallion screening programme. In addition, qPCR provided a sensitive method to test cryopreserved semen samples, which resulted in the identification of an additional *T. equigenitalis*-positive stallion during the South African outbreak as described in Chapter 3 (section 3.2). The threat posed by cryopreserved semen is currently largely undefined [39; 89]. Viable *T. equigenitalis* organisms capable of causing clinical disease are reported to persist in cryopreserved semen samples despite the inclusion of various

antibiotics such as amphotericin, gentamicin and penicillin [89] and have recently been shown to cause clinical endometritis following AI with *T. equigenitalis*-contaminated semen [89]. Therefore, qPCR has important future application in preventing the introduction of *T. equigenitalis* into non-endemic countries by the international movement of cryopreserved gametes.

The second objective of this study was to develop an efficacious treatment protocol for *T. equigenitalis.* Our findings showed that the recommended international treatment protocols [5] provided limited efficiency in eliminating the *Taylorellae* spp. Although a small sample, a prescribed five-day treatment protocol [6] failed to eliminate *T. equigenitalis* from five positive stallions and an extended nine-day programme only cleared 5/10 (50%) stallions. The institution of daily in-treatment qPCR monitoring allowed treatment duration to be tailored to individual stallions, although treatment duration was still prolonged (range 8- 51 days, see Table 4-6 and Table 4-7) [5]. The reasons for this variable and commonly prolonged interval are unclear, although the SALC was identified as a long-standing nidus of infection which may have affected the duration of treatment required for individual stallions.

As described in Chapter 3 (section 3.3), the experience of these protracted treatment periods while treating the Lipizzaner stallions [105] prompted the introduction of a bovine topical intramammary antimicrobial formulation applied topically to the clitoral or urethral fossae and sinuses during treatment of subsequent *T. equigenitalis*-positive horses [7]. The fine-bore intra-mammary applicator was able to reach these otherwise-elusive anatomical predilection sites and we speculated that the 'dry cow' formulation provided prolonged mucosal contact time. Although a small sample size, the introduction of the dry cow intramammary preparation did appear to shorten the interval to elimination of the organism (see Table 4-8) [7]. The use of the in-treatment qPCR and a topically applied bovine intramammary antimicrobial preparation requires further studies on a larger cohort of animals to determine treatment efficacy and may provide a valuable alternative to current recommended protocols. Preliminary data using this novel treatment regimen showed its practicality in both successfully decreasing the time to elimination of the micro-organism and eliminating the need for repeat treatments. As described in Chapter 3 (section 3.3), two *T. asinigenitalis*-positive donkey stallions were also treated according to this modified treatment regimen which resulted in rapid elimination of colonization compared to other

reports (see Table 4-4) [70; 73]. Since the introduction of the combination of in-treatment monitoring and the modified treatment regimen, no repeat treatments (as were previously frequently observed) have been required for elimination of *T. equigenitalis* or *T. asinigenitalis* in South African equids.

The National Horseracing Authority implemented a mandatory stud health scheme for all Thoroughbred breeding stock [170] in South Africa in 1978, with a specific focus on CEM and dourine. In 2019, a pilot scheme was introduced to include swabbing for *Pseudomonas aeruginosa* and *Klebsiella pneumoniae,* both considered to be venereally transmitted pathogens responsible for causing endometritis, the primary cause of infertility in mares [40]. An interesting corollary of the treatment of 23 Lipizzaners at the SALC was the effect of antimicrobials on the genital microflora. Unsurprisingly, 39% of treated stallions showed alterations of the microbial biome of the genitalia post treatment and cultured positive for either *P. aeruginosa* and, or *K. pneumoniae* on genital swabs taken one month post-treatment endpoint as seen in Table 4-9. Interestingly, 78% of these stallions had cleared themselves of both pathogens by the second month post-treatment and all had cleared themselves by four months post-treatment without additional interventions, bringing into question the associated carrier state, or at least the duration of its persistence. Of even greater interest, 44% of nine untreated stallions swabbed positive for *K. pneumoniae* one year later, with only one of those stallions having been used for breeding purposes during that time. Although a small sample size, these findings warrant further investigation of the relevance of applying extensive population surveillance exercises for these two pathogens.

Implementation of the national stallion screening programme has allowed ongoing surveillance of the national herd and provides continuing epidemiological assurance of South Africa's CEM-free status. Globally, this was the first legislated CEM programme of its kind to be implemented on such an extensive scale. The incorporation of a duplex qPCR and multiple sample opportunities to post arrival quarantine testing of equids has provided an effective barrier to the reintroduction of *T. equigenitalis* or the introduction of *T. asinigenitalis* into South Africa. Post-arrival quarantine testing for *T. equigenitalis* was introduced in 1978 and this control point's findings over the decades combined more recently with approximately a decade's annual surveillance data derived from all breeding stallions within the country, has supported no further observed incursions into South Africa.

Although bacterial culture is the current OIE gold standard method [101] for identification of *T. equigenitalis*, this method is laborious and does not permit differentiation from *T. asinigenitalis.* Further investigation and possible validation of a duplex qPCR assay for international movement purposes will alleviate reliance on bacterial culture with its associated limitations. Molecular diagnostic methods such as qPCR confer significant advantages in terms of practicality, capacity and turnaround times and have demonstrated superior sensitivity and specificity compared to traditional bacterial culture.

The *T. asinigenitalis*-positive donkey stallion identified during post-arrival quarantine in South Africa had been tested prior to export using bacterial culture and was only identified on the third of three sampling opportunities. Regulatory reliance solely on bacterial culture appears injudicious and due to the currently undefined risk posed by *T. asinigenitalis* and the potential for misidentification as *T. equigenitalis,* there is a need to validate a diagnostic duplex qPCR test that is reliable, robust and readily able to distinguish *T. asinigenitalis* from *T. equigenitalis.* There are limited reports available on *T. asinigenitalis* and additional studies are indicated to more clearly define both its associated disease risk in horses and its effect on equine reproductive performance. These findings also support multiple sampling opportunities for the identification of *Taylorella* spp.

Chapter 6
Conclusions

From these studies the following conclusions could be made:

- The index case identified during the 2011 South African outbreak was not the source of infection.
- *Taylorella equigenitalis* was introduced into South Africa as a point introduction prior to 1996 and had been present but undetected in the equine population prior to outbreak identification in 2011.
- qPCR provides a robust, highly sensitive test that can be used on large number of samples and is also useful in screening cryopreserved semen.
- The introduction of a duplex qPCR during post-importation screening proved invaluable in detecting a case of *T. asinigenitalis* in an imported miniature donkey.
- Targeted surveillance of stallions and exposed mares should be performed during epidemiological screening.
- Multiple sampling opportunities increased test sensitivity.
- MLST can be used on crude extracts directly from clinical samples which has important implications for diagnostic and epidemiological testing in future outbreaks.
- Newer molecular technologies such as MLST and whole genome sequencing have standardized epidemiological analysis of *Taylorellae* isolates.
- Online tools for genomic analysis such as PATRIC (https://www.patricbrc.org) facilitated investigation of the origin and evolution of the South African *T. equigenitalis* strain, ST4.
- The common ancestor of all South African isolates was a 1992 US importation from Austria (USDA_92_0920).
- The *T. equigenitalis* strain, ST4, identified in South Africa was most likely introduced by importation of Lipizzaner horses from Austria.
- There have been multiple incursions of ST4 from Europe identified in post-importation quarantine in the US.
- The strains (n=9) isolated in the US in 2013 are linked with a clade of Austrian strains (n=5).
- Fomite transmission is an important source of infection for both *Taylorellae* spp.
- A modified treatment regimen including the use of in-treatment qPCR monitoring and a topical intramammary antimicrobial formulation hastened treatment endpoint.

References

[1] World Organisation for Animal Health. (2011) Contagious equine metritis: South Africa, Immediate notification (09/05/2011). https://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?reportid=10553. Accessed 1 March 2020.

[2] May, C.E., Guthrie, A.J., Keys, B., Joone, C., Monyai, M. and Schulman, M.L. (2015) Polymerase chain reaction-based national surveillance programme to determine the distribution and prevalence of *Taylorella equigenitalis* in South African horses. *Equine Vet. J.* **48**, 307-311. https://doi.org/10.1111/evj.12439

[3] Hébert, L., Moumen, B., Duquesne, F., Breuil, M.-F., Laugier, C., Batto, J.-M., Renault, P. and Petry, S. (2011) Genome sequence of *Taylorella equigenitalis* MCE9, the causative agent of contagious equine metritis. *J. Bacteriol.*, 1785. DOI: 10.1128/JB.01547-10

[4] Duquesne, F., Hébert, L., Breuil, M.-F., Matsuda, M., Laugier, C. and Petry, S. (2013) Development of a single multi-locus sequence typing scheme for *Taylorella equigenitalis* and *Taylorella asinigenitalis*. *Vet. Microbiol.* **167**, 609-618. https://doi.org/10.1016/j.vetmic.2013.09.016

[5] Luddy, S. and Kutzler, M. (2010) Contagious equine metritis within the United States: a review of the 2008 outbreak. *J. Equine Vet. Sci.* **30**, 393-400. https://doi.org/10.1016/j.jevs.2010.07.006

[6] Kristula, M.A. and Smith, B.I. (2004) Diagnosis and treatment of four stallions, carriers of the contagious metritis organism-case report. *Theriogenology* **61**, 595-601. https://doi.org/10.1016/S0093-691X(03)00228-0

[7] May, C., Guthrie, A. and Schulman, M. (2016) Addition of an intramammary antimicrobial formulation markedly decreased the intervals to elimination of *Taylorella equigenitalis* in a carrier mare and gelding. *J. Equine Vet. Sci.*, S62. 10.1016/j.jevs.2016.02.134

[8] Directorate Animal Health: Department of Agriculture, Forestry and Fisheries. Veterinary procedural notice: Surveillance for contagious equine metritis in South Africa (2019). https://www.daff.gov.za/daffweb3/Branches/Agricultural-Production-Health-Food-Safety/Animal-Health/information/dahpolicy. Accessed August 2019.

[9] Directorate Animal Health: Department of Agriculture, Forestry and Fisheries. Veterinary Procedural Notice: The confirmation of diagnosis and treatment of contagious equine metritis (2019). https://www.daff.gov.za/daffweb3/Branches/Agricultural-Production-Health-Food-Safety/Animal-Health/information/dahpolicy. Accessed August 2019.

[10] Duquesne, F., Merlin, A., Pérez-Cobo, I., Sedlák, K., Melzer, F., Overesch, G., Fretin, D., Iwaniak, W., Breuil, M.-F. and Wernery, U. (2020) Overview of spatio-temporal distribution inferred by multi-locus sequence typing of *Taylorella equigenitalis* isolated worldwide from 1977 to 2018 in equidae. *Vet. Microbiol.* **242**, 108597. https://doi.org/10.1016/j.vetmic.2020.108597

[11] Hébert, L., Touzain, F., de Boisséson, C., Breuil, M.-F., Duquesne, F., Laugier, C., Blanchard, Y. and Petry, S. (2014) Draft genome sequence of *Taylorella equigenitalis* strain MCE529, isolated from a Belgian Warmblood horse. *Genome Announc.* **2**, e01214-01214. 10.1128/genomeA.01214-14

[12] Hicks, J., Stuber, T., Lantz, K., Erdman, M., Robbe-Austerman, S. and Huang, X. (2018) Genomic diversity of *Taylorella equigenitalis* introduced into the United States from 1978 to 2012. *PLoS One* **13**, e0194253. 10.1371/journal.pone.0194253

[13] Sluyter, F. (2001) Traceability of Equidae: a population in motion. *Revue scientifique et technique-OIE.* **20**, 500-509.

[14] Herholz, C., Fussel, A.E., Timoney, P., Schwermer, H., Bruckner, L. and Leadon, D. (2008) Equine travellers to the Olympic Games in Hong Kong 2008: a review of worldwide challenges to equine health, with particular reference to vector-borne diseases. *Equine Vet. J.* **40**, 87-95.

[15] Timoney, P.J. (2014) Infectious diseases and international movement of horses. In: *Equine Infectious Diseases*, Ed: D.C.a.L. Sellon, M.T, Elsevier. pp 544-551.

[16] American Horse Council Foundation. Economic impact study of the US horse industry (2017). https://www.equinebusinessassociation.com/2017-economic-impact-study-u-s-horse-industry/. Accessed January, 2021.

[17] The Thoroughbred Breeders' Association. (2018) The contribution of thoroughbred breeding to the UK economy and factors impacting the industry's supply chain. https://www.thetba.co.uk/wp-content/uploads/2018/09/TBA-Economic-Impact-Study-2018.pdf. Accessed January, 2021.

[18] British equestrian trade association. National equestrian survey 2019 (2019). https://www.beta-uk.org/pages/industry-information/market-information.php. Accessed January, 2021.

[19] HM Revenue and Customs. Overseas trade data table (2021). https://www.uktradeinfo.com/trade-data/ots-custom-table/. Accessed February, 2021.

[20] CNN Sports. World's richest horse races: The top seven prize pots in racing https://edition.cnn.com/2018/12/19/sport/winning-post-breeders-cup-dubai-world-cup-pegasus-japan-cup-prix-de-larc-de-triomphe-spt-intl/index.html. Accessed January, 2021.

[21] International Federation for Equestrian Sports. (2021) FEI events 2009-2018. https://inside.fei.org/fei/about-fei/publications/fei-annual-report/2018/feifactsandstats/. Accessed January, 2021.

[22] Unt, V. (2014) Science-in-brief: Sports Forum-international movement of horses. *Equine Vet. J.* **46**, 262-263.

[23] World Organisation for Animal Health. High health, high performance (HHP) horses (2021). https://www.oie.int/scientific-expertise/specific-information-and-recommendations/international-competition-horse-movement/high-health-high-performance-hhp-horses/. Accessed January, 2021.

[24] World Organisation for Animal Health. Chapter 4.17. High health status horse subpopulation (2017). https://www.oie.int/index.php?id=169&L=0&htmfile=chapitre_high_level.htm. Accessed January, 2021.

[25] World Organisation for Animal Health. Handbook for the management of high health, high performance horses (2016). https://www.oie.int/fileadmin/Home/eng/Our_scientific_expertise/docs/pdf/Chevaux/HHP_Handbook_December_2016_V3.pdf. Accessed January, 2021.

[26] TRACES (2021). https://ec.europa.eu/food/animals/traces_en. Accessed January, 2021.

[27] Slater, J. (2013) Ensuring equine biosecurity at London 2012. *Vet. Rec.* **172**, 117-119.

[28] International Federation of Horseracing Authorities. Annual reports (2021). https://www.ifhaonline.org/Default.asp?section=Resources&area=2. Accessed January, 2021.

[29] Rosanowski, S., Cogger, N. and Rogers, C. (2013) An investigation of the movement patterns and biosecurity practices on Thoroughbred and Standardbred stud farms in New Zealand. *Prev. Vet. Med.* **108**, 178-187.

[30] Digby, J. (1996) The likely impact of dual hemisphere serving (shuttle) stallions on the stallion population and on covering patterns in Australia. *Aust. Vet. J.* **1996**, 109-111.

[31] Lane, E., Bijnen, M., Osborne, M., More, S., Henderson, I., Duffy, P. and Crowe, M. (2016) Key factors affecting reproductive success of thoroughbred mares and stallions on a commercial stud farm. *Reprod. Domest. Anim.* **51**, 181-187.

[32] Watson, J., Daniels, P., Kirkland, P., Carroll, A. and Jeggo, M. (2011) The 2007 outbreak of equine influenza in Australia: lessons learned for international trade in horses. *Revue Scientifique et Technique-OIE* **30**, 87.

[33] Rogers, C.W., Gee, E.K., Bolwell, C.F. and Rosanowski, S.M. (2020) Commercial equine production in New Zealand. 2. Growth and development of the equine athlete. *Anim. Prod. Sci.* **60**, 2155-2163.

[34] Timoney, P.J. (2000) Factors influencing the international spread of equine diseases. *Vet. Clin. North Am. Equine Pract.* **16**, 537-551.

[35] World organisation for animal health. Terrestrial Animal Health Code (2019). https://www.oie.int/en/standard-setting/terrestrial-code/access-online/. Accessed January, 2021.

[36] European Union. Live animals – trade and imports (Equine animals) (2021). https://ec.europa.eu/food/animals/live_animals/equine_en. Accessed January, 2021.

[37] Dominguez, M., Münstermann, S., Guindos, I. and Timoney, P. (2015) Equine disease events resulting from international horse movements: Systematic review and lessons learned. *Equine Vet. J.* **48,** 641-653. https://doi.org/10.1111/evj.12523

[38] Schulman, M.L., May, C.E., Keys, B. and Guthrie, A.J. (2013) Contagious equine metritis: artificial reproduction changes the epidemiologic paradigm. *Vet. Microbiol.* **167**, 2-8. https://doi.org/10.1016/j.vetmic.2012.12.021

[39] Tibary, A., Pearson, L.K. and Cheryl L. Fite (2014) Chapter 8 - Reproductive Tract Infections. In: *Equine Infectious Diseases (Second Edition)*, Ed: M.T.L. Debra C. Sellon. pp 84-106.

[40] World Organisation for Animal Health. OIE-Listed diseases, infections and infestations in force in 2020 (2021). https://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2021/. Accessed January, 2021.

[41] Horserace betting levies board. International codes of practice 2021 (2021). https://codes.hblb.org.uk/. Accessed January, 2021.

[42] United States Department of Agriculture, Animal and Plant Health Inspection Service. Equine Information https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/animal-disease-information/equine. Accessed January, 2021.

[43] Taylor, C., Rosenthal, R., Brown, D., Lapage, S., Hill, L. and Legros, R. (1978) The causative organism of contagious equine metritis 1977: proposal for a new species to be known as *Haemophilus equigenitalis*. *Equine Vet. J.* **10**, 136-144.

[44] Sugimoto, C., Isayama, Y., Sakazaki, R. and Kuramochi, S. (1983) Transfer of H*aemophilus equigenitalis* Taylor et al. 1978 to the genus *Taylorella* gen. nov. as *Taylorella equigenitalis* comb. nov. *Curr. Microbiol.* **9**, 155-162. https://doi.org/10.1007/BF01567289

[45] Crowhurst, R. (1977) Genital infection in mares. *Vet. Rec.* **100**, 476-476.

[46] Platt, H., Atherton, J., Simpson, D., Taylor, C., Rosenthal, R., Brown, D. and Wreghitt, T. (1977) Genital infection in mares. *Vet. Rec.* **101**, 20-20. http://dx.doi.org/10.1136/vr.101.1.20

[47] Timoney, P.J., Ward, J. and Kelly, P. (1977) A contagious genital infection of mares. *Vet. Rec.* **101**, 103. http://dx.doi.org/10.1136/vr.101.5.103-a

[48] Greenwood, R. and Allen, W. (2020) Memories of contagious equine metritis 1977 in Newmarket. *Equine Vet. J.* **52**, 344-346.

[49] Eaglesome, M. and Garcia, M. (1979) Contagious equine metritis: a review. *Can. Vet. J.* **20**, 201.

[50] Swerczek, T. (1978) Contagious equine metritis in the USA. *Vet. Rec.* **102**, 512-513.

[51] Holden, C. (1978) Outbreak of equine VD stirs fear in Kentucky *Science* **200**, 181-185. 10.1126/science.200.4338.181

[52] Blobel, H., Bruckler, J., Kitzrow, D. and Blobel, K. (1980) Contribution to the knowledge of Contagious Equine Metritis in the Federal Republic of Germany. *Comp. Immunol. Microbiol. Infect. Dis.* **2**, 551-554. https://doi.org/10.1016/0147-9571(79)90096-1

[53] Breuil, M., Duquesne, F., Leperchois, E., Laugier, C., Ferry, B., Collin, G. and Petry, S. (2015) Contagious equine metritis cases reported in France since 2006. *Vet. Rec.* **177**, 340-340. http://dx.doi.org/10.1136/vr.103349

[54] Rocha, T. (2016) Contagious equine metritis in Portugal: A retrospective report of the first outbreak in the country and recent contagious equine metritis test results. *Open Vet. J.* **6**, 263-267. 10.4314/ovj.v6i3.18

[55] Zdovc, I., Ocepek, M., Gruntar, I., Pate, M., Klobučar, I. and Krt, B. (2005) Prevalence of *Taylorella equigenitalis* infection in stallions in Slovenia: bacteriology compared with PCR examination. *Equine Vet. J.* **37**, 217-221. https://doi.org/10.2746/0425164054530696

[56] Parlevliet, J., Bleumink-Pluym, N., Houwers, D., Remmen, J., Sluijter, F. and Colenbrander, B. (1997) Epidemiologic aspects of *Taylorella equigenitalis*. *Theriogenology* **47**, 1169-1177. https://doi.org/10.1016/S0093-691X(97)00097-6

[57] Stritof, Z., Habus, J., Perko, V.M., Zdovc, I., Majhut, M., Lucic, K. and Turk, N. (2016) First report of *Taylorella equigenitalis* and *Taylorella asinigenitalis* natural infections in horses in Croatia. *J. Equine Vet. Sci.* **39**, S63. 10.1016/j.jevs.2016.02.136

[58] Sting, R., Seeh, C., Mauder, N., Maurer, M., Loncaric, I., Stessl, B., Kopp, P., Banzhaf, K., Martin, B. and Melzer, F. (2016) Genotyping of German and Austrian *Taylorella equigenitalis* isolates using repetitive extragenic palindromic (REP) PCR and pulsed-field gel electrophoresis (PFGE). *Res. Vet. Sci.* **109**, 101-106. https://doi.org/10.1016/j.rvsc.2016.09.017

[59] World Organisation for Animal Health. (2019) Contagious equine metritis: Denmark, Immediate notfication (11/07/2019). https://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?page_refer=MapFullEventReport&reportid=31059. Accessed 3 March 2020.

[60] Roest, H., Heath, J., Dawson, D., Markey, G., Dijkstra, Y., Engelsma, M. and van Zijderveld, F. (2008) Summary of the first international conference on contagious equine metritis. *Tijdschr. Diergeneeskd.* **132**, 936-938.

[61] Hughes, K., Bryden, J. and MacDonald, F. (1978) Equine contagious metritis. *Aust. Vet. J.* **54**, 101. https://doi.org/10.1111/j.1751-0813.1978.tb00365.x

[62] Sugimoto, C., Isayama, Y., Kashiwazaki, M., Fujikura, T. and Mitani, K. (1980) Detection of *Haemophilus equigenitalis*, the causal agent of contagious equine metritis, in Japan. *Natl. Inst. Anim. Health Q. (Tokyo)* **20**, 118-119.

[63] May, C., Schulman, M., Gerstenberg, C., Grobler, A., Mphele, A. and Guthrie, A. (2012) Confirmation of the first outbreak of contagious equine metritis in South Africa. *J. Equine Vet. Sci.* **32**, S77. 10.1016/j.jevs.2012.08.164

[64] Pourmahdi Borujeni, M., Gharibi, D., Gooraninejad, S. and Memari, S. (2014) Detection of *Taylorella equigenitalis* in Arabian horse in Khuzestan province by culture and PCR. Food and Agriculture Organisation of the United Nations. https://agris.fao.org/agris-search/search.do?recordID=IR2015002390. Accessed January 2021.

[65] Jeoung, H.-Y., Lee, K.-E., Yang, S.-J., Park, T., Lee, S.K., Lee, J.-H., Kim, S.-H., Kim, B., Kim, Y.-J. and Park, J.-Y. (2016) First isolation of *Taylorella equigenitalis* from thoroughbred horses in South Korea. *J. Equine Vet. Sci.* **47**, 42-46. https://doi.org/10.1016/j.jevs.2016.07.022.

[66] Jang, S.S., Donahue, J.M., Arata, A.B., Goris, J., Hansen, L.M., Earley, D.L., Vandamme, P., Timoney, P.J. and Hirsh, D.C. (2001) *Taylorella asinigenitalis* sp. nov., a bacterium isolated from the genital tract of male donkeys (*Equus asinus*). *Int. J. Syst. Evol. Microbiol.* **51**, 971-976. https://doi.org/10.1099/00207713-51-3-971

[67] Katz, J.B., Evans, L.E., Hutto, D.L., Schroeder-Tucker, L.C., Carew, A.M., Donahue, J.M. and Hirsh, D.C. (2000) Clinical, bacteriologic, serologic, and pathologic features of infections with atypical *Taylorella equigenitalis* in mares. *J. Am. Vet. Med. Assoc.* **216**, 1945-1948. https://doi.org/10.2460/javma.2000.216.1945

[68] Donahue, J.M., Timoney, P.J., Carleton, C.L., Marteniuk, J.V., Sells, S.F. and Meade, B.J. (2012) Prevalence and persistence of *Taylorella asinigenitalis* in male donkeys. *Vet. Microbiol.* **160**, 435-442. https://doi.org/10.1016/j.vetmic.2012.06.016

[69] Meade, B., Timoney, P., Donahue, J., Branscum, A., Ford, R. and Rowe, R. (2010) Initial occurrence of *Taylorella asinigenitalis* and its detection in nurse mares, a stallion and donkeys in Kentucky. *Prev. Vet. Med.* **95**, 292-296. https://doi.org/10.1016/j.prevetmed.2010.04.010

[70] Reilly, T.J., Calcutt, M.J., Ganjam, I.K., Erdman, M.M., Aalsburg, A.M., Blyden, J.F. and Fales, W.H. (2012) Isolation and identification of *Taylorella asinigenitalis* from a mare in Oklahoma, USA. *Clinical Theriogenology.* **4**, 163-167.

[71] Båverud, V., Nyström, C. and Johansson, K.-E. (2006) Isolation and identification of *Taylorella asinigenitalis* from the genital tract of a stallion, first case of a natural infection. *Vet. Microbiol.* **116**, 294-300. https://doi.org/10.1016/j.vetmic.2006.04.027

[72] Franco, A., Donati, V., Lorenzetti, R., Zini, M., Autorino, G., Battisti, A., Troiano, P., Petrella, A. and Maggi, A. (2009) Detection of *Taylorella asinigenitalis* in donkey jacks in Italy. *Vet. Rec.* **165**, 540-541. http://dx.doi.org/10.1136/vr.165.18.540

[73] Breuil, M.-F., Duquesne, F., Laugier, C. and Petry, S. (2011) Phenotypic and 16S ribosomal RNA gene diversity of *Taylorella asinigenitalis* strains isolated between 1995 and 2008. *Vet. Microbiol.* **148**, 260-266. https://doi.org/10.1016/j.vetmic.2010.08.028

[74] Mawhinney, I., Florea, L., Torrens, N., Thurston, L. and Poynter, R. (2018) *Taylorella asinigenitalis* in an onager in a UK zoo. *Vet. Rec.* **183**, 266. DOI:10.1136/vr.k3687

[75] Bleumink-Pluym, N.M., van Dijk, L., van Vliet, A.H., van der Giessen, J.W. and van der Zeijst, B.A. (1993) Phylogenetic position of *Taylorella equigenitalis* determined by analysis of amplified 16S ribosomal DNA sequences. *Int. J. Syst. Bacteriol.* **43**, 618-621. https://doi.org/10.1099/00207713-43-3-618

[76] Matsuda, M., Tazumi, A., Kagawa, S., Sekizuka, T., Murayama, O., Moore, J.E. and Millar, B.C. (2006) Homogeneity of the 16S rDNA sequence among geographically disparate isolates of *Taylorella equigenitalis*. *BMC Vet. Res.* **2**, 1. https://doi.org/10.1186/1746-6148-2-1

[77] Ricketts, S., Rossdale, P. and SAMUEL, C.A. (1978) Endometrial biopsy studies of mares with contagious equine metritis 1977. *Equine Vet. J.* **10**, 160-166. https://doi.org/10.1111/j.2042-3306.1978.tb02247.x

[78] Timoney, P. (2011) Horse species symposium: contagious equine metritis: an insidious threat to the horse breeding industry in the United States. *J. Anim. Sci.* **89**, 1552-1560. https://doi.org/10.2527/jas.2010-3368

[79] Taylor, C. and Rosenthal, R. (1978) Agglutinins to the causative organism of contagious equine metritis 1977, in human serum. *The Lancet*. 1038.

[80] Bleumink-Pluym, N., Werdler, M., Houwers, D.J., Parlevliet, J.M., Colenbrander, B. and Van der Zeijst, B. (1994) Development and evaluation of PCR test for detection of *Taylorella equigenitalis*. *J. Clin. Microbiol.* **32**, 893-896.

[81] Matsuda, M. and Moore, J.E. (2003) Recent advances in molecular epidemiology and detection of *Taylorella equigenitalis* associated with contagious equine metritis (CEM). *Vet. Microbiol.* **97**, 111-122. https://doi.org/10.1016/j.vetmic.2003.08.001

[82] Duquesne, F., Pronost, S., Laugier, C. and Petry, S. (2007) Identification of *Taylorella equigenitalis* responsible for contagious equine metritis in equine genital swabs by direct polymerase chain reaction. *Res. Vet. Sci.* **82**, 47-49. https://doi.org/10.1016/j.rvsc.2006.05.001

[83] Tazumi, A., Petry, S., Hayashi, K., Moore, J.E., Millar, B.C. and Matsuda, M. (2012) Molecular identification and characterization of the intervening sequences (IVSs) within 23S ribosomal RNA (rRNA) genes of *Taylorella asinigenitalis* isolated in France. *Res. Vet. Sci.* **92**, 45-52. https://doi.org/10.1016/j.rvsc.2010.10.004

[84] Tazumi, A., Saito, S., Sekizuka, T., Murayama, O., Takamiya, S., Moore, J.E., Millar, B.C. and Matsuda, M. (2007) Molecular characterization of the full-length 23S and 5S ribosomal RNA (rRNA) genes of *Taylorella asinigenitalis*. *Antonie Van Leeuwenhoek* **92**, 257-264. https://doi.org/10.1007/s10482-007-9144-8

[85] Tazumi, A., Sekizuka, T., Moore, J.E., Millar, C.B., Taneike, I. and Matsuda, M. (2008) Intervening sequences in 23S rRNA genes and 23S rRNA fragmentation in *Taylorella asinigenitalis* UCD-1(T) strain. *J. Basic Microbiol.* **48**, 284-292. https://doi.org/10.1002/jobm.200700386

[86] Tazumi, A., Sekizuka, T., Moore, J., Millar, B., Taneike, I. and Matsuda, M. (2008) Molecular characterization of intervening sequences in 23S rRNA genes and 23S rRNA fragmentation in *Taylorella equigenitalis*. *Folia Microbiol. (Praha)* **53**, 486-492. https://doi.org/10.1007/s12223-008-0076-0

[87] Tazumi, A., Nakanishi, S., Hayashi, K., Petry, S., Tasaki, E., Nakajima, T., Ueno, H., Moore, J.E., Millar, B.C. and Matsuda, M. (2012) Demonstration of the absence of intervening sequences (IVSs) within 16S rRNA genes of *Taylorella equigenitalis* and *Taylorella asinigenitalis* isolates. *Res. Vet. Sci.* **92**, 435-437. https://doi.org/10.1016/j.rvsc.2011.04.010

[88] Delerue, M., Breuil, M.-F., Duquesne, F., Bayon-Auboyer, M.-H., Amenna-Bernard, N. and Petry, S. (2019) Acute endometritis due to *Taylorella equigenitalis* transmission by insemination of cryopreserved stallion semen. *J. Equine Vet. Sci.* **78**, 10-13. https://doi.org/10.1016/j.jevs.2019.03.217

[89] Ricketts, S., Crowhurst, J., Newton, R. and Gibbens, N. (2012) Contagious equine metritis organism confirmed in Gloucestershire. *Vet. Rec.* **170**, 398-398. 10.1136/vr.e2647

[90] Erdman, M.M., Creekmore, L.H., Fox, P.E., Pelzel, A.M., Porter-Spalding, B.A., Aalsburg, A.M., Cox, L.K., Morningstar-Shaw, B.R. and Crom, R.L. (2011) Diagnostic and epidemiologic analysis of the 2008-2010 investigation of a multi-year outbreak of contagious equine metritis in the United States. *Prev. Vet. Med.* **101**, 219-228. https://doi.org/10.1016/j.prevetmed.2011.05.015

[91] Knowles, R. (1983) Contagious equine metritis (CEM) situation report, 1983. *Proc. Am. Assoc. Equine Pract.*

[92] Aalsburg, A.M. and Erdman, M.M. (2011) Pulsed-field gel electrophoresis genotyping of *Taylorella equigenitalis* isolates collected in the United States from 1978 to 2010. *J. Clin. Microbiol.* **49**, 829-833. 10.1128/JCM.00956-10

[93] Timoney, P. and Powell, D. (1988) Contagious equine metritis-epidemiology and control. *J. Equine Vet. Sci.* **8**, 42-46. https://doi.org/10.1016/S0737-0806(88)80109-6

[94] Lu, K.G. and Morresey, P.R. (2007) Infectious diseases in breeding stallions. *Clin. Tech. Eq. P.* **6**, 285-290. https://doi.org/10.1053/j.ctep.2007.09.008

[95] Timoney, P., McArdle, J., O'reilly, P. and Ward, J. (1978) Infection patterns in pony mares challenged with the agent of contagious equine metritis 1977. *Equine Vet. J.* **10**, 148-152. https://doi.org/10.1111/j.2042-3306.1978.tb02244.x

[96] Timoney, P. (1996) Contagious equine metritis. *Comp. Immunol. Microbiol. Infect. Dis.* **19**, 199-204. https://doi.org/10.1016/0147-9571(96)00005-7

[97] Wood, J., Kelly, L., Cardwell, J. and Park, A. (2005) Quantitative assessment of the risks of reducing the routine swabbing requirements for the detection of *Taylorella equigenitalis*. *Vet. Rec.* **157**, 41-46. http://dx.doi.org/10.1136/vr.157.2.41

[98] Platt, H., Atherton, J. and Simpson, D. (1978) The experimental infection of ponies with contagious equine metritis. *Equine Vet. J.* **10**, 153-159. https://doi.org/10.1111/j.2042-3306.1978.tb02245.x

[99] Arata, A.B., Cooke, C.L., Jang, S.S. and Hirsh, D.C. (2001) Multiplex polymerase chain reaction for distinguishing *Taylorella equigenitalis* from *Taylorella equigenitalis*-like organisms. *J. Vet. Diagn. Invest.* **13**, 263-265. https://doi.org/10.1177/104063870101300316

[100] Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Chapter 2.5.2 Contagious equine metritis (2018). http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.05.02_CEM.pdf. Accessed August 2019.

[101] Wakeley, P., Errington, J., Hannon, S., Roest, H., Carson, T., Hunt, B., Sawyer, J. and Heath, P. (2006) Development of a real time PCR for the detection of *Taylorella equigenitalis* directly from genital swabs and discrimination from *Taylorella asinigenitalis*. *Vet. Microbiol.* **118**, 247-254. https://doi.org/10.1016/j.vetmic.2006.08.007

[102] United States Department of Agriculture, Animal and Plant Health Inspection Service. Equine imports and exports (2020). https://www.aphis.usda.gov/aphis/ourfocus/importexport/animal-import-and-export/equine. Accessed 17 March, 2020.

[103] Horserace Betting Levy Board. International codes of practice – Contagious equine metritis (2020). https://codes.hblb.org.uk/index.php/page/19. Accessed 17 March, 2020.

[104] Keys, B., Schulman, M., May, K., Joone, C., Monyai, M., Mpofu, D., Marais, H. and Guthrie, A. (2012) Comparison of two treatment methods for the elimination of Contagious Equine Metritis in stallions. *J. Equine Vet. Sci.* **32**, S76. 10.1016/j.jevs.2012.08.162

[105] Keys, B. (2014) *Evaluation of two topical treatment methods for the elimination of Taylorella equigenitalis in stallions*, University of Pretoria, Pretoria.

[106] Anzai, T., Kamada, M., Hidekazu, N., Eguchi, M. and Nishi, H. (2012) Contagious equine metritis eradicated from Japan. *J. Vet. Med. Sci.* **74**, 519-522.

[107] Gunson, R., Collins, T. and Carman, W. (2006) Practical experience of high throughput real time PCR in the routine diagnostic virology setting. *J. Clin. Virol.* **35**, 355-367.

[108] Kaltenboeck, B. and Wang, C. (2005) Advances in real-time PCR: application to clinical laboratory diagnostics. *Adv. Clin. Chem.* **40**, 219-259.

[109] Hu, Y. (2016) Regulatory concern of polymerase chain reaction (PCR) carryover contamination. In: *Polymerase chain reaction for biomedical applications*, Ed: A. Samadikuchaksaraei, OpenAccess. pp 57-68.

[110] Anzai, T., Eguchi, M., Sekizaki, T., Kamada, M., Yamamoto, K. and Okuda, T. (1999) Development of a PCR test for rapid diagnosis of contagious equine metritis. *J. Vet. Med. Sci.* **61**, 1287-1292. https://doi.org/10.1292/jvms.61.1287

[111] Premanandh, J., George, L., Wernery, U. and Sasse, J. (2003) Evaluation of a newly developed real-time PCR for the detection of *Taylorella equigenitalis* and discrimination from *T. asinigenitalis*. *Vet. Microbiol.* **95**, 229-237. https://doi.org/10.1016/S0378-1135(03)00065-8

[112] Ousey, J., Palmer, L., Cash, R., Grimes, K., Fletcher, A., Barrelet, A., Foote, A., Manning, F. and Ricketts, S. (2009) An investigation into the suitability of a commercial real-time PCR assay to screen for *Taylorella equigenitalis* in routine prebreeding equine genital swabs. *Equine Vet. J.* **41**, 878-882. https://doi.org/10.2746/042516409X474275

[113] Léon, A., Despois, L., Castagnet, S., Versmisse, Y., Gracieux, P. and Blanchard, B. (2016) Validation of an easy handling sample preparation and a triplex real-time polymerase chain reaction for rapid detection of contagious equine metritis organisms. *J. Equine Vet. Sci.* **39**, S12.

[114] Nadin-Davis, S., Knowles, M.K., Burke, T., Böse, R. and Devenish, J. (2015) Comparison of culture versus quantitative real-time polymerase chain reaction for the detection of *Taylorella equigenitalis* in field samples from naturally infected horses in Canada and Germany. *Can. J. Vet. Res.* **79**, 161-169.

[115] Belloy, L., Ferreira, M. and Waldvogel, A.S. (2012) Diagnosis of *Taylorella equigenitalis* by culture or by real time PCR? *Schweiz. Arch. Tierheilkd.* **154**, 87-88. 10.1024/0036-7281/a000301

[116] Mawhinney, I., Errington, J., Stamper, N., Torrens, N., Engelsma, M.Y. and Roest, H.I.J. (2019) Pooling of genital swabs for detection by PCR of *Taylorella equigenitalis*, the cause of contagious equine metritis. *Equine Vet. J.* **51**, 227-230. https://doi.org/10.1111/evj.12986

[117] Anzai, T., Wada, R., Okuda, T. and Aoki, T. (2002) Evaluation of the field application of PCR in the eradication of contagious equine metritis from Japan. *J. Vet. Med. Sci.* **64**, 999-1002. https://doi.org/10.1292/jvms.64.999

[118] Moore, J.E., Millar, B.C. and Buckley, T.C. (2000) Potential misidentification of *Taylorella asinigenitalis* as *Taylorella equigenitalis*: implications for the epidemiology of CEM. *J. Equine Vet. Sci.* **20**, 479. 10.1016/S0737-0806(00)70204-8

[119] Kinoshita, Y., Hidekazu, N., Katayama, Y. and Hariu, K. (2015) Development of loop-mediated isothermal amplification methods for detecting *Taylorella equigenitalis* and *Taylorella asinigenitalis*. *J. Equine Vet. Sci.* **26**, 25-29. https://doi.org/10.1294/jes.26.25

[120] Karami, A., Gill, P., Motamedi, M.H.K. and Saghafinia, M. (2011) A review of the current isothermal amplification techniques: applications, advantages and disadvantages. *J Global Infect Dis* **3**, 293-292.

[121] United States Department of Agriculture, Animal and Plant Health Inspection Service. Contagious equine metritis (2021). https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/animal-disease-information/equine/cem/contagious-equine-metritis. Accessed April, 2021.

[122] Kristula, M. (2014) Contagious equine metritis. In: *Equine Infectious Diseases 2nd Edition*, Ed: D.C. Sellon, Saunders Elsevier, St Louis, Missouri.

[123] Matsuda, M., Asami, Y., Miyazawa, T., Sugawara, T., Kumano, M., Isayama, Y. and Honda, M. (1994) Estimation of the size of the genome of *Taylorella equigenitalis* by crossed-field gel electrophoresis. *Vet. Res. Commun.* **18**, 99-102.

[124] Kagawa, S., Klein, F., Corboz, L., Moore, J., Murayama, O. and Matsuda, M. (2001) Demonstration of heterogeneous genotypes of *Taylorella equigenitalis* isolated from horses in six European countries by pulsed-field gel electrophoresis. *Vet. Res. Commun.* **25**, 565-575. https://doi.org/10.1023/A:1017925216350

[125] Matsuda, M., Asami, Y., Miyazawa, T., Samata, T., Isayama, Y., Honda, M. and Ide, Y. (1994) Analysis of chromosome-sized DNA and genome typing of isolated strains of *Taylorella equigenitalis*. *Vet. Res. Commun.* **18**, 93-98.

[126] Matsuda, M., Kagawa, S., Sakamoto, Y., Miyajima, M., Barton, M. and Moore, J. (2000) Detection of heterogeneous genotypes among Australian strains of *Taylorella equigenitalis*. *Aust. Vet. J.* **78**, 56-57. https://doi.org/10.1111/j.1751-0813.2000.tb10362.x

[127] Matsuda, M., Miyazawa, T. and Anzai, T. (1999) Detection of a common genotype among strains of *Taylorella equigenitalis* isolated from thoroughbred horses in Japan between 1994 and 1996. *J. Basic Microbiol.* **39**, 127-130. https://doi.org/10.1002/(SICI)1521-4028(199905)39:2<127::AID-JOBM127>3.0.CO;2-Q

[128] Matsuda, M., Miyazawa, T., Ishida, Y. and Moore, J. (1997) Profiles of fragments after pulsed-field gel electrophoresis of cleaved genomic DNA from strains of *Taylorella equigenitalis* isolated from horses in Norway. *Microbiol. Res.* **152**, 217-220. https://doi.org/10.1016/S0944-5013(97)80015-8

[129] Matsuda, M., Miyazawa, T., Moore, J., Buckley, T. and Thomas, L. (1998) Molecular genotyping by pulsed-field gel electrophoresis of restricted genomic DNA of strains of *Taylorella equigenitalis* isolated in Ireland and in the United States. *Vet. Res. Commun.* **22**, 217-224. https://doi.org/10.1023/A:1006002212604

[130] Miyazawa, T., Matsuda, M., Isayama, Y., Samata, T., Ishida, Y., Ogawa, S., Takei, K., Honda, M. and Kamada, M. (1995) Genotyping of isolates of *Taylorella equigenitalis* from thoroughbred brood mares in Japan. *Vet. Res. Commun.* **19**, 265-271. https://doi.org/10.1007/BF01839309

[131] Bleumink-Pluym, N., Ter Laak, E. and Van der Zeijst, B. (1990) Epidemiologic study of *Taylorella equigenitalis* strains by field inversion gel electrophoresis of genomic restriction endonuclease fragments. *J. Clin. Microbiol.* **28**, 2012-2016.

[132] Heller, C. (1994) Field Inversion Gel Electrophoresis. In: *Protocols for Gene Analysis. Methods in Molecular Biology*, Ed: H. A.J., Humana Press. pp 135-146.

[133] Thoresen, S.I., Jenkins, A. and Ask, E. (1995) Genetic homogeneity of *Taylorella equigenitalis* from Norwegian trotting horses revealed by chromosomal DNA fingerprinting. *J. Clin. Microbiol.* **33**, 233-234.

[134] Hébert, L., Moumen, B., Pons, N., Duquesne, F., Breuil, M.-F., Goux, D., Batto, J.-M., Laugier, C., Renault, P. and Petry, S. (2012) Genomic characterization of the *Taylorella* genus. *PLoS One* **7**, 1-13. 10.1371/journal.pone.0029953

[135] Enright, M.C. and Spratt, B.G. (1999) Multilocus sequence typing. *Trends Microbiol.* **7**, 482-487. https://doi.org/10.1016/S0966-842X(99)01609-1

[136] Feil, E.J., Li, B.C., Aanensen, D.M., Hanage, W.P. and Spratt, B.G. (2004) eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J. Bacteriol.* **186**, 1518-1530. 10.1128/JB.186.5.1518-1530.2004

[137] Smith, A.M., Tau, N.P., Smouse, S.L., Allam, M., Ismail, A., Ramalwa, N.R., Disenyeng, B., Ngomane, M. and Thomas, J. (2019) Outbreak of *Listeria monocytogenes* in South Africa, 2017-2018: Laboratory activities and experiences associated with whole-genome sequencing analysis of isolates. *Foodborne Pathog. Dis.* **16**, 524-530.

[138] Melzer, F., Rassbach, A., Koenig-Mozes, A., Elschner, M.C., Tomaso, H. and Busch, A. (2018) Draft Genome Sequence of *Taylorella equigenitalis* Strain 210217RC10635, Isolated from a Pony Stallion in Germany. *Microbiol. Resour. Announc.* **7**. 10.1128/mra.01112-18

[139] Hwang, J.Y. and Cho, G.J. (2018) First identification of *Taylorella equigenitalis* from genital tracts of Thoroughbred horses from the inland area of South Korea by multilocus sequence typing. *J. Equine Vet. Sci.* **60**, 16-22. https://doi.org/10.1016/j.jevs.2017.09.011

[140] Hara, Y., Hayashi, K., Nakajima, T., Kagawa, S., Tazumi, A., Moore, J.E. and Matsuda, M. (2013) Molecular identification and characterization of clustered regularly interspaced short palindromic repeat (CRISPR) gene cluster in *Taylorella equigenitalis*. *Folia Microbiol. (Praha)* **58**, 375-384. https://doi.org/10.1007/s12223-012-0217-3

[141] Hara, Y., Nakajima, T., Akamatsu, M., Yahiro, M., Kagawa, S., Petry, S., Matsuda, M. and Moore, J.E. (2015) Development of a novel molecular detection method for clustered regularly interspaced short palindromic repeats (CRISPRs) in *Taylorella* organisms. *J. Med. Microbiol.* **64**, 782-787. https://doi.org/10.1099/jmm.0.000079

[142] Springer, B., Kidan, Y.G., Prammananan, T., Ellrott, K., Böttger, E.C. and Sander, P. (2001) Mechanisms of streptomycin resistance: selection of mutations in the 16S rRNA gene conferring resistance. *Antimicrob. Agents Chemother.* **45**, 2877-2884. 10.1128/AAC.45.10.2877-2884.2001

[143] Nair, J., Rouse, D.A., Bai, G.H. and Morris, S.L. (1993) The rpsL gene and streptomycin resistance in single and multiple drug-resistant strains of Mycobacterium tuberculosis. *Mol. Microbiol.* **10**, 521-527. https://doi.org/10.1111/j.1365-2958.1993.tb00924.x

[144] Hauser, H., Richter, D.C., van Tonder, A., Clark, L. and Preston, A. (2012) Comparative genomic analyses of the *Taylorellae*. *Vet. Microbiol.* **159**, 195-203. https://doi.org/10.1016/j.vetmic.2012.03.041

[145] Overbeek, R., Begley, T., Butler, R.M., Choudhuri, J.V., Chuang, H.-Y., Cohoon, M., de Crécy-Lagard, V., Diaz, N., Disz, T. and Edwards, R. (2005) The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. *Nucleic Acids Res.* **33**, 5691-5702.

[146] Davis, J.J., Wattam, A.R., Aziz, R.K., Brettin, T., Butler, R., Butler, R.M., Chlenski, P., Conrad, N., Dickerman, A., Dietrich, E.M., Gabbard, J.L., Gerdes, S., Guard, A., Kenyon, R.W., Machi, D., Mao, C., Murphy-Olson, D., Nguyen, M., Nordberg, E.K., Olsen, G.J., Olson, R.D., Overbeek, J.C., Overbeek, R., Parrello, B., Pusch, G.D., Shukla, M., Thomas, C., VanOeffelen, M., Vonstein, V., Warren, A.S., Xia, F., Xie, D., Yoo, H. and Stevens, R. (2019) The PATRIC Bioinformatics Resource Center: expanding data and analysis capabilities. *Nucleic Acids Res.* **48**, D606-D612. 10.1093/nar/gkz943

[147] Guthrie, A., Howell, P., Hedges, J., Bosman, A.M., Balasuriya, U., McCollum, W., Timoney, P. and MacLachlan, N. (2003) Lateral transmission of equine arteritis virus among Lipizzaner stallions in South Africa. *Equine Vet. J.* **35**, 596-600. https://doi.org/10.2746/042516403775467162

[148] Blacket, M.J., Robin, C., Good, R.T., Lee, S.F. and Miller, A.D. (2012) Universal primers for fluorescent labelling of PCR fragments – an efficient and cost-effective approach to genotyping by fluorescence. *Mol. Ecol. Resour.* **12**, 456-463. https://doi.org/10.1111/j.1755-0998.2011.03104.x

[149] Cleenwerck, I., Vandemeulebroecke, K., Janssens, D. and Swings, J. (2002) Re-examination of the genus Acetobacter, with descriptions of Acetobacter cerevisiae sp. nov. and *Acetobacter malorum* sp. nov. *Int. J. Syst. Evol. Microbiol.* **52**, 1551-1558. https://doi.org/10.1099/00207713-52-5-1551

[150] (2011) Immediate notification: Contagioue Equine Metritis. http://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?reportid =10553. Accessed August 2019.

[151] World Organisation for Animal Health. (2011) Contagious equine metritis: South Africa, Follow up report 1 (12/07/2011). https://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?reportid=10790. Accessed 1 March 2020.

[152] World Organisation for Animal Health. (2017) Contagious equine metritis: South Africa, Follow up report 9 (03/02/2017). https://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?reportid=22620. Accessed 1 March 2020.

[153] Baez-Ortega, A., Lorenzo-Diaz, F., Hernandez, M., Gonzalez-Vila, C.I., Roda-Garcia, J.L., Colebrook, M. and Flores, C. (2015) IonGAP: integrative bacterial genome analysis for Ion Torrent sequence data. *Bioinformatics* **31**, 2870-2873. https://doi.org/10.1093/bioinformatics/btv283

[154] Quail, M.A., Smith, M., Coupland, P., Otto, T.D., Harris, S.R., Connor, T.R., Bertoni, A., Swerdlow, H.P. and Gu, Y. (2012) A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers. *BMC Genomics* **13**, 341.

[155] Crosby, A.W. (1967) Conquistador y pestilencia: the first New World pandemic and the fall of the great Indian empires. *Hispanic American Historical Review* **47**, 321-337.

[156] Balasuriya, U., Carossino, M. and Timoney, P. (2018) Equine viral arteritis: a respiratory and reproductive disease of significant economic importance to the equine industry. *Equine Vet. Edu.* **30**, 497-512.

[157] Wood, J., Chirnside, E., Mumford, J. and Higgins, A. (1995) First recorded outbreak of equine viral arteritis in the United Kingdom. *Vet. Rec.* **136**, 381-381.

[158] Metcalf, E.S. (2001) The role of international transport of equine semen on disease transmission. *Anim. Reprod. Sci.* **68**, 229-237.

[159] Bolwell, C.F., Rogers, C.W., Gee, E.K. and Rosanowski, S.M. (2020) Commercial equine production in New Zealand. 3. The racing and sport industries. *Anim. Product. Sci.* **60**, 2164-2174.

[160] Rosanowski, S., Cogger, N., Rogers, C., Benschop, J. and Stevenson, M. (2012) A description of the demographic characteristics of the New Zealand non-commercial horse population with data collected using a generalised random-tessellation stratified sampling design. *Prev. Vet. Med.* **107**, 242-252.

[161] May, C.E., Guthrie, A.J. and Schulman, M.L. (2019) Direct culture-independent sequence typing of *Taylorella equigenitalis* obtained from genital swabs and frozen semen samples from South African horses. *J. Vet. Diagn. Invest.* **31**, 792-794. https://doi.org/10.1177/1040638719871089

[162] Maiden, M.C., Bygraves, J.A., Feil, E., Morelli, G., Russell, J.E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K. and Caugant, D.A. (1998) Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *PNAS.* **95**, 3140-3145.

[163] Ankenbrand, M.J. and Keller, A. (2016) bcgTree: automatized phylogenetic tree building from bacterial core genomes. *Genome* **59**, 783-791.

[164] Francisco, A.P., Vaz, C., Monteiro, P.T., Melo-Cristino, J., Ramirez, M. and Carriço, J.A. (2012) PHYLOViZ: phylogenetic inference and data visualization for sequence based typing methods. *BMC Bioinformatics* **13**, 87. 10.1186/1471-2105-13-87

[165] Gagetti, P., Wattam, A.R., Giacoboni, G., De Paulis, A., Bertona, E., Corso, A. and Rosato, A.E. (2019) Identification and molecular epidemiology of methicillin resistant Staphylococcus pseudintermedius strains isolated from canine clinical samples in Argentina. *BMC Vet. Res.* **15**, 264. 10.1186/s12917-019-1990-x

[166] Timoney, P., Kristula, M., Ford, E., Short, M., Flynn, K., Broaddus, C., Tamassia, M., Donahue, J. and Meade, B. (2016) Contagious equine metritis: Efficacy of US post-entry testing protocols for identifying carrier stallions and mares. *J. Equine Vet. Sci.* **39**, S60.

[167] May, C.E., Schulman, M.L. and Guthrie, A.J. (2013) The distribution of *Taylorella equigenitalis* on the external genitalia of carrier stallions in South Africa. *Clinical Theriogenology.* **5**, 399.

[168] HBLB. International Codes of Practice 2021 – CEM Notification (2021). https://codes.hblb.org.uk/index.php/page/36. Accessed February, 2021.

[169] Moore, J., Buckley, T., Millar, B., Gibson, P., Cannon, G., Egan, C., Cosgrove, H., Stanbridge, S., Anzai, T. and Matsuda, M. (2001) Molecular surveillance of the incidence of *Taylorella equigenitalis* and *Pseudomonas aeruginosa* from horses in Ireland by sequence-specific PCR [SS-PCR]. *Equine Vet. J.* **33**, 319-322. https://doi.org/10.2746/042516401776249750

[170] The National Horseracing Authority. Stud Health Scheme (2021). https://www.nhra.co.za/index.php/studbook/stud-health/stud-health-scheme. Accessed January, 2021.

Appendix A: Draft Genome sequence of *Taylorella equigenitalis* strain ERC_G2224 isolated from the semen of a Lipizzaner stallion in South Africa

Draft Genome Sequence of *Taylorella equigenitalis* Strain ERC_G2224 Isolated from the Semen of a Lipizzaner Stallion in South Africa

Catherine E. May,[a] Martin L. Schulman,[a] Peter G. Howell,[b] Carina W. Lourens,[b] Johan Gouws,[c] Christopher Joone,[b] Mpho S. Monyai,[b] Misha le Grange,[b] Oliver K. I. Bezuidt,[d] Cindy K. Harper,[e] Alan J. Guthrie[b]

Section of Reproduction, Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa[a]; Equine Research Centre, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa[b]; Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa[c]; Centre for Microbial and Environmental Genomics, Department of Genetics, Faculty of Natural Sciences, University of Pretoria, Hatfield, South Africa[d]; Veterinary Genetics Laboratory, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa[e]

Taylorella equigenitalis **is the causative agent of contagious equine metritis (CEM), a sexually transmitted infection of horses. We report here the genome sequence of *T. equigenitalis* strain ERC_G2224, isolated in 2015 from a semen sample collected in 1996 from a Lipizzaner stallion in South Africa.**

Received 4 September 2015 **Accepted** 11 September 2015 **Published** 15 October 2015

Citation May CE, Schulman ML, Howell PG, Lourens CW, Gouws J, Joone C, Monyai MS, le Grange M, Bezuidt OKI, Harper CK, Guthrie AJ. 2015. Draft genome sequence of *Taylorella equigenitalis* strain ERC_G2224 isolated from the semen of a Lipizzaner stallion in South Africa. Genome Announc 3(5):e1205-15. doi:10.1128/genomeA.01205-15.

Copyright © 2015 May et al. This is an open-access article distributed under the terms of the Creative Commons Attribution 3.0 Unported license.

Address correspondence to Alan J. Guthrie, alan.guthrie@up.ac.za.

Downloaded from http://genomea.asm.org/ on July 20, 2016 by guest

Taylorella equigenitalis is a slow-growing microaerophilic Gram-negative coccobacillus, classified in the *Burkholderiales* order and the *Alcaligenaceae* family (1). It is the etiological agent of contagious equine metritis (CEM), a highly contagious sexually transmitted infection of horses characterized in infected mares by mucopurulent vaginal discharge and various degrees of vaginitis, cervicitis, and endometritis, and it may result in temporary infertility or early embryonic death (2). In stallions, the long-term presence of *T. equigenitalis* as a colonist of the external genitalia does not cause clinical signs, and asymptomatic carrier mares have also been reported (2). CEM is a World Organisation for Animal Health (OIE)-notifiable disease and is considered part of veterinary certification for international trade purposes (2). The multilocus sequence typing (MLST) scheme for taylorellae (3) recently provided a comprehensive overview of the genetic diversity of taylorellae. To date, the genome sequences of only four *T. equigenitalis* strains have been reported (4–6), and the genome sequences of most *T. equigenitalis* sequence types (STs) remain to be characterized.

We report here the genome sequence of *T. equigenitalis* ERC_G2224, which was isolated in 2015 from a stored frozen (-80ºC) semen sample collected in 1996 from an asymptomatic carrier Lipizzaner stallion from a property in Gauteng (South Africa). Sequence typing of this strain using the *Taylorella* MLST Databases (http://pubmlst.org/taylorella/) revealed its membership in the previously nonsequenced ST4, which is not linked to one of the existing clonal complexes (CC1 to CC4) (3).

The genome of *T. equigenitalis* strain ERC_G2224 was sequenced using the Ion Torrent (Life Technologies) platform. High-molecular-weight DNA was extracted, and the size, quantity, and quality were checked using previously described methods (7). The library was constructed from 1 μg of genomic DNA using the Ion Xpress Plus fragment library kit (Life Technologies). The size selection was performed on a 2% E-Gel SizeSelect gel (Invitrogen) using the 400-bp selection criterion. Fragments were not amplified during the library-building process. Template amplification was performed using the Ion OneTouch 2 system (OT2) with the Ion PGM Hi-Q OT2 kit, and the templated particles were enriched on the Ion OneTouch ES system (Life Technologies). The samples were loaded on an Ion 316 Chip version 2 and sequenced on the Ion PGM system (Life Technologies) using the Ion PGM Hi-Q sequencing kit (Life Technologies) for 400-bp chemistry. In total, 2.25 million reads (mean length, 314 bp) generated 706 Mb of data, of which 1,123,016 reads were assembled (estimated coverage, <80×) into 18 large contigs (>500 bp), giving a consensus length of 1,670,247 bp; the contigs were ordered and compared with the genome of *T. equigenitalis* strain MCE9 (accession no. CP002456) using IonGAP: integrative bacterial genome analysis for Ion Torrent sequence data, released in 2015 (http://iongap.hpc.iter.es/iongap/) (8). Annotation was added by the NCBI Prokaryotic Genome Annotation Pipeline, released in 2013 (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/), yielding 1,540 genes, 38 tRNAs, and 12 rRNAs. One clustered regularly interspaced short palindromic repeat (CRISPR)/Cas loci was detected. The average G+C content of the draft genome sequences is 37.4%.

Nucleotide sequence accession numbers. This whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession no. LIYJ00000000. The version described in this paper is the first version LIYJ01000000.

ACKNOWLEDGMENTS

This work was supported by the Equine Research Centre and the National Research Foundation of South Africa.

REFERENCES

1. Sugimoto C, Isayama Y, Sakazaki R, Kuramochi S. 1983. Transfer of *Haemophilus equigenitalis* Taylor et al. 1978 to the genus *Taylorella* gen. nov. as *Taylorella equigenitalis* comb. nov. Curr Microbiol 9:155–162. http://dx.doi.org/10.1007/BF01567289.
2. Schulman ML, May CE, Keys B, Guthrie AJ. 2013. Contagious equine

metritis: artificial reproduction changes the epidemiologic paradigm. Vet Microbiol 167:2–8. http://dx.doi.org/10.1016/j.vetmic.2012.12.021.
3. Duquesne F, Hébert L, Breuil M, Matsuda M, Laugier C, Petry S. 2013. Development of a single multi-locus sequence typing scheme for *Taylorella equigenitalis* and *Taylorella asinigenitalis*. Vet Microbiol 167:609–618. http://dx.doi.org/10.1016/j.vetmic.2013.09.016.
4. Hauser H, Richter DC, van Tonder A, Clark L, Preston A. 2012. Comparative genomic analyses of the taylorellae. Vet Microbiol 159:195–203. http://dx.doi.org/10.1016/j.vetmic.2012.03.041.
5. Hébert L, Moumen B, Duquesne F, Breuil M-, Laugier C, Batto J-, Renault P, Petry S. 2011. Genome sequence of *Taylorella equigenitalis* MCE9, the causative agent of contagious equine metritis. J Bacteriol 193:1785. http://dx.doi.org/10.1128/JB.01547-10.
6. Hébert L, Touzain F, de Boisséson C, Breuil M-F, Duquesne F, Laugier C, Blanchard Y, Petry S. 2014. Draft genome sequence of *Taylorella equigenitalis* strain MCE529, isolated from a Belgian Warmblood horse. Genome Announc 2(6):e01214-14. http://dx.doi.org/10.1128/genomeA.01214-14.
7. Cleenwerck I, Vandemeulebroecke K, Janssens D, Swings J. 2002. Reexamination of the genus *Acetobacter*, with descriptions of *Acetobacter cerevisiae* sp. nov. and *Acetobacter malorum* sp. nov. Int J Syst Evol Microbiol 52:1551–1558. http://dx.doi.org/10.1099/ijs.0.02064-0.
8. Baez-Ortega A, Lorenzo-Diaz F, Hernandez M, Gonzalez-Vila CI, Roda-Garcia JL, Colebrook M, Flores C. 2015. IonGAP: integrative bacterial genome analysis for Ion Torrent sequence data. Bioinformatics 31:2870–2873. http://dx.doi.org/10.1093/bioinformatics/btv283.

Downloaded from http://genomea.asm.org/ on July 20, 2016 by guest

Appendix B: Direct culture-independent sequence typing of *Taylorella equigenitalis* obtained from genital swabs and frozen semen samples from South African horses

Brief Communication

Journal of Veterinary Diagnostic Investigation
2019, Vol. 31(5) 792–794
© 2019 The Author(s)
Article reuse guidelines:
sagepub.com/journals-permissions
DOI: 10.1177/1040638719871089
jvdi.sagepub.com

Direct culture–independent sequence typing of *Taylorella equigenitalis* obtained from genital swabs and frozen semen samples from South African horses

Catherine E. May,[1] Alan J. Guthrie, Martin L. Schulman

Abstract. We report herein the use of crude extracts obtained from samples of *Taylorella equigenitalis*–infected horses for the purpose of multi-locus sequence typing (MLST). Samples ($n = 36$) were collected from horses in South Africa from 1996 to 2017: 34 from genital swabs (stored at −20°C for 2–3 y) and 2 from cryopreserved raw semen aliquots (stored at −70°C for 18 y) prior to assay. The MLST assay showed a single sequence type (ST), designated ST4, that supported a point introduction and thus a common source for the South African outbreak of contagious equine metritis.

Key words: contagious equine metritis; direct culture–independent strain typing; *Taylorella equigenitalis*.

Taylorella equigenitalis, a gram-negative, microaerophilic, frequently pleomorphic bacterium,[18] is the causative agent of contagious equine metritis (CEM), a non-systemic, venereally transmitted disease of horses. *T. equigenitalis* is one of only 2 species of the *Taylorella* genus, a member of the *Alcaligenaceae* family.[9] This organism causes an inapparent carrier status without associated clinical signs in stallions and temporary infertility in affected mares characterized by mucopurulent vaginitis, cervicitis, or endometritis.[19] Although the majority of mares rid themselves of infection, 20–40% may become carriers.[11] *T. equigenitalis* is spread by natural mating or artificial insemination with contaminated semen and by fomite transmission.[1,16,19] The effect on equine reproductive efficiency, especially in systems utilizing natural mating, as well as international trade restrictions, can result in substantial economic losses to the equine industry. These outcomes have resulted in CEM being one of the most regulated equine bacterial diseases in the world.[1,19]

The 2018 World Organisation for Animal Health (OIE) gold standard for identification of *T. equigenitalis* is bacterial culture (http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.05.02_CEM.pdf). However, given associated difficulties, including the fastidious transport and growth requirements required for isolating *T. equigenitalis*, various PCR-based methods have been developed to detect and additionally differentiate *T. equigenitalis* from the other member of the genus, *T. asinigenitalis*.[5,12,20]

Since the first reported outbreak in the United Kingdom in 1977,[3] CEM has attained worldwide distribution. The most recently reported outbreaks in non-endemic countries include a 2008 outbreak in Portugal,[15] a 2008–2011 outbreak in the United States,[6] a 2012 outbreak in the United Kingdom,[14] and a 2015 outbreak in South Korea.[10] South Africa was considered to be free of CEM until the first reported case in May 2011 (OIE. Immediate notification (09/05/2011) of contagious equine metritis, http://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?reportid=10553). Since identification of the South African outbreak, development of molecular techniques such as MLST and genome sequencing have allowed further characterization of *Taylorella* spp.[4,8,9] We report herein the application of an MLST assay to crude extracts from both genital swabs and cryopreserved semen samples in the absence of stored isolates.

T. equigenitalis–positive genital swab samples were collected from mares and male horses in South Africa from 2011 to 2017 ($n = 33$). This inventory originated from samples obtained during both a nationwide stallion screening program, legislated subsequent to outbreak recognition, and an epidemiologic traceback.[13] Heightened awareness of the CEM-associated risk in South Africa prompted additional voluntary submissions of cryopreserved semen straws derived from ejaculates collected and processed from stallions prior to outbreak identification in 2011. In 2015, this submission of cryopreserved semen identified an additional *T. equigenitalis*–positive case linked to a stallion that had

Section of Reproduction, Department of Production Animal Studies (May, Schulman) and Equine Research Centre (Guthrie), Faculty of Veterinary Science, University of Pretoria, Onderstepoort, Pretoria, Gauteng, Republic of South Africa.

[1]Corresponding author: Catherine E. May, University of Pretoria, Faculty of Veterinary Science, Private Bag X4, Onderstepoort, Pretoria, Gauteng, 0110, South Africa. Kate.may@up.ac.za

semen collected and cryopreserved in 2008. The stallion was subsequently gelded and on follow-up was found to be positive 7 y later (case 34).

The traceback exercise had furthermore identified a subpopulation focus at the South African Lipizzaner Centre and, based on this finding, archived semen samples (n = 2; cases 1 and 2) were accessed in August 2014. These samples had been collected from Lipizzaner stallion residents of this Centre during an equine viral arteritis investigation[7] in 1996 and were included in the MLST analysis.

Swabs were agitated for 5 s in 0.4 mL of 0.1 M phosphate-buffered saline (PBS; pH 7.4) in separate 1.5-mL microfuge tubes. In the case of semen samples, after thawing, 0.1 mL was added to 0.4 mL of 0.1 M PBS as described above. All samples were then pelleted by centrifugation (18,000 × g, 60 s), and the supernatant was aspirated. The resultant pellet was resuspended in 0.1 mL of nuclease-free water (MilliporeSigma, St. Louis, MO) and then heated at 95°C for 15 min. Insoluble material was pelleted by centrifugation (18,000 × g, 60 s) and the supernatant stored at −20°C.

Lysates were amplified using specific primers for 7 loci (*gltA*, citrate synthase; *gyrB*, gyrase subunit B; *fh*, putative hydratase; *shmt*, serine hydroxymethyltransferase; *tyrB*, tyrosine aminotransferase; *adk*, adenylate kinase; *txn*, thioredoxin) as described previously.[4] Primers for the PCR[2] were standardized with universal tail C and tail D sequences (Integrated DNA Technology, Whitehead Scientific, Cape Town, South Africa). PCR amplification (KAPA SYBR FAST qPCR master mix, ABI Prism; Kapa Biosystems, Cape Town, South Africa) following the manufacturer's instructions on a PCR machine (StepOnePlus real-time; Applied Biosystems, Thermo Fisher Scientific, Johannesburg, South Africa) was performed as follows: 95°C for 5 min followed by 35 cycles of 95°C for 30 s and 60°C for 60 s. We visualized the PCR outcome using commercial software (StepOne software v.2.3; Thermo Fisher Scientific). PCR products with a cycle threshold value <25 were purified (illustra ExoProStar 1-step; GE Healthcare, Little Chalfort, Buckinghamshire, UK) and diluted in half with Tris-EDTA buffer (MilliporeSigma) before sequencing. A commercial sequencing kit (ABI Prism BigDye terminator v.3.1 cycle; Applied Biosystems) was used for the sequencing reactions with C and D tail primers using a quarter of the recommended concentration of BigDye terminator v.3.1. Sequencing products were purified with an ethanol precipitation and analyzed (3130*xl* genetic analyzer; Applied Biosystems).

Geneious v.8.0.4 (https://www.geneious.com) was used to assemble forward and reverse sequences, and the resulting consensus sequences were uploaded to the relevant PubMLST database (http://pubmlst.org/perl/bigsdb/bigsdb.pl?db=pubmlst_taylorella_seqdef&page=batchSequenceQuery) for strain type identification. No ambiguous sequencing results were observed.

A single sequence type (ST4) was identified from all South African samples, with 34 of 36 (94%) obtained from male and 2 of 36 (6%) from female horses (Table 1). Sample distribution by breed was as follows: Lipizzaner (23), Warmblood (10), Holsteiner (1), Andalusian (1), and Thoroughbred (1).

Table 1. *Taylorella equigenitalis* isolates (n = 36) obtained from crude extract of samples (genital swabs, n = 34; frozen semen samples, n = 2) collected in South Africa in 1996–2017. All samples had an identical allelic profile* (1-2-5-3-2-2-3) and strain type (ST4).

Cases	Sample type	Sex	Breed	Year
1, 2	FSS	Stallion	Lipizzaner	1996
3	GS	Stallion	Holsteiner	2011
4, 5, 6, 8, 31, 32	GS	Stallion	Warmblood	2011
7	GS	Stallion	Lipizzaner	2011
9	GS	Mare	Warmblood	2012
10	GS	Gelding	Lipizzaner	2011
11–29	GS	Stallion	Lipizzaner	2011
30	GS	Stallion	Andalusian	2011
33	GS	Mare	Thoroughbred	2011
34	GS	Gelding	Warmblood	2015
35	GS	Stallion	Warmblood	2017
36	GS	Stallion	Warmblood	2017

FSS = frozen semen sample; GS = genital swab.
* Allelic profile of *gltA-gyrB-fh-shmt-tyrB-adk-txn*.

The successful outcome of our study using MLST analysis for *T. equigenitalis* supported the utilization of crude extracts direct from clinical samples in cases in which bacterial isolates are unavailable. The samples examined in our study were from 14 of 15 properties at which *T. equigenitalis* was identified. However, evidence of the point introduction, the epidemiologic link between cases, and an identical ST of samples isolated from all other cases, strongly suggested that this remaining property would most likely have been associated with the same ST4.

A study reporting genotyping of German and Austrian *T. equigenitalis* isolates using repetitive extragenic palindromic (REP) PCR and pulsed-field gel electrophoresis (PGFE) included a South African Holsteiner stallion isolate, which was the index case (case 3) in our study and which was allocated the REP/PGFE genotype rep-E1/TE-A5.[17] In that report,[17] rep-E1/TE-A5 was present exclusively in the majority of Austrian Lipizzaners (n = 38) and additionally was identical to the TE011 genotype reported from 4 stallions imported from Austria and Germany as part of the U.S. outbreak from 1978 to 2010.[1] These data show that ST4, rep-E1/TE-A5, and TE011 represent a single *T. equigenitalis* type. These findings clearly support both a common source and breed of origin associated with the South African outbreak.

The epidemiologic findings during the initial outbreak investigation suggested a point introduction of *T. equigenitalis* into South Africa prior to outbreak identification in May 2011.[13] It also indicated that current stringent pre- and post-entry CEM importation screening procedures of all equids entering the country, implemented in the early 1980s, have to

date proved to be an effective barrier against the reintroduction of *T. equigenitalis* into South Africa.

We have shown that MLST assays for *T. equigenitalis* can be successfully performed on a crude extract originally prepared for PCR assays, negating the need for prior culture. This finding has important potential benefits for future large-scale investigation of CEM outbreaks in which organism identification is time-sensitive or isolates are not available. The analysis of MLST results from South Africa over a 21-y period showed the presence of a single ST, namely ST4, strongly suggesting a common, as yet unconfirmed, point introduction.

Acknowledgments

We thank Susan Miller and the staff of the Veterinary Genetics Laboratory, Faculty of Veterinary Science, Onderstepoort, for assistance with data analysis.

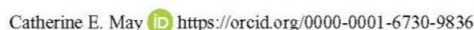
Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors received no financial support for the research, authorship, and/or publication of this article.

ORCID iD

Catherine E. May  https://orcid.org/0000-0001-6730-9836

References

1. Aalsburg AM, et al. Pulsed-field gel electrophoresis genotyping of *Taylorella equigenitalis* isolates collected in the United States from 1978 to 2010. J Clin Microbiol 2011;49:829–833.
2. Blacket MJ, et al. Universal primers for fluorescent labelling of PCR fragments—an efficient and cost-effective approach to genotyping by fluorescence. Mol Ecol Resour 2012;12:456–463.
3. Crowhurst R. Genital infection in mares. Vet Rec 1977;100: 476–476.
4. Duquesne F, et al. Development of a single multi-locus sequence typing scheme for *Taylorella equigenitalis* and *Taylorella asinigenitalis*. Vet Microbiol 2013;167:609–618.
5. Duquesne F, et al. Identification of *Taylorella equigenitalis* responsible for contagious equine metritis in equine genital swabs by direct polymerase chain reaction. Res Vet Sci 2007;82:47–49.
6. Erdman MM, et al. Diagnostic and epidemiologic analysis of the 2008–2010 investigation of a multi-year outbreak of contagious equine metritis in the United States. Prev Vet Med 2011;101:219–228.
7. Guthrie A, et al. Lateral transmission of equine arteritis virus among Lipizzaner stallions in South Africa. Equine Vet J 2003;35:596–600.
8. Hébert L, et al. Genome sequence of *Taylorella equigenitalis* MCE9, the causative agent of contagious equine metritis. J Bacteriol 2011:1785.
9. Hébert L, et al. Genomic characterization of the *Taylorella* genus. PLoS One 2012;7:1–13.
10. Jeoung H-Y, et al. First isolation of *Taylorella equigenitalis* from thoroughbred horses in South Korea. J Equine Vet Sci 2016;47:42–46.
11. Luddy S, et al. Contagious equine metritis within the United States: a review of the 2008 outbreak. J Equine Vet Sci 2010;30:393–400.
12. Matsuda M, et al. Recent advances in molecular epidemiology and detection of *Taylorella equigenitalis* associated with contagious equine metritis (CEM). Vet Microbiol 2003;97: 111–122.
13. May CE, et al. Polymerase chain reaction-based national surveillance programme to determine the distribution and prevalence of *Taylorella equigenitalis* in South African horses. Equine Vet J 2015;48:307–311.
14. Ricketts S, et al. Contagious equine metritis organism confirmed in Gloucestershire. Vet Rec 2012;170:398–398.
15. Rocha T. Contagious equine metritis in Portugal: a retrospective report of the first outbreak in the country and recent contagious equine metritis test results. Open Vet J 2016;6:263–267.
16. Schulman ML, et al. Contagious equine metritis: artificial reproduction changes the epidemiologic paradigm. Vet Microbiol 2013;167:2–8.
17. Sting R, et al. Genotyping of German and Austrian *Taylorella equigenitalis* isolates using repetitive extragenic palindromic (REP) PCR and pulsed-field gel electrophoresis (PFGE). Res Vet Sci 2016;109:101–106.
18. Sugimoto C, et al. Transfer of *Haemophilus equigenitalis* Taylor et al. 1978 to the genus *Taylorella* gen. nov. as *Taylorella equigenitalis* comb. nov. Curr Microbiol 1983;9:155–162.
19. Timoney P. Horse species symposium: contagious equine metritis: an insidious threat to the horse breeding industry in the United States. J Anim Sci 2011;89:1552–1560.
20. Wakeley P, et al. Development of a real time PCR for the detection of *Taylorella equigenitalis* directly from genital swabs and discrimination from *Taylorella asinigenitalis*. Vet Microbiol 2006;118:247–254.

Appendix C: In-treatment monitoring and use of a novel topical therapy shortens the duration to eliminate *Taylorella asinigenitalis* in donkey stallions

Veterinary Record Case Reports

HORSES AND OTHER EQUIDS

In-treatment monitoring and use of a novel topical therapy shorten the duration to eliminate *Taylorella asinigenitalis* in donkey stallions

Catherine May [1], Alan John Guthrie,[2] Martin Lance Schulman[1]

[1]Department of Production Animal Studies, University of Pretoria Faculty of Veterinary Science, Pretoria, South Africa
[2]Equine Research Centre, University of Pretoria Faculty of Veterinary Science, Pretoria, South Africa

Correspondence to
Dr Catherine May;
kate.may@up.ac.za

Received 17 January 2020
Revised 3 May 2020
Accepted 11 May 2020

SUMMARY
Postarrival quarantine testing of a consignment of imported miniature donkeys (*Equus asinus*) with a duplex *Taylorella equigenitalis/asinigenitalis* real-time quantitative PCR (qPCR) assay and bacteriological culture identified *T asinigenitalis* in one donkey stallion from the third set of samples collected at weekly intervals. Following transportation to an approved quarantine station, further testing showed the second companion donkey stallion to be additionally positive for *T asinigenitalis*. Daily topical treatment included the off-label application of a bovine intramammary antimicrobial preparation to the predilection sites with concurrent in-treatment sampling for qPCR to determine treatment end point. Treatment successfully eliminated colonisation with the organism in both donkey stallions within seven days. This report strongly supported multiple sampling opportunities and the inclusion of a duplex qPCR during postimportation screening. The modified treatment regimen appeared to facilitate the rapid interval to elimination of the organism and the prompt determination of treatment end point.

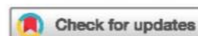
BACKGROUND

In the USA, an atypical strain of *Taylorella equigenitalis*, the causative agent of contagious equine metritis (CEM), was identified in donkey stallions in California (1987) and Kentucky (1988) following routine regulatory testing.[1] The organism was further characterised and shown to be a new (second) member of the genus *Taylorella*, and based on taxonomic studies was classified as *T asinigenitalis*.[1,2] Since then, *T asinigenitalis* has been reported from other regions in the USA,[3–5] Sweden (2004),[6] Italy (2008),[7] France (2011)[8] and the UK (2018).[9]

T asinigenitalis is considered non-pathogenic,[1,4,10] but has been cultured from the reproductive tract of mares following natural service with infected donkey stallions,[1,4] and shown to cause a transient metritis and cervicitis in mares following intrauterine infusion.[2] In that study, all experimentally infected mares (n=4) cleared themselves of clinical signs by day 35 postinoculation, although the organism was still cultured from one mare throughout the 111-day observation period.[2] In another study, *T asinigenitalis* persisted in two out of seven nurse mares despite two rounds of treatment, with one that remained culture-positive more than 300 days.[4] In mares, this organism seemingly showed a tropism for the proximal (uterus, cervix) rather than the distal (clitoral fossa and sinuses) reproductive tract,[3,4] which is dissimilar to the chronic carrier state seen in mares with *T equigenitalis*.[11] In male equids, the organism has a reported tropism for the urethral fossa, urethral sinus and urethra,[3,9] similar to *T equigenitalis*.[12] Transmission was reported via direct or indirect venereal routes[3] and fomites,[4,6] with two proposed cases of transplacental transmission.[3] A long-term carrier state[3,4] exists in male equids, although treatment appears to be curative.[4]

The main importance of *T asinigenitalis* is the difficulty in differentiating the two species using conventional methods,[13,14] with the possibility of misidentification of the organism as *T equigenitalis*, with subsequent grave implications for international trade[7] and the currently undefined pathogenicity in mares following natural infection.

In South Africa, CEM is a controlled animal disease requiring mandatory notification of the state regulatory authorities. Experience gained during the first South African outbreak of CEM in 2011[15] informed several modifications to the national diagnostic and treatment protocols,[16] including the use of a modified duplex quantitative PCR (qPCR) assay[17,18] able to distinguish *T equigenitalis* from *T asinigenitalis* for initial screening.[19] A molecular method was chosen in preference to bacterial culture due to the associated difficulty in differentiating between these two organisms owing to their similar colony morphology, growth rate and immunofluorescence characteristics when using the latter technique.[2] Internationally recognised treatment protocols[11] were applied during the initial response to the CEM outbreak in 2011. The experience of prolonged treatment periods with frequent retreatments and a reliance on bacteriology with an associated delay for detecting treatment end point prompted subsequent modifications to these protocols. These included strategically applied in-treatment qPCR testing in infected horses to determine treatment end point as defined by elimination of target nucleic acid. In addition, topical treatment included the off-label application of dry cow intramammary antimicrobial preparations.[20] The application of these preparations facilitated focused deposition and prolonged contact time within the predilection sites in affected female (clitoral fossae and sinuses) and male (urethral fossae and sinuses) horses. These modifications appeared to be key in significantly reducing the duration of treatment of four horses (authors' experience). These treatment



© British Veterinary Association 2020. No commercial re-use. See rights and permissions. Published by BMJ.

To cite: May C, Guthrie AJ, Schulman ML. *Vet Rec Case Rep* Published Online First: [*please include* Day Month Year]. doi:10.1136/vetreccr-2020-001082

modifications subsequently informed the protocol implemented in the two affected miniature donkey stallions.

CASE PRESENTATION

During the course of routine postarrival testing in 2015 of five miniature donkey (*Equus asinus*) mares and two donkey stallions originating from the USA, one donkey stallion tested positive for *T asinigenitalis* using a duplex *T equigenitalis/asinigenitalis* qPCR assay.[17] This positive sample was obtained on the third of three prescribed weekly tests. Following its reporting, the state regulatory authority transported this donkey stallion (jack 1: born 2015, 37 kg) accompanied by the second donkey stallion (jack 2: born 2007, 92 kg) as a companion to an additional quarantine station approved for further management.

INVESTIGATIONS

For confirmatory sample collection, the donkey stallions were sedated intravenously with detomidine hydrochloride (Domosedan, Pfizer Laboratories) at a dose of 0.005 mg/kg (jack 1) and 0.01 mg/kg (jack 2) in combination with butorphanol tartrate (Torbugesic, Zoetis) at 0.005 mg/kg (jack 1) and 0.01 mg/kg (jack 2) to facilitate penile extrusion. Standard dry swabs (Labchem) were obtained sequentially from each of the prescribed predilection sites: the distal urethra, urethral fossa (including sinus) and preputial *lamina interna*. A duplicate set of swabs were collected concurrently and transported in Amies charcoal medium at 2°C–8°C for bacterial culture.

Swabs for qPCR assay were transferred to the Veterinary Genetics Laboratory, University of Pretoria for testing. A TaqMan PCR assay was used based on the method described by Wakeley and others.[17] The DNA was extracted from the swabs and qPCR conducted as previously described.[18] Positive samples were subjected to multilocus sequence typing (MLST) analysis as previously described.[21]

The swabs for bacterial culture were transferred to the Agricultural Research Council, Onderstepoort Veterinary Institute, Onderstepoort, Gauteng within 24 hours of collection for culture according to the World Organisation for Animal Health Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.[22]

In order to monitor treatment efficacy and to determine treatment end point, diagnostic samples were obtained before the daily commencement of treatment. The three predilection sites were swabbed as previously described and samples immediately transferred for subsequent testing by qPCR assay. A positive test was defined as a cycle threshold (Ct) less than 40.[18]

TREATMENT

The two miniature donkey stallions were managed indoors under quarantine conditions. They were housed in the same stable (3 m x 3.4 m) on straw bedding with access to grass hay (*Eragrostis tef*) supplemented with concentrates via a pelleted ration.

The donkey stallions were treated daily following the revised method as described in the legislation.[16] Briefly, the donkey stallions were sedated as previously described, and following extrusion the penis was thoroughly cleansed by irrigation with 4 per cent chlorhexidine gluconate solution (Bioscrub, Medinox) applied with dampened cotton wool swabs. Additionally, plain cotton-tipped swabs dipped in 4 per cent chlorhexidine gluconate were applied to the urethral sinus and fossa to thoroughly cleanse these sites. Thereafter the penis and prepuce were dried using disposable paper towel. A commercially available dry cow intramammary preparation containing 200-mg sodium cloxacillin and 75-mg sodium ampicillin (Curaclox DC intramammary, Norbrook Laboratories) was applied directly via its applicator into the urethral fossa and its sinus. Thereafter a silver sulphadiazine cream (Silbecor, Biotech Laboratories) was applied topically to the mucosal surfaces of the penile shaft and the preputial *lamina interna*.

OUTCOME AND FOLLOW-UP

Assay results are summarised in table 1.

The most commonly affected site was the urethral fossa and sinus—jack 1: four out of eight (50 per cent); jack 2: six out of eight (75 per cent)—followed by the urethra in both donkey stallions: two out of eight (25 per cent). The interval to elimination or treatment end point was four days (jack 1: 15–18 October) and seven days (jack 2: 15–21 October), respectively.

An MLST assay using the seven prescribed housekeeping genes[13] showed the following allelic profile: new allele-unable to type-17-10-8-10-15 (*gltA*, citrate synthase; *gyrB*, gyrase subunit B; *fh*, putative hydratase; *shmt*, serine hydroxymethyltransferase; *tyrB*, tyrosine aminotransferase; *adk*, adenylate kinase; *txn*, thioredoxin, respectively).

Treatment end point was defined when all animals were confirmed negative on all sites for two consecutive days, after which they were treated for an additional three days to confirm negative status. The donkey stallions were released from the quarantine centre and transported to their owner's farm in the Free State Province, where they remained under state-supervised quarantine conditions pending follow-up testing. All results from the follow-up testing at five and six weeks post-treatment by both qPCR and bacterial culture were negative.

DISCUSSION

As initial diagnosis of *T asinigenitalis* was made only on the third (and final) sampling attempt, this strongly supported multiple sampling opportunities for effective screening. Interestingly, the donkey stallion initially diagnosed positive was a yearling and probably prepubertal. The second, mature donkey stallion, initially negative, became positive only following its transfer as a companion to the affected donkey stallion. This followed their housing in close proximity within the same stable during treatment, where previously during postarrival quarantine all donkey mares and stallions were separated. Rigorously applied biosecurity protocols and the observation that jack 2 became positive before commencement of treatment reduced the possibility of this finding being due to contamination of samples or cross-contamination during treatment and suggested possible lateral fomite transmission. This non-venereal route is recognised as an important mode in transmission of *T asinigenitalis* in male equids.[3 4 6] As their reproductive history was unknown, the initial source of infection of jack 1 remains unidentified.

MLST is a molecular technique which uses conserved housekeeper genes[13] to accurately identify bacterial genotypes. Housekeeper genes are chosen based on their stable allelic profile and relatively slow accumulation of nucleotide changes.[23] MLST analysis is highly discriminatory and allows direct, unambiguous interlaboratory comparison of results.[24] An MLST scheme has been compiled for the *Taylorella* genus and is available at http://pubmlst.org/taylorella/. The MLST analysis of the *T asinigenitalis* isolate in this study most closely matched the strain isolated from a male horse in Sweden (8-10-17-10-8-10-16).[6]

May C, *et al. Vet Rec Case Rep* 2020;**8**:e001082. doi:10.1136/vetreccr-2020-001082

Table 1 Summary of the results of a qPCR assay for *Taylorella equigenitalis/asinigenitalis* and bacteriological culture from swabs obtained from the predilection sites on the external genitalia of two miniature donkey stallions

Animal	Assay	Timeline (2015)																													
		Postarrival quarantine				In-treatment testing																								Follow-up testing	
		16 September	23 September	30 September	9 October	15 October			16 October			17 October			18 October			19 October			20 October			21 October			22–26 October			1 December	8 December
						A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C		
Jack 1	Culture				+																									–	–
	qPCR	–	–	30.8		–	24.3	–	31.6	28.5	–	35.3	32.0	–	–	34.6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Jack 2	Culture				–																									–	–
	qPCR	–	–	–		–	37.5	–	–	30.6	–	–	32.3	–	35.9	32.3	–	32.5	–	–	–	33.8	–	–	39.3	–	–	–	–	–	–

'+': positive culture; '–': negative culture/qPCR.
A: urethra; B: urethral fossa and sinus; C: lamina interna.
qPCR, quantitative PCR.

The organism was isolated mainly from the urethral fossa, sinus and urethra, supporting previous reports in donkeys and horses.[1 3 6 9] On commencement of treatment in both donkey stallions, Ct values on daily qPCR testing initially showed high then lower (increased nucleic acid 'load') values before increasing again to above the threshold value at elimination. The authors speculated that this pattern of Ct values before organism elimination was due to mechanical agitation and exposure of the organism from within its smegma-associated mucosal predilection sites during the treatment process. Following initial negative qPCR results, due prudence dictated continuation of treatment for several days to ensure elimination of the organism.

Previously described treatment protocols for *T asinigenitalis* described either a 10-day protocol with multiplex PCR screening at 14, 21 and 28 days after the beginning of treatment,[7] or a 10-day protocol with follow-up bacteriology at 21, 28 and 35 days after treatment end point followed by test breeding.[4] In comparison, the donkey stallions in this study required up to seven days of treatment for elimination of *T asinigenitalis* as determined by qPCR assay with bacteriological confirmation five and six weeks post-treatment. Selection of a topical antimicrobial formulated as an intramammary product was suggested to have facilitated this outcome. The authors speculated this to be associated with both its fine-bore intramammary applicator able to reach the elusive anatomical predilection sites and the 'dry cow' formulation that provided prolonged mucosal contact time. A bovine intramammary antimicrobial was used off-label, based on the prescribing cascade, due to the lack of an equivalent registered product for use in equids.[25] The animals were monitored closely for any adverse reactions. A shortcoming of this study was that an antibiogram to determine antimicrobial susceptibility was not performed before commencement of treatment. Few studies have documented antimicrobial susceptibility in *T asinigenitalis* isolates; however, the majority of strains show resistance to streptomycin.[4–7] A study of 43 French *T asinigenitalis* isolates did however report resistance to a number of antimicrobials including ampicillin.[8] Although an antibiogram was not performed during this study, the short interval to treatment end point strongly suggested that the South African *T asinigenitalis* isolate was susceptible to penicillin.

Although *T asinigenitalis* is considered non-pathogenic in horses, further epidemiological investigation is indicated to define the disease risk associated with this organism in equids. *T asinigenitalis* has been reported in association with a transient cervicitis and metritis in mares[2]; however, culture-positive, *T asinigenitalis*-infected mares do not appear to exhibit clinical signs following natural service.[4] Unfortunately this study[4] did not report the number of breedings per pregnancy in affected mares as this may have given an indication of the impact of *T asinigenitalis* on reproductive performance. During the 2011 South African outbreak of *T equigenitalis*, the actual number of mares that might have become infected remained undefined largely due the prolonged interval between potential exposure and traceback implementation and their ability to clear themselves of infection.[18] Due to similar pathogenicity, the actual number of *T asinigenitalis*-positive mares in an outbreak will remain largely undefined, which poses a risk in systems using natural cover. The potential misidentification of *T asinigenitalis* as *T equigenitalis* when using traditional bacterial culture methods[13] has potentially far-reaching implications for both local and international trade.

Limited experience of successful treatment of *T equigenitalis* in four horses (three males and one female) and *T asinigenitalis* in two donkey stallions using bovine intramammary antimicrobial

preparations applied to the genital predilection sites provides a direction for further research to define an enhanced treatment regimen for these cases.

Learning points

- Duplex quantitative PCR provides a sensitive, rapid screening method to identify *Taylorella asinigenitalis*.
- Multiple sampling opportunities are required to identify the organism.
- In-treatment quantitative PCR testing allows monitoring of treatment end point.
- Bovine antimicrobial intramammary preparation allows targeted, sustained treatment.

Contributors All authors contributed equally to this manuscript.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests None declared.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon request.

ORCID iD
Catherine May http://orcid.org/0000-0001-6730-9836

REFERENCES

1 Jang SS, Donahue JM, Arata AB, *et al*. *Taylorella asinigenitalis* sp. nov., a bacterium isolated from the genital tract of male donkeys (*Equus asinus*). *Int J Syst Evol Microbiol* 2001;51:971–6.
2 Katz JB, Evans LE, Hutto DL, *et al*. Clinical, bacteriologic, serologic, and pathologic features of infections with atypical *Taylorella equigenitalis* in mares. *J Am Vet Med Assoc* 2000;216:1945–8.
3 Donahue JM, Timoney PJ, Carleton CL, *et al*. Prevalence and persistence of *Taylorella asinigenitalis* in male donkeys. *Vet Microbiol* 2012;160:435–42.
4 Meade BJ, Timoney PJ, Donahue JM, *et al*. Initial occurrence of *Taylorella asinigenitalis* and its detection in nurse mares, a stallion and donkeys in Kentucky. *Prev Vet Med* 2010;95:292–6.
5 Reilly TJ, Calcutt MJ, Ganjam IK, *et al*. Isolation and identification of *Taylorella asinigenitalis* from a mare in Oklahoma, USA. *Clin Theriogenol* 2012;4:163–7.
6 Båverud V, Nyström C, Johansson K-E. Isolation and identification of *Taylorella asinigenitalis* from the genital tract of a stallion, first case of a natural infection. *Vet Microbiol* 2006;116:294–300.
7 Franco A, Donati V, Troiano P, *et al*. Detection of *Taylorella asinigenitalis* in donkey jacks in Italy. *Vet Rec* 2009;165:540–1.
8 Breuil M-F, Duquesne F, Laugier C, *et al*. Phenotypic and 16S ribosomal RNA gene diversity of *Taylorella asinigenitalis* strains isolated between 1995 and 2008. *Vet Microbiol* 2011;148:260–6.
9 Mawhinney I, Florea L, Torrens N, *et al*. *Taylorella asinigenitalis* in an onager in a UK zoo. *Vet Rec* 2018;183:266.
10 Arata AB, Cooke CL, Jang SS, *et al*. Multiplex polymerase chain reaction for distinguishing *Taylorella equigenitalis* from *Taylorella equigenitalis*-like organisms. *J Vet Diagn Invest* 2001;13:263–5.
11 Luddy S, Kutzler MA. Contagious equine metritis within the United States: a review of the 2008 outbreak. *J Equine Vet Sci* 2010;30:393–400.
12 Timoney PJ. Contagious equine metritis. *Comp Immunol Microbiol Infect Dis* 1996;19:199–204.
13 Duquesne F, Hébert L, Breuil M-F, *et al*. Development of a single multi-locus sequence typing scheme for *Taylorella equigenitalis* and *Taylorella asinigenitalis*. *Vet Microbiol* 2013;167:609–18.
14 Moore JE, Millar BC, Xu J, *et al*. Possible misidentification of *Bacteroides sp.*, probably *B. ureolyticus* as *Taylorella equigenitalis*: implications for the laboratory diagnosis of CEM. *Vet Res* 2001;32:611–6.
15 OIE. Contagious equine Metritis: South Africa, immediate notification, 2011. Available: https://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?reportid=10553
16 Directorate Animal Health: Department of Agriculture, forestry and fisheries. *Veterinary procedural notice: the confirmation of diagnosis and treatment of contagious equine Metritis*, 2019. https://www.daff.gov.za/daffweb3/Branches/Agricultural-Production-Health-Food-Safety/Animal-Health/information/dahpolicy
17 Wakeley PR, Errington J, Hannon S, *et al*. Development of a real time PCR for the detection of *Taylorella equigenitalis* directly from genital swabs and discrimination from *Taylorella asinigenitalis*. *Vet Microbiol* 2006;118:247–54.
18 May CE, Guthrie AJ, Keys B, *et al*. Polymerase chain reaction-based national surveillance programme to determine the distribution and prevalence of *Taylorella equigenitalis* in South African horses. *Equine Vet J* 2016;48:307–11.
19 Directorate Animal Health: Department of Agriculture, forestry and fisheries. *Veterinary procedural notice: surveillance for contagious equine Metritis in South Africa*, 2019. https://www.daff.gov.za/daffweb3/Branches/Agricultural-Production-Health-Food-Safety/Animal-Health/information/dahpolicy
20 May CE, Guthrie AJ, Schulman ML. Addition of an intramammary antimicrobial formulation markedly decreased the intervals to elimination of *Taylorella equigenitalis* in a carrier mare and gelding. *J Equine Vet Sci* 2016;39:S62.
21 May CE, Guthrie AJ, Schulman ML. Direct culture-independent sequence typing of *Taylorella equigenitalis* obtained from genital swabs and frozen semen samples from South African horses. *J Vet Diagn Invest* 2019;31:792–4.
22 Chapter 2.5.2 contagious equine Metritis. Manual of diagnostic tests and vaccines for terrestrial animals, 2018. Available: http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.05.02_CEM.pdf [Accessed Aug 2019].
23 Enright MC, Spratt BG. Multilocus sequence typing. *Trends Microbiol* 1999;7:482–7.
24 Feil EJ, Li BC, Aanensen DM, *et al*. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J Bacteriol* 2004;186:1518–30.
25 Teale CJ, Moulin G. Prudent use guidelines: a review of existing veterinary guidelines. *Rev Sci Tech* 2012;31:343–54.

Copyright 2020 British Veterinary Association. All rights reserved. For permission to reuse any of this content visit
http://www.bmj.com/company/products-services/rights-and-licensing/permissions/
Veterinary Record Case Reports subscribers may re-use this article for personal use and teaching without any further permission.

Subscribe to Vet Record Case Reports and you can:
- Submit as many cases as you like
- Enjoy fast sympathetic peer review and rapid publication of accepted articles
- Access all the published articles
- Re-use any of the published material for personal use and teaching without further permission

For information on Institutional Fellowships contact consortiasales@bmjgroup.com

Visit vetrecordcasereports.bvapublications.com for more articles like this and to become a subscriber