Epidemiological investigation of the first reported outbreak of Contagious Equine Metritis in South Africa

By

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Declaration

I, Catherine Edith May, do hereby declare that the research presented in this dissertation, was conceived and executed by myself, and apart from the normal guidance from my supervisor and co-supervisor, I have received no assistance.

Neither the substance, nor any part of this dissertation has been submitted in the past, or is to be submitted for degree at this University or any other University.

This dissertation is presented in partial fulfillment of the requirements for the degree in MMedVet: Theriogenology.

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Signed,

Catherine Edith May

Date: 13th November 2012

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Summary

This dissertation describes the epidemiological investigation and management of the first outbreak of Contagious Equine Metritis (CEM) reported in South Africa. In addition, the subsequent implementation of a nationwide quantitative polymerase chain reaction (qPCR)-based stallion screening programme and traceback of exposed animals to define the spread of CEM in South Africa is described.

The first South African outbreak of CEM caused by the bacterium, *Taylorella equigenitalis* was reported on the 9th May 2011 to the World Health Organisation for Animal Health (OIE). The outbreak was recognized subsequent to the importation of a young Warmblood stallion from Germany. The outbreak initially appeared confined to a single index property (focus property), an equine breeding facility in Midrand, Gauteng, South Africa with a single confirmed case of transmission involving the index stallion and a Thoroughbred mare.

The initial response was rapidly instituted following the suspicion of *T. equigenitalis* on the index property. This included an inspection of the index property and its records. A risk-classification of in-contact animals allocated them to "high," "moderate" or "low"-risk categories. The classification was dependent on the temporal relationship of their presence on the index property relative to the period of residence of the index cases. After *T. equigenitalis* infection was confirmed from both index cases, the breeding facility was placed under state–administered quarantine and all exposed mares and the index cases were transferred to a quarantine facility. The animals were re-tested by genital swabbing for bacterial culture following a standard protocol according to internationally-accepted practice (OIE Terrestrial Manual on Contagious Equine Metritis). Additional duplicate swabs were obtained for real time qPCR. None of the mares were shown to be positive on either bacterial culture or qPCR. All animals were however treated according to an accepted protocol for *T. equigenitalis* infection (Luddy and Kutzler, 2010, UK Horseracing Betting Levy Board (HBLB) Code of Practice, 2011).

All stallions that had been present on the index property and undergone semen collection on the same day as the index stallion were classified as "moderate risk" and were similarly tested. This identified two additional *T. equigenitalis*-positive stallions, which were confirmed on bacterial culture (World Organization for Health (OIE) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Chapter 2.5.1, Contagious Equine Metritis version adopted 20/10/2011) and which were subsequently quarantined and successfully treated.

Following recognition of this outbreak, the Department of Agriculture, Forestry and Fisheries (DAFF) in consultation with the Equine Research Centre (ERC) of the Faculty of Veterinary Science at the University of Pretoria promulgated a nationwide qPCR-based screening programme that aimed to establish the prevalence and distribution of *T. equigenitalis* in South Africa. This required certification of all South African stallions used for breeding either by natural cover or assisted reproductive techniques. The compliance for certification was based on submission of two sets of genital swabs that both tested negative for *T. equigenitalis* on qPCR-testing. The process was coordinated by a web-based platform (http://www.cemsa.co.za)

As of 31st October 2013, an additional 33 carrier stallions have been identified by this screening programme. Of these stallions, 23 have been confirmed on bacteriology according

to the prescribed World Organisation for Health (OIE) method (Terrestrial Manual on Contagious Equine Metritis). All stallions apart from one could be linked to the index property.

As of 31st October 2013, two additional *T. equigenitalis*-positive females have been identified, following a traceback of all identified potentially-exposed mares and their offspring that was instituted in September 2012. An "exposed mare" was defined as a mare that had been bred, either by natural breeding or assisted breeding techniques to the index stallion or any other *T. equigenitalis*-positive stallion. These mares were distributed across five provinces of South Africa at the time the traceback was initiated.

During the nationwide traceback, a subpopulation focus was identified when 24 of the 33 resident stallions at the South African Lipizzaner Centre, Midrand, Gauteng tested positive for *T. equigenitalis*. Six of these stallions had visited the index property for semen collection over the course of several years prior to the arrival of the index stallion. This suggested the possible albeit undetected presence of *T. equigenitalis* on these premises prior to the arrival of the first reported index case. We strongly suspected that there may have been undetected CEM incursions into South Africa in the past. The index case in this outbreak may either have introduced a new infection or was infected by a pre-existing source of infection subsequent to his arrival. It is hoped that future strain-typing of the isolates from the positive cases identified during this intervention will further clarify this scenario.

The use of qPCR-based screening proved to be a highly specific and sensitive method for detecting *T. equigenitalis* and helped to define the distribution and prevalence of *T. equigenitalis* in breeding stallions and exposed mares in South Africa. In addition, this method had significant practical advantages with respect to the associated costs, turn-around times and in-the-field application when compared with bacterial culture. The institution of a web-based platform from which the national screening programme was launched and co-ordinated proved to be indispensable in managing stakeholder access and information availability.

To date, 31st October 2013, a total of 39 horses (36 males and 3 females) have been identified as *T. equigenitalis*-positive and have all subsequently been successfully treated.

Chapter 1: Literature review

1.1. Historical context

Contagious equine metritis is a non-systemic, venereally-transmitted disease of horses caused by the bacterium, *Taylorella equigenitalis.* The organism was first described as *Haemophilis equigenitalis* by Taylor et al. (1978) but was later renamed *Taylorella equigenitalis* (Int. J. Syst. Bacteriol., 1984). It was first described in Thoroughbred horses during the 1977 breeding season in the United Kingdom (Crowhurst, 1977; Platt and Atherton, 1977; Timoney et al, 1977) following the introduction of Thoroughbred mares from Ireland to breed with stallions in the Newmarket area. The infection was thought to have originated from France in 1976 after Thoroughbred mares were shipped internationally to Ireland. (Eaglesome and Garcia, 1979; Luddy and Kutzler, 2010). 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 infected (Eaglesome and Garcia, 1979; Luddy and Kutzler, 2010). This resulted in closure of the National Stud and other stud farms in the Newmarket area, resulting in massive losses in both stud fees and foal sales, and a reported decrease in the foaling rate to 42% from 72% the previous year (Holden 1978; Luddy and Kutzler 2010).

Despite the rapid imposition of a ban on the importation of horses from the United Kingdom and Europe into the USA and Canada, *T. equigenitalis* was confirmed in Kentucky (USA) in 1978 (Holden 1978; Swerczek, 1978), and in Missouri (USA) and Canada in 1979 (Eaglesome and Garcia, 1979). The 1978 outbreak was estimated to have cost the Kentucky Thoroughbred breeding industry one million dollars a day and the estimated cost of eradicating *T. equigenitalis* from the United States was 13.5 million dollars. (Kristula and Smith, 2004; Timoney 2011)

The disease has attained worldwide significance, and the regulatory restrictions associated with CEM continue to limit the movement and trade of horses, affecting the Thoroughbred and other breeding industries. The costs associated with CEM are not only due to direct economic loss as a result of infertility, but also due 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 (Schulman et al. 2013)

Following the first reported cases of CEM in the United Kingdom and Ireland, the disease has been found to have a worldwide distribution and has been confirmed in various countries including several within Europe, the USA, Australia and Japan (Hughes et al, 1978; Swerczek, 1978; Blobel et al, 1980; Knowles, 1983; Parlevliet et al, 1997; Kristula 2007; Luddy and Kutzler, 2010).

1.2. Bacterial culture and molecular genotyping

T. equigenitalis is a non-motile, microaerophilic Gram-negative, frequently pleiomorphic bacterium (Platt and Atherton, 1977; Ricketts et al, 1977; Timoney et al, 1977; Taylor et al, 1978; Timoney, 2011). It is fastidious and slow growing and is rapidly overgrown by other bacteria present in the reproductive tract of horses during bacterial culture (Bleumink-Pluym et al, 1994; Matsuda and Moore, 2003; Duquesne et al, 2007). It is sensitive to a broad range of antimicrobials and disinfectants (Taylor et al, 1978; Timoney, 2011; Schulman et al, 2013)

In 1997, the National Veterinary Services Laboratory in California identified a bacterial isolate (UCD-1^T) from a donkey jack (*Equus asinus*) that showed similar colony morphology, growth rate and immunofluorescence characteristics to *T. equigenitalis.* Interestingly, serum from the jack reacted with antibodies in the Complement Fixation (CF) test for *T. equigenitalis*, a feature that is absent in the sera of *T. equigenitalis*-positive stallions (Jang et al, 2001). In early 1998, Katz et al (2000) confirmed two atypical isolates from two donkey jacks in Kentucky (UK-1 and UK-2). The CF test for antibodies to *T. equigenitalis* was also positive on the sera from both the Kentucky jacks. Mares bred naturally (Kentucky) or artificially (California) to the infected jacks did not develop clinical signs of disease, although the micro-organism was cultured from the mares bred by natural service (Jang et al, 2001). Further studies by Katz et al (2000) showed that intrauterine inoculation of mares with the Kentucky strain of the atypical isolate resulted in transient clinical signs of an abnormal vaginal discharge, cervicitis and shortened oestrus cycles. These mares responded several days later and with less intensity than mares inoculated with classic *T. equigenitalis* isolates. Sequence analysis of the 16S rDNA (97.6% similarity), DNA-DNA hybridization studies (23% similarity) and G + C composition analysis of the genomic DNA between *T. equigenitalis* and these three donkey isolates confirmed that the isolates were closely related but not identical to *T. equigenitalis* strains (Matsuda and Moore, 2003) and a new species of the genus *Taylorella*¸ *T. asinigenitalis* was proposed (Jang et al, 2001). This new species had slower growth rates and a weaker positive reaction in the immunofluorescent antibody test than *T. equigenitalis.*

Two biotypes (strains) of *T. equigenitalis* are recognised based on their sensitivity or resistance to streptomycin (Taylor et al, 1978; Matsuda and Moore, 2003). All strains of *T. equigenitalis* isolated in the United Kingdom, Australia and Ireland have been streptomycin-resistant (Timoney and Powell, 1988). Given the relative ease with which the organism may acquire streptomycin resistance, and based on the isolation of streptomycin-sensitive strains of the organism from several European countries, it is likely that CEM originated in these countries and was introduced into the equine populations of other countries following the importation of unapparent carrier animals (Timoney and Powell, 1988).

Aalsburg and Erdman (2011) reported that streptomycin sensitivity values can differ within a pulsed-field gel electrophoresis (PFGE) genotype. Their results showed that among five isolates displaying one genotype (identified as TE015) that three were streptomycin-resistant and two were streptomycin-susceptible.

Hébert et al (2011) first described the genomic sequence for *T. equigenitalis* and it is available on Genbank via accession number CP002456.

Bleumink-Pluym et al (1990) compared 20 Dutch isolates and 12 isolates from other countries by Field Inversion Gel Electrophoresis (FIGE) of genomic restriction endonuclease fragments. They determined that all 32 strains could be designated into one of five different restriction patterns, that were designated A-E. Group A contained strains that were isolated from Thoroughbreds only (1977-1982). Group B contained the strain isolated from the Belgian outbreak (1978), the streptomycin-susceptible strain isolated from a horse in the United States (1979) and the strain isolated from a Dutch saddle horse (1985). Group C contained strains from Austria (1982) and Switzerland (1988). Group D contained the strain from Germany (1979) and strains from Dutch Halflinger horses (1988) and Dutch saddle horses (1986 and the first two outbreaks of 1987). Group E contained all Dutch strains

isolated from trotters (1988) and the only strain isolated from the third outbreak of 1987 in a Dutch saddle horse.

Matsuda et al (1999, 2003) employed crossed-field gel electrophoresis (CFGE) to describe the molecular genotyping of various isolates of *T. equigenitalis* from around the world. They showed that only a single genotype of *T. equigenitalis,* designated 'Genotype J' was present among all the isolates found in Japan over a 16 year period. Their research also showed that amongst various isolates from Ireland, the USA and Southern Australia, the strain Kentucky 188 was common to all countries and was detected in 22 of 34 isolates in these three countries. Similarly, the Kentucky 188 strain was found to be identical to 2 English and 4 French isolates, suggesting a common source.

Aalsburg and Erdman (2011) analysed a total of 82 strains of *T. equigenitalis* isolated in the United States. Twenty-eight of those strains were isolated from horses in the 2009 U.S. outbreak (CEM09). Using the restriction enzyme ApaI, the 82 isolates could be clustered into 15 different genotypes that clearly defined epidemiologically related groups of horses. These results suggested a common source for the outbreak that was not linked to previous occurrences of CEM in the United States.

1.3. Pathogenesis and clinical signs

Clinical signs seen in naïve mares exposed to *T. equigenitalis* can vary significantly from overt signs of the disease to a subclinical infection. Mares typically show a muco-purulent vaginal discharge and irregular interoestrus periods. The incubation period varies from 2-12 days (Timoney and Powell, 1988; Lu and Morresey, 2007). Mares may suffer from endometritis, cervicitis or vaginitis, or may show no clinical signs (Platt et al, 1978; Ricketts et al, 1978; Timoney and Powell, 1988; Timoney, 1996; Luddy and Kutzler, 2010; Erdman et al, 2011). Bleumink-Pluym et al (1996) studied the kinetics of invasion and replication of four strains of *T. equigenitalis*. The strains belonged to two difference FIGE groups: A and E. They showed a marked difference in *in vitro* invasion of and intracellular replication in equine derm cells amongst the 4 strains observed. Invasiveness of strains from the same outbreak occurred at similar levels. Two early isolates were considered highly contagious and showed high invasion levels in the study. There was, however, no association between invasiveness and replication index of the strains. There was also considerable variation in intracellular replication ability within each group, leading to the observation that the milder clinical signs of CEM observed in Thoroughbreds since the disease was first reported may be associated with the loss of intracellular reproducibility.

Most mares rid themselves of infection and develop a short-lived humoral antibody response. Subsequent reinfection does not appear to result in such severe clinical signs (Timoney et al, 1978; Timoney and Powell, 1988; Luddy and Kutzler, 2010; Timoney, 2011). However, it has been reported that up to 20-25% of aymptomatically infected mares may attain carrier status which may last for many months or even years (Timoney, 1996; Matsuda and Moore, 2003; Luddy and Kutzler, 2010; Timoney, 2011). Most commonly, carrier mares harbour the organism in the clitoral fossa and sinuses, although (rarely) the organism may be carried in the endometrium. It has been reported in some *T. equigenitalis*-positive mares to persist in the endometrium throughout pregnancy, resulting in the birth of *T. equigenitalis*-positive foals, through apparent vertical transmission (Timoney and Powell, 1988; Luddy and Kutzler, 2010, Timoney, 2011). Rarely, abortions have been reported (Nakashiro et al 1981, cited by Timoney 2011). Wood et al (2005) reported a difference in the distribution of *T. equigenitalis*

between the initial infection and subsequent carrier status; a detection rate of 93% (clitoral) and 31% (cervix) was reported in chronically affected mares versus 69% (clitoral) and 84% (cervix) in acutely infected mares.

Stallions are unapparent carriers of the organism and this smegma-associated commensal merely colonises the predilection sites of the external genitalia without eliciting an immune response or clinical signs. The organism shows a tropism for the urethra, the urethral sinus and the *lamina interna* (Platt et al, 1978; Timoney and Powell, 1988; Kristula and Smith, 2004, Timoney, 1996; Luddy and Kutzler, 2010; Erdman et al, 2011). Affected stallions are the principal source of infection and the carrier status may persist for months or years (Timoney and Powell, 1988; Schulman et al, 2013). There has been one reported case describing the isolation of *T. equigenitalis* from the testis, epididymis, seminal vesicles and urethra of an experimentally infected stallion at necropsy (Schluter et al, 1991, cited by Timoney, 2011)

1.4. Transmission

T. equigenitalis is a venereally-transmitted organism spread directly or indirectly during natural mating or artificial insemination with fresh, cooled or extended semen from a carrier stallion (Timoney, 2011). An important trend of increasing significance especially in recent outbreaks, 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 (Aalsburg and Erdman, 2011; Timoney, 2011; Schulman et al, 2013). *T. equigenitalis* has been reported to persist in the reproductive tract of pregnant mares without appearing to compromise the health of the unborn foal. Such foals may apparently be born *T. equigenitalis*-positive on their external genitalia, or may be exposed shortly after birth through contact with infected placenta, foetal fluids or the contaminated clitoral area of the mare at foaling (Timoney and Powell, 1988; Luddy and Kutzler, 2010; Timoney, 2011)

The rate of transmission is believed to be related to the concentration of *T. equigenitalis* on the external genitalia at the time of breeding and also the susceptibility or resilience of the mare exposed to the infection (Timoney, 2011).

Due to the increasing importance of assisted reproductive techniques such as artificial insemination (AI) in the transmission of *T. equigenitalis,* Olivieri et al (2011) investigated the effect of antibiotic-containing semen extenders on the growth of *T. equigenitalis* in extended semen. They found that two semen extenders VMDZ (Reproduction Resources, Walworth, WI) and INRA 96 (INRA 96 extender; IMV International Corp., Maple Grove, MN) with the additional of various concentrations of a ticarcillin-clavulanate antibiotic reduced the presence of *T. equigenitalis* in extended semen when compared to INRA 96 as supplied by the manufacturer or the extender E-Z Mixin® BF (Animal Reproductive Systems, Chino, CA) which contains no antibiotic.

The risks associated with cryopreserved semen from a *T.* equigenitalis-positive stallion have not yet been established, although they are believed to be minimal (Timoney, 2011). Klein and co-workers (personal communication reported by Timoney, 2011) failed to achieve

transmission of *T. equigenitalis* in a limited number of mares using AI with cryopreserved semen collected and processed from a *T. equigenitalis*-positive stallion.

Survival of *T. equigenitalis* outside the body is reported to be brief, as the organism is susceptible to many disinfectants, ultraviolet light, high temperatures and reduced humidity (Timoney 2011; Schulman et al 2013). The organism does, however, survive as a surface contaminant associated with various fomites if environmental conditions are favourable, such as those associated with stallion handling and breeding activities (Timoney, 1996, 2011). Survival of the organism is suspected to be enhanced by the presence of smegma on the external genitalia of carrier stallions and mares which presumably acts as a protective barrier.

Of the 1005 animals included in a traceback of the outbreak investigated in the USA between December 2008 and March 2010, 23 carrier stallions and 5 carrier mares were identified as positive for *T. equigenitalis* (Erdman et al, 2011, Timoney, 2011). This constituted a prevalence of 2.2% in stallions and 0.49% in mares in the animals tested. In comparison, in the South African outbreak, between April 2011 and January 2013, a total of 2659 male and 68 exposed female horses in South Africa were screened for CEM using qPCR. Of these, 36 carrier stallions and 3 mares were identified and confirmed using bacterial culture. An overall prevalence of 1.43%, with 36/2659 (1.35%) of males and 3/68 (4.41%) of females among tested animals was observed (May et al, 2013).

1.5. Detection and diagnostic methods

The current gold standard test for *T. equigenitalis* which is recognized by the OIE is bacterial culture. According to the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Chapter 2.5.1, Contagious Equine Metritis version adopted 20/10/2011); swabs are obtained in stallions from the urethra, urethral fossa and *lamina interna*, and in mares from the non-pregnant endometrium, clitoral sinus and clitoral fossa. The swabs are transferred in charcoal Amies medium and placed on ice to reach the laboratory within 24 h. *T. equigenitalis* is fastidious, slow growing and easily overgrown by commensal bacteria. According to the OIE Manual, the samples are inoculated onto Chocolate agar with antibiotics. The plates are incubated at 37^{o}C in 5-10% (v/v) CO_2 in air. At least 72 h is normally required before colonies of *T. equigenitalis* become visible, after which inspection at one day intervals is recommended. An incubation time of at least 7 d 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 are performed. *T. equigenitalis* is a Gram-negative pleiomorphic rod that is sometimes pleiomorphic and exhibits bipolar staining. It produces catalase and is strongly oxidase positive. A related organism, *T. asinigenitalis* has similar colony morphology and can be a confused with *T. equigenitalis*. *T asinigenitalis* is isolated from donkeys and is not associated with disease manifestation in naturally bred mares (Jang et al, 2001). Recent genomic analysis has shown that these two Taylorellae are closely related, but that *T. asinigenitalis* shares surprisingly little DNA sequence homology with *T. equigenitalis* (Hauser et al, 2012).

Due to the difficulties in isolating *T. equigenitalis* owing to it fastidious culture requirements such as micro-aerophilia, its drug sensitivity and slow growth, as well as its tendency to be overgrown by other contaminants, a real-time quantitative PCR test was developed and reported in 1994 by Bleumink-Pluym et al. Bleumink-Pluym et al (1994) derived a genus-specific primer-probe from the 16S ribosomal DNA sequence. They compared the results of qPCR to culture using 191 genital swabs from horses of different breeds. They concluded that

the sensitivity of qPCR was superior to that of culture. These findings were similar to those of Anzai et al (2002) who screened 7534 genital swabs from 4026 Thoroughbred broodmares and stallions in Japan from 1998-2001. They detected *T. equigenitalis* in twelve mares and one stallion using qPCR, but were only able to isolate the organism from two-qPCR-positive mares. Wakeley et al (2006) developed and validated a real time TaqMan® PCR for the detection and discrimination of *T. equigenitalis* from *T. asinigenitalis.* Similarly, Duquesne et al (2007) developed a direct qPCR assay that they described as "rapid, specific and sensitive" for the detection of *T. equigenitalis* and with the ability to discriminate it from *T. asinigenitalis.* qPCR shows enhanced sensitivity and therefore fewer false negatives than traditional bacterial culture and has greater practicality, quicker turnaround times and lower costs that bacterial culture. There are also fewer constraints from the sample taking viewpoint including media, temperatures and transfer interval.

Serology has limited application in the detection of *T. equigenitalis*-affected animals, with sero-conversion being reported as a transient feature associated with the acute phase of endometritis in the mare, and being absent in stallions. Antibody titres rise from 7 d post exposure to reach a peak at 3 weeks, before declining at 6-10 weeks (Timoney, 1996; Luddy and Kutzler, 2010). In certain countries, serology is still currently applied for pre-export or import screening and post-treatment quarantine test-breeding protocols in mares (Luddy and Kutzler, 2010, Erdman et al, 2011).

1.6. Recently-reported outbreaks

The most recent CEM outbreaks in non-endemic countries include the 2008-2010 outbreak in the USA and the 2012 outbreak in Gloucestershire, United Kingdom. Over 1000 animals were included in the traceback of the outbreak investigated in the USA between December 2008 and March 2010, which identified 22 stallions, one gelding and five mares as being positive for *T. equigenitalis* (Erdman et al, 2011). Epidemiologic analysis indicated that all positive cases could be linked to a single common source, and that the organism had been spread between stallions by undetermined indirect mechanisms at shared breeding facilities and to mares by live cover and AI.

The UK outbreak in 2012 was confined to two mares (one thoroughbred and one non-thoroughbred) and one non-thoroughbred stallion on a single property (Rickets et al, 2012).

1.7. Status of CEM in South Africa

CEM is a controlled animal disease in South Africa in terms of the Animal Diseases Act (Act No 36 of 1984). South Africa was considered free of CEM until 3rd May 2011 when an outbreak was confirmed following submission of samples for qPCR-testing to the Animal Health Veterinary Laboratory Agency (AHVLA), UK. The OIE was informed on 9th May 2011 and South Africa lost its CEM-free status. Epidemiological evidence suggested that CEM entered South Africa by importation of a Warmblood stallion from Germany. Initially, the outbreak was confined to one property (a veterinary facility) with one confirmed case of transmission involving two animals (one stallion and one mare). The veterinary facility was placed under quarantine and all "high" and "moderate" risk in-contact horses were screened under the direction of the State Authorities to detect additional positive cases. Following the confirmation of two additional *T. equigenitalis*-positive stallions, including one never present on the index property, a nationwide stallion-screening programme was instituted to determine the extent of the outbreak. A nationwide-traceback of all exposed mares that had been bred to

T.equigenitalis-positive stallions and the resultant offspring was additionally instituted. The screening and traceback programmes used qPCR as a test for the detection of *T. equigenitalis* and were described in the Procedure Manual Screening of Stallions for Contagious Equine Metritis with effect from the 2012/2013 breeding season commencing 1st July 2012 (Appendix I). Any suspect *T. equigenitalis*-positive horses were then confirmed positive using prescribed bacterial culture techniques and treated according to the Procedure Manual for the Confirmation of Diagnosis and Treatment of Positive Cases of Contagious Equine Metritis dated 13 April 2011, Updated June 2012 (Appendix II). The ultimate aim of the screening programme and traceback was to re-establish South Africa's *T. equigenitalis*-free status.

Chapter 2: Introduction

The following two articles describe the initial response, management and epidemiological investigation followed during the first reported outbreak of CEM in South Africa. Following the confirmation of a positive case of *T. equigenitalis* in Gauteng Province, the index property was placed under State-authorised quarantine and an investigation of all breeding activities was performed. This identified the index case and 11 associated mares linked to the index case by assisted reproductive techniques. These animals were sent to a specialized quarantine facility for follow-up swabbing and treatment.

Further investigation of the index property revealed the existence of an additional property in the Free State Province, where breeding animals linked with the index property were resident. Genital swabbing of all animals on this property revealed no additional positive animals.

Inspection of the breeding records of the index case showed that a first round of AI and embryo transfer (ET) had been a success, but the subsequently unsuccessful second round had raised the suspicion of a venereally transmitted pathogen. This fact combined with the identification of an additional *T. equigenitalis*-positive stallion that had been classified as "moderate risk," but had been present on the index property before the arrival of the index case, raised the suspicion that the index case had not been the initial case, but had, in fact, been infected subsequent to his importation into South Africa, most likely via horizontal transmission by fomites. This identified the index property as the geographical focus of the epidemiological investigation.

The institution of a nationwide screening programme resulted in the identification of an additional 33 stallions that were positive for *T. equigenitalis.* Of these horses, 12 had visited the index property. Most of these suspected animals were confirmed on bacterial culture and were subsequently treated and have re-tested negative.

During the national screening programme, a subpopulation focus was identified at the South African Lipizzaner Centre, Midrand, Gauteng where 24 of 33 resident stallions were found to be *T. equigenitalis*-positive. Initially, this was thought to be due to iatrogenic cross-contamination during sample collection, but subsequent swabbing following strict biosecurity protocols confirmed these positive animals. Due to the fact that only a limited number of these stallions had been used for breeding activities, horizontal transmission via fomites was suspected to have spread the disease to other stallions resident at the South African Lipizzaner Centre. The Lipizzaner mares and youngstock are kept at a separate facility and genital swabbing of all these animals revealed no additional positive cases.

The use of qPCR as a screening method provided rapid results that allowed for in-treatment testing and monitoring of treatment efficacy and identification of the point of eradication of the disease.

Chapter 3: The first reported outbreak of Contagious Equine Metritis in South Africa: Initial response and treatment protocol

3.1. Background

CEM was initially suspected on the 4th April 2011, associated with the importation into South Africa of a two-year old Warmblood stallion from Germany. This stallion, the eventual index stallion, had been released from post-arrival quarantine at the Kempton Park State Quarantine Station (GPS -26.13705, 28.26450) on 22nd February 2011 and was transported directly by road to a private veterinary facility (-25.95881, 28.14755) in Midrand, Gauteng Province. He remained at the facility for approximately 5 weeks and semen was collected to AI donors for subsequent ET into recipient mares. Thereafter, movement of the index stallion and these exposed mares to their owner's farm (-28.15150. 25.09433) in the Free State Province occurred prior to the disease being suspected. This resulted in quarantine orders being served on both premises only subsequent to the animals' translocation from the veterinary facility to the farm.

Following an unsuccessful round of AI and ET, where no embryos were recovered and the donor mares developed a mucopurulent vaginal discharge, a Thoroughbred mare (the index mare) had been inseminated with semen collected from the index stallion at the veterinary facility in an attempt to establish venereal transmission of an unknown infectious organism. Genital swabs were taken of both the index stallion and the mare. The uterine swab taken from this mare yielded a heavy pure-growth of suspected *T. equigenitalis* (reported 11th April 2011). Following this result, re-culture of swabs taken from the stallion yielded a single colony similar to that of the mare (reported 15th April 2011). The attending veterinarian and State authorities were informed of the suspicion of *T. equigenitalis* pending confirmation. The breeding facility was placed under state-administered quarantine (13th April 2011).

In response to a suspected disease outbreak, the Director of Animal Health (DAH) authorized Profs ML Schulman and AJ Guthrie of the University of Pretoria on 18th April 2011 to direct all necessary diagnostic and therapeutic measures to control the disease in cooperation with the State authorities. This was followed by their visits to both the veterinary facility in Gauteng (18th April 2011) and the farm of residence (-28.149503, 25.103652) of the index case in the Free State on 19th April 2011 to investigate the circumstances associated with the outbreak. Consultation of the breeding records of the index stallion identified a group of Warmblood mares (n=6) suspected of exposure via AI to the index stallion. These mares were used as donors for ET into other, recipient mares. All donors (n=6) and recipients (n=3) were classified as "high risk". The index mare and her paddock companion were also classified as "high risk." Further investigation showed that all mares had been treated for an apparent infectious endometritis of unknown origin prior to confirmation of *T. equigenitalis* at the breeding facility. All "high risk" mares and the two index cases were transferred to a quarantine facility (-25.65015, 28.18114) for follow up testing and treatment.

At the private veterinary facility, all stallions (n = 21) that had been either resident or had visited the index property during the period of residence of the index stallion (22nd February 2011- 31st March 2011) were identified. These stallions were allocated to "moderate" or "low"-risk categories, dependent on the temporal relationship of their presence on the index property relative to the period of residence of the index case. Stallions identified as "moderate risk" had been resident on the index property at the same time as the index stallion and had semen collected on the same days as the index stallion and those identified as "low

risk" were stallions that were resident on the index property at the same time as the index stallion but had semen collected on alternate days to the index stallion. At the time the index property was placed under quarantine (13th April 2011) all but one of the suspect stallions had returned to their home properties.

Further investigation of the breeding records at the index property, identified the existence of an additional associated satellite facility near Clarens in the Free State Province, where breeding stock was resident. The entire horse population of over 100 animals including stallions, geldings, mares and young stock on this farm was screened using genital swabs for qPCR. No additional positive animals were identified.

A trace-back of the stallions classified as "moderate risk" (n=11) identified one *T. equigenitalis*- positive stallion, CEM_2011_02_03. Investigation of the breeding records at the index property indicated that this stallion had been present on the index property prior to the arrival of the index case. This, combined with the fact that the initial round of ET was successful, but the second was unsuccessful and associated with an endometritis, strongly suggested that the index case may have been infected with *T. equigenitalis* subsequent to his importation into South Africa during his stay on the index property. The subsequent quarantine and swabbing of other horses resident on the home property (-25.990182, 28.027686) of CEM_2011_02_03, yielded another *T. equigenitalis*-positive stallion, CEM_2011_02_04.

3.2. Hypotheses

1. With the exception of a single documented venereal route of transmission via AI between the index mare and the index stallion, transmission of T *equigenitalis* occurred via fomites such as the breeding phantom, to other stallions visiting the index property.
2. The index stallion was not the source of the outbreak of CEM due to *T. equigenitalis* in South Africa.

3.3. Materials and methods

3.3.1. Initial response

3.3.1.1. Outbreak identification

Following suspicion of an outbreak of CEM in South Africa and pending confirmation by qPCR, the index property was visited by Professors Guthrie and Schulman on 18th April 2011 and the breeding records examined. It was confirmed that the index stallion had been used for semen collection to AI six ET donor mares. These mares were sub-divided into two separate groups for AI and ET. According to records from the breeding facility, three mares, "Donor A" (AI on 2nd March 2011), "Donor B" (AI on 3rd and 4th March 2011) and "Donor C" (AI on 6th and 8th March 2011) were inseminated with semen previously diluted with an antibiotic-containing extender 'INRA 96" (IMV Technologies, L'Aigle Cedex, France) and, were flushed to retrieve embryos nine days after the last insemination, in the interval 11th-16th March 2011. One embryo was retrieved from each donor and placed into a recipient mare as follows: "Donor A" into "Recipient G", "Donor B" into "Recipient H" and "Donor C" into "Recipient I."

Two of the three recipients ("Recipients H and I") were confirmed pregnant one week later (17th March 2011). However, when "Recipient H" was re-examined later at her farm of residence (5th May 2011), she was found non-pregnant. "Donor C" died of African Horse Sickness on 25th March 2011.

The second batch of mares for ET consisted of four donors which were inseminated using semen without prior addition of an antibiotic-containing extender. These mares and their AI dates were as follows: "Donor A" (19th March 2011), "Donor D" (10th March 2011), "Donor E" (13th March 2011) and "Donor F" (18th March 2011). These four donors were flushed 8 to 10 d post-AI in the interval 20th-28th March 2011. Suspicion of uterine infections was first aroused when no embryo flushes produced an embryo and an abnormal-appearing, mucoid fluid was recovered from all donor mares. The donor mares were therapeutically flushed and each mare received either two or three uterine instillations of a suspension of 20 ml gentamicin, "Genta 50" Phenix (Virbac RSA (Pty) Ltd, Halfway House) with 20 ml 4.2% sodium bicarbonate and 20 ml of sodium penicillin "Depocillin" (Intervet SA (Pty) Ltd, Edenvale). The mares were discharged and returned to the owner's stud farm (-28.149503, 25.103652) in the Free State Province on the 29th March 2011.

According to records examined at the veterinary facility, a pre-export examination of the index stallion indicated that a beta-haemolytic *Streptococcus* had been cultured from swabs taken in Germany. Due to this history, the veterinarian at the index facility suspected the possibility of an unknown pathogen and obtained genital swabs from the index stallion for bacterial culture on the 29th March 2011. The swabs from the penile shaft and the urethal fossa were transferred in transport media (Kyron AM, Kyron Laboratories (Pty) Ltd, Johannesburg) and submitted to Idexx Laboratories, Johannesburg, Gauteng for bacterial culture. In addition, a semen sample was collected directly into a handheld bottle using an open ended artificial vagina lined with a disposable plastic liner and submitted for culture. No pathogens were cultured according to the Idexx report dated 4th April 2011 (Appendix III).

A four-year-old Thoroughbred mare, subsequently the index mare, was inseminated using semen collected from the index stallion on the 29th and again on 31st March 2011 without the addition of semen extender. An examination performed on the mare on the 1st April 2011 showed no vaginal discharge and no uterine fluid was seen on trans-rectal ultrasound. A uterine swab was obtained and submitted to Idexx Laboratories, Johannesburg, Gauteng for bacterial culture (Appendix IV).

The index stallion was discharged to his owner's stud farm in the Free State Province on the 31st March 2011.

On the 11th April 2011, Idexx Laboratories informed the attending veterinarian that the isolates cultured from the index mare were suspicious for *T. equigenitalis.* The laboratory then re-incubated and further cultured the isolates obtained from the index stallion (Appendix III).

The index mare was isolated and treated with the same intra-uterine suspension previously used on the in-contact ET donors. The clitoral fossa was washed repeatedly with 2.5% chlorehexidine (Dismed Bioscrub, Dismed Pharma (Pty) Ltd, Halfway House) and packed with nitrofurazone ointment (Aspen Pharmacare, Sandton). The index mare was kept together with a companion mare ("Companion mare").

On the 15th April 2011, repeat culture of the urethral fossa swab from the index stallion yielded a single colony similar to that isolated from the index mare. The State Veterinarian, Gauteng was informed of a possible outbreak of CEM, pending qPCR results from the Animal Health and Veterinary Laboratories Agency (AHVLA), Bury St Edmunds, United Kingdom.

At that stage, the stallion's owner was informed of the suspicion regarding CEM and all in-contact animals were placed in isolation on their home property and treated. This included the index stallion and all mares used for ET. The mares were treated with a similar protocol applied to the index mare comprising of uterine irrigation as described above, and the genitalia were additionally washed with chlorhexidine and thereafter silver sulfadiazine ointment (Silbecor Biotech, South Africa) was applied on alternate days for 5 days. The index stallion commenced treatment on the 11th April 2011 for 5 d. His penis and sheath were washed with chlorhexidine and silver-sulfadiazine ointment was applied.

In response to a suspected outbreak of CEM, the veterinary facility was inspected by state officials on the 18th April 2011 The identities of all additional horses (mares and stallions), either resident or visiting in the interval from the suspect stallion's arrival on 22nd February 2011 until the facility was placed under quarantine (18th April 2011) were established.

The index mare and stallions' isolates were dispatched to the AHVLA on the 18th April 2011 for qPCR conformation of suspect *T. equigenitalis* (Appendix V). The four swabs (2 from the stallion and 2 from the mare) submitted to the AHVLA (Wakeley et al, 2006) were confirmed positive by real time PCR for *T. equigenitalis* on 3rd May 2011.

In the interim, the farm of residence (-28.149503, 25.103652) of the index case in the Free State Province was visited on 19th April 2011. Following a meeting with various State authorities, the farm owner and management, the premise was inspected by Profs Schulman and Guthrie. A total of 120 in-contact animals exposed to the newly- repatriated mares had venous blood samples collected for serology. No tests were performed on these samples as it was subsequently not indicated.

Following this visit it was decided to transfer the index stallion and all in-contact mares, excluding the pregnant recipient, "Recipient I," to the Onderstepoort Veterinary Academic Hospital (OVAH), Gauteng (-25.65015, 28.18114) for testing and treatment under quarantine conditions following a period of 3 weeks since the last treatment date. This was conducted in accordance with the current legislation regarding CEM in South Africa (Procedural Manual for Contagious Equine Metritis for the Suspect Outbreak Identified April 2011, Appendix VI). The nine in-contact mares, including one with a foal at foot and the index stallion arrived at the OVAH under Red Cross permit conditions on 13th May 2011. The mares included the surviving ET donors (n=5), the non-pregnant ET recipients (n=2), the index mare and her paddock companion ("Companion mare"). The horses were transferred to a previously-prepared quarantine facility at the OVAH that was kept locked and sealed to prevent unauthorized access and potential for fomite transmission, where they were subsequently screening and treated. The horses remained within this facility until 31st May 2011 when they were repatriated to their farm of residence.

The pregnant embryo recipient, "Recipient I", remained at the stud farm in the Free State Province under quarantine conditions in the pregnant mare quarantine facility until she foaled

down on the 30th January 2012. She was reportedly subsequently swabbed for *T. equigenitalis* and tested negative.

One month (28th June 2011) following the end of treatment, the index stallion together with a gelding companion, was readmitted to the OVAH. Genital swabs from the index stallion were obtained and assayed as previously described on the 28th June, 1st July and 4th July 2011. The index stallion tested positive on qPCR from all three swabs obtained on the 1st and 4th July 2011 and he was re-treated for 8 d (8th-15th July 2011).

Following re-treatment, samples were taken on the 14th July 2011 for qPCR from both the index stallion and his gelding companion. The index stallion's samples were negative for *T equigenitalis*, however, the gelding companion's samples from the urethral sinus and *lamina interna* were weakly positive on qPCR. Following this positive result, the gelding was treated from the 15th-19th July 2011. He was retested on the 15th July 2011 and tested negative on qPCR.

The index stallion and his gelding companion were retested 5 d after the cessation of the stallion's treatment and both tested negative on qPCR and were discharged and returned to their farm of residence on the 20th July 2011.

3.3.1.2. Traceback and stallion screening

All in-contact horses, including mares and stallions, on the index property were risk-categorised into "high," "moderate" or "low" risk, dependent on both the temporal relationship of their presence on the index property and their level of potential exposure via breeding-related procedures relative to the index stallion. The "high" risk horses included mares that had been bred to the index stallion and those involved in assisted reproductive techniques such as ET involving semen collected from the index stallion. The stallions (n = 21) that had either been resident or visited the index property, during the same period as the index stallion (22nd February-31st March 2011) were identified. Five of these suspect stallions had departed prior to any semen collection attempts from the index stallion. The "moderate" risk stallions (n=11) were those resident on the index property at the same time as the index stallion and having undergone semen collection on the same day as the index stallion. The "low" risk stallions (n=4) were those resident on the index property at the same time, but having undergone semen collection on different days to the index stallion.

A follow-up of the stallions identified as "moderate risk" identified one stallion, CEM-2011_02_03 that was *T. equigenitalis*-positive on bacterial culture and qPCR (Table1). Quarantine and swabbing of other horses resident on his home property yielded an additional *T. equigenitalis*-positive stallion, CEM_2011_02_04.

3.3.1.3. Diagnostic protocol

A diagnostic and therapeutic protocol was adapted from recently-published references (UK Horseracing Betting Levy Board (HBLB) Code of Practice, 2011, Luddy and Kutzler, 2010) and used in conjunction with the South African Procedural Manual for Contagious Equine Metritis for the Suspect Outbreak Identified April 2011 (Appendix VI).

It was decided that for the index stallion and "high risk" mares quarantined at the OVAH, a series of three tests within 7 d, with a two-day interval between tests would be conducted.

The stallion traceback and screening consisted of two sets of swabs taken at a 7 d interval as described below according to the Procedural Manual for Contagious Equine Metritis for the Suspect Outbreak Identified April 2011.

The tests were conducted as follows:

i) "High risk" mares in quarantine: duplicate swabs were taken of each of the following sites: clitoral fossa using standard dry swabs (Labchem, Johannesburg, South Africa), clitoral sinuses using paediatric swabs (Copan Innovation, Brescia, Italy), and endometrium using a double-guarded endometrial swab (Easy Guard, Section of Reproduction, Onderstepoort, SA). One replicate swab of each site was transferred in Amies charcoal medium for storage at 5 °C prior to transfer for culture at the Agricultural Research Council, Onderstepoort Veterinary Institute (ARC-OVI), Onderstepoort, Gauteng within 2 h of collection. The other replicate was transferred to a 5 ml eppendorf tube (Scientific Group, Vorna Valley, South Africa) containing phosphate buffered saline, swirled for several seconds then sealed, marked and transferred to the Veterinary Genetics Laboratory, University of Pretoria within 2 h for qPCR testing.

ii) Stallions: After intravenous sedation with a combination of 12 µg/kg detomidine hydrochloride (Domosedan, Novartis SA (Pty) Ltd, Animal Health, Isando) and 25 µg/kg butorphanol tartrate (Torbugesic, Fort Dodge, USA) to allow extrusion of the penis, duplicate swabs were similarly obtained and treated from the following three sites: urethra, urethral fossa and *lamina interna*

The real time qPCR assay applied in this study was a modified Taqman® PCR method. The Taqman® PCR uses common amplimers for both *Taylorella* species to produce an 112bp fragment encompassing a region of the 16S rDNA of *T. equigenitalis* and *T. asinigenitalis* in which 8bp differences between the two base pairs were identified (Wakeley et al., 2006). The Taqman® PCR is specific for the 16S ribosomal DNA of the *Taylorella* species and does not cross-hybridise with other bacteria tested. The real time Taqman® PCR can directly identify *Taylorella* spp. from genital swabs, and discriminates between *T. equigenitalis*, and *T. asinigenitalis* (Wakeley et al., 2006). Modifications made by the Veterinary Genetics Laboratory included using the mastermix supplied by Kappa Biotechnologies (Kapabiosystems, Cape Town, South Africa and changing both the fluorescent dye on the *T. asinigenitalis* probe from HEX to NED and the quencher from Black Hole Quencher 1 to a MGB probe (Applied Biosystems, Cheshire, UK). This allowed shortening on both the primers and probes. Samples were classified as positive if the normalized fluorescence for the *T. equigenitalis* assay exceeded a 0.1 threshold within 40 PCR cycles.

The method used for bacterial culture is according to that described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.

3.3.2. Treatment protocol

i) "High risk" mares in quarantine

Immediately following the final testing (23rd May 2011), all mares were treated topically and parenterally for 5 d. Topical treatment consisted of once daily thorough irrigation of the vestibulum, clitoral fossa, *glans clitoridis* and adjacent tissues with a 4% chlorhexidine solution and removal of any accumulated smegma with disposable paper toweling. Thereafter

the area was dried and a copious application of silver-sulfadiazine ointment was used to pack and coat the area. Antimicrobial therapy consisted of oral trimethoprim sulphamethoxazole (Purbac Adult Tablets, Aspen Pharmacare, Sandton) at a dose rate of 30 mg/kg twice daily for 5 d. All biohazardous waste was collected in biohazard bags and incinerated.

ii) Index stallion

Immediately following the final testing (23rd May 2011), the stallion was treated topically for 5 d. Exteriorizing of his penis was facilitated by intravenous sedation (detomidine hydrochloride and butorphanol tartrate) and 4% chlorhexidine solution was used to irrigate the exposed penis and prepuce. Disposable paper towel was used to remove accumulated smegma from the urethra, *glans penis*, *fossa glandis* and exposed penile and preputial laminae. Thereafter, the area was dried and a copious application of silver-sulfadiazine ointment was used to pack and coat the area. Antimicrobial therapy consisted of oral trimethoprim sulphamethoxazole at 30 mg/kg twice daily for 10 d. All biohazardous waste was collected in biohazard bags and incinerated.

The index stallion and a gelding companion were re-admitted at the OVAH for follow-up testing one month after the end of initial treatment. The stallion retested positive for *T. equigenitalis* and was re-treated. Treatment consisted of topical treatment for 8 d (8th-15th July 2011) as described above, however a 2% chlorhexidine spray was used following cleaning of the penis with a surfactant containing 2.5% w/w lactic acid and 0.1% w/w salicylic acid ("Epiotic ®" Virbac RSA (Pty) Ltd, Centurion). The chlorhexidine spray was left *in situ* for 10 min. The penis was then dried and packed with silver sulfadiazine. After 3 d the stallion developed erosive lesions on the cranial aspect of the penis caudal to the *glans penis*. The treatment protocol was changed to omit the use of both the surfactant and 2% chlorhexidine and substitute acriflavine glycerine (Kyron, Benrose, Johannesbueg) solution followed by the application of silver sulfadiazine ointment.

Following the gelding companion's positive result on the 14th July 2011, he was treated as described above for the re-treatment protocol for a period of 5 d (15th-19th July 2011).

iii) Stallion traceback

Both stallions, CEM_2011_02_03 and CEM_2011_02_04 identified on the stallion traceback were quarantined at a designated quarantine facility and were treated by a private veterinarian for 5 d (28th June -2nd July 2011) according to the method described in the Procedural Manual for Contagious Equine Metritis for the Suspect Outbreak Identified April 2011 (Appendix IV) under the supervision of the State Authorities.

3.3. Results

3.3.1. Initial outbreak

After testing and treatment at the OVAH, no positive test results were obtained on either qPCR or bacterial culture from any of the 9 sets of swabs obtained per mare. Thus, apart from the single documented case of venereal transmission between the index stallion and index mare via AI, no other venereal transmission between the index stallion and in-contact mares was shown. All 9 sets of swabs from the index stallion were positive on both qPCR and bacterial culture (Table 1).

A month after the completion of initial treatment, the index stallion and a gelding companion were re-admitted to the OVAH for follow-up testing. These test results are shown in Table 1.

3.3.2. Stallion screening

The two stallions, CEM_2011_02_03 and CEM_2011_02_04 were identified on qPCR during the stallion traceback and later confirmed on bacterial culture (Table 1).

Table 1: Anatomical distribution of *T. equigenitalis* using qPCR testing and bacterial culture on genital swabs obtained from predilection sites in male animals in the course of the initial outbreak and stallion traceback.

Stallion	sample date dd/mm/yy	urethra		urethral fossa		*lamina interna*		pooled sample
		qPCR	bacter-iology	qPCR	bacter-iology	qPCR	bacter-iology	qPCR
Index stallion CEM_2011_01_01	17/05/11	+	+	+	+	+	+	n/s
	20/05/11	+	+	+	+	+	+	n/s
	23/05/11	+	+	+	+	+	+	n/s
	28/06/11	-		-	-	-		n/s
	01/07/11	+	n/s	+	n/s	+	n/s	n/s
	04/07/11	+	n/s	+	n/s	-	n/s	n/s
	14/07/11	n/s	n/s	n/s	n/s	n/s	n/s	-
	20/07/11	n/s	n/s	n/s	n/s	n/s	n/s	-
Gelding companion	14/07/11	-	n/s	+	n/s	+	n/s	n/s
	15/07/11	n/s	n/s	n/s	n/s	n/s	n/s	-
	20/07/11	n/s	n/s	n/s	n/s	n/s	n/s	-
CEM_2011_02_03	20/06/11	n/s	-	n/s	+	n/s	-	n/s
	23/06/11	-	-	+	+	+	-	n/s
	28/06/11	-	-	+	-	-	-	n/s
	04/07/11	-	n/s	-	n/s	-	n/s	n/s
	25/07/11	n/s	-	n/s	-	n/s	-	n/s
CEM_2011_02_04	28/06/11	-	-	-	+	-	-	n/s
	04/07/11	+	n/s	+	n/s	+	n/s	n/s
	25/07/11	n/s	-	n/s	-	n/s	-	n/s

Key:
+ = positive result; - = negative result; n/s = not sampled

3.4. Discussion

The reported predilection of *T. equigenitalis* to colonise the urethral fossa and glans of the stallion and the clitoris and endometrium of the mare and for a more prolonged duration of infection in stallions than in mares was supported by our findings (Platt et al, 1978; Timoney and Powell, 1988; Kristula and Smith, 2004; Timoney, 1996; Luddy and Kutzler, 2010; Erdman et al, 2011). No *T. equigenitalis*-positive mares, including the index mare, were identified subsequent to their treatment. The organism persisted on the genitalia of the index stallion despite prior treatment on his home property and the later implementation of a recommended treatment protocol for a prescribed duration (Luddy and Kutzler, 2010; UK Horseracing Betting Levy Board (HBLB) Code of Practice for CEM 2011) while under quarantine at the OVAH. This necessitated a second round of treatment, after which he tested negative for *T. equigenitalis*.

Follow-up swabbing of the index stallion for *T. equigenitalis* one month after his initial treatment showed the first of three tests to be negative but both the second and third swabs to be positive for *T. equigenitalis*, similar to the findings of Zdovc et al (2005) (Table1). The reason for these results was unclear. We speculated that the smegma-associated organism may be shielded within the predilection sites, and is only exposed to sampling following mechanical disturbance e.g. by swabbing or cleaning, resulting in positive-identification on subsequent testing. This observation supported serial testing to enhance sensitivity for identification of this organism

The detection of *T. equigenitalis* from the companion gelding raised the question of horizontal fomite transmission from the index stallion. This may have occurred e.g. via grooming equipment or human contact. However, as only one of a sequence of three tests was positive and showed a high Ct value, this suggested either a low level of contamination, or more probably, the possibility of cross contamination at sampling or laboratory error.

In the case of the “high risk” mares, *T. equigenitalis* was confirmed in only one animal, the index mare, and prior to their presentation at the OVAH quarantine facility. In the first group of ET mares, there were no reported clinical signs to suggest CEM. There was suspicion of an endometritis of infectious origin in the second batch of ET donors but these animals were treated prior to a diagnosis and no samples were taken to confirm the presence of *T. equigenitalis*. It has been reported that up to 20-25% of aymptomatically-infected mares may attain carrier status which may last for many months or even years (Timoney, 1996; Matsuda and Moore, 2003; Luddy and Kutzler, 2010; Timoney, 2011), however, no positive results were obtained from any of the swabs obtained from each mare, therefore indicating *T. equigenitalis*-negative status. The organism, *T. equigenitalis* was reported to be susceptible to many disinfectants, ultraviolet light, high temperatures and reduced humidity (Timoney, 2011; Schulman et al, 2013). This susceptibility combined with prior treatment as well as the known transient contamination of mares may all have contributed to the negative results in the “high risk” mares.

An important aspect of this outbreak was its association with assisted reproductive techniques such as AI and not natural mating, with documented evidence of transmission via AI from the index stallion to the index mare.

The epidemiological investigation during the initial outbreak response was useful in defining that the index stallion was unlikely to have been the probable source of infection. The

evidence strongly suggested that the index stallion was not the original source, but was in fact infected on the index property. This supportive evidence included: (i) serial bacterial culture following internationally accepted methods in both the country of export and the country of import, all of which were negative, (ii) the documented account of an initially successful AI and ET programme followed by an unsuccessful second attempt after which the presence of *T. equigenitalis* was identified in the index stallion and mare, and (iii) the presence of the confirmed *T. equigenitalis*-positive stallion, CEM_2011_02_03 on the index property prior to arrival of the index stallion with breeding records indicating that CEM_2011_02_03 and the index stallion had semen collected on the same day using the same breeding equipment. This final point strongly suggested horizontal transmission via fomites, to or from the index stallion, therefore supporting our hypothesis that with the exception of a single documented venereal route of transmission via AI between the index mare and the index stallion, transmission of *T. equigenitalis* occurred solely via fomites such as the breeding phantom, to other stallions visiting the index property. However, there was insufficient evidence to conclusively support the hypothesis that the index stallion was not the source of the South African outbreak of *T. equigenitalis.* Future planned strain typing of the South African isolates of *T. equigenitalis* will provide further information in this regard.

The identification of a second stallion, CEM_2011_02_04 that had reportedly never been used for breeding or visited the index property, but was resident on the home property of CEM_2011_02_03, suggested an additional direct association with the index property that implied possible horizontal transmission of *T. equigenitalis* had occurred by fomites, possibly grooming equipment or personnel between stallions on their home property.

The qPCR method was shown to have a sensitivity of 100% in the sample population (n=4) when compared with the gold standard of bacterial culture prescribed by the OIE (Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Table 1). In addition, the qPCR assay was rapid, reliable and robust with a quick turnaround time that facilitated effective monitoring of treatment efficacy.

Due to the limited diagnostic potential of serology to identify *T. equigenitalis*-positive animals, it was not utilized during this intervention. *T. equigenitalis* is a colonist of the external genitalia and therefore, stallions do not mount a serological response to the organism. In mares, sero-conversion is reported as a transient feature associated with the acute phase of endometritis with antibody titres rising from 7 days post exposure to reach a peak at 3 weeks, before declining at 6-10 weeks (Timoney, 1996; Luddy and Kutzler, 2010). The “high-risk” mares were presented at the OVAH quarantine facility six weeks after their suspected exposure to *T. equigenitalis* and may have shown declining antibody titres to *T. equigenitalis.* However, this would only have indicated exposure to *T. equigenitalis* and not their current infection status.

The findings during the initial outbreak recognition and response suggested that the index stallion was not indisputably the likely source of introduction. This raised the question of the extent and prevalence of *T. equigenitalis* in the national population particularly if its introduction had pre-dated the importation of the index stallion. Due to the reported pathogenesis of the organism and its observed persistence on the external genitalia of stallions gained during the initial outbreak intervention, the stallion was shown to be the most likely source of diagnostic material. This supported the targeted surveillance of stallions in assessing a large population of horses. Furthermore, the role of assisted reproductive techniques, particularly including the potential for wide-spread dispersal via shipped semen

for AI, highlighted the importance of extending the screening to mares that were bred by stallions with semen collected at the index property during the period of residence of the index stallion.

Chapter 4: Epidemiological investigation of the South African Outbreak of Contagious Equine Metritis and stallion screening programme

4.1. Background

The aims of the traceback exercise identifying in-contact or exposed stallions and mares following the identification of the CEM outbreak were to further define observations made during the initial outbreak recognition and intervention. Evidence strongly suggested the possibility that the index stallion may have become infected by horizontal transmission at the index property subsequent to his arrival in South Africa. This implied that *T. equigenitalis* had been present in South Africa prior to initial recognition in April 2011. The identification of a *T. equigenitalis*-positive stallion (CEM_2011_02_04) that had reportedly neither visited the index property nor been used for breeding, but was a stable mate of the "moderate risk" stallion, CEM_2011_02_03, which had been resident on the index property concurrent with the index stallion strongly suggested (i) horizontal transmission by fomites between stallions and, (ii) *T. equigenitalis* had been present in South Africa prior to the importation of the index stallion.

The probable pre-existing occurrence of *T. equigenitalis* in South Africa indicated the necessity to ascertain its extent and prevalence in the national horse population. This required the institution of a nationwide traceback and screening programme that ultimately resulted in the identification of an additional 33 stallions, including a subpopulation focus of 24 Lipizzaner stallions, and 2 female horses that were positive for *T. equigenitalis.*

Defining the distribution and prevalence of *T. equigenitalis* in South Africa were deemed essential aims in order to facilitate the process of disease eradication. This background was used to develop three hypotheses for further investigation.

4.2. Hypotheses

1. The outbreak was limited to one geographical focus.
2. qPCR was an appropriate method to determine the spread of an outbreak of CEM when used in a screening programme for breeding stallions.
3. Currently advocated treatment methods and prescribed durations would be effective to treat animals confirmed positive for *T. equigenitalis*.

4.3. Materials and Methods

4.3.1. Focus property traceback

All horses (stallions and mares) categorised on risk classification as having been exposed to the index stallion were traced to their current owners using a variety of data sources including breeding records from the veterinary facility (index property) and stallion owners, and information gained by State animal health officials.

i) Stallions
During the 6 months following the initial outbreak identification, all stallions identified as "moderate" (n=11) or "low risk" (n=4) were screened for *T. equigenitalis* using qPCR testing of genital swabs collected from the following three sites: urethra, urethral fossa, and *lamina*

interna (Platt et al, 1978; Timoney and Powell, 1988; Kristula and Smith, 2004; Timoney, 1996; Luddy and Kutzler, 2010; Erdman et al, 2011) according to the prescribed method described in the Procedure Manual Screening of Stallions for Contagious Equine Metritis, dated 3 August 2011 (Appendix VIII). These swabs were identified using a unique barcode system and transferred to the Veterinary Genetics Laboratory, University of Pretoria for qPCR testing. Stallions identified as "moderate risk" had been resident on the index property at the same time as the index stallion, and had semen collected on the same days as the index stallion and those identified as "low risk" were stallions that were resident on the index property at the same time as the index stallion but had semen collected on alternate days to the index stallion.

Stallions found to be *T. equigenitalis*-positive during this qPCR-screening were then confirmed positive by using the OIE recognized bacterial culture method (Manual of Diagnostic Tests and Vaccines for Terrestrial Animals) from swabs obtained in accordance with the prescribed method and transferred in charcoal Amie's medium within 24 h to a State-designated bacteriology laboratory, the Agricultural Research Council, Onderstepoort Veterinary Institute (ARC-OVI).

An additional 10 home properties with *T. equigenitalis*-positive stallions were identified on the screening programme. This gave a total of 12 properties nationwide on which *T. equigenitalis*-positive stallions were identified. These properties were placed under State administered quarantine and all other stallions on the property were screened for *T. equigenitalis* as previously described.

As per the Animal Diseases Act, 1984 (Act no. 35 of 1984), all confirmed *T. equigenitalis*-positive stallions would be euthanized, castrated or treated under State supervision. If the owners elected treatment, this would need to occur under supervised quarantine by means of topical treatment using appropriate antimicrobials for a period of five days according to the method prescribed in the Procedural Manual for the Confirmation of, Diagnosis and Treatment of Positive Cases of Contagious Equine Metritis, 13 April 2011, updated October 2012 (Appendix VII) by a private veterinarian of the owner's choice under the supervision of the relevant State authority. Follow-up testing by genital swabs on two occasions to confirm *T. equigenitalis*-negative status was prescribed at 21 d and at 28 d post- treatment.

For a stallion to be certified free of *T. equigenitalis* and to resume breeding, following successful treatment and confirmation of negative status, test breeding was required as prescribed in the Procedure Manual for the Confirmation of Diagnosis and Treatment of Positive Cases of Contagious Equine Metritis, 13 April 2011, updated October 2012 (Appendix VII).

If an owner requested an exemption or deferment of the test mating procedure for a particular stallion, for example in the case of a competition horse, then the owner would need to apply in writing to the Director: Animal Health with detailed reasons for the exemption or deferment.

ii) Mares

The trace-back of exposed mares commenced in September 2012, approximately 18 months after the index case was reported. Mares were classified as "exposed" if their associated data indicated that they had been bred, either by natural breeding or assisted breeding techniques to any confirmed *T. equigenitalis*-positive stallion. The data sources used were:

(i) breeding facility records from the index property,
(ii) breeding data kept by stallion owners, and
(iii) information gained by State animal health officials.

The trace-back was conducted by staff affiliated with the Equine Research Centre (ERC) of the Faculty of Veterinary Science, University of Pretoria.

In addition to the exposed mares, a trace-back of all offspring resultant from *T. equigenitalis*-positive sires was conducted, based on the reported potential for vertical transmission, either *in utero* or intra-partum (Timoney, 2011).

Mares were screened via genital swabbing for qPCR-testing using swabs taken of each of the following sites: clitoral fossa using standard dry swabs (Labchem. Johannesburg, South Africa), clitoral sinuses using paediatric swabs (Copan Innovation, Brescia, Italy) and endometrium (in non-pregnant mares) using a double-guarded endometrial swab (Easy Guard, Section of Reproduction, Onderstepoort, SA). Clitoral swabs only were obtained from pregnant mares and from young fillies < 1 year of age. Swabs were identified as previously described.

Two mares were subsequently identified as *T. equigenitalis*-positive. These mares were transferred and housed under quarantine conditions for treatment and testing at the OVAH. On admission, genital swabs were obtained in duplicate from each site as described above. One replicate swab per site was transferred in Amie's charcoal medium for storage at 5 °C prior to transfer for culture at the ARC-OVI, Onderstepoort, Gauteng within 2 h of collection. The second replicate was transferred to the Veterinary Genetics Laboratory within 2 h for qPCR-testing. During the treatment period in order to monitor treatment efficacy, dry swabs were taken daily for qPCR from the clitoral sinus and clitoral fossa as previously described. One set of swabs was taken before treatment commenced, and one set was taken after irrigation as described below, but prior to treatment with antibiotics.

Depending on the daily qPCR-test results monitoring treatment efficacy, topical treatment was applied for a duration ≥7 d. Treatment consisted of once daily copious irrigation of the vestibulum, clitoral fossa, *glans clitoridis* and adjacent tissues with a 4% chlorhexidine solution and removal of any accumulated smegma with disposable cotton wool and dry swabs dipped in the 4% chlorhexidine solution. In order to remove smegma from the clitoral sinuses, paediatric swabs dipped in chlorhexidine solution were used. Thereafter, the area was dried and copious application of silver-sulfadiazine ointment was used to pack and coat the area (mare 1). In the second case (mare 2), a dry cow intramammary treatment of 500 mg cloxacillin and 250 mg ampicillin per syringe (Curaclox, Norbrook, Biotech Vet, South Africa) was instilled into the clitoral sinuses in addition to using silver-sulfadiazine ointment to pack and coat the area.

All biohazardous waste was collected in biohazard bags and incinerated.

Following treatment the mares were held in quarantine and swabbed for qPCR-testing post-treatment to establish *T equigenitalis*-status.

4.3.2. Nationwide screening programme

The South African Directorate: Animal Services (DAFF) instituted a nationwide screening programme and issued an instruction or order to every owner or manager of stallions which

stated that 'no stallion, irrespective of breed, may be used or allowed to be used to breed (natural mating or artificial insemination), unless an official CEM clearance certificate has been issued. An official CEM clearance certificate will only be issued by the Faculty of Veterinary Science, Equine Research Centre, once two negative tests have been done.'

The aims of the stallion screening programme as set down in the Procedure Manual Screening of Stallions for Contagious Equine Metritis, dated 3 August 2011 (Appendix VIII) were threefold:

i) to determine the prevalence of *T. equigenitalis* in stallions in South Africa,
ii) to prevent *T. equigenitalis* from becoming endemic within South Africa, and
iii) to eradicate *T. equigenitalis* and apply to regain South Africa's CEM-free status

To facilitate compliance with the screening programme, no offspring from a stallion lacking a CEM clearance certificate valid for that year would be eligible for registration by the relevant Breed Society.

The measures to be followed in the case of a suspect or confirmed case of CEM, the swabbing procedure and sample submission were described in the Procedure Manual Screening of Stallions for Contagious Equine Metritis, dated 3 August 2011 (Appendix VIII).

The screening programme utilized qPCR as a screening test for CEM. Horses positive on qPCR were retested using a combination of both qPCR and bacterial culture to confirm the presence of *T. equigenitalis*. The qPCR was chosen as the screening test due to its associated properties that included minimal transport requirements, lack of a stringent time limit within which the samples needed to reach the laboratory and the turn-around time. This permitted the provision of results within 24 h with obvious benefits to the effective management of suspect cases.

The advocated methodology advised that a stallion was sedated to allow extrusion of the penis. Dry bacterial culture swabs without medium would be obtained from three predilection sites: urethra, urethral fossa and *lamina interna* ensuring strict adherence to biosecurity measures at all times. The swabs would be identified with the date, stallion's name and the swabbing site. The swabs, sealed correctly, were to be placed in a separate sealed bag and submitted to the Veterinary Genetics Laboratory for qPCR testing.

The instruction or order took effect from the 31st August 2011, and was still valid in October 2013.

Under the guidance of Professors Guthrie and Schulman, a web-based platform (http://www.cemsa.co.za) was developed with the aim of coordinating the key aspects of the stallion screening programme. The components of the platform were to achieve the following aims.

i) Dissemination of information
The official DAFF letter to owners/managers of stallions and the Procedure Manual Screening of Stallions for Contagious Equine Metritis, dated 3 August 2011 (Appendix VIII) was available to download off the website. Mare owners would be able to view the list of certified *T. equigenitalis*–free stallions before considering breeding their mare to a specific stallion.

ii) Submission of samples for qPCR testing
Veterinarians, breeders and concerned parties would have access to the legislation pertaining to CEM in the Procedure Manual Screening of Stallions for Contagious Equine Metritis, dated 3 August 2011 (Appendix VIII), in addition to the submission forms and instructions on how to submit samples. The submission form available to download from the website included all relevant owner, stallion and submitting veterinarian details as well as methods of payment for submitting samples to the Veterinary Genetics Laboratory. (Appendix IX)

iii) Data capture and analysis
Data captured included the stallion's name, breed, country of origin, microchip and passport number. The owner's name and the private veterinary practice information were also recorded. The first and second dates that swabs were taken and reported and the results of negative tests were displayed on the database. Positive cases were not displayed on the database, and the stallions were not listed under the CEM-free listing. Stallions with outstanding fees for swabbing were also not certified free until payment had been made. Access to the database was limited; however, the certified CEM-free stallion list was freely available.

iv) Reporting results via certification of CEM-free status
Following testing of the two required sets of swabs, a clearance certificate was issued to the owner of the stallion and simultaneously displayed on the website. Mare owners were able to check the website for a list of certified stallions, to ensure that their mares were bred by certified CEM-free stallions and any resultant offspring would be eligible for registration with the relevant breed society.

4.3.3. Subpopulation focus intervention

In accordance with the State-instituted national stallion screening programme, all stallions that were resident at the South African Lipizzaner Centre, Midrand, Gauteng (-25.970064, 28.054358) had genital swabs taken for qPCR-testing in accordance with the Procedure Manual Screening of Stallions for Contagious Equine Metritis, dated 3 August 2011 (Appendix VIII). Twenty four of the 33 stallions resident at the South African Lipizzaner Centre, Midrand, Gauteng had tested positive on qPCR that indicated a subpopulation focus associated with, but separated from the geographical focus at the index property. The Lipizzaner Centre was placed under State administered quarantine.

Initially the housing management at the SA Lipizzaner Centre was changed to ensure that there was a spatial division between the *T. equigenitalis*-positive stallions and the negative stallions. Following this, the *T. equigenitalis*-positive stallions were divided into batches for the purpose of expediting their treatment. The first batch of stallions was treated according to Procedural Manual for Contagious Equine Metritis for the Suspect Outbreak Identified April 2011 (Appendix VI). Following this treatment period, several of the treated stallions remained positive for *T. equigenitalis* and a more far-reaching depopulation-repopulation protocol was implemented. All resident stallions, including the negative animals were removed under State Veterinary permits to quarantine facilities. One positive stallion was transported to the owner's property in Kwazulu Natal Province for quarantine and treatment. The *T. equigenitalis*-negative stallions were removed to an additional isolation facility and underwent weekly genital swabbing for qPCR-testing for three consecutive weeks to confirm their negative status before being allowed to return to the Lipizzaner Centre. During the

depopulation phase, the Lipizzaner Centre's facilities were subjected to rigorous cleaning, decontamination and disinfection. All bedding material was removed followed by steam-cleaning, disinfection and repainting. All wooden doors and partitions were replaced. All items that were in-contact with the horses, such as tack and grooming kits, were either discarded or disinfected. Concurrent with this decontamination and disinfection programme, the positive stallions were again treated in batches at the quarantine facility according to a newly-revised treatment protocol as described in the Procedure Manual for the Confirmation of Diagnosis and Treatment of Positive cases of Contagious Equine Metritis, 13 April 2011, updated October 2012 (Appendix VII). The primary innovation to the treatment protocol was to include in-treatment swabs for qPCR-testing to monitor treatment efficacy and determination of the point of eradication of *T. equigenitalis.*

Following confirmation of negative status by in-treatment qPCR results, stallions were removed to an additional quarantine facility and underwent post-treatment testing 21 and 28 d after the end of treatment, according to the prescribed method in the Procedure Manual for the Confirmation of Diagnosis and Treatment of Positive cases of Contagious Equine Metritis, 13 April 2011, updated October 2012 (Appendix VII). After confirmation of negative status, the stallions were allowed to return to the Lipizzaner Centre. Follow-up swabs for qPCR-testing from the sub-population were taken at monthly intervals over the period of a year to monitor their post-treatment disease status.

4.4. Results

4.4.1 Focus property traceback

i) Stallions
Traceback of all stallions identified as "moderate" (n=11) or "low risk" (n=4) during the initial risk assessment of the index property yielded one "moderate risk" stallion (CEM_2011_02_03) reported in Chapter 3 and two "low risk" stallions (CEM_2011_03_05 and CEM_2011_04_06) as positive for *T. equigenitalis*.

ii) Mares
The traceback of 69 in-contact mares and their 51 offspring identified two *T. equigenitalis*-positive females. This indicated a prevalence rate of 1.67% in the animals tested in this trace-back of exposed mares and offspring of *T. equigenitalis*-positive stallions.

The first female identified was a Thoroughbred mare bred by AI using chilled semen collected from the *T. equigenitalis*-positive stallion, CEM_2011_02_03 and shipped to her home property in the North West Province (-26.20485, 26,24371) in February 2011. The pregnancy that resulted produced a *T. equigenitalis*-negative mare. The mare was swabbed and identified as positive using qPCR on the 21st February 2012, almost a year after the presumed time of infectious exposure. This result could not be confirmed on bacterial culture despite serial sampling on 2 occasions according to the previously described method.

The second female identified was a three-year-old Warmblood mare in Gauteng Province (-26.271711, 28.004538). Her dam had been bred by AI using semen collected from the *T. equigenitalis*-positive stallion, CEM_2011_08_10. The mare was identified during the trace-back of offspring from *T. equigenitalis*-positive stallions and was swabbed and identified as positive using qPCR on the 30th November 2012 when she was just under three years-old.

This was confirmed on bacterial culture. The dam herself was *T. equigenitalis*-negative on testing.

Both animals were successfully quarantined and treated at the OVAH on separate occasions and confirmed negative on follow up qPCR post treatment before being repatriated to their home properties. The mare took 25 d of treatment before testing negative on qPCR and the mare took 7 d, therefore mares in this study required an average of 17 d of treatment to regain *T. equigenitalis*-negative status.

The initial identification of the *T. equigenitalis*-positive mares and their duration of treatment is summarised in Table 2.

Table 2: Confirmation of *T. equigenitalis*-positive status by genital swabbing for qPCR-testing and bacterial culture and subsequent duration of treatment in two mares

Mare	Date and result of 1st PCR test	Date and result of additional qPCR test	Date and result of 1st bacterial culture	Date and result of additional bacterial culture	Treatment dates dd/mm/yy	Treatment interval (days)
CEM_2011_13_38 (TB)	21/02/12 positive	03/04/12 positive	04/04/12 negative	03/07/12 negative	06/07/12-30/07/12	25
CEM_2011_08_39 (WB)	30/11/12 positive	22/03/13 positive	26/02/13 positive	22/03/13 positive	24/04/13-30/04/13	7

Key:
TB = Thoroughbred; WB = Warmblood

4.4.2. Nationwide screening programme

The national qPCR screening programme for breeding stallions tested more than 1800 stallions in all 9 provinces of South Africa and identified an additional 33 positive stallions, including 24 Lipizzaners resident on the same property during the interval July 2011 to July 2013. The most common site from which *T. equigenitalis* was isolated on both qPCR testing and bacterial culture was the urethral fossa. All positive stallions with a single exception (CEM_2011_10_35) could be traced either directly or indirectly to the index property. Of these stallions, 12 were linked directly to the index property; this included two stallions that had been identified as "low risk" stallions (CEM_2011_03_05 and CEM_2011_04_06), two stallions (CEM_2011_08_10 and CEM_2011_12_37) that were resident on the index property concurrent with the index stallion, but prior to any semen collection from the index stallion and eight stallions (CEM_2011_05_07; CEM_2011_07_09, CEM_2011_11_36 and five Lipizzaner stallions) that were linked with previous semen collection on the index property, but prior to the arrival of the index case, including several years prior in certain cases (Table 2).

All *T. equigenitalis*-positive stallions were identified from Gauteng Province, with the exception of one stallion (CEM_2011_06_08) identified in the Western Cape Province. This stallion, a Lipizzaner, had initially been resident in Gauteng before relocation to the Western Cape. Subsequent to their identification, two stallions were transferred from Gauteng for quarantine and treatment: one Lipizzaner to Kwazulu Natal Province and a Warmblood (CEM_2011_11_36) to the North West Province.

Several stallion-owners have made application to the State authorities to defer their stallion's test-mating programme to avoid interference with a competition schedule and three stallions have been castrated following their successful treatment and negative post-treatment swabbing.

Table 3 summarises the association of the index property with all *T. equigenitalis*-positive stallions. It also shows the dates of initial identification and the duration of treatment required to regain *T. equigenitalis*-negative status.

For the *T. equigenitalis*-positive stallions, excluding those stallions resident at the Lipizzaner Centre, average treatment duration of 7.45 d was required to regain *T. equigenitalis*-negative status.

The owners of three of the *T. equigenitalis*-positive stallions elected to castrate the stallions after successful treatment and negative post-treatment testing.

The stallions tested represented 85 breeds associated with 1635 owners and 307 veterinary practices originating from all 9 provinces in South Africa.

The distribution by breed of horses testing positive during this period was: Lipizzaner (n=25), Warmblood (n=9), Connemara (n=1), Thoroughbred (n=2), Andalusian (n=1) and Celle Francais (n=1). The highest proportion of *T. equigenitalis*-positive stallions by breed were Lipizzaner at 69.4% (25/36) of the overall breed distribution; Warmbloods accounted for the second highest proportion at 22.2% (8/36) and all other breeds (n=3) each accounted for 2.8% (1/36). The distribution of positive mares by breed was Thoroughbred (n=2) and Warmblood (n=1)

Figure 1 shows the geographical association of the index property with 13 other properties (n=13) where *T. equigenitalis*-positive horses were identified. This included 11 properties with only *T. equigenitalis*-positive stallions present, one property with only a *T. equigenitalis*-positive mare, and one property where a *T. equigenitalis*-positive mare and stallion were present.

Figure 2 shows a schematic representation of the geographic and temporal association and the hypothesized routes of transmission of *T. equigenitalis* amongst *T. equigenitalis*-positive horses identified during the South African outbreak between 2011-2013.

Table 3: Association of the index property with the 20 stallions concurrently present with the index stallion and all other stallions identified as *T. equigenitalis*-positive carriers by a nationwide stallion screening programme

Presence on index property Yes/No	Stallion			Testing		Treatment			
	Identity	Breed	Import Yes/No	Date and result of 1st test for qPCR or culture dd/mm/yy	Date and result of 2nd test for qPCR dd/mm/yy	Dates for positive stallions dd/mm/yy	Re-treatment dates for positive stallions dd/mm/yy	Additional re-treatment dates dd/mm/yy (additional notes)	Total treatment interval days
Yes	Stallion 1**	WB	Yes	20/06/11 negative	23/06/11 negative	N/A	N/A	N/A	N/A
Yes	Stallion 2**	Boer	No	14/11/11 negative	21/11/11 negative	N/A	N/A	N/A	N/A
Yes	Stallion 3**	WB	Yes	27/06/11 negative	04/07/11 negative	N/A	N/A	N/A	N/A
Yes	Stallion 4**	WB	No	04/10/11 negative	11/10/11 negative	N/A	N/A	N/A	N/A
Yes	Stallion 5**	WB	Yes	20/06/11 negative	23/11/11 negative	N/A	N/A	N/A	N/A
Yes	Stallion 6**	Dutch WB	Yes	19/09/11 negative	26/09/11 negative	N/A	N/A	N/A	N/A
Yes	CEM_2011_02_03**	WB	No	20/06/11 Positive culture only	24/06/11 positive	28/06/11-02/07/11	N/A	castrated	5
Yes	Stallion 7**	WB	Yes	20/06/11 negative	23/06/11 negative	N/A	N/A	N/A	N/A
Yes	Stallion 8**	Arab	Yes	25/05/11 negative	30/05/11 negative	N/A	N/A	N/A	N/A

Presence on index property Yes/No	Stallion			Testing		Treatment			
	Identity	Breed	Import Yes/No	Date and result of 1st test for qPCR or culture dd/mm/yy	Date and result of 2nd test for qPCR dd/mm/yy	Dates for positive stallions dd/mm/yy	Re-treatment dates for positive stallions dd/mm/yy	Additional re-treatment dates dd/mm/yy (additional notes)	Total treatment interval days
Yes	Stallion 9**	Dutch WB	Yes	25/05/11 negative	30/05/11 negative	N/A	N/A	N/A	N/A
Yes	Stallion 10** – Died of AHS	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Yes	CEM_2011_04_06*	Con	Yes	24/08/11 negative	31/08/11 positive	03/10/11-07/10/11	N/A	test mated	5
Yes	Stallion 11*	WB	No	11/10/11 negative	18/10/11 negative	N/A	N/A	N/A	N/A
Yes	CEM_2011_03_05*	WB	Yes	22/08/11 positive	29/08/11 positive	21/09/11-25/09/11	N/A	castrated	5
Yes	Stallion 12*	Dutch WB	Yes	21/08/11 negative	28/08/11 negative	N/A	N/A	N/A	N/A
Yes	Stallion 13†	Dutch WB	Yes	23/09/11 negative	28/09/11 negative	N/A	N/A	N/A	N/A
Yes	CEM_2011_08_10†	Celle	Yes	23/09/11 positive	30/09/11 positive	07/11/11-11/11/11	N/A	exempted	5
Yes	Stallion 14†	Arab	Yes	30/08/11 negative	06/09//11 negative	N/A	N/A	N/A	N/A
Yes	CEM_2011_12_37†	Belg WB	Yes	11/01/12 positive	19/01/12 positive	18/04/12-25/04/12	N/A	test mated	8
Yes	Stallion 15†	WB	Yes	29/09/11 negative	06/10/11 negative	N/A	N/A	N/A	N/A
Yes	CEM_2011_05_07†	WB	Yes	22/08/11 positive	29/08/11 positive	21/09/11-25/09/11	N/A	test mated	5

Presence on index property Yes/No	Stallion			Testing		Treatment			
	Identity	Breed	Import Yes/No	Date and result of 1st test for qPCR or culture dd/mm/yy	Date and result of 2nd test for qPCR dd/mm/yy	Dates for positive stallions dd/mm/yy	Re-treatment dates for positive stallions dd/mm/yy	Additional re-treatment dates dd/mm/yy (additional notes)	Total treatment interval days
Yes	CEM_2011_07_09†	Belg WB	Yes	24/08/11 positive	05/09/11 positive	21/09/11-25/09/11	N/A	N/A	5
Yes	CEM_2011_11_36†	Bel WB	Yes	01/12/11 positive	08/12/11 positive	28/05/12-01/06/12	17/7/12-25/7/12	test mated	14
Yes	CEM_2011_06_08†	Lip	No	29/08/11 positive	05/09/11 positive	07/03/11-13/03/11	N/A	exempted	7
No	Lipizzaner stallion 1†	Lip	No	11/10/11 positive	13/10/11 positive	16/01/12-24/01/12	07/02/12-16/02/12	N/A	19
Yes	Lipizzaner stallion 9†	Lip	No	11/10/11 positive	13/10/11 positive	28/02/12-07/03/12	N/A	N/A	9
Yes	Lipizzaner stallion 15†	Lip	Yes	11/10/11 positive	13/10/11 positive	06/03/12-11/04/12	N/A	N/A	36
Yes	Lipizzaner stallion 20†	Lip	No	11/10/11 positive	13/10/11 positive	05/12/11-09/12/11	16/01/12-24/01/12	07/02/12-05/03/12	43
No	CEM_2011_10_35†	And	Yes	25/01/12 positive	21/02/12 positive	15/03/12-20/03/12	26/02/13-11/03/13	N/A	18
No	CEM_2011_02_04†	WB	No	28/06/11 positive culture only	04/07/11 positive	28/06/11-02/07/11	N/A	castrated	5
No	20 Lipizzaner stallions†	Lip	No	11/10/11 positive	13/10/11 positive	see Table 4	see Table 4	see Table 4	19.25 (avg)

Key:
* = "Low risk" : collected on separate days to index case; ** = "Moderate risk" : collected on same day as index case; † = nationwide stallion screening programme; AHS : African horse sickness; And : Andalusian; Belg WB : Belgian Warmblood; Boer : Boerperd; Celle : Celle Francais; Con : Connemara; Dutch WB: Dutch Warmblood; Lip : Lipizzaner; WB : Warmblood; N/A : Not applicable

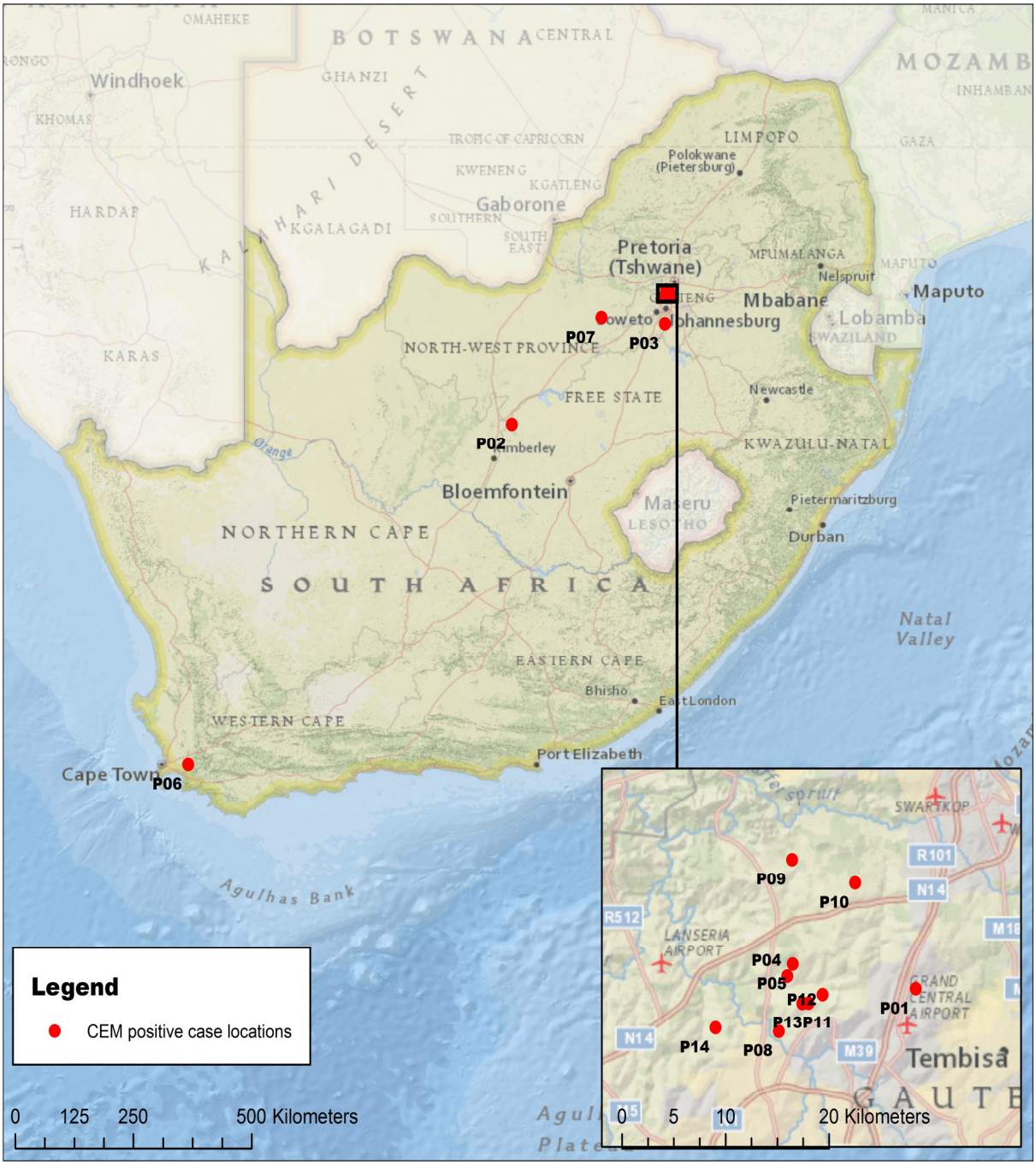

Fig 1: The geographical association of the index property (P01) with 13 other properties (P02-P14) where *T. equigenitalis*-positive horses were identified in South Africa

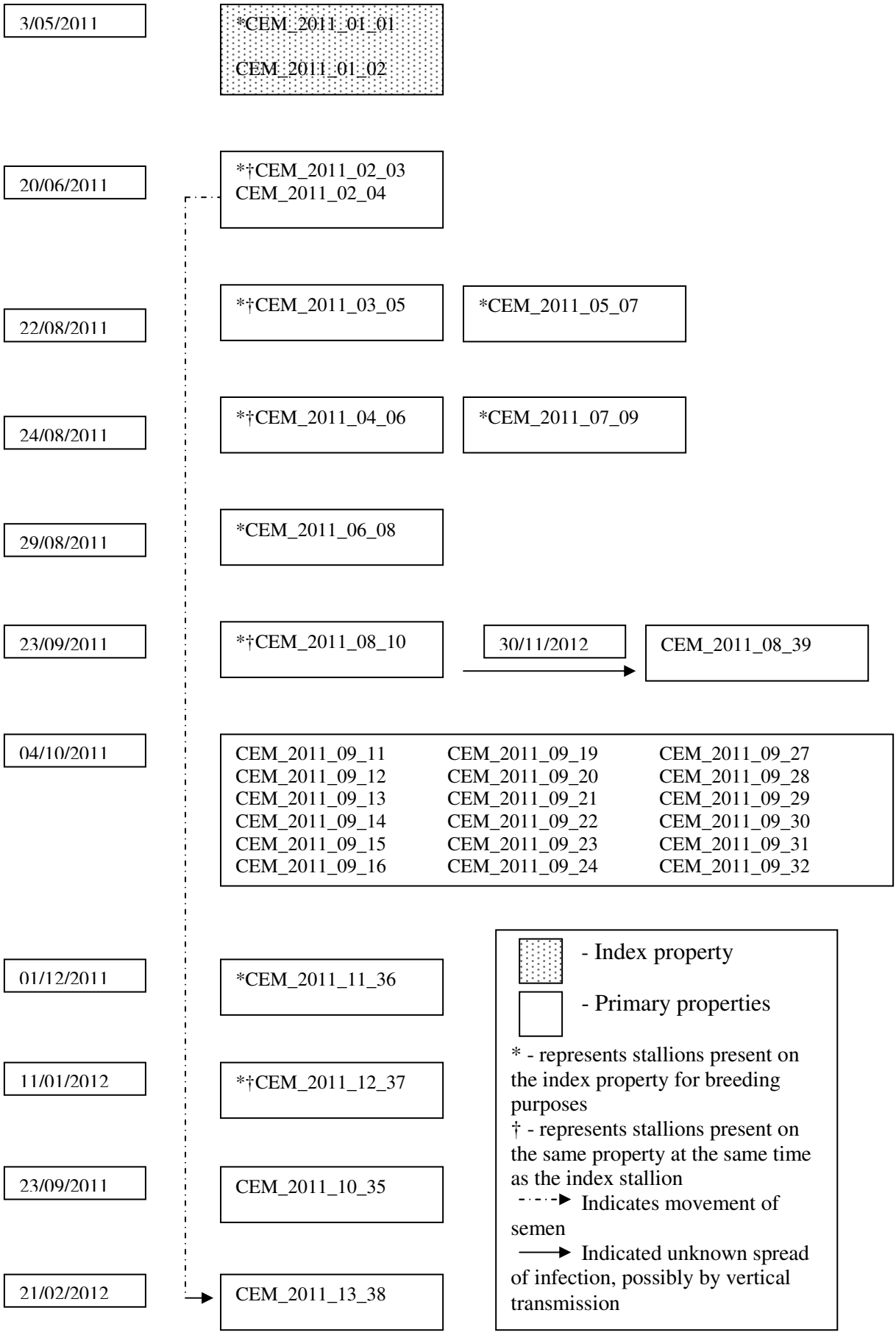

Fig 2. Geographic and temporal association and hypothesized routes of transmission of *T. equigenitalis* amongst *T.equigenitalis*- positive horses during the South African Outbreak 2011-2013

4.4.3. Epidemiological focus subpopulation

Of the 24 *T. equigenitalis*-positive stallions resident at the Lipizzaner Centre, 14 were confirmed positive on bacterial culture according to the OIE prescribed method (Manual of Diagnostic Tests and Vaccines for Terrestrial Animals). In total, 6 Lipizzaner stallions had visited the index property for semen collection for AI since 2001. This number included one Lipizzaner stallion subsequently identified in the Western Cape during the stallion screening programme. From the offspring of stallions that had visited the index property, 7 stallions were found to be *T. equigenitalis*-positive. A total of 11 of the 33 resident stallions had been used for breeding either by natural cover or AI. The average period required to successfully treat a Lipizzaner stallion was 19.96 d. Monthly follow-up swabbing of the subpopulation for qPCR was performed as previously described to monitor long-term status. To date, October 2013, no relapses of Lipizzaner stallions following treatment have been identified.

Table 4 summarises the association of the *T. equigenitalis*-positive Lipizzaner stallions with the index property as well as their applicable breeding history and the treatment duration required to eliminate the infection.

Table 4: Association of *T. equigenitalis*-positive Lipizzaners (n=25) with the index property and their breeding and treatment records

Stallion				**Breeding**			**Treatment**			
Identification	**Date of birth**	**Import Yes/No**	**Resident province**	**Breeding use**	**Breeding on index property**	***T. equigenitalis*-positive offspring (Yes/no)**	**Dates and duration (days)**	**Re-treatment dates and duration (days)**	**Additional Re-treatment dates and duration (days)**	**Overall treatment duration (days)**
Stallion 1	22/12/00	No	Gauteng	Yes	Yes	No	16/01/12-24/01/12 (9)	07/02/12-16/02/12 (10d)	N/A	19
Stallion 2	03/12/00	No	Gauteng	No	N/A	No	30/01/12-14/02/12 (16)	N/A	N/A	16
Gelding	27/11/03	No	Gauteng	No	N/A	N/A	30/01/12-08/02/12 (10)	N/A	N/A	10
Stallion 3	10/11/06	No	Gauteng	No	N/A	N/A	16/01/12-24/01/12 (9)	N/A	N/A	9
Stallion 4	09/10/04	No	Gauteng	No	N/A	N/A	16/01/12-24/01/12 (9)	N/A	N/A	9
Stallion 5	21/11/95	No	Gauteng	No	N/A	N/A	07/02/12-16/02/12 (10)	N/A	N/A	10
Stallion 6	25/12/93	No	Gauteng	No	N/A	N/A	30/01/12-10/02/12 (12)	N/A	N/A	12
Stallion 7	04/12/95	No	Gauteng	No	N/A	N/A	16/01/12-24/01/12 (9)	N/A	N/A	9

Stallion				**Breeding**			**Treatment**			
Identification	**Date of birth**	**Import Yes/No**	**Resident province**	**Breeding use**	**Breeding on index property**	***T. equigenitalis* -positive offspring (Yes/no)**	**Dates and duration (days)**	**Re-treatment dates and duration (days)**	**Additional Re-treatment dates and duration (days)**	**Overall treatment duration (days)**
Stallion 8	04/11/06	No	Gauteng	Yes – 2010	No	No	05/12/11-09/12/11 (5)	16/01/12-24/01/12 (9)	07/02/12-29/03/12 (52)	66
Stallion 9	10/08/93	No	Gauteng	Yes- 2005	Yes	Yes Stallion 8	28/02/12-07/03/12 (9)	N/A	N/A	9 d
CEM_2011_06_08	1995	No	WP	Yes -2004	Yes	Yes Stallion 5 Yes Stallion 4	07/03/11-13/03/11 (7)	N/A	N/A	7
Stallion 10	04/12/95	No	Gauteng	No	N/A	N/A	05/12/11-09/12/11 (5)	16/01/12-24/01/12 (9)	N/A	14
Stallion 11	10/01/08	No	Gauteng	Yes	No	No	30/01/12-24/02/12 (25)	N/A	N/A	25
Stallion 12	14/02/97	No	Gauteng	No	N/A	N/A	05/12/11-09/12/11 (5)	28/02/12-07/03/12 (9)	N/A	14
Stallion 13	09/10/02	No	Gauteng	No	N/A	N/A	16/01/12-24/01/12 (9)	11/02/12-07/03/12 (26)	N/A	35
Stallion 14	18/12/03	No	Gauteng	Yes	No	No	30/01/12-10/02/12 (12)	N/A	N/A	12

Stallion				**Breeding**			**Treatment**			
Identification	**Date of birth**	**Import Yes/No**	**Resident province**	**Breeding use**	**Breeding on index property**	***T. equigenitalis* -positive offspring (Yes/no)**	**Dates and duration (days)**	**Re-treatment dates and duration (days)**	**Additional Re-treatment dates and duration (days)**	**Overall treatment duration (days)**
Stallion 15	18/01/96	Yes-Austria	Gauteng	Yes	Yes	Yes Stallion 16 Yes Stallion 14	06/03/12-11/04/12 (36)	N/A	N/A	36
Stallion 16	25/11/03	No	Gauteng	No	N/A	N/A	05/12/11-09/12/11 (5)	30/01/12-20/02/12 (22)	N/A	27
Stallion 17	25/11/07	No	Gauteng	Yes	No	No	30/01/12-17/02/12 (19)	N/A	N/A	19
Stallion 18	18/01/08	No	Gauteng	Yes	No	No	30/01/12-20/02/12 (22)	13/03/12-20/03/12 (8)	N/A	30
Stallion 19	08/10/05	No	Gauteng	Yes	No	No	30/01/12-10/02/12 (12)	N/A	N/A	12
Stallion 20	03/12/00	No	Gauteng	Yes	Yes	No	05/12/11-09/12/11 (5)	16/01/12-24/01/12 (9)	07/02/12-05/03/12 (29)	43
Stallion 21	31/12/02	No	Gauteng	No	N/A	N/A	30/01/12-27/02/12 (28)	N/A	N/A	28

Stallion				**Breeding**			**Treatment**			
Identification	**Date of birth**	**Import Yes/No**	**Resident province**	**Breeding use**	**Breeding on index prop**	***T. equigenitalis* -positive offspring (Yes/no)**	**Dates and duration (days)**	**Re-treatment dates and duration (days)**	**Additional Re-treatment dates and duration (days)**	**Overall treatment duration (days)**
Stallion 22	28/10/06	No	Gauteng	No	N/A	N/A	16/01/12-24/01/12 (9)	07/02/12-16/02/12 (10)	N/A	19
Stallion 23	27/10/05	No	Gauteng	No	N/A	N/A	16/01/12-24/01/12 (9)	N/A	N/A	9

4.5. Discussion

The initial outbreak recognition and response identified four animals that included the index Warmblood stallion, index Thoroughbred mare and two additional Warmblood stallions. Following recognition of an outbreak of CEM in South Africa, the nation-wide screening programme and exposed mare traceback scheme tested more than 1900 horses across the country and ultimately identified an additional 33 infected stallions and 2 mares between 18th April 2011 and 31st October 2013.

Subsequent to the identification of the index property, *T. equigenitalis*-positive animals were identified at an additional 12 primary properties including 10 properties in Gauteng Province, one property in the Western Cape and one property in the North West Province. Of these 12 primary properties, all but one was directly linked with the index property. This refuted our hypothesis that the outbreak had been limited to one geographical focus.

During the South African outbreak, no incidence of natural cover was associated with transmission of *T. equigenitalis*, however, there were two documented incidences of transmission to mares by assisted reproductive techniques, namely AI. No stallions were infected venereally, but it was suspected that they were contaminated by undefined fomites at the index property (visiting stallions) or alternatively on their home premises (non-visiting stallions). The candidate fomites on the index property included the phantom, the artificial vagina (Timoney and Powell, 1988; Timoney 1996) or personnel during semen collection and the handling processes. (Erdman et al, 2011). These findings substantiated our hypothesis that with the exception of a single documented case of venereal transmission (via AI) from the index stallion to the index mare, the most likely transmission of T *equigenitalis* on the index property to visiting stallions occurred via fomites.

The question of whether the index case was infected prior to his arrival in South Africa, or more likely, that he was infected subsequent to his arrival at the index property in Midrand from a *T. equigenitalis* strain that was already present in South Africa is currently unanswered. Future planned strain typing of the *T. equigenitalis* isolates should assist in further defining the origin of the South African outbreak. Therefore there is currently insufficient evidence to definitively refute the hypothesis that the index stallion was not the source of the South African outbreak of CEM due to *T. equigenitalis.*

Screening of the South African Lipizzaner Centre identified a subpopulation focus associated with, but separated from the geographical focus at the index property. The stallions are permanently housed at the Centre for training and performance purposes, while the mares and foals are resident at a separate satellite property. Approximately a third of the resident stallion population was reported to have previously been used for breeding. This suggested that fomite transmission at the Lipizzaner Centre was the probable primary route of transmission of *T. equigenitalis* among the resident stallions. No evidence of *T equigenitalis* was detected among the Lipizzaner mares and their offspring at the satellite property. It may be speculated that any infected mares had spontaneously resolved the infection over time. It may be further speculated that a degree of immunity may have developed in this population of mares during the course of prolonged and sporadic exposure within this subpopulation to the organism as has previously been reported (Timoney et al, 1978; Timoney and Powell, 1988; Luddy and Kutzler, 2010; Timoney, 2011). Lipizzaners accounted for the highest proportion of *T. equigenitalis*-positive stallions in the overall breed distribution, arguably suggesting a possible breed association with *T. equigenitalis* infection. It may also be speculated that a

familial association is present as there appears to be a link between *T. equigenitalis*-positive sires and their sons.

The average treatment duration for successful elimination of *T. equigenitalis* in stallions resident at the Lipizzaner Centre (19.96 d) and for other *T. equigenitalis*-positive stallions (7.45 d) was far longer than the currently advocated treatment protocols (Luddy and Kutzler, 2010; Procedural Manual for Contagious Equine Metritis for the Suspect Outbreak Identified April 2011, Appendix VI). These findings failed to support our hypothesis that the currently-advocated treatment methods including the prescribed duration would be effective in treating *T. equigenitalis*-positive stallions. The limitations of the legislation current at the time of outbreak recognition were overcome by introducing in-treatment qPCR monitoring in order to determine treatment efficacy and point of disease eradication. These new recommendations were utilized and were incorporated in the Procedure Manual for the Confirmation of Diagnosis and Treatment of Positive Cases of Contagious Equine Metritis, 13 April 2011, updated October 2012 (Appendix VII).

Of interest in this study was the observation in two cases (Index stallion and CEM_2011_04_06) that the initial swab taken from stallions for qPCR-testing was negative for *T. equigenitalis,* but follow-up swabs taken $\geq$ 7 d after the initial swab were positive for *T. equigenitalis*. The reason for these findings are unclear, but we speculated that this smegma-associated organism may be shielded within the predilection sites and was only exposed following mechanical disturbance e.g. by swabbing, resulting in positive-identification on subsequent testing. A less likely possibility is that this could be ascribed to laboratory error. This observation supported serial-testing to enhance sensitivity for identification of this organism in future surveillance exercises.

The total number of mares that were infected overall was unknown due to a combination of factors, primarily the reported potential for self-cure over time (Timoney et al, 1978; Timoney and Powell, 1988; Luddy and Kutzler, 2010; Timoney, 2011) and additionally the lag interval from potential exposure to the institution of the trace-back testing of individual mares. The higher prevalence of *T. equigenitalis*-positive mares (4.41%) when compared to stallions (1.35%) that were tested as reported by May et al (2013), was affected by the bias of the population screened but supported the inclusion of exposed mares during targeted epidemiologic surveillance of stallions. One of the *T. equigenitalis*-positive mares identified on trace-back could be definitively linked to a known *T. equigenitalis*-positive stallion via contaminated chilled semen shipped to her farm of residence for AI. A commercial semen extender containing antibiotics failed to prevent the transmission of *T. equigenitalis* to this mare in support of other reports (Olivieri et al, 2011; Timoney, 2011; Schulman et al, 2013). This mare conceived and established a pregnancy that carried to term, with delivery of a foal that was negative on testing. The possibility that the foal may have been infected *in utero*, at birth or in the post-partum stage and spontaneously resolved the infection cannot be entirely excluded (Luddy and Krutzler, 2010; Timoney, 2011).

The second *T. equigenitalis*-positive mare could be linked via her dam that had been bred by contaminated fresh semen collected from a known *T. equigenitalis*-positive stallion at the index property. The dam was herself negative at the time of screening. The mare had never been moved off her farm of birth, therefore transmission may have resulted either from the rarely-reported route of vertical transmission from her dam, or by horizontal fomite transmission from the dam subsequent to the mare's birth but prior to the dam clearing herself of *T. equigenitalis.* These findings in exposed mares demonstrate the critical role of

both fresh and chilled contaminated semen in transmission of this disease, despite the addition of antimicrobial-containing semen extenders (Timoney and Powell, 1988) and highlight the importance of assisted reproductive techniques as a risk factor in transmission of *T. equigenitalis.*

Due to the long lag phase between initial outbreak identification and instigation of the trace-back, serology was not performed on the exposed mares as the antibody response following infection is reported to decrease by 10 weeks (Timoney, 1996, Luddy and Kutzler, 2010).

Of the 39 *T. equigenitalis*-positive animals identified on qPCR, 27 of them were confirmed using bacterial culture, indicating that the rate of detection using qPCR is higher than bacterial isolation in support of previous reports (Zdovc et al, 2005). The qPCR-test proved to be an appropriate method for the nationwide screening programme in South Africa, a country characterised by extensive distances, limited postal services and periodic extremes of environmental temperatures. These factors complicate CEM diagnostics reliant on bacterial culture and qPCR therefore provided a practical, reliable and sensitive method that facilitated the ability to detect *T. equigenitalis.* This supported our hypothesis that qPCR is an appropriate method to determine the spread of an outbreak of CEM in a screening programme for breeding stallions.

All the positive horses from this outbreak were successfully quarantined and treated. With the exception of several Lipizzaners, three of these stallions; the index stallion (CEM_2011_01_01), CEM_2011_11_36 and CEM_2011_10_35 remained positive after initial treatment and required an additional treatment cycle. These stallions were all initially treated by private veterinarians under state-supervised quarantine. We observed that without in-treatment monitoring as was available at the OVAH, it was problematic to define the treatment endpoint. Following current protocols, if an animal remained positive after treatment, this would only be recognized after an interval of 21 d had elapsed to permit traditional testing by bacterial culture, in accordance with current legislation. The successful application of daily in-treatment monitoring has resulted in this being made mandatory according to the Procedure Manual for the Confirmation of Diagnosis and Treatment of Positive Cases of Contagious Equine Metritis, 13 April 2011, updated October 2012 (Appendix VII). In order for a stallion to be released from quarantine, an additional set of swabs taken $\geq$ 21 d post-treatment (two sets of swabs taken 7 d apart) for qPCR-testing and bacterial culture must be negative. Currently all known positive horses have been successfully treated based on results of follow-up qPCR screening.

In order for a stallion to receive a CEM-clearance certificate, he must have been test-bred by natural cover to two mares as stipulated in the Procedure Manual for the Confirmation of Diagnosis and Treatment of Positive Cases of Contagious Equine Metritis, 13 April 2011, updated October 2012 (Appendix VII). The owners of various individual competition stallions requested deferment of this test-mating until the stallion's sporting career was at an end.

The nationwide stallion screening programme was facilitated via the development of a dedicated website which provided pertinent information and relevant resources for horse owners and veterinarians (www.cemsa.co.za). The overwhelming compliance by stallion owners indicated by the numbers of swabs received for qPCR analysis supported the practicality and efficacy of the process.

4.6. Conclusions

A notable feature of the South African outbreak that supported recent outbreak reports from other countries was the apparent exposure to and subsequent infection of stallions by *T.equigenitalis* that occurred via fomites at an artificial breeding facility. This observation and other features supported the importance of applying informed-biosecurity protocols at breeding facilities that should include the effective cleaning and disinfection of all facilities and equipment used during breeding and semen collection procedures. Further recommendations would include proper hand-washing and the use of gloves by attending personnel. Our findings also supported previous reports that antibiotic-containing semen extenders were unreliable in preventing transmission via shipped semen.

Horizontal fomite-associated spread was the most probable route of transmission at the South African Lipizzaner Centre and other properties housing groups of stallions. When considering groups of stallions housed in close proximity, the amplified risk of transmission by fomites such as personnel and grooming equipment must be recognised and stringent biosecurity measures must be applied to mitigate these risks. The potential for cross-contamination during diagnostic procedures involving large groups was also recognized and must be managed accordingly to prevent this possibility.

Previous reports that the organism persisted on the genitalia of carrier stallions for a longer duration than in mares were supported by the study. The higher proportion of positive mares versus stallions identified during the trace-back however, probably reflected a sample population bias, but supported the inclusion of exposed mares during target epidemiologic surveillance for *T. equigenitalis.* Speculatively, this proportion may have been greater if the interval between potential exposure and sampling of the mares during the trace-back exercise was shorter. Also, the observation that most exposed mares in this sample population were bred by AI using semen diluted with antimicrobial-containing extender and not by natural service, as well as the reported potential for self-cure may have contributed to the lower proportion of *T. equigenitalis*-positive mares identified during the trace-back exercise.

The determination of the original source of *T. equigenitalis* in South Africa remains undefined. Strain-typing of *T. equigenitalis* isolates will further define both the origins of the South African outbreak and whether the index stallion was the original source or not. In addition, there may have been a concurrent introduction of the same or a unique strain by the index stallion. Strain-typing will further enable the determination of any previously undiagnosed incursions of *T. equigenitalis* into the country via importation of horses such as the Lipizzaners.

This outbreak and the observations made during its investigation and response has resulted in a number of potential benefits to the equine industry in South Africa and internationally. The experience gained has enhanced the development and implementation of more effective and practical testing, treatment and surveillance protocols. Notable during our outbreak response was the fact that currently prescribed treatment durations were ineffective in treating animals confirmed positive for *T. equigenitalis.* This resulted in unique modifications of current treatment protocols and resulted in the inclusion of in-treatment qPCR testing to monitor treatment efficacy and the point of disease eradication. The South African authorities redrafted the legislation pertinent to CEM that included a derogation that permitted the treatment of any infected equines provided that the measures stipulated in the Procedure Manual for the Confirmation of Diagnosis and Treatment of Positive Cases of Contagious

Equine Metritis, 13 April 2011, updated October 2012 (Appendix VII) were complied with. It also resulted in the development of a nationwide stallion screening programme able to effectively determine the extent and prevalence of *T. equigenitalis* in the national population of commercial breeding stallions and provided a means for ongoing yearly surveillance. Our findings during this outbreak response supported the targeted surveillance of stallions and exposed mares during epidemiologic investigation. The observation that in certain stallions, initial swabs for qPCR-testing were negative, followed by subsequent positive follow-up swabs for qPCR-testing, supported serial-testing to enhance sensitivity for identification of this organism in future surveillance exercises The web-based platform to co-ordinate the surveillance exercise was unique to South Africa and proved to be practical and effective in coordinating the nationwide stallion screening programme. The use of qPCR as the screening test supported its application as a practical and sensitive method for surveillance in large horse populations. In addition, its value for in-treatment monitoring was recognized by its inclusion as a mandatory component in revised legislation. Finally, this outbreak created awareness of CEM within the horse-owning population of South Africa. This had previously been restricted to awareness of post-arrival quarantine testing of imported horses. This awareness included the risk-association of *T. equigenitalis* with fomite transmission and assisted reproductive techniques. This has resulted in heightened awareness of biosecurity at breeding facilities and properties housing groups of stallions.

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Appendices

Appendix I: Procedure Manual Screening of Stallions for Contagious Equine Metritis with effect from the 2012/2013 breeding season commencing 1st July 2012

Appendix II: Procedure Manual for the Confirmation of Diagnosis and Treatment of Positive Cases of Contagious Equine Metritis , 13 April 2011, updated June 2012

Appendix VI: Procedural Manual for Contagious Equine Metritis for the Suspect Outbreak Identified April 2011

Appendix VII: Procedure Manual for the Confirmation of Diagnosis and Treatment of Positive Cases of Contagious Equine Metritis, 13 April 2011, updated October 2012

Appendix VIII: Procedure Manual Screening of Stallions for Contagious Equine Metritis, dated 3 August 2011

Appendix III: Initial and appended Idexx laboratory report on "Index stallion" cultures

APPENDIX 1

IDEXX LABORATORIES

JOHANNESBURG
Tel: (011) 803-3001/2/3
Fax: (011) 803-3006

PRETORIA
Tel: (012) 529-6345/6
Fax: (012) 529-8318

CAPE TOWN
Tel: (021) 671-5146
Fax: (021) 671-5011

PORT ELIZABETH
Tel: (041) 365-1649
Fax: (041) 365-3818

PATHOLOGY REPORT

Owner:
Patient:
Gender:
Specie: Equine
Age: 3Y 0M 0W
Breed: Holsteiner

Lab number: 3B354402
Received: 29 March 2011 14:18:17
Printed: 2011/04/15
Referring vet:

Telephone: 011314 5338
Account: 873

CLINICAL HISTORY:

Testname	Test value	Units	Flag	Range	Low	Normal	High
Sample Materials And Evaluation	Completed						
Aerobic culture	Completed						

Antibiogram (General)
SWAB (Aerobic culture)

Bacterial Identification	Corynebacterium xerosis	Suspected Taylorella equigenitalis		
Penicillin	Sensitive	Sensitive		
Oxacillin	Sensitive	Sensitive		
Ampicillin / Amoxycillin	Sensitive	Sensitive		
Augmentin/Synulox	Sensitive	Sensitive		
Tetracycline/Doxycycline	Sensitive	Sensitive		
Quinolones	Sensitive	Sensitive		
Erythromycin	Sensitive	Sensitive		
Moxifloxacin	Sensitive	Sensitive		
Clindamycin / Lincomycin	Resistant	Resistant		
Cephalothin / Cephalexin 1	Sensitive	Sensitive		
Cefotaxime 3	Sensitive	Sensitive		
Colistin/Polymyxin B	Resistant	Sensitive		
Trimethoprim Sulpha	Sensitive	Sensitive		
Gentamicin	Sensitive	Sensitive		
Amikacin	Sensitive	Sensitive		
Neomycin	Sensitive	Sensitive		

Testname	Test value	Units	Flag	Range	Low	Normal	High

Microbiology Comment:

Only normal flora chiefly Corynebacterium xerosis were isolated from all 3 samples. No Streptococcus was isolated from any of the 3 samples nor any other obvious pathogens. Were any of the discharges from the mares cultured?

Addendum (15 April 2011):

After the isolate was made from the mare, the stallion's cultures were reincubated and cultured further. A careful search was made for colonies suspicious for Taylorella equigenitalis. No such colonies were found from the penis and semen swabs, but the urethral fossa yielded a single colony similar to the isolate from the mare. The isolate also confirms biochemically with Taylorella equigenitalis, but this needs to be confirmed serologically and/or with PCR. I will therefore send the stallion's isolate as well as the mare's isolate to Dr. Rebollar on Monday for despatch to the VLA, Suffolk, United Kingdom.

Comment by: Dr. Marijke Heuton, Tel: 011 - 803 3001 (available weekdays 10am-2pm), E-mail: maryke@idexxsa.co.za

Appendix IV: Idexx laboratories report on "Index mare"

IDEXX LABORATORIES

JOHANNESBURG
Tel: (011) 803-3001/2/3
Fax: (011) 803-3006

PRETORIA
Tel: (012) 529-8345/6
Fax: (012) 529-8318

CAPE TOWN
Tel: (021) 671-5146
Fax: (021) 671-5011

PORT ELIZABETH
Tel: (041) 365-1649
Fax: (041) 365-3818

PATHOLOGY REPORT

Owner:
Patient:
Gender: Female
Specie: Equine
Age: 4Y 0M 0W
Breed: Thoroughbred

Lab number: JB354771
Received: 01 April 2011 14:05:58
Printed: 2011/04/11
Referring vet:

Telephone: 011314 5338
Account: 873

CLINICAL HISTORY:

Testname	Test value	Units	Flag	Range	Low	Normal	High
Sample Materials And Evaluation	Completed						
F ne pre-breeding	Completed						

Antibiogram (General)
SWAB (Equine pre-breeding)

Bacterial Identification	Taylorella equigenitalis
Penicillin	Sensitive
Oxacillin	Sensitive
Ampicillin / Amoxycillin	Sensitive
Augmentin/Synulox	Sensitive
Tetracycline/Doxycycline	Sensitive
Quinolones	Sensitive
Erythromycin	Sensitive
Moxifloxacin	Sensitive
Clindamycin / Lincomycin	Resistant
Cephalothin / Cephalexin 1	Sensitive
Cefotaxime 3	Sensitive
Colistin/Polymyxin B	Sensitive
Trimethroprim Sulpha	Sensitive
...tamicin	Sensitive
Amikacin	Sensitive
Neomycin	Sensitive
Rifampicin	Sensitive
Fosfomycin	Sensitive
Tilmicosin	Sensitive
Streptomycin	Resistant
Florfenicol	Sensitive
Kanamycin	Sensitive

Testname	Test value	Units	Flag	Range	Low	Normal	High

Microbiology comment:

A heavy pure growth of isolate, biochemically Taylorella equigenitalis, was cultured. This result needs to be confirmed serologically and or by PCR. Neither test is available is in south africa, as far as I am aware.

Comment by: Dr. Marijke Henton, Tel: 011 - 803 3001 (available weekdays 10am-2pm), E-mail: maryke@idexxsa.co.za

Appendix V: AHVLA report

Animal Health and Veterinary Laboratories Agency – Bury St Edmunds
Rougham Hill, Bury St Edmunds, Suffolk IP33 2RX United Kingdom
Telephone +44 (0) 1284 724499 Facsimile +44 (0) 1284 724500
E-Mail VLA.BuryStEdmunds@ahvla.gsi.gov.uk
Web site http://www.defra.gov.uk/corporate/vla/vla

AHVLA
Animal Health and Veterinary Laboratories Agency

3rd May 2011

Dr Laura Lopez
ARC Onderstepoort Veterinary Institute
100 Old Soutpan Road
Onderstepoort 0110
Johannesburg

REPORT 1 (Final) OUR REFERENCE 14-M0543-04-11

4 swabs were received for confirmatory testing for *Taylorella equigenitalis*. The swabs were paired and labeled JB354771 (Mare) and JB354771 (Mare – duplicate) and similarly JB354402 (Stallion) and JB354402 (Stallion duplicate).

The duplicate swabs were retained and PCR testing carried out on the 'original' swabs from the mare and stallion.

JB354771 (Mare) - **POSITIVE** by real time PCR for *Taylorella equigenitalis*.
JB354402 (Stallion) - **POSITIVE** by real time PCR for *Taylorella equigenitalis*.

Paul Todd
Workgroup Leader
Animal Health and Veterinary Laboratories Agency(AHVLA)
Regional Laboratory
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Bury St Edmunds
Suffolk
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F: +44(0)1284 724500
E: paul.todd@ahvla.gsi.gov.uk

www.defra.go.uk/ahvla

The charges for this laboratory work will be 869.10 (GBP) TC0687 x 2 and will be invoiced separately

Appendix IX: CEM Submission form available on www.cemsa.co.za

SUBMISSION FORM FOR SWABS FOR PCR SCREENING OF STALLIONS FOR CEM

PART A: IDENTIFICATION OF ANIMAL
TO BE COMPLETED AND SIGNED BY STALLION OWNER / AGENT

NAME OF STALLION: ..... **BREED** .....
Passport number:..... **Date of birth** ...../...../.....
Country of origin (if not born in South Africa).....
Microchip number: .....
Name of owner:.....
Farm of origin:.....
Address:.....
Tel:..... **Cell:**..... **Email:** .....
State Veterinary district: .....
SIGNATURE: .....
.....

PART B: CEM SWAB DETAILS
TO BE COMPLETED AND SIGNED BY A VETERINARIAN

Date of collection: ...../...../..... **Place of collection:** .....
Sites of swabbing of external genitalia:
Note: A separate dry swab must be taken from each of the following three sites:
1. **urethral fossa including sinus** ☐ **2. urethra** ☐ **3. lamina** ☐

Note: Each dry swab must be clearly labeled (Stallion name; site; date) and securely sealed
Swabs obtained during course of penile erection / complete exteriorization: YES ☐ NO ☐
Swab obtained with the aid of sedation: YES ☐ NO ☐
Additional remarks: .....
.....

NAME OF VETERINARIAN..... Qualification: .....
Signature:.....
Tel: .....
Fax: .....
Cell: .....
Email: .....

PRACTISE STAMP

Submission:
By Speed Post: Equine Research Centre; Private Bag X04, Onderstepoort, 0110
By Courier: Equine Research Centre, Faculty of Veterinary Science, University of Pretoria, Old Soutpan Road, Onderstepoort, 0110

Cost:
The cost of testing is R200.00 (VAT Incl) for each set of 3 swabs

Payment Options (Mark One of the Following):
1. EFT Payment to ABSA, Account: 2140000038, Branch: 335545 (Hatfield), Reference: CEM/Name/A2373 (Proof of EFT Payment attached)
2. Invoice Owner
3. Invoice Veterinarian

(NOTE: Veterinarian will be Invoiced if neither of the options above are marked or if owner details are not provided)