

**Evaluation of
two topical treatment methods
for the elimination of
Taylorella equigenitalis
in stallions**

by
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A dissertation submitted in fulfilment of the requirements for the degree

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Declaration

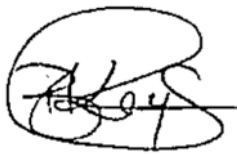
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Bronwyn Keys

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Abbreviations

AI	Artificial insemination
CEM	Contagious equine metritis
CF	Complement fixation
CFGE	Cross-field gel electrophoresis
Ct	Cycle threshold
DAFF	South African Department of Agriculture, Forestry and Fisheries
DAH	Director of Animal Health
ERC	Equine Research Centre
HBLB	Horserace Betting Levy Board
MLST	Multilocus sequence typing
OVAH	Onderstepoort Veterinary Academic Hospital
PBS	Polysulphated buffer solution
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SRS	Spanish Riding School
US	United States
USDA	The United States Department of Agriculture
VGL	Veterinary Genetics Laboratory

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Summary

Taylorella equigenitalis, the aetiological agent for contagious equine metritis (CEM) is sensitive to a wide range of antimicrobial drugs *in vitro*. Current treatments are anecdotal and validated protocols are undefined.

The objective of this study was to compare the efficacy of two topical antimicrobial agents and the duration of treatment required to eliminate *T. equigenitalis* in stallions. The treatment process was strictly monitored with daily quantitative polymerase chain reaction (qPCR) sampling and testing of the colonised stallion to determine an effective treatment duration and to note trends in cycle threshold (Ct) values. The study was approved by the University of Pretoria's Animal Use and Care Committee (V 068/12) according to the South African National Standard (SANS 10386: 2008) for the care and use of animals for scientific purposes.

Twenty three stallions colonised with *Taylorella equigenitalis* were randomly assigned to either one of two topical treatment categories: 1% silver sulphadiazine (n=11) and 0.2% nitrofurazone (n=12). A standardised treatment protocol consisting of topical cleansing and antimicrobial application was performed.

Descriptive statistical analysis of the treatment duration and cycle threshold (Ct) values obtained from qPCR was performed to compare the two antimicrobial agents and to determine trends in the data. This study can provide a validated treatment and monitoring protocol for the topical treatment of CEM in stallions.

Chapter 1: General introduction

1.1 Introduction

Contagious equine metritis (CEM) was first described in 1977 in the United Kingdom and Ireland (Crowhurst, 1977; Platt *et al.*, 1977; Ricketts *et al.*, 1977; Timoney *et al.*, 1977), and is currently described as a venereal disease of horses caused by the bacterium *Taylorella equigenitalis*. The primary means by which CEM is transmitted is through unapparent disease-carrier mares or stallions (Kristula and Smith, 2004).

CEM remains a clinically and economically important disease of horses due to the losses that have been associated with the disease as result of its profound effects on reproductive efficiency (Kristula, 2007). These losses are related both directly to temporary infertility in acutely-infected mares and indirectly to the additional costs of testing, quarantining and treatment in outbreaks, international movement regulatory protocols and ongoing surveillance. This disease has a significant effect on the free trade and movement of horses internationally.

Stallions become colonised by *Taylorella equigenitalis* and subsequently act as clinically-unaffected carriers of the organism by harbouring the bacteria in predilection sites associated with their external genitalia (Swerczek, 1978). Standardised treatment protocols report that the stallion's external genitalia should be cleaned for five consecutive days with 4% chlorhexidine gluconate followed by the topical application of an antibacterial ointment such as 0.2% nitrofurazone (Zirkle, 1998). For effective topical treatment, the penis must be fully erect and exteriorised (Luddy and Kutzler, 2010). Treatment response and its duration are currently undefined. Single five-day topical treatment duration was reported sufficient to eliminate the organism from the stallion's reproductive tract (Timoney, 1996). The

bacterium was shown to be sensitive to most of the antibiotics commonly tested *in vitro* (Ensink *et al.*, 1993; Sugimoto *et al.*, 1981).

The literature is conflicting about treatment protocols with regard to factors including: inclusion of systemic antimicrobial therapy as an adjunct to topical antimicrobial therapy, choice of topical antimicrobial agent, treatment duration and surgical ablation of the clitoris (Luddy and Kutzler, 2010; Anzai *et al.*, 2011; Erdman *et al.*, 2011). In addition, treatment protocols are anecdotal and reports of validated protocols are lacking, thus warranting further investigation.

Chapter 2: Literature review

2.1 Aetiology and epidemiology

The aetiological agent responsible for contagious equine metritis (CEM) was established as *Taylorella equigenitalis* (originally classified as *Haemophilus equigenitalis*) (Taylor *et al.*, 1978). The bacterium is a Gram-negative coccobacillus with fastidious growth requirements (Ousey *et al.*, 2009; Timoney, 2011). Two biotypes of the organism were identified, those either sensitive to or resistant to streptomycin (Swerczek, 1978; Platt and Taylor, 1982). 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 carrier animals colonised by *Taylorella equigenitalis* (Timoney and Powell, 1988). It has also been demonstrated that there are biological differences between strains of *Taylorella*, and these proposed strains differ in their pathogenicity based on field observations of naturally occurring outbreaks of *T. equigenitalis* and also on the outcome of early experimental studies (Timoney *et al.*, 1978b, 1979; Kanemaru *et al.*, 1988). Any correlation between specific genotypes and the ability to induce clinical infection in the mare is currently undefined (Timoney, 2011).

A highly-transmissible venereal disease of the horse, CEM was first reported in Thoroughbreds in 1977 in Newmarket, United Kingdom (Crowhurst, 1977; Timoney and Powell, 1988). Apparently CEM was introduced by unapparent disease-carrying Thoroughbred mares being shipped from Ireland to be covered by Newmarket stallions in the spring of 1977 (Knowles, 1983). The disease was speculated to have entered Ireland via

Thoroughbred mares shipped internationally from France in 1976 (Knowles, 1983). Thoroughbred stallions imported from France are thought to have introduced the disease into the United States (US) in 1978 (Kristula, 2007). The disease has been reported in numerous countries since then, including several European countries, Asia, Australia, North and South America (Timoney, 1996; Swerczek, 1978; Erdman *et al.*, 2011; Timoney, 2011; Ricketts *et al.*, 2012). In 1977, the UK Horserace Betting Levy Boards's (HBLB) Voluntary Code of Practice (2011) was adopted by many countries as a “standard operating procedure” for the prevention and control of CEM and other sexually transmitted diseases. The widespread adoption of this code eradicated the disease in the UK (Ousey *et al.*, 2009). In the non-Thoroughbred population throughout mainland Europe, CEM has become endemic and is assumed to be the current source for episodic outbreaks (Ousey *et al.*, 2009; Schulman *et al.*, 2013; Ricketts *et al.*, 2012). Most recently, an outbreak was reported in the USA (California) involving a singular positive mare which was confirmed with bacterial culture in February 2013 (www.promedmail.org). One of the more recent and significantly important was the first outbreak of CEM in South Africa, a country previously considered as free of the disease that was reported in 2011 (May *et al.*, 2012; Schulman *et al.*, 2013).

2.2 Pathogenesis

CEM is transmitted by direct or indirect venereal contact (Timoney, 1996). The carrier state has been confirmed in both mares and stallions, with these animals providing a focus for initiating fresh outbreaks of the disease by dissemination of the causal organism primarily by the venereal route (Timoney and Powell, 1988). Once predilection sites associated with the external genitalia are colonised, stallions become carriers of *T.*

equigenitalis (Swerczek, 1979; Timoney, 2011). The bacterium may be isolated from the following reported predilection sites: urethral fossa and its sinus, distal urethra, *lamina interna* of the prepuce, and the surface of the penis (Timoney and Powell, 1988). Schluter *et al.* (1991) isolated *T. equigenitalis* at slaughter from the prepuce, urethra, testis, epididymis and seminal vesicles of an infected stallion, demonstrating that *T. equigenitalis* may not always be confined to the external genitalia of the stallion.

The clinical picture in the mare is different as *T. equigenitalis* may cause temporary infertility, albeit without concurrent evidence of systemic illness. Acute infection in mares may result in a failure to conceive, and if conception does occur, mares may either abort or produce foals at term which could potentially be congenital carriers of the organism (Nakashiro *et al.*, 1981; Timoney and Powell, 1982). Abortion is however a reportedly rare sequel to infection, in two cases the bacterium was recovered not only from the placenta but also from several sites in the aborted foetus (Nakashiro *et al.*, 1981; Timoney, 1996). Clinical signs in acutely-infected mares range from a copious purulent or mucopurulent vaginal discharge for up to 14 days after mating or artificial insemination (AI) to shortened dioestrus intervals unaccompanied by other clinical signs (Powell, 1981; Kristula, 2007). Affected mares may then become carriers of *T. equigenitalis*. Endometritis, cervicitis and vaginitis are associated with the vaginal discharge. The incubation period varies from 2-12 days (Timoney, 1996). No long-term adverse effects on fertility have been reported (Timoney, 2011). Re-exposure of mares previously infected with *T. equigenitalis* is associated with the development of minimal or no clinical evidence of CEM (Timoney and Powell, 1988). An undefined percentage of mares may become chronically infected (Wood *et al.*, 2005), with bacteria localising most commonly in the clitoral sinuses and fossa and occasionally in the uterus (Kristula, 2007), but this does not seem to interfere with normal

pregnancy maintenance. The shedding pattern of *T. equigenitalis* by carrier mares can vary, with significant fluctuations in the number of detectable organisms being observed in certain animals over time (Timoney *et al.*, 1978; Timoney and Powell, 1988).

Direct, horizontal transmission of *T. equigenitalis* via the venereal route from the stallion to the mare, occurs primarily through natural service or AI (Timoney, 1996). The risk of infection with the use of fresh or chilled semen for AI via contaminated semen collected from a carrier stallion, even where appropriate antibiotics are included in the semen extender is considerably less when compared with exposure by natural service (Timoney, 2011). The risk associated with cryopreserved semen from a carrier stallion is thought to be minimal but has not yet been established (Timoney, 2011). The organism can also be mechanically transmitted by indirect means (fomites) through the inadvertent contamination of equipment, e.g. vaginal specula, examination sleeves, insemination equipment, etc., or by personnel failing to observe adequate hygienic precautions in handling mares and stallions at time of breeding (Powell, 1981; Timoney, 1996). As previously stated the organism may also be transmitted *in utero* (Powell and Whitwell, 1979) and may result in foals becoming congenitally infected or contaminated with the bacterium at birth (Timoney and Powell 1982; Timoney, 2011).

2.3 Diagnosis

T. equigenitalis can be isolated by bacterial culture from samples obtained from the external genitalia of the stallion and the proximal and distal reproductive tract of the mare (Erdman *et al.*, 2011). The bacteriological culture and identification of *T. equigenitalis* is the current “gold standard”, mandatory for pre-export and pre-import certification and

detection of the carrier state for all international trade and movement protocols (Zdovc *et al.*, 2005; Kristula, 2007; Luddy and Kutzler, 2010; Aalsburg and Erdman, 2011).

Diagnostic tests currently required in South Africa involve a combination of bacterial culture and qPCR testing. Stallions, colts and geldings require duplicate swab sampling of the following sites: urethra, urethral fossa including urethral sinus and the *lamina interna* of an exteriorized penis. Mares and fillies also require duplicate swab sampling of the following sites: clitoral fossa, clitoral sinus and endometrium (non-pregnant mares) (Appendix 1).

The most appropriate sites to culture for *T. equigenitalis* in the maiden, barren or post-parturient mare include the uterus (endometrium) or cervix, clitoral sinuses and fossa. The use of swabs small enough to effectively sample the clitoral sinuses is also required (Platt and Taylor, 1982; Timoney and Powell, 1988). Endometrial or cervical swabs should ideally be obtained during early oestrus (Timoney, 1996). The swabbing of pregnant mares is restricted to sampling from the clitoral sinuses and fossa (Timoney, 1996). Samples of a vaginal exudate, if present, should additionally be submitted for bacterial culture.

For stallions, the most common sites from which swabs are obtained include: the distal urethra, the *lamina interna* of the prepuce and surface of the penis, the urethral fossa (*fossa glandis*) and the urethral sinus, and a sample of pre-ejaculatory fluid. The penis should be fully extruded and erect to facilitate effective sampling of these sites (Timoney, 1996). The regulatory reliance on bacterial culture and its variable sensitivity has required additional testing as a further diagnostic safe-check (Zdovc *et al.*, 2005; Schulman *et al.*, 2013). Certain regulatory protocols have thus required that a stallion found negative for the bacterium on initial testing should subsequently mate two test mares of known *T. equigenitalis*-negative status (by bacteriological and serological methods). Following this, the stallion's test mares undergo a series of tests using a combination of serology by a

complement fixation (CF) test and bacterial culture of samples obtained from the clitoral sinuses and fossa and intra-uterine samples (Kristula, 2007). Confirmation of the carrier state in a stallion is based on demonstration of *T. equigenitalis* in the reproductive tract of the test mares and, or development of antibodies to the organism within 15-40 days after breeding (Timoney, 1996).

Bacterial culture for *T. equigenitalis* as a diagnostic method has several reported disadvantages: genital swabs must be collected and transported in Amies' charcoal medium, refrigerated at 4-6°C during transit, must reach a laboratory officially licensed by the health authorities to carry out the *T. equigenitalis* bacteriological culture within 24-48 h, *T. equigenitalis* must be grown in an atmosphere of 7-10% carbon dioxide at 37°C for 6-10 d, and genital swabs may be strongly contaminated by saprophytic bacteria and fungi which conceal and, or inhibit *T. equigenitalis* growth (Duquesne *et al.*, 2007). In addition, Jang *et al.*, (2001) confirmed the presence of a second species within the genus *Taylorella* named *Taylorella asinigenitalis* and isolated from genital tracts of donkeys. The colony morphology and growth rate of these two species were too similar to allow differentiation through standard bacteriological isolation (Duquesne *et al.*, 2007).

No serologic test is reliable for the diagnosis and control of CEM (Kristula, 2007). Serology has limited application and sero-conversion was reported as a transient feature associated with acute endometritis in the mare, and absent in stallions (Timoney, 1996; Schulman *et al.*, 2013). The CF test is a variably-useful diagnostic adjunct for detecting infection in the acutely infected mare. Antibodies are detected at approximately seven days and usually reach maximal titres at about three weeks and then start to decline between six and ten weeks after the primary infection with *T. equigenitalis*. No serological differences

have been observed among the isolates of *T. equigenitalis* examined throughout the world (Croxtton-Smith *et al.*, 1978; Taylor *et al.*, 1978; Benson *et al.*, 1977; Fernie *et al.*, 1979).

Various polymerase chain reaction (PCR) tests have been developed to detect *T. equigenitalis* (Matsuda and Moore, 2003; Zdovc *et al.*, 2005; Wakeley *et al.*, 2006; Duquesne *et al.*, 2007; Kristula, 2007; Ousey *et al.*, 2009; Anzai *et al.*, 2011). A genus-specific primer set and a probe for confirmation was derived from previously determined 16S ribosomal DNA sequences (Bleumink-Pluym *et al.*, 1993; Matsuda and Moore, 2003). Real-time PCR has been an important development in providing a method of detection of *T. equigenitalis* with enhanced specificity, sensitivity and speed of identification when compared to culture. In addition, this test is able to differentiate between the two species of phenotypically indistinguishable *Taylorella sp.* i.e. *T. equigenitalis* and *T. asinigenitalis*. PCR analysis on genital swabs has been shown to be more sensitive than culture (Bleumink-Pluym *et al.*, 1994; Zdovc *et al.*, 2005). Taqman® labelled probe and fluorescent dye systems are considered superior to conventional gel-based PCR as they provide a fast and efficient method for quantification of target DNA, are highly specific, thereby eliminating DNA carryover and the occurrence of false positives, and the results are readily reproduced (Pusterla *et al.*, 2006; Ousey *et al.*, 2009).

The *Taylorella* genus comprises two species: *Taylorella equigenitalis*, associated with contagious equine metritis (CEM) and *Taylorella asinigenitalis*, a closely-related species mainly found in donkeys (Jang *et al.*, 2001; Hebert *et al.*, 2012). The *T. equigenitalis* MEC9 genome which was isolated in 2005 from a stallion was sequenced recently (Hebert *et al.*, 2011). Furthermore, the *T. asinigenitalis* genome has also been sequenced by comparing it to the previously-sequenced *T. equigenitalis* genome (Hebert *et al.*, 2012). Molecular

379	CCCTGATCCAGCCATTCCGCGTGTGCGATTGAAGGCCTTCGGGTTGTAAAGCACTTTTGT	T. asinigenitalis UCD-1
361	CCCTGATCCAGCCATTCCGCGTGTGCGATTGAAGGCCTTCGGGTTGTAAAGCACTTTTGT CCGCGTGTGCGATTGA	T. equigenitalis K- 188 Taylorella 377 for
439	CAGGGAAGAAAAG <u>I</u> TTT <u>A</u> GG <u>A</u> TAAATACCCTAGGATGCTGACGGTACCTGAAGAATAAGCA	T. asinigenitalis UCD-1
421	CAGGGAAGAAAAG <u>G</u> TTT <u>G</u> T <u>I</u> TAAATACC <u>A</u> T <u>G</u> G <u>A</u> CTGCTGACGGTACCTGAAGAATAAGCA	T. equigenitalis K- 188
	AAAGTTTTAGGATAATACCCTAGGATGCTGACGG	TasiniHEX
	AAAGTTTGTGTTAATACCATGGACTGCTGACGG	TequiFAM
	TGAAGAATAAGCA	Taylorella 488 rev
499	CCGGCAAACACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCGGAATT	T. asinigenitalis UCD-1
481	CCGGCAAACACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCGGAATT CCGGCAAA	T. equigenitalis K- 188 Taylorella 488 rev

Figure 1: Alignment of representative *T. equigenitalis* (K-188) and *T. asinigenitalis* (UCD-1) 16S rDNA sequences with primers and probes used in the Taylorella Taqman® PCR assay. The eight divergent residues covered by the Taqman® probes for the two species are underscored (Wakeley *et al.*, 2006).

genotyping of *T. equigenitalis* strains by pulsed-field gel electrophoresis (PFGE) after digestion with the suitable restriction enzymes enabled the effective discrimination of strains, thus allowing the examination of the mechanisms for both its occurrence and transmission of contagious equine metritis (Matsuda and Moore, 2003). The differentiation of the *Taylorella* genus was confirmed by 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 isolates obtained from donkeys (Matsuda and Moore, 2003). Both PFGE and cross-field gel electrophoresis (CFGE) reportedly provide a more useful discriminatory genotyping method than several other DNA genotyping techniques for the epidemiological study of CEM (Matsuda and Moore, 2003). It was recommended that almost all the strains of *T. equigenitalis* that have been isolated and are currently archived in various institutes world-wide should be analysed by PFGE genotyping

in order to help elucidate the reservoirs and to clarify the mechanisms of occurrence and transmission of this bacterial organisms in equines (Matsuda and Moore, 2003).

Most recently, Duquesne *et al.*, 2013 described the development of a multilocus sequence typing (MLST) scheme for *Taylorella equigenitalis* and *Taylorella asinigenitalis*. Molecular typing tools include PFGE genotyping (Aalsburg and Erdman, 2011), field inversion gel electrophoresis (Bleumink-Pluym *et al.*, 1990), chromosomal DNA fingerprinting (Thoresen *et al.*, 1995), CFGE (Miyazawa *et al.*, 1995). These molecular epidemiological tools are poorly portable because of their index of variation and it is difficult to compare results between laboratories (Maiden *et al.*, 1998). MLST is based on nucleotide sequence and is therefore highly discriminatory and provides unambiguous results that are directly comparable among laboratories (Enright *et al.*, 2000) and is thus more appropriate for large-scale epidemiologic investigations and population biology studies (Duquesne *et al.*, 2013).

2.4 Treatment

Treatment protocols in both mares and stallions albeit reportedly successful are however currently undefined (Schulman *et al.*, 2013). Standardised protocols for the treatment of *T. equigenitalis*-positive mares include flushing the clitoral sinuses using a cerumenolytic agent before irrigating with warmed saline solution (Luddy and Kutzler, 2010). The clitoral sinuses and fossa are subsequently scrubbed for an additional four days using 4% chlorhexidine gluconate and packed with either 0.2% nitrofurazone ointment, 1% silver sulphadiazine, gentamicin ointment or other suitable antimicrobial preparation efficacious against *T. equigenitalis* (Timoney, 1996; Kristula, 2007; Luddy and Kutzler, 2010;

Anzai *et al.*, 2011). While *T. equigenitalis* is sensitive to a wide range of antimicrobial drugs *in vitro*, currently no one treatment will ensure both a rapid resolution of clinical signs associated with CEM and clearance of the organism from the sites of persistence in the carrier mare (Platt and Taylor, 1982). In individual mares, several courses of treatment may be required before the organism is successfully eliminated (Timoney, 1996). Mares undergoing repeated treatments may undergo surgical ablation of the clitoral sinuses, however this is currently rarely performed due to the reported efficacy of topical treatment (Luddy and Kutzler, 2010; Anzai *et al.*, 2011).

The reported standardised treatment protocol for stallions is similar, consisting of scrubbing the external genitalia for five days with 4% chlorhexidine and packing with 0.2% nitrofurazone ointment or any of the aforementioned antimicrobial preparations. In contrast to the mare however, treatment of colts or stallions for elimination of *T. equigenitalis* from the external genitalia has been reported as being apparently straightforward and frequently successful (Platt and Taylor, 1982; Timoney and Powell, 1988). More recently-reported case studies have however demonstrated varying treatment success in stallions (Kristula and Smith, 2004; Erdman *et al.*, 2011).

The standardised treatment protocol was not always effective in stallions (Kristula and Smith, 2004) and a more aggressive combination of both topical and systemic antibiotics may be required to rid some infected mares and stallions of *T. equigenitalis*. A treatment regimen with either oral trimethoprim sulphamethoxale, 30mg per kg twice daily, or an antibiotic choice based on sensitivity, along with cleaning of the genitalia (as previously described) and packing with a suitable antimicrobial agent may be required. It is imperative when initiating treatment of infected horses that, a treatment protocol be

discussed with an experienced veterinarian concerning drugs of choice, optimal administration routes and duration of treatment (Kristula and Smith, 2004).

2.5 Prevention and Control

The bacteriological culture and identification of *T. equigenitalis* is the current “gold standard”, mandatory for pre-export and pre-import certification and detection of the carrier state (Kristula, 2007). The United States Department of Agriculture (USDA) stipulates a protocol for use at a certified CEM quarantine station (Kristula and Smith, 2004).

Integral to the success of any national program for the prevention and control of CEM has been the ability to classify the disease as either a “notifiable” or a “non-notifiable but reportable” condition (Platt and Taylor, 1982; Timoney and Powell, 1988). A laboratory able to provide a reliable and rapid diagnostic service for *T. equigenitalis* is paramount to any successful control program (Kristula, 2007).

Countries that are CEM-free require the pre-importation screening of all imported horses from countries in which CEM is known or suspected to exist (Timoney and Powell, 1988; Blanchard *et al.*, 1992). Pre- and post-import quarantine screening following the US protocol should include both bacteriological examination of the stallion together with test-breeding of the stallion to two susceptible mares that are subsequently monitored for evidence of transmission of *T. equigenitalis* (Timoney, 1996). All mares should be rigorously screened both bacteriologically and serologically for the presence of *T. equigenitalis* before being released from quarantine stations. Screening with a PCR-assay could potentially both expedite and improve the sensitivity of bacterial culture to diagnose the presence of *T. equigenitalis* (Kristula and Smith, 2004). Any mares or stallions that culture positive for *T.*

equigenitalis must be isolated and not bred until being treated and confirmed free of the organism (Timoney, 1996).

Codes of practice have been developed by various countries, including South Africa, which provide guidelines for veterinarians, horse owners and breeders alike for the prevention and control of CEM in horse breeding populations (Platt and Taylor, 1982; Timoney and Powell, 1988; <http://www.nda.agric.za/vetweb/pamphlets&Information/Policy/CEM%20Screening%20manual-%20signed%2021%20May%202012.pdf>). It is imperative as emphasized in these codes, to keep in mind improved hygiene standards on breeding farms and minimise the risks of indirect spread of *T. equigenitalis*. Stringent control methods and regulations for equine movement are currently deemed necessary to minimize and control the spread of *T. equigenitalis* (Kristula, 2007). It has been reported that it is possible to prevent outbreaks and to eradicate this disease in countries where effective control programs have been instituted (Anzai *et al.*, 2011)

2.6 Status in South Africa

CEM is a controlled animal disease according to the South African Animal Diseases Act, No. 35 of 1984. CEM was first confirmed in South Africa in April 2011 and reported to the Organisation Internationale Epizootique (OIE) on 9 May 2011 (http://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review/viewsummary?fupser=&dothis=&reportid=10553). Prior to this, it had never been reported in South Africa (or indeed anywhere in Africa). In terms of Table 2 of the Animal Diseases Regulations (1986) the following control measures for CEM were prescribed:

- i) In respect of a susceptible animal: “Serving of infected mares or by infected stallions shall be prevented”.
- ii) In respect of contact animals: “Contact animals shall be isolated and tested under the supervision of or by an officer or authorized person”.
- iii) In respect of infected animals: “Infected animals shall be isolated, and in the case of:
 - a. mares, destroyed under the supervision of or by an officer or authorized person; and
 - b. stallions, castrated or destroyed under the supervision of an officer or authorized person”.

However, subsequently scientific information indicated that CEM could be treated successfully in both stallions and mares that have become colonized by *T. equigenitalis*. The Director of Animal Health (DAH) therefore made a derogation (exemption) in terms of Regulation 11 (2) (b) that was issued to each owner or manager of an infected animal by means of an official order. This order stipulated the treatment measures that should be complied with in full, as defined in the South African Procedural Manual for Contagious Equine Metritis for the Outbreak Identified April 2011 (Appendix 2). This manual was compiled by the DAH, in consultation with designated experts from the University of Pretoria’s Faculty of Veterinary Science. The purpose of the Manual was to provide guidelines for the management of suspected or confirmed cases of CEM in terms of quarantine, sample collection, testing and treatment. The Manual was issued on the 18th April 2011 by the DAH to all the Provincial Directors of Veterinary Service, and was distributed to and via the South African Veterinary Council (SAVC), South African Veterinary Association (SAVA), equine breed societies and other relevant stakeholders. In order to

determine the extent of infection and to protect the Equine Industry, every breeding stallion in South Africa, irrespective of breed, was required to be tested twice (using real-time qPCR) at an interval of no less than seven days and have negative results for both *Taylorella* dual assay qPCR tests prior to breeding or semen collection. This was described in the Procedure Manual Screening of Stallions for Contagious Equine Metritis dated 3 August 2011 (<http://www.nda.agric.za/vetweb/pamphlets&Information/Policy/CEM%20Screening%20Manual-%20signed%2021%20May%202012.pdf>) and became effective for the 2012/2013 breeding season that commenced from the 1st July 2012.

An official CEM clearance certificate would be issued by the Equine Research Centre (ERC), Faculty of Veterinary Science once two negative PCR test results had been obtained (DAFF, 2011). In terms of Section 11 of the Animal Diseases Act, the DAH issued an instruction or order to every owner or manager of stallions stipulating, that no stallion (irrespective of breed) could be used or allowed to breed (natural mating or artificial insemination), unless an official CEM clearance certificate had been issued. This instruction or order took effect from the 31 August 2011. All stallions were obliged to be screened for *T. equigenitalis* annually from 1 July-31 September, prior to the breeding season. A website, www.cemsa.co.za was created in May 2012, which formed a database for all breeding stallions in the country and their corresponding testing and results status for CEM (May, 2012).

2.7 The Lipizzaners

The Lipizzaner breed dates back to around 1562, when Archduke Maximilian first bred Spanish horses in Lipica, a village in modern-day Slovenia (<http://www.lipizzaners.co.za/Modules-FE/layout1/displayfull.asp>). It was the need for military horses of unusual

strength, loyalty and courage that inspired him to import Spanish, Italian and Arab-oriental horses for his breeding programme. The modern-day Lipizzaner is thus well suited to the discipline of classical horsemanship.

The Spanish Riding School (SRS) in Vienna as it is known today has been in existence since the 19th century (<http://www.srs.at/en/tradition/the-history>). It is the only riding academy in the world where the Renaissance tradition of classical horsemanship is preserved and cultivated to this day. Just over 400 Lipizzaners are maintained at the SRS (<http://blogs.equisearch.com/horsehealth/2007/04/16/when-bad-things-happen-to-nice-horses-equine-venereal-disease-at-austrias-spanish-riding-school>). Their performances are fondly known as “the ballet of the dancing white stallions”. The stud farm in Piber was established in 1920 (<http://www.srs.at/en/tradition/the-history>).

Meier (2010) reported that late in 2006, a three year-old stallion from the SRS tested positive for *T. equigenitalis* during the export screening process to the USA. The following triple screen (PCR and culture method) of all 68 stallions at the SRS revealed 48 (71%) positive results. Testing of all Lipizzaner horses at the stud farm in Piber was performed. All six stallions used for breeding purposes were negative. While all yearling colts were negative, 44% of the 50 two-, three- and four-year old colts and stallions and five geldings tested positive. *T. equigenitalis* was also detected in eight non-breeding mares and two of the 50 broodmares. The main method used for breeding was by semen collection for AI. No clinical signs were observed. The origin of the infection was unknown and *T. equigenitalis* appears to have been endemic in both Vienna and Piber for at least six years. Transmission appears to have taken place mostly within the stallions through direct contact or contamination (DEFRA, 2010). Most of the infected stallions were never used for breeding, nor was semen ever collected from them.

Following World War 2, a number of Lipizzaners were rescued from war-torn Europe and imported to South Africa by Count Jankovich Besan (<http://www.lipizzaners.co.za/Modules-FE/layout1/displayfull.asp>). The *T. equigenitalis*-positive stallions included in this study were all direct descendants of these horses, with the exception of a single stallion that was directly imported from the SRS in 2000. At the time of his importation, this stallion had tested negative by bacterial culture for *T. equigenitalis* during his post-importation quarantine.

Chapter 3: Evaluation of two topical treatment methods for the elimination of *Taylorella equigenitalis* in stallions

3.1 Abstract

Reasons for performing study: *Taylorella equigenitalis*, the aetiological agent for contagious equine metritis is reportedly sensitive to a wide range of antimicrobial drugs *in vitro*. Treatment regimens have been used with an antibiotic choice based on sensitivity, together with cleaning of the genitalia and application of a topical antimicrobial agent. Controversy surrounds treatment protocols including the choice of antimicrobial therapy to apply topically, whether or not to include systemic antimicrobials as an adjunct to the topical antimicrobial therapy, and the treatment duration. Current treatments are anecdotal and undefined and validated protocols are lacking.

Objectives: The objective of this study was to compare the efficacy of two topical antimicrobial agents and duration of treatment required to eliminate *T. equigenitalis* in colonised stallions.

Methods: Twenty-three Lipizzaner stallions that tested positive for *T. equigenitalis* on a combination of bacterial culture and duplex qPCR assay from swabs of the external genitalia were included and randomly assigned to either of two topical antimicrobial-ointment treatment categories: Category 1 (1% silver sulphadiazine, n=11) or Category 2 (0.2% nitrofurazone, n= 12). Stallions were sedated to allow effective extrusion of the penis. These treatments did not include the administration of systemic antimicrobials. The topical treatment protocol consisted of an initial two consecutive days of once-daily topical

cleansing of the penis and prepuce with a surfactant solution that contained 5% docusate sodium to remove all smegma and external debris. Thereafter, treatment consisted of once-daily topical cleansing with 5% docusate sodium and irrigation with a disinfectant solution containing 4% chlorhexidine gluconate before applying the topical antimicrobials until the individual stallion tested negative for *T. equigenitalis* on three consecutive days. This testing was by quantitative polymerase chain reaction (qPCR) assay of daily genital swabs obtained prior to commencing treatment.

Results: All stallions were effectively treated and three weeks thereafter all tested negative for *T. equigenitalis* on both bacterial culture and qPCR. Eleven of 23 stallions (48%) tested negative at the conclusion of the defined minimum treatment interval regardless of antimicrobial selection. Median values for the interval to elimination were 10.5 d (Category 2) and 11 d (Category 1). Individual treatment response showed great variability between stallions. There was no significant difference between the two antimicrobial agents for the duration of treatment ($P= 0.396$).

Conclusions: The application of either 1% silver sulphadiazine or 0.2% nitrofurazone as topical antimicrobial agents was equally effective for eliminating *T. equigenitalis* from the external genitalia of stallions. The use of qPCR was demonstrated to be a valuable adjunct in monitoring treatment.

Potential relevance: This study demonstrated an effective topical antimicrobial treatment of *T. equigenitalis* in stallions.

3.2 Introduction

Treatment regimens for *T. equigenitalis* in stallions that have been most-recently reported include the parenteral administration of an antibiotic agent with the choice based

on sensitivity, together with cleaning of the genitalia and application of various topical antimicrobial agents (Luddy and Kutzler, 2012; Timoney, 2011; Anzai *et al.*, 2012). The duration and response of treatment in stallions however, remains anecdotal and reported durations were: five (Luddy and Kutzler, 2012; Anzai *et al.*, 2012; Erdman *et al.*, 2011), seven (Samper, 2006) and 10 days (Kristula and Smith, 2004) all with varying degrees of success. The United States Department of Agriculture (USDA) prescribes a treatment protocol for stallions of scrubbing the external genitalia for five days with 4% chlorhexidine and packing with 0.2% nitrofurazone ointment (Zirkle, 1998). Treatment of colts and stallions for elimination of *T. equigenitalis* is often considered uncomplicated and frequently successful (Platt and Taylor, 1982; Timoney & Powell, 1988). The USDA treatment protocol was reportedly not consistently effective and a more aggressive combination of both topical and systemic antibiotics may be required to effectively eliminate *T. equigenitalis* in individual stallions (Schluter *et al.*, 1991; Kristula and Smith, 2004).

The purpose of this study was to determine which topical antimicrobial ointment (0.2% nitrofurazone or 1% silver sulphadiazine) was most effective in treating *T. equigenitalis*-positive stallions and concurrently determining the treatment duration required for successful elimination of *T. equigenitalis*.

3.3 Materials and Methods

Background

Twenty four of the 33 (73%) Lipizzaner stallions resident at the South African Lipizzaner Centre (Midrand, Gauteng, South Africa) tested positive for *T. equigenitalis* by a combination of bacterial culture and a duplex-qPCR assay from swabs obtained from their

external genitalia (<http://www.nda.agric.za/vetweb/pamphlets&Information/Policy/CEM%20Screening%20manual-%20signed%202021%20May%202012.pdf>). Two sets (Set 1 and Set 2) of three duplicate swabs were obtained from the following predilection sites: distal urethra (Fig 1), urethral fossa including sinus (Fig 2) and the *lamina interna*. The swabs from Set 1 were transported in Amies charcoal medium on ice for bacterial culture within 6 h of collection. The method used for bacterial culture was according to the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Set 2 were dry swabs (Labchem, Johannesburg, South Africa) submitted on the day of collection for a duplex-qPCR assay for *T. equigenitalis* and *T. asinigenitalis* (Wakeley *et al.*, 2006).



Figure 2: Swabbing the distal urethra in the stallion



Figure 3: Swabbing the urethra fossa and sinus in the stallion

A depopulation-repopulation exercise at the Lipizzaner Centre was implemented. The entire stallion population was removed from the Lipizzaner Centre. The *T. equigenitalis*-positive stallions were moved under State Veterinary Permits and were housed in an isolation facility under strict quarantine measures supervised by the South African Department of Agriculture Forestry and Fisheries (DAFF). The *T. equigenitalis*-negative stallions were removed to an additional isolation facility and were tested weekly for three consecutive weeks to confirm maintenance of a negative status before being returned to

the Lipizzaner Centre. In the interim depopulation phase, the Lipizzaner Centre's stabling facilities were subjected to a rigorous cleaning, decontamination and disinfection process. This included removal of all bedding materials prior to steam-cleaning, disinfection and repainting. All wooden doors and partitions were replaced. All tack, grooming kits and items in contact with the horses were either discarded or disinfected.

Study population

Twenty three of the 24 *T. equigenitalis*-positive stallions were included in this study and were randomly assigned to either of two categories: Category 1 (n=11) treated with 1% silver sulphadiazine (Silbecor Biotech, South Africa) and Category 2 (n=12) treated with 0.2% nitrofurazone (Aspen Pharmacare, Sandton) as topical agents on the external genitalia (Table 1). Category 1 ages ranged from 4-19 years (median: 9 years) and Category 2 ages ranged from 5-19 years (median: 12 years). The nine *T. equigenitalis*-negative stallions aged 5-21 years (median: 10 years) received no treatment. The *T. equigenitalis*-positive stallion not included in this study was successfully treated at a separate isolation facility under State supervision at the owner's request. Individual animals were readily identified by both their names and freeze-brand markings visible on their bodies. The owners of the stallions provided consent based on an informed decision to allow their animals to participate in this study.

Treatment protocol

Stallions were sedated with an intravenous combination of detomidine hydrochloride (Domosedan, Novartis SA (Pty) Ltd, Animal Health, Isando) at 0.01mg/kg and butorphanol tartrate (Torbugesic, Fort Dodge Animal Health, Iowa, USA) at 0.01-0.02 mg/kg which allowed effective extrusion of the penis. The treatment protocol consisted of topical treatment alone without the additional administration of systemic antimicrobials. The first

two consecutive days consisted of topical cleansing of the penis and prepuce with a surfactant solution containing 5 % docusate sodium (Docusol, Kyron Laboratories, Benrose, RSA) once daily to remove all smegma and external debris.

Table 1: Summary of the resident population at the South African Lipizzaner Centre by their *T. equigenitalis* status, age, sex and allocation to topical antimicrobial treatment category: Category 1 (1% silver sulphadiazine) or Category 2 (0.2% nitrofurazone).

Animal identity	qPCR assay	Age (years)	Sex	Category
1	Positive	12	Stallion	1
2	Positive	12	Stallion	2
3	Positive	9	Gelding	1
4	Positive	6	Stallion	1
5	Positive	8	Stallion	1
6	Positive	19	Stallion	2
7	Positive	17	Stallion	2
8	Positive	17	Stallion	2
9	Positive	6	Stallion	1
10	Positive	19	Stallion	1
11	Positive	17	Stallion	2
12	Positive	4	Stallion	1
13	Positive	15	Stallion	2
14	Positive	10	Stallion	1
15	Positive	9	Stallion	1
16	Positive	9	Stallion	2
17	Positive	5	Stallion	2
18	Positive	4	Stallion	1
19	Positive	7	Stallion	2
20	Positive	12	Stallion	2
21	Positive	10	Stallion	1
22	Positive	6	Stallion	2
23	Positive	7	Stallion	2
24	Positive	16	Stallion	-
25	Negative	11	Stallion	-
26	Negative	17	Stallion	-
27	Negative	21	Gelding	-
28	Negative	10	Stallion	-
29	Negative	5	Stallion	-
30	Negative	10	Stallion	-
31	Negative	20	Stallion	-
32	Negative	10	Stallion	-
33	Negative	9	Stallion	-

Thereafter daily treatment consisted of topical cleansing of the external genitalia with the 5 % docusate sodium solution and irrigation with a disinfectant solution containing

4 % chlorhexidine gluconate (Dismed Bioscrub, Dismed Pharma (Pty) Ltd, Halfway House), before drying of the area with paper towelling and application of either one of the two topical antimicrobial ointments: 1 % silver sulphadiazine (Category 1) or 0.2 % nitrofurazone (Category 2) until testing negative on qPCR on three consecutive days. Two sets of swabs were obtained on the first day: the first set for bacterial culture, and the second set for qPCR analysis as detailed above. Thereafter, prior to commencement of daily treatment, a set of genital swabs was similarly obtained and additionally, following irrigation with 4% chlorhexidine gluconate and drying, an additional set was obtained from the same three aforementioned sites. All swabs were submitted for qPCR analysis and reporting on the same day.

All positive stallions remained at the isolation facility until testing negative on daily qPCR and confirmation by both bacterial culture and qPCR testing ≥ 21 d post-treatment, whereupon individuals were returned to the Lipizzaner Centre. Following the repatriation of the last negative post-treatment stallion, two sets of swabs from the three sites were similarly obtained from all repatriated stallions: Set 1 for bacterial culture and Set 2 for qPCR assay to confirm their negative status upon return. Thereafter all resident stallions were tested monthly by swabbing for qPCR assay alone for a period of twelve months.

All persons engaged in testing and treatment of the stallions wore appropriate protective clothing and ensured the necessary precautions to prevent spread of *T. equigenitalis* between individual animals. A double-gloving technique was used for persons treating the stallions and a single-gloving technique was used for persons handling the samples. Gloves were discarded and a new set used for each stallion to both prevent cross-contamination and confounding of the highly-sensitive qPCR assay. All clothing, equipment

and refuse were appropriately disinfected or securely bagged on site and removed for incineration.

Molecular assay

The method of qPCR analysis used in this study was based on the Taqman[®] PCR method with several modifications instituted by the Equine Research Centre (ERC, University of Pretoria, Gauteng, South Africa) in combination with the Veterinary Genetics Laboratory (VGL, University of Pretoria, Gauteng, South Africa). The Taqman[®] PCR was designed using common amplimers for all the *Taylorella* sp. to produce a 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 (Figure 1) (Wakeley *et al.*, 2006). The Taqman[®] PCR has been shown to be specific for the 16S ribosomal DNA of the two species of *Taylorella* and does not cross-hybridise with the 16S ribosomal DNA of other bacteria tested. The real time Taqman[®] PCR can be used for the detection of *T. equigenitalis* directly from genital swabs, and the assay can discriminate the causative agent of CEM, *T. equigenitalis*, from *T. asinigenitalis* (Wakeley *et al.*, 2006). Modifications made by the ERC were as follows: the fluorescent dye on the *T. asinigenitalis* probe was changed from HEX to NED; the quencher was changed from Black Hole Quencher 1 to a MGB probe (Applied Biosystems, Cheshire, UK) allowing shortening on the primers and probes; and the mastermix was changed to that supplied by Kappa Biotechnologies (Kapabiosystems, Cape Town, South Africa). This allowed shortening on both the primers and probes (Figure 4). Samples were classified as positive if the normalized fluorescence for the *T. equigenitalis* assay exceeded a 0.1 threshold within 40 PCR cycles.

Data analysis

Variables considered for this study were age and treatment duration for the two antimicrobial agents: Category 1 (1% silver sulphadiazine) or Category 2 (0.2% nitrofurazone). The SigmaStat 12 programme provided basic descriptive statistics and the Mann-Whitney Rank Sum Test was used for the treatment duration. Statistical significance was defined as $P < 0.05$.

379	CCCTGATCCAGCCATTCCGCGTGTGCGATTGAAGGCCTTCGGGTTGTAAAGCACTTTTGT	T. asinigenitalis UCD-1
361	CCCTGATCCAGCCATTCCGCGTGTGCGATTGAAGGCCTTCGGGTTGTAAAGCACTTTTGT	T. equigenitalis K- 188
	CCGCGTGTGCGATTGA	Taylorella 377 for
	TCGGGTTGTAAAGCACTTTTGT	TayFERC
	C	
439	CAGGGAAGAAAAGITTTAGGATAATACCCTAGGATGCTGACGGTACCTGAAGAATAAGCA	T. asinigenitalis UCD-1
421	CAGGGAAGAAAAGGTTTGTGTAATACCATGGACTGCTGACGGTACCTGAAGAATAAGCA	T. equigenitalis K- 188
	TAGGATAATACCCTAGGATGC	TasiniPERC (NED)
	AAAGTTTTAGGATAATACCCTAGGATGCTGACGG	TasiniHEX
	AAAGGTTTGTGTTAATACCATGGACTGCTGACGG	TequiFAM
	TGTGTTAATACCATGGACTGC	TequiPERC (FAM)
	TACCTGAAGAATAAGCA	TayRERC
	TGAAGAATAAGCA	Taylorella 488 rev
499	CCGGCAAACACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCGGAATT	T. asinigenitalis UCD-1
481	CCGGCAAACACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCGGAATT	T. equigenitalis K- 188
	CCGGCAAA	Taylorella 488 rev
	CCGGC	TayRERC

Figure 4: Alignment of representative *Taylorella equigenitalis* (K- 188) and *T. asinigenitalis* (UCD-1) 16S r DNA sequences with primers and probes published in Wakeley *et al.* 2006 and those used by ERC in the taylorella TaqMan® PCR assay. The ERC TaqMan® probes only cover seven (compared to eight in Wakeley *et al.*, 2006) divergent residues for the two species which are underscored.

3.4 Results

Eleven of 23 (48%) of the stallions were negative for *T. equigenitalis* after nine days of treatment independent of the antimicrobial agent selected (Figure 5). Five of 11 (45%) stallions from Category 1 and six of 12 (50%) of stallions in Category 2 were negative within

this interval. The median values for effective treatment interval for Category 1 was 11 d (range: 9-52 d) and for Category 2 was 10.5 d (range: 9-26 d).

Table 2: Summary statistics for the treatment duration of stallions treated for *T. equigenitalis* with 1% silver sulphadiazine and 0.2% nitrofurazone.

	Category 1 1% silver sulphadiazine (n=11)	Category 2 0.2% nitrofurazone (n=12)	P value
Median	11d	10.5d	0.396
First Quartile	9d	9d	
Second Quartile	26d	18.25d	
Range	9-52d	9-26d	

No statistically significant difference ($P= 0.396$) was observed in the treatment duration between the two topical antimicrobial agents. No age effect on treatment duration could be demonstrated ($P= 0.414$). The difference in the median values between the two treatment categories was however insufficient to exclude possible differences due to random sampling variability.

The treatment interval varied greatly between individual stallions (Figure 5). All 23 stallions were effectively treated and remained negative for *T. equigenitalis* on once-monthly testing by qPCR assay at twelve months post-treatment.

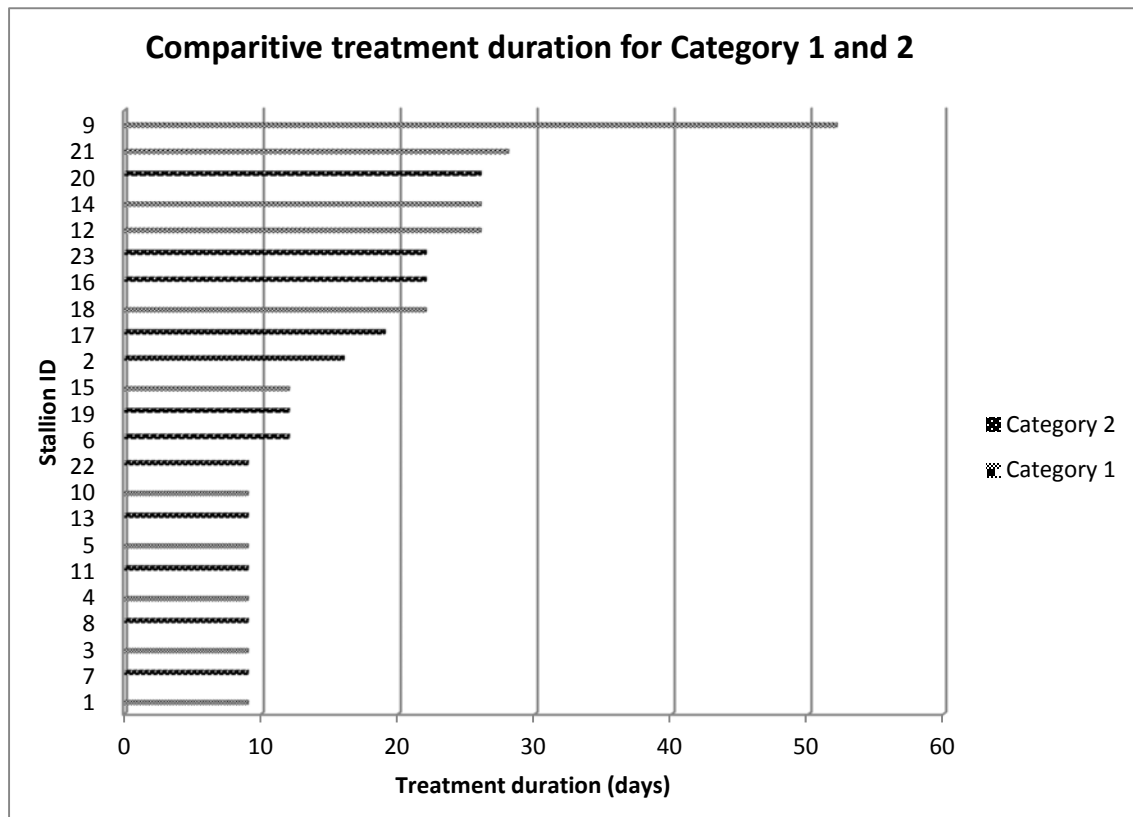


Figure 5: Graphic representation of the number of treatment days required to eliminate *T. equigenitalis* in Category 1 (n=11) and Category 2 (n=12) stallions

3.5 Discussion

There was no difference in the interval to elimination of *T. equigenitalis* between the two topical antimicrobial agents selected for this study. Clinically, however 0.2% nitrofurazone seemed to have superior adhesive properties compared with silver sulphadiazine thus potentially increasing the contact time of this antimicrobial agent.

Due to the marked individual variation observed within this study, a standardised treatment interval in days could not be defined. The median values obtained however, suggest that a 10 to 11 d treatment duration programme was adequate in the majority of cases. This duration must be considered in association with the concomitant application of

appropriate biosecurity measures and daily sampling for qPCR analysis to monitor treatment. The great variation in individual response to treatment does however suggest that additional factors may play a role in maintaining the organism for longer intervals than was anticipated based on previous reports. These factors included: individual anatomical characteristics of the external genitalia, the duration of the organism's colonisation of the external genitalia prior to detection, strain-variation in *T. equigenitalis* associated with antimicrobial resistance, and re-colonisation of the external genitalia via fomites.

The qPCR assay was shown for the first time to provide a rapid, practical and highly sensitive monitoring method that permitted precise determination of the point of organism elimination and treatment completion. This supports the value of its inclusion for monitoring treatment of carrier stallions, avoiding the shortcomings inherent in bacteriology. The use of qPCR in determining the end point of treatment assisted in eliminating the possibility of stallions requiring re-treatment following previously advocated protocols.

Chapter 4: Trends in Ct values of RT-qPCR during the treatment of stallions for *Taylorella equigenitalis*

4.1 Abstract

Reasons for performing study: Various quantitative polymerase chain reaction (qPCR) tests have been developed to detect *T. equigenitalis* (Kristula, 2007). Real-time qPCR (RT-qPCR) has been an important development in providing a method of detection of *T. equigenitalis* with enhanced specificity, sensitivity and speed of identification, allowing, in some instances, detection of bacteria where routine culture methods have failed (Wakeley *et al.*, 2006; Matsuda and Moore, 2003; Zdovc *et al.*, 2005; Ousey *et al.*, 2009). The use of RT-qPCR to monitor the treatment of stallions for *T. equigenitalis* and to determine the treatment duration required to effectively eliminate the organism from colonised stallions has never previously been reported.

Objectives: To analyse and note trends in the cycle threshold (Ct) values of RT-qPCR during the topical treatment of stallions colonised with *T. equigenitalis*.

Methods: Data was collected during the treatment process of 23 Lipizzaner stallions colonised by *T. equigenitalis*. Prior to commencement of daily treatment, a set of genital swabs was obtained and following irrigation with 4% chlorhexidine gluconate and drying an additional set of swabs was obtained from the same sites. All swabs were submitted for qPCR analysis and reporting on the same day.

The duplex RT-qPCR assay used in this study was based on a previously-described Taqman® PCR method (Wakeley *et al.*, 2006), with modifications by the Veterinary Genetics Laboratory (VGL, University of Pretoria) that allowed shortening on both 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.

Results: The silver sulphadiazine category (n=11) pre-wash Δ Ct value mean (8.465391, +/- SD 7.9162182) was lower than the post-wash Δ Ct value mean (9.5710822, +/- SD 6.9109895). The nitrofurazone category (n=12) pre-wash Δ Ct value mean (4.3336031, +/- SD 7.1744782) was similar to the post-wash Δ Ct value mean (4.2463156, +/- SD 6.3330246). When comparing the two categories: the nitrofurazone category had a lower mean Δ Ct value for both pre-wash and post-wash samples.

The minimum Δ Ct values for the pre-wash and post-wash samples obtained from both categories were zero which would effectively become the end point stage of treatment duration. The maximum Δ Ct values for the pre-wash and post-wash samples obtained from both categories were similar.

Conclusions: The general trend in Δ Ct values showed a gradual decrease. This rate of decrease in Δ Ct values was unpredictable.

Potential relevance: The use of RT- qPCR proved effective for monitoring the topical treatment of *T. equigenitalis* in stallions and in determining the end point of treatment that resulted in elimination of the organism.

4.2 Introduction

Various quantitative polymerase chain reaction (qPCR) tests have been developed to detect *T. equigenitalis* (Kristula 2007). Real-time qPCR (RT-qPCR) has been an important development in providing a method of detection of *T. equigenitalis* with enhanced specificity, sensitivity and speed of identification, allowing, in some instances, detection of

bacteria where routine culture methods have failed (Wakeley *et al.*, 2006; Matsuda and Moore, 2003; Zdovc *et al.*, 2005; Ousey *et al.*, 2009). In addition, this test was able to differentiate between the two species of phenotypically indistinguishable *Taylorella sp.* i.e. *T. equigenitalis* and *T. asinigenitalis* (Wakeley *et al.*, 2006; Duquesne *et al.*, 2007).

Treatment periods required to eliminate *T. equigenitalis* from the external genitalia of stallions is variable (Samper, 2006; Kristula and Simth, 2004; Luddy and Kutzler, 2012; Anzai *et al.*, 2012; Erdman *et al.*, 2011). The use of RT- qPCR to monitor the treatment of stallions for *T. equigenitalis* and to determine the treatment duration required to effectively eliminate the organism from colonised stallions has never been reported.

4.3 Materials and Methods

Data was collected prospectively during the treatment process of 23 Lipizzaner stallions (Mean age: 10.4 years; Range: 4-19 years) colonised with *T. equigenitalis* that were randomly assigned to two treatment categories: Category 1 (n=11) were treated with 1% silver sulphadiazine (Silbecor Biotech, South Africa) and Category 2 (n=12) were treated with 0.2% nitrofurazone (Aspen Pharmacare, Sandton) as topical agents applied to the external genitalia. While sedated, all stallions were subjected to a standardised topical treatment protocol consisting of cleansing of the external genitalia with 5% docusate sodium solution, irrigation with a disinfectant solution containing 4% chlorhexidine gluconate (Dismed Bioscrub, Dismed Pharma (Pty) Ltd, Halfway House) and finally the once daily application of either one of the selected antimicrobial agents. The procedure was repeated daily until testing negative for three consecutive days on dry swabs (Labchem, Johannesburg, South Africa) for a duplex-qPCR assay for *T. equigenitalis*. The three genital sites used for swabbing

were the distal urethra, urethral fossa and sinus and the *lamina interna*. Prior to commencement of daily treatment, a set of genital swabs was obtained and following irrigation with 4% chlorhexidine gluconate and drying an additional set of was obtained from the same three aforementioned sites.

The duplex RT-qPCR assay used in this study was based on the Taqman® PCR method previously described by Wakeley *et al.*, (2006), with several modifications instituted by the VGL (Figure 4).

Preparation of DNA samples from genital swabs

The preparation of bacterial lysates from material captured on dry genital swabs was based on the method described in Wakeley *et al.*, (2006) and Bleumink-Pluym *et al.*, (1993). Swabs were agitated for 5 s in 300 µl 0.1 M PBS solution in a 1.5 ml Eppendorf tube (Scientific Group, Vorna Valley, South Africa). Centrifugation of the sample was performed at 13 000 rpm in a microcentrifuge for 30 s to form a pellet. The supernatant was aspirated and the pellet resuspended in 100 µl of molecular grade water and vortexed for 5 s. The resuspended pellet was placed in a heat block for heating at 95 °C for 15 min. Insoluble material was pelleted by centrifugation at 13 000 rpm for 1 min in a microcentrifuge. The lysate was either used immediately or after storage at -21 °C.

Primers and probes

Discriminatory TaqMan® probes were used and conjugated with different fluorophores; the *T. equigenitalis* specific probe (TequiPERC) with FAM™, and the *T. asinigenitalis* specific probe (TasiniPERC) with NED™. PCR primers capable of amplifying Taylorella 16S rDNA amplicons suitable for use in real time PCR were designed around these probes by the ERC). The primers TayFERC (position 399-421, *T. equigenitalis* strain K -188; position 417-439, *T. asinigenitalis* strain UCD- 1) and TayRERC (position 485-464, *T.*

equigenitalis strain K -188; position 503-482, *T. asinigenitalis* strain UCD- 1) primed the amplification of the PCR product. All primers and probes were manufactured by Applied Biosystems supplied by Life Technologies (Cape Town, RSA).

Real time qPCR

Amplifications were performed in a total volume of 20 μ l containing 3 μ l of bacterial lysate, 2 μ l of the specific primer/probe mix, 10 μ l of Kapa Master Mix, 0.4 μ l High ROX and 5 μ l molecular grade water.

The qPCR was performed on a StepOnePlus real-time qPCR system (Applied Biosystems, Cheshire, UK) real time PCR machine. The sequences were TayFERC, TCGGGTTGTAAAGCACTTTTGTC; TayRERC, CCGGTGCTTATTCTTCAGGTA; TasiniPERC, TAGGATAATACCCTAGGATGC; TequiPERC, TGTGTTAATACCATGGACTGC. Following initial denaturation of the template and primers, at 95 °C for 20s, a total of 40 cycles of the following regimen was used for amplification; primer annealing, 10s at 60 °C; extension and data collection, 10 s at 60 °C. A positive and negative control was run concurrently with the samples. A positive reaction in a real time qPCR assay is detected by the accumulation of a fluorescent signal. The cycle threshold (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold. Ct levels are inversely proportional to the amount of target nucleic acid in the sample (i.e. the lower the Ct level the greater the amount of target nucleic acid in the sample). Samples were classified as positive if the normalized fluorescence for the *T. equigenitalis* assay exceeded a 0.1 threshold within 40 PCR cycles. Therefore, a Ct value of ≥ 40 would be considered a negative result.

Data analysis

The Δ Ct values were calculated by subtracting the qPCR Ct value from 1 (1= 40 PCR cycles). Descriptive statistics were calculated of the highest Δ Ct value obtained (regardless

of site: distal urethra, urethral fossa and *lamina interna*) for each treatment category of stallions, using SAS® 9.3 (SAS Institute Inc, Cary NC, 2011). Stallions were divided into three groups according to the number of treatment days: short (9-15d, n=14), medium (16-30d, n=8) and long (>30d, n=1). The short and medium groups were further subdivided by antimicrobial agent (1% silver sulphadiazine or 0.2% nitrofurazone) used for the elimination of *T. equigenitalis*. The highest pre-wash and post-wash Δ Ct values for these groups of stallions were plotted on individual graphs using SAS® 9.3 (Figures 6-10).

4.4 Results

Category 1 (n=11) pre-wash Δ Ct value mean (8.465391, +/- SD 7.9162182) was lower than the post-wash Δ Ct value mean (9.5710822, +/- SD 6.9109895). Category 2 (n=12) pre-wash Δ Ct value mean (4.3336031, +/- SD 7.1744782) was similar to the post-wash Δ Ct value mean (4.2463156, +/- SD 6.3330246). When comparing the two categories, Category 2 had a lower mean Δ Ct value for both pre-wash and post-wash samples.

The minimum Δ Ct values for the pre-wash and post-wash samples obtained from both categories were zero. The maximum Δ Ct values for the pre-wash and post-wash samples obtained from both categories were similar: Category 1 pre-wash (26.9745979) value being slightly higher than the post-wash (25.4526043) value; Category 2 pre-wash (23.0588341) being slightly lower than the post-wash (24.017292) value.

Table 3: Summary statistics for the Δ Ct values obtained during treatment of both categories of stallions.

Category no	Variable	No	Mean	Standard deviation	Min	Max	Lower quartile	Median	Upper quartile	Lower 95% CL for mean	Upper 95% CL for mean
1	Pre-wash	52									
	Δ Ct value		8.465391	7.9162182	0	26.9745979	0	8.2542	15.91468143	6.261501795	10.66928023
	Post-wash										
	Δ Ct value		9.5710822	6.9109895	0	25.4526043	4.653968811	8.8723125	15.39314842	7.647050378	11.49511393
2	Pre-wash	52									
	Δ Ct value		4.3336031	7.1744782	0	23.0588341	0	0	6.517707825	2.336215555	6.330990549
	Post-wash										
	Δ Ct value		4.2463156	6.3330246	0	24.017292	0	0	8.024554253	2.483190239	6.009440866

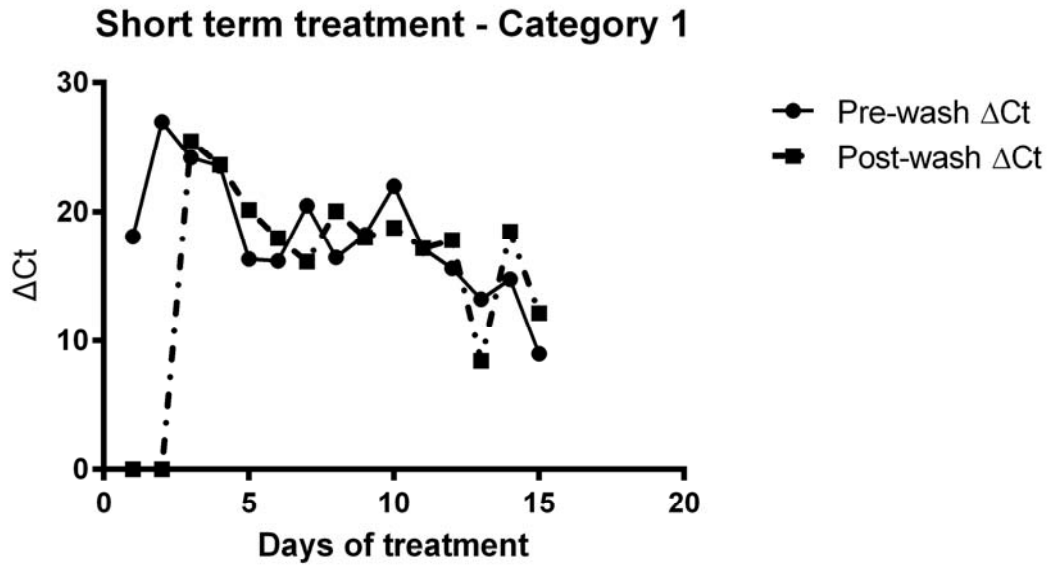


Figure 6: Mean pre- and post-wash ΔCt values for the short Category 1 of stallions (n=6)

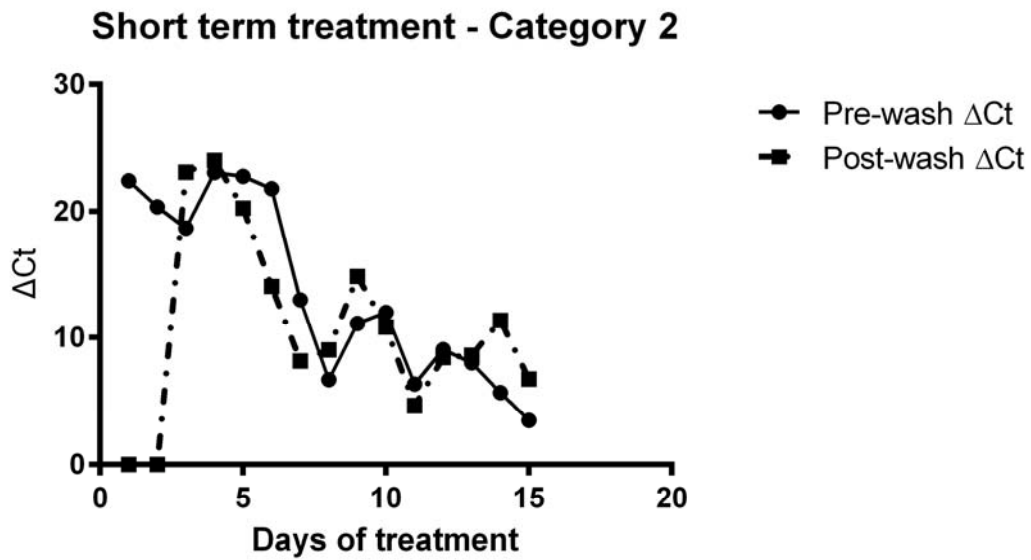


Figure 7: Mean pre- and post-wash ΔCt values for the short Category 2 of stallions (n=8)

Medium term treatment - Category 1

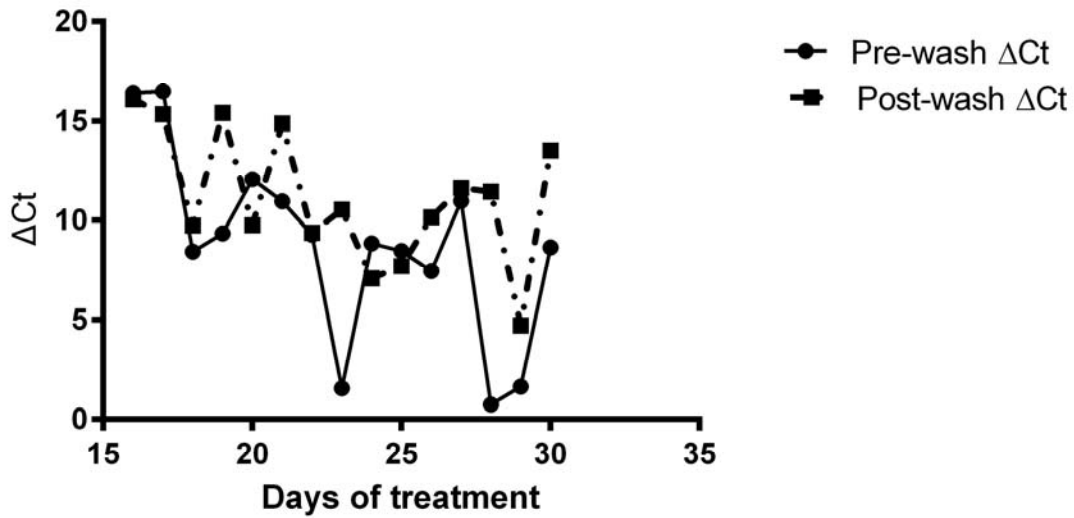


Figure 8: Mean pre- and post-wash ΔCt values for the medium Category 1 of stallions (n=4)

Medium term treatment - Category 2

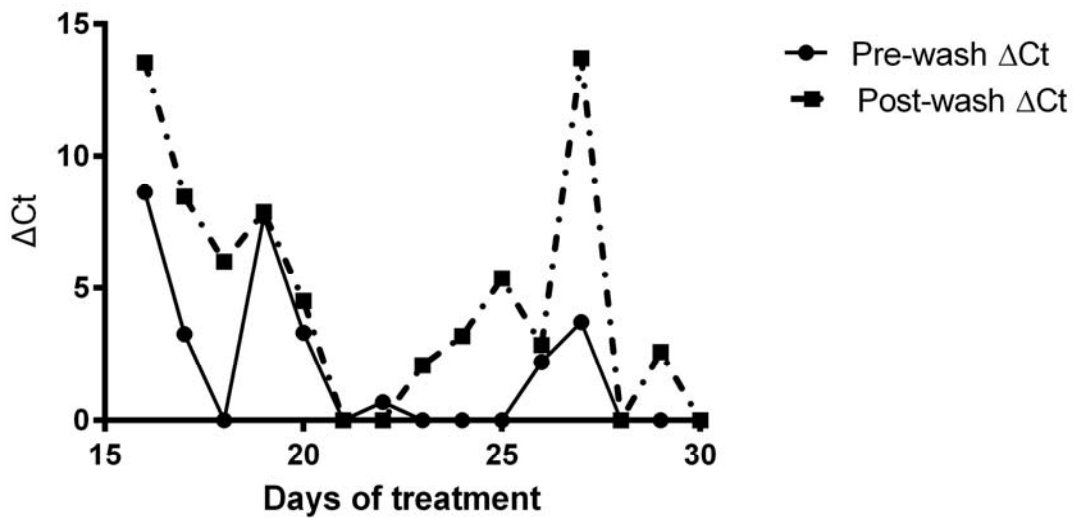


Figure 9: Mean pre- and post-wash ΔCt values for the medium Category 2 of stallions (n=4)

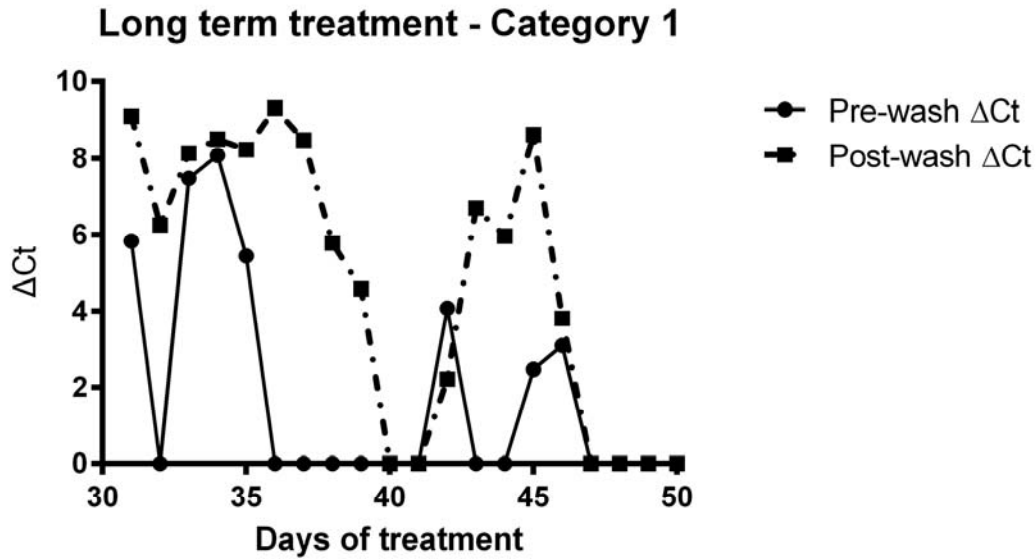


Figure 10: Mean pre- and post-wash ΔCt values for the single long Category 1 stallion.

4.5 Discussion

To the author's knowledge, this was the first study to analyse the qPCR Ct values obtained during the treatment of stallions for *T. equigenitalis*. The post-wash samples showed a similar trend to the pre-wash samples with the exception of the medium Category 2 (nitrofurazone) and long Category 1 (silver sulphadiazine). All categories demonstrated an increase in bacterial load after the 4% chlorhexidine gluconate wash. It was hypothesised that the action of washing disrupts the bacterial surface film (biofilm) (Kanemaru *et al.*, 1992; Hebert *et al.*, 2011) and thus a sample with a higher bacterial count was obtained.

The general trend observed was a gradual decrease in ΔCt values. The rate of this decrease in ΔCt values was however unpredictable. The application of a qPCR assay was shown to be effective in determining the end-point of successful treatment with a 100% success rate. Successful treatment was defined as the external genitalia swabs testing negative on both bacterial culture and qPCR. A factor which may have contributed to the

comparatively long treatment duration observed for stallion ID 9 may be associated with a variation in strain-type. Future strain-typing may further define the observed wide variation in treatment duration.

This treatment protocol did not include systemic antimicrobials. A randomized additional category receiving systemic antimicrobials may be considered in any future study to serve as a means of comparison. Andresen (1987) has emphasized the use of both systemic and local therapy as a requirement for the treatment of mares and stallions with *T. equigenitalis*. However, the current study showed that local (topical) therapy was effective without the inclusion of systemic antimicrobials. The addition of systemic antimicrobials is unlikely to be essential for the elimination of *T. equigenitalis*, suggested by the report that colonisation of the external genitalia of stallions is not associated with any systemic immune response (Timoney, 1996).

Serial qPCR testing proved invaluable in this study as it eliminated the potential complications of individual stallions requiring repeat treatments and the prolonged intervals previously required before lifting quarantine restrictions on animals undergoing treatment. This innovation consequently decreased the overall costs and management inputs required for effectively eliminating *T. equigenitalis* from a population of stallions. This simple, cost-effective adjunct is indicated for implementation during future treatment protocols with potential for worldwide adoption.

Conclusions

Contagious equine metritis continues to be a problematic disease in terms of its economic importance worldwide (Kristula, 2007). Sporadic outbreaks have reportedly cost millions of dollars to eliminate the organism from affected populations (Kristula and Smith, 2004). Many countries have instituted mandatory regulatory testing of mares and stallions for *T. equigenitalis* featuring bacterial culture as the gold standard. False negatives are common when bacterial culture is used for detecting *T. equigenitalis* (Sugimoto *et al.*, 1981). The implementation of a dual testing protocol with the addition of a qPCR assay for inclusion in current regulatory testing procedures will greatly improve the overall sensitivity and specificity of detecting horses colonised with *T. equigenitalis* (Erdman *et al.*, 2011; Hebert *et al.*, 2011) and may serve to reduce the incidence of CEM outbreaks.

The sample population in this study was solely represented by the Lipizzaner breed, consequently no breed differences, if present, were detectable. No breed predisposition has been reported previously, although breeds at highest risk currently are apparently the Thoroughbred and Warmblood populations due to the epidemiological nature of recent outbreaks (Schulman *et al.*, 2013).

Further limitations of this study were the absence of a control group of stallions and the age group represented. No young stallions (< 4 years old) were colonised by *T. equigenitalis* and differences in treatment duration were undetermined.

Stallions colonised by *T. equigenitalis* should be individually treated and currently standardized treatment protocols are problematic to institute. The in-treatment monitoring of the point of elimination by molecular methodology will assist in mitigation of this shortcoming.

The inclusion of systemic antimicrobials in treatment protocols remains undefined as it has neither been shown to be essential nor does it decrease the number of treatment days required and warrants further investigation.

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Appendices

Appendix 1: Procedure Manual for the confirmation of diagnosis and treatment of positive cases of contagious equine metritis

	<p style="text-align: center;">DEPARTMENT OF AGRICULTURE, FORESTRY AND FISHERIES DIRECTORATE ANIMAL HEALTH</p>
	<p style="text-align: center;">PROCEDURE MANUAL FOR THE CONFIRMATION OF DIAGNOSIS AND TREATMENT OF POSITIVE CASES OF CONTAGIOUS EQUINE METRITIS</p>
<p>COMPILED BY:</p>	<p>Dr Ayanda Mpehle , Prof Alan Guthrie, Prof Martin Schulman, Dr Cornelia Gerstenberg, Dr Romona Naidoo, Dr Bev Zietsman, Dr Kate May</p>
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A. PURPOSE OF THIS PROCEDURE MANUAL

To lay down the procedures to be followed for the confirmation of diagnosis and treatment of positive cases of Contagious Equine Metritis (CEM) with the aim of:

- A.1. Preventing the disease from becoming endemic within South Africa
- A.2. Eradicating the disease (South Africa was considered free of CEM prior to an outbreak diagnosed in April 2011).

B. SCOPE

This Procedure Manual is applicable to any **suspect** or **confirmed** CEM cases.

Of major concern is:

- B.1. The risk of CEM becoming endemic within South Africa and its detrimental effect on equine breeding.
- B.2. The potential negative impact of the disease on the equine industry.

C. LEGISLATION

- C.1 CEM is a controlled animal disease in terms of the Animal Diseases Act, 1984 (Act No. 35 of 1984).
- C.2. Any suspect or confirmed CEM cases must be reported to the Director in terms of the Animal Diseases Act, 1984 (Act No. 35 of 1984):
 - (i) Section 11(1)(b)(ii) of the Animal Diseases Act states: “Any owner or manager of land on which there are animals, and any owner in respect of animals, shall, whether or not such owner or manager has obtained advice regarding the health, or any certificate of fitness or health of the animals in terms of section 13(1)(c), from the director, whenever such animals have become or can reasonably be suspected of having become infected with any controlled animal disease, immediately report such incidence in the prescribed manner to the director.”
 - (ii) Section 11(2) of the Animal Diseases Act as amended states: “A veterinarian or any other person who finds the incidence or suspected incidence of any controlled animal disease in any animal or progeny or product thereof, shall immediately report such incidence to the director.”
- C.3. In terms of Table 2 of the Animal Diseases Regulations the following control measures for CEM are described.
 - (i) In respect of susceptible animals: “Serving of infected mares or by infected stallions shall be prevented.”
 - (ii) In respect of contact animals: “Contact animals shall be isolated and tested under the supervision of or by an officer or authorized person.”
 - (iii) In respect of infected animals: “Infected animals shall be isolated, and in the case of (a) mares, destroyed under the supervision of or by an officer or authorized person; and (b) stallions, castrated or destroyed under the supervision of or by an officer or authorized person.”
- C.4. The Director: Animal Health will issue a derogation (exemption) in terms of Regulation 11(2) (b) to each owner/manager by means of an official order to allow the treatment of any infected equines on condition that the measures as stipulated in this Procedure Manual for the Confirmation of Diagnosis and Treatment of Positive Cases of Contagious Equine Metritis (CEM) will be complied with in full.

D. CONTAGIOUS EQUINE METRITIS PRESENTATION IN EQUINES

CEM is an acute, highly contagious and venereal disease of equines caused by gram negative bacteria, *Taylorella equigenitalis*. It is characterised by a mucopurulent vaginal discharge and early return to oestrus in most affected mares. Infected stallions and chronically infected mares may not show clinical signs and may become carriers.

E. TRANSMISSION OF CONTAGIOUS EQUINE METRITIS

Infection can be transmitted to equines in any of the following ways:

- E.1 Direct transmission primarily during mating;
- E.2 Indirect transmission via fomites, including hands, clothing, artificial vaginas, vaginal speculums, other instrumentation and breeding dummies or phantoms used for semen collection;
- E.3 Transmission to mares by means of artificial insemination using semen obtained from a CEM infected stallion, or semen contaminated by CEM bacteria during semen collection, processing or storage;
- E.4 Direct or indirect transmission during teasing. Direct transmission when an infected teaser transmits disease to mares through contact with the teaser's genitalia. Indirect transmission by a teaser after contact with infected vulval discharge and subsequent contact with mares through genital or naso-genital contact.

F. PROCEDURES TO BE FOLLOWED IN ALL SUSPECT OR CONFIRMED CASES OF CEM

F.1. NOTIFICATIONS

Any owner, laboratory or veterinarian who is aware of a suspect or confirmed positive test result is obliged to report this immediately to the local Provincial State Veterinarian or Provincial Veterinary Director. The Provincial Veterinary Authorities have to notify the Directorate Animal Health, Department of Agriculture, Forestry and Fisheries (DAFF) of the **suspect** or **confirmed** CEM infected animals via an immediate notification (SR1 report) and also include the incidence in monthly animal disease reporting. These reports must be sent to:

- (a) Sub-Directorate: Epidemiology
Dr Hannes Pienaar
Fax: 012 319 7470
Email: HannesP@daff.gov.za

For more information, please contact:

- (b) Sub-Directorate: Disease Control
Dr Romona Naidoo
Tel: 012 319 7630
Fax: 012 329 0499
Email: RomonaN@daff.gov.za

F.2. QUARANTINE

The responsible state veterinarian shall place the land on which suspect CEM infected animals are located under quarantine. A quarantine notice/official order will be served on the responsible person in terms of Section 15 (Orders) of the Animal Diseases Act, 1984 (Act No. 35 of 1984) (Annex A), with due reference to Regulation 28 (Serving of documents) (Annex B) as well as the provisions of the Animal Diseases Act Regulations Table 2 pertaining to CEM (Annex C) and this Procedure Manual.

- (a) As from the date on which the land is placed under quarantine, no animal shall, without the written permission of the responsible state veterinarian, be moved from, to or through the land on which the equines (horses, donkeys and mules) and zebras concerned are quarantined.
- (b) Suspect cases may only be moved under cover of a red cross permit to an isolation facility approved by the Director: Animal Health or the appropriate Provincial Director of Veterinary Services to facilitate treatment. (The state veterinarian may issue permits for non-contact non-suspect horses to be moved temporarily, for example to a show, provided the conditions on the permit state that the animal may not be involved in any natural mating, artificial insemination, embryo transfer or veterinary examination of equine genitalia while it is absent from the approved isolation facility.)
- (c) No natural mating, artificial insemination, embryo transfer, veterinary examination of equine genitalia or treatment for CEM may be undertaken while the land is under quarantine, except by a veterinarian under the supervision of a state veterinary official.
- (d) The responsible state veterinarian will personally serve the order on the owner or manager of the relevant equines and zebras.
- (e) Two copies of the order must be signed. One signed copy of the order should be retained by the owner and the other signed copy must be kept by the responsible state veterinarian.
- (f) The responsible person shall without delay identify all the susceptible and in-contact equines and zebras by means of individual equine passports or equivalent documents.
 - The responsible person shall keep a register in respect of all the identified equines and zebras
- (g) The owner or manager shall:
 - Declare all natural breeding activities and all veterinary activities such as semen collection, semen freezing, artificial insemination, teasing, embryo collection and transfers, vaginal examinations at any date past or present.
 - Disclose all information on other equines and zebras that were on the premises at any date past or present.
 - In each case the responsible state veterinarian will make a decision as to which equines and zebras are considered suspect for CEM.
- (h) CEM confirmed equines shall be relocated immediately to an approved isolation facility as determined by the Director: Animal Health or put into an isolation facility approved by the Director: Animal Health on the premises for the purpose of further diagnostic testing and treatment as required.
- (i) All costs during isolation including boarding, all treatment and other related veterinary costs must be borne by the owner.
- (j) The equines will be released from the isolation facilities **ONLY** after they are certified CEM negative by the Director: Animal Health.
- (k) The state veterinarian shall conduct a full forward and backward tracing exercise and identify all possible in contact equines and zebras that may be present on other properties or premises or pieces of land. The

- state veterinarian responsible for the quarantined land shall without delay inform the state veterinarian responsible for the area where the other possible in-contact animals may reside.
- (l) Possible in contact equines and zebras (direct or indirect contact) that have been moved or are present on other properties or premises or pieces of land as determined by the state veterinarian may not necessarily be put under full quarantine by the state veterinarian responsible for the area where these possible in-contact animals reside but shall undergo diagnostic testing under state veterinary supervision as described under H of this document.
 - (m) All surfaces and equipment that may have been in contact with CEM suspect equines or zebras must be properly cleaned and then disinfected with F10 or Vircon (according to the manufacturer's specifications for disease control).
 - (n) After all suspect CEM infected equines or zebras have been removed to an isolation facility approved by the Director: Animal Health and all surfaces properly disinfected, quarantine may be lifted.
 - (o) ALL test swabbing, treatments and test matings of suspect or in contact horses MUST be witnessed by a State Veterinary /Para-Veterinary official.

G. APPROVAL OF ISOLATION FACILITIES

The Director: Animal Health will only consider the approval of an isolation facility following the receipt of an application letter (Annex D) with a detailed Standard Operating Procedure (SOP) for the isolation facility. The following must be included in the SOP:

- (a) The name of the isolation facility.
- (b) A map detailing the layout of the isolation facility including all access points.
- (c) A description of how the CEM confirmed equines and zebras will be housed and managed to prevent further transmission of CEM.
- (d) A statement confirming that no teasing, mating (except mating for CEM diagnostic purposes), artificial insemination, embryo collection or transfer will be undertaken within the isolation facility.
- (e) A description of the biosecurity measures that will be applied to all equines and zebras within the isolation facility.
- (f) A description of the biosecurity measures that will be applied for people that have access to the isolation facility.
- (g) The name and contact details of the private veterinarian who will collect all samples, treat all horses and test mate mares at the isolation facility under supervision of the provincial state veterinary authorities.
- (h) A letter from the private veterinarian proposed in (g) above confirming that he/she is prepared to perform the duties and follow all prescriptions outlined in the SOP and in this Procedure Manual and that he/she will ensure that all the actions will be performed under supervision of the state veterinary authorities.
- (i) The name and contact details of the local State Veterinary/Para-veterinary official who will observe /monitor the CEM sample collection, CEM treatments and CEM test mating of mares.
- (j) Each isolation facility must be approved by the Director: Animal Health (DAH), DAFF, prior to testing and treatment procedures commencing. The completed application forms (Annex D) should be submitted together with the required SOP via the State Veterinarian to the Provincial Director of Veterinary Services (PDVS) and by the PDVS to the DAH for this purpose.

H. DIAGNOSTIC PROCEDURES FOR SUSPECT CEM INFECTED EQUINES AND ZEBRAS

Only a veterinarian authorised by the Director: Animal Health shall test CEM suspect or infected equines and zebras **in the presence of a local State Veterinary/Para-veterinary official. The samples shall be collected only at an approved isolation facility (Annex D).**

- (a) Serology is not considered a useful test for CEM diagnostics.
- (b) Two sets of swabs (duplicate swabs of each site) for CEM diagnostics shall be taken in the manner prescribed below.
- (c) One set of swabs for culture, taken in the prescribed manner below, must be placed in Amie's charcoal transport medium. The other set of swabs must be kept dry and must be replaced in the plastic sleeves for PCR testing.
- (d) All swabs must be clearly labelled to show the date and time of collection, the identification of the equine or zebra as well as the site of swabbing.
- (e) For endometrial swabs, guarded swabs must be used.
- (f) For the clitoral fossa standard swabs may be used. However for clitoral sinus swabs, paediatric swabs **MUST** be used.
- (g) Duplicate swabs must be packed separately as separate sets.
- (h) All swabs for culture must reach the Onderstepoort Veterinary Institute (OVI) within 24 hours of collection, and must be transported on ice or at 4°C. The sample submission form (Annex E) must clearly indicate that the swabs are submitted for CEM culture. All samples taken should be coordinated with the OVI in advance:
Dr Laura Lopez at OVI
Tel: 012 529 9272
- (i) Samples for PCR must be sent by speedpost to: Equine Research Centre, Private Bag X04, Onderstepoort, 0110; or sent by courier to: Equine Research Centre, Faculty of Veterinary Science, University of Pretoria, Old Soutpan Road, Onderstepoort, 0110. The sample submission form for PCR testing can be downloaded from www.cemsa.co.za and tracking numbers must be emailed to info@cemsa.co.za.

H.1 Stallions, colts and geldings

- (a) Duplicate swabs of each site must be taken (two swabs taken from the same site at the same time). In stallions, colts and geldings, swabs must be taken from the following sites:
 - Urethra
 - Urethral fossa including urethral sinus
 - Penile sheath (*lamina interna*) with an exteriorised penis (it is important to choose a site with an accumulation of smegma).
- (b) Swabs from all three sites must be collected twice at an interval of at least seven days.

H.2 Mares and fillies

- (a) Duplicate swabs of each site must be taken at all times (two swabs taken from the same site at the same time) In mares and fillies, swabs must be taken not less than ten days after initial suspect contact from the following sites:
 - Clitoral fossa

- Clitoral sinus using paediatric swabs
 - Endometrium using guarded swabs.
- (b) Swabs from all three sites must be collected twice at an interval of at least seven days.
- (c) In the case of pregnant mares, clitoral fossa and clitoral sinus swabs only must be collected twice at an interval of at least seven days.
- (d) Suspect pregnant mares must foal in isolation and testing of the mare must be repeated within 7 days after foaling as described in point H.2.(a) above. The foal must be tested within 7 days of foaling as described in point H.1(a) for colts and point H.2(a) for fillies.
- (e) For in contact pregnant mares, the Director: Animal Health will decide whether to declare these animals negative based only on the clitoral fossa and clitoral sinus swab results.

I. TREATMENT OF CONFIRMED CEM INFECTED EQUINES AND ZEBRAS

Only the private veterinarian who has signed the application for the isolation facility that was approved by the Director: Animal Health (Annex D) shall treat CEM infected equines and zebras and only **in the presence of a local State Veterinary/Para-veterinary official. The treatment shall only be conducted at an approved isolation facility (Annex D).**

Biosecurity during the treatment process is essential as there is a high risk of contaminating the environment. All irrigation fluids must be disposed of through secure drainage directed away from horse-traffic and after each treatment cotton wool, gloves, needles and syringes etc. must be placed in sealed biohazard bags and incinerated. Dedicated clothing and boots must be worn during the treatment process and generally when handling suspect or positive animals. During treatment, long plastic rectal sleeves as well as latex examination gloves must be worn (“double glove”). Particular attention should be paid to hand-washing and the use of footbaths. Effective disinfectants must be used on hands and clothing as well as tack and other items e.g. “F10” in aerosol spray solution and hand-gel form. Animals must be handled by dedicated grooms and disposable gloves must be worn at all times. Feed and tack must be kept isolated in the quarantine facility.

After the treatment process, all protective clothing and consumables must be bagged in biohazard bags and incinerated. Wet shavings must be collected by a “double gloved” person and disposed of in biohazard bags and the stable must be thoroughly cleaned with a pressure hose using F10 solution. All of the above requirements must be dealt with fully and must be included in the SOP that is submitted together with the application for approval of the isolation facility (Annex D).

ONLY THE VETERINARIAN WHO HAS SIGNED THE APPLICATION FOR THE ISOLATION FACILITY THAT WAS APPROVED BY THE DIRECTOR: ANIMAL HEALTH MAY TREAT POSITIVE CEM CASES AND SUCH TREATMENTS MAY COMMENCE ONLY AFTER THE APPROVAL BY THE DAH HAS BEEN RECEIVED.

ALL TREATMENTS MUST BE DONE UNDER THE SUPERVISION OF THE STATE VETERINARIAN.

I.1 Stallions, colts and geldings

- (a) Treatment must be done under sedation. Effective sedation and penile extrusion is obtained with an i/v injection of Detomidine HCL (“Domosedan®” @ 5mg IV (= 0,5ml)) and Butorphanol Tartrate (“Torbugesic®” @10mg IV (=1ml)).
- On Day One of treatment, a set of duplicate swabs must be taken for both PCR and culture prior to any therapeutic intervention. The bacteriology swabs must be placed in Amie’s charcoal medium and transferred on ice to reach the ARC-OVI laboratory within 24 hours. Swabs must be co-ordinated with the OVI in advance: Dr Laura Lopez, Tel: 0125299272. The swabs for PCR are dry swabs, and must be replaced in the plastic sleeve and have no special temperature requirement. They may be sent as a batch to the ERC after Day Nine of treatment – see 4th bullet point below.
 - For the first two days of treatment, the prepuce and exteriorised penis must be thoroughly irrigated once a day with a topical surfactant solution: 5% docusate sodium (Ducasol, Kyron) and then thoroughly cleaned with a disinfectant solution of 2% chlorhexidine gluconate. (Dismed, Bioscrub)
 - On the third day of treatment, the prepuce and exteriorised penis must be thoroughly irrigated with 5% docusate sodium and then cleaned with a disinfectant solution of 2% chlorhexidine gluconate, completely dried and a topical application of 1% silver sulphadiazene (Silbecor, Biotech Laboratories), must be liberally applied. This process is repeated for seven days i.e. a total of Nine (9) treatment days. A new tube of antimicrobial cream must be used every day to prevent cross contamination.
 - After cleansing and prior to application of the antimicrobials, dry swabs must be taken daily from the urethra, urethral fossa including sinus and *lamina interna* and submitted for PCR assay. The three swabs are labelled as follows: urethra = A; urethral fossa including sinus = B; *lamina* = C. The stallion’s name and sample date must also be recorded on each swab. The dry swabs require no special storage temperature and they may be sent to the Equine Research Centre as a batch.
 - After the nine days of treatment the stallion, colt or gelding must remain in isolation for a further 21 days.
- (b) The stallion, colt or gelding must be retested as prescribed under H and H.1 above (i.e. two sets of swabs must be taken 7 days apart) at least 21 days after completion of treatment.
- (c) If treatment was unsuccessful and the stallion, colt or gelding tests positive for CEM upon retesting, treatment may be repeated or the stallion, colt or gelding destroyed at the discretion of the Director: Animal Health.
- (d) If treatment was successful, in order to obtain breeding clearance, the stallion or colt must be test mated to two mares as described in (J) below. If the stallion is not intended to be used for breeding, two additional sets of duplicate swabs must be taken 7 days apart as described in point H.1(a) and H.1(b) for culture and PCR.
- e) Any successfully treated stallion, even if not intended for breeding must be re-tested annually (culture + PCR) for at least three successive years.

I.2 Mares and fillies

- (a) Treatment must be done under appropriate physical and/or chemical restraint.
- (b) Mares and fillies must be treated daily for 5 successive days.
- (c) On Day One of treatment, prior to any therapeutic intervention, duplicate swabs must be taken from the clitoral fossa and clitoral sinuses using paediatric swabs (Copan Innovation, Brescia, Italy) and additionally from the endometrium using guarded endometrial swabs. (Easy Guard, Section of Reproduction, University of Pretoria, SA) The swabs for bacteriology must be placed in Amie's charcoal medium and transferred on ice at 4°C to reach the Agricultural Research Council, Onderstepoort Veterinary Institute (ARC-OVI), laboratory within 24 hours. Swabs must be coordinated with the OVI in advance: Dr Laura Lopez, Tel: 0125299272. The second set of swabs for PCR have no special temperature requirement. They must be kept dry (i.e. no transport medium), sealed in individual plastic sleeves and sent by courier to the Equine Research Centre, University of Pretoria. The swabs for PCR may be collected, kept at room temperature and sent as one batch to the Equine Research Centre after Day 5 of treatment.
- (d) The vestibulum, clitoral fossa, *glans clitoridis*, clitoral sinuses and adjacent tissue must be thoroughly flushed with a surfactant solution of 5% docusate sodium (Docusol, Kyron) and rinsed with warm saline. Remove any accumulated smegma with disposable paper towelling. Use cotton-tipped swabs dipped in 5% docusate sodium to clean the clitoris thoroughly.
- (e) Following the above flushing, the vestibulum, clitoral fossa, *glans clitoridis*, clitoral sinuses and adjacent tissue must be thoroughly cleaned with 2% chlorhexidine surgical scrub (Dismed, Bioscrub) applied with damp cottonwool.
- (f) In order to evaluate the effect of daily cleaning on positive mares, dry swabs for PCR must be taken daily from the clitoral fossa and clitoral sinuses using paediatric swabs immediately before flushing and washing and then additionally after flushing and washing but before treatment with silversulphadiazine. There will be no charge for this PCR testing. Please label these swabs carefully and indicate on the submission form "CEM swabs to evaluate the effect of cleaning mares prior to treatment".
- (g) After flushing and washing, the area must be dried and a copious application of 1% silversulfadiazine (Silbecor, Biotech, SA) must be used to pack and coat the area. The above flushing, cleaning and antibiotic treatment must be repeated on a daily basis for 5 days. To prevent cross contamination a new tube of antimicrobial must be used each day.
- (h) The mare must be retested according to H.2.above at least 21 days after completion of the 5 day treatment.
- (i) If treatment was unsuccessful and the mare tests positive for CEM upon retesting, treatment may be repeated or the mare may be destroyed at the discretion of the Director: Animal Health.

J. TEST MATING

If treatment was successful, in order to obtain breeding clearance, the stallion or colt must be test mated with two CEM-negative mares in the presence of a local State Veterinary/Para-veterinary Official at an approved isolation facility (Annex D) under quarantine conditions. The test matings must be conducted within a three month

period after the second set of negative swabs post-treatment unless otherwise authorised by the Director: Animal Health.

The stallion or colt and the test mares must be swabbed for PCR testing after entry into the test breeding programme. One set of swabs from all three sites as described under H.1 for stallions, colts and geldings and H.2 for mares and fillies must be collected twice at an interval of at least seven days prior to test mating and at least 7 days following any previous swabbing for CEM. Test mating can only proceed following confirmation that all swabs have tested negative.

- a) No disinfectant or detergent cleaners may be used on the test mares prior to breeding.
- b) Test breeding consists of live cover of two confirmed negative mares. At a minimum each test mare must experience at least two (2) complete penetrations from the stallion, one of which must result in ejaculation.
- c) Duplicate endometrial, clitoral fossa and clitoral sinus swabs must be taken from the mares that were test mated. The first set of swabs must be taken 3-5 days after test mating, the second set 3-5 days later and the third set 3-5 days after the second set. Paediatric swabs (Copan Innovation, Brescia, Italy) must be used to swab the clitoral fossa and clitoral sinuses and guarded endometrial swabs (Easy Guard, Section of Reproduction, University of Pretoria, SA) must be used to swab the endometrium. The bacteriology swabs must be placed in Amie's charcoal medium and transferred on ice at 4°C to reach the Agricultural Research Council, Onderstepoort Veterinary Institute (ARC-OVI) laboratory within 24 hours. Swabs must be coordinated with the OVI in advance: Dr Laura Lopez, Tel: 0125299272. The second set of swabs for PCR require no special temperature, must be kept dry (i.e. no transport medium) and sealed in individual plastic sleeves and must be sent by courier to the Equine Research Centre, University of Pretoria. The swabs for PCR may be collected, kept at room temperature and sent as one batch to the ERC.
- d) A serum sample must be collected from each mare 15 days after test mating and tested using a Complement Fixation (CF) test for CEM.
- e) The stallion or colt and the mares used for test mating must remain in the isolation facility until such time as negative results have been returned on all samples collected.
- f) Quarantine will be lifted from those stallions or colts that have been through a test mating programme with negative results and a CEM Clearance Certificate will be issued. At this stage the quarantine will also be lifted for the test mares.
- g) An individual owner must apply in writing to the Director: Animal Health if he/she wants a particular stallion to be exempted from test breeding, with detailed reasons as to why the exemption is requested. Such an application should be accompanied by a suggested appropriate alternative testing procedure recommended by the local state veterinarian and endorsed by the Provincial Director of Veterinary Services. Refer to I.1(e)

K. HANDLING AND TESTING OF FROZEN SEMEN.

Frozen semen obtained from all suspect or confirmed cases of CEM should be placed under quarantine by the local state veterinarian until either the suspicion has been removed or the semen has been tested as described below.

- a) If the stallion is still alive, the stallion must be tested for CEM according to the procedure outlined under (H) above.

- At least one (1) straw per batch of the frozen semen must be tested using qPCR.
- b) If the stallion is deceased, records of breeding (semen collection facility etc.) must be evaluated.
- At least two (2) straws per batch of frozen semen must be tested by qPCR.
- c) With imported frozen semen, the import documents should be checked and evaluated for CEM tests done.
- At least one (1) straw per batch of frozen semen must be tested by qPCR.

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ANNEX A

15. Orders

- (1) The director may, in order to achieve a controlled purpose, serve an order in the prescribed manner on any owner of animals or things, or any owner or manager of land, wherein he is directed, in respect of-
 - (a) any specified controlled animal or thing; or
 - (b) land defined therein; or
 - (c) any such animal or thing and such land,to comply with a specified provision of this Act, or to perform, or abstain from performing, any other defined act.
- (2) An order shall, subject to subsections (3) and (4), be binding on the person concerned and any other person who is his legal successor in respect of the relevant animals, things or land.
- (3) Notwithstanding any provision to the contrary in any order regarding the movement or removal of any controlled animal or thing, the director may at any time on written application of a person concerned grant him written authority to move or remove any such animal or thing in accordance with the conditions of the authority (if any).
- (4) The director may by written notice served on an owner or a manager, or his legal successor, in the prescribed manner-
 - (a) amend any order; or
 - (b) if the director is satisfied after such inspections or examinations as he may deem necessary that the provisions of the order have been properly complied with and that the objects thereof have been achieved, withdraw any order.
- (5) A document purporting to have been signed by the director and framed in a manner which has in respect of the serving of orders and notices been prescribed for purposes of this subsection, shall be conclusive proof that an order under subsection (1), or a notice under subsection (4), has been served on a person concerned.

ANNEX B

28. Serving of Documents [14, 15]

- (1) A written notification in terms of section 14 or 15(4) of the Act, and an order referred to in section 15(1) of the Act shall be served by-
 - (a) forwarding it by registered post to the person concerned;
 - (b) delivering it to the person concerned personally or to his authorised representative;
 - (c) delivering it at the regular or most recently known residence or place of business of the person concerned, to some person who is apparently not younger than 16 years of age and apparently resides or is employed there; or
 - (d) in the case of a juristic person, delivering it at the registered office of that juristic person.
- (2) When such notification or order is served in terms of subregulation (1)(a) the director shall -
 - (a) place the direction in an envelope addressed to the person concerned at his last known postal address and forward it by pre-paid registered post; and
 - (b) at the time of registration thereof make application to be provided with an acknowledgement by the addressee of the receipt thereof as provided in regulation 44(5) of the Post Regulations published by Government Notice R550 of 14 April 1960: Provided that-
 - (i) a receipt form completed as provided in regulation 44(8) of the said regulations shall be sufficient acknowledgement of receipt for the purposes hereof; and
 - (ii) if no such acknowledgement is received, this fact shall be recorded by the director on a copy of the notification or order concerned.
- (3) When a notification or order has been served in terms of subregulation (1)(b), (c) or (d), the person by whom it was delivered shall immediately after delivery thereof, make an entry on a copy of that notification or order to indicate the manner in which, the person to whom, the place at which, and the date on and approximate time at which it was thus delivered.
- (4) The entries referred to in subregulation (3) shall immediately after being made, be signed by the person by whom the notification or order was delivered.
- (5) If the same land or animals are owned by two or more responsible persons, a notification or order referred to in subregulation (1) may be served to any one of them.

ANNEX C

GOVERNMENT GAZETTE, 26 SEPTEMBER 1986

No. 10469 29

Animal disease	Nature, causal organism and symptoms	Susceptible animals	Controlled veterinary act to be performed in respect of—		
			susceptible animals	contact animals	infected animals
1 Contagious equine metritis ...	2 Contagious venereal disease caused by the bacterium <i>Haemophilus equigenitalium</i> , transmitted by coitus and characterised in mares by metritis, abortions, and low fertility	3 Equines and Zebra	4 Serving of infected mares or by infected stallions shall be prevented	5 Contact animals shall be isolated and tested under the supervision of or by an officer or authorised person	6 Infected animals shall be isolated and, in the case of— (a) mares, destroyed under the supervision of or by an officer or authorised person; and (b) stallions, castrated or destroyed under the supervision of or by an officer or authorised person.
Contagious haematopoietic necrosis	A contagious viral disease of fish, characterised by sudden deaths, dark discolouration, anaemia, eye-bulging with distention of the abdomen and hard faeces	Trout	—	All fish in infected dams shall be destroyed under the supervision of or by an officer or authorised person	All fish in infected dams shall be destroyed under the supervision of or by an officer or authorised person.
Contagious pancreatic necrosis	A contagious viral disease of fish, characterised by a sudden high mortality rate especially under young fish, rotating swimming movements, dark discolouration, distention of the abdomen with milky slime in the stomach and intestine	Trout	—	All fish in infected dams shall be destroyed under supervision of or by an officer or authorised person	All fish in infected dams shall be destroyed under supervision of or by an officer or authorized person.
Corridor or buffalo disease ...	Acute communicable animal disease caused by the protozoan <i>Theileria parva lawrencei</i> , transmitted by the brown ear tick (<i>Rhipicephalus appendiculatus</i>), occurring after contact with African buffaloes, and characterised by respiratory distress, enlarged lymph nodes, occasional blindness in one or both eyes, and marked oedema and sometimes emphysema in the lungs.	Cattle and African buffaloes	1. Contact between cattle and African buffaloes shall be prevented 2. All cattle in a controlled area shall be dipped or sprayed regularly by the responsible person with an efficient remedy 3. No animal shall be chemotherapeutically treated without the written authorisation of the director	Contact animals shall be isolated and dipped or sprayed with an efficient remedy under the supervision of an officer or an authorised person in the manner and at the intervals determined by the responsible State Veterinarian	Infected animals shall be isolated and clipped or sprayed with an efficient remedy under the supervision of an officer or an authorised person in the manner and at the intervals determined by the responsible State Veterinarian.
Dourine	Chronic contagious venereal disease caused by the protozoan <i>Trypanosoma equiperdum</i> , transmitted by coitus and characterised by swelling of the genitalia, legs and abdomen and sometimes by nervous symptoms	Equines and Zebra	1. Serving of infected mares or by infected stallions shall be prevented 2. Susceptible animals may be tested by an officer, an authorised person or a veterinarian	Contact animals shall be isolated and tested by an officer or authorised person	Infected animals shall be isolated and, in the case of— (a) mares, sterilised, slaughtered or destroyed under the supervision of or by an officer or authorised person; and (b) stallions, castrated, slaughtered or destroyed under the supervision of or by an officer or authorised person.

Annex D

Application for approval of an isolation facility for the testing, treatment and test mating of CEM suspect and infected equines

**Identification of property, isolation facility and biosecurity measures:
Agreement to do CEM sample collection, treatment and test mating**

I, the undersigned registered veterinarian authorised thereto by the farm owner/manager,....., agree to :

1. Collect all CEM samples as described in the **Procedure Manual for the confirmation of diagnosis and treatment of positive cases of CEM**
2. Treat all horses as described in the **Procedure Manual for the confirmation of diagnosis and treatment of positive cases of CEM**
3. Test mate mares as described in the **Procedure Manual for the confirmation of diagnosis and treatment of positive cases of CEM**

I (Private Veterinarian),..... hereby confirm that :

- a. I will comply with and uphold the requirements and the standards of the isolation facility as stipulated/outlined in the DAFF approved CEM isolation facility SOP.
- b. I will ensure that I have all the knowledge and expertise to follow all the procedures described in both the attached SOP for this isolation facility as well as those in the Procedure Manual for the confirmation of diagnosis and treatment of positive cases of CEM.
- c. I will not come into contact with any horse involved in breeding activities for a period of at least 24 hours following any visit to the isolation facility.
- d. All the activities stated under points 1; 2 and 3 above will be done under the supervision/observation of the local State Veterinary/Para veterinary Official, whose contact details and signature appear below.

Signature.....
Date.....

PRIVATE VETERINARIAN

Name in print: **Official Stamp**

Address:

.....

Signature.....

Date.....

STATE VETERINARIAN

Name in print:

Official Stamp

Address:

.....

.....

Description:

RECOMMENDED

Signature.....

Date.....

Provincial Director of Veterinary Services

Name in print:

Official Stamp

Address:

.....

.....

APPROVED

Signature.....

Date.....

Director: Animal Health

Name in print:

Official Stamp

Address:

ANNEX E



ONDERSTEPSOORT VETERINARY INSTITUTE

100 Old Soutpan Road, ONDERSTEPSOORT, 0110, SOUTH AFRICA
Private Bag X5, ONDERSTEPSOORT, 0110 RSA
Tel (012) 529 9272
Fax (012) 529 9275
E-mail: diagreg@arc.agric.za



SUBMISSION FORM FOR SAMPLE TESTING

Sender Ref:		Date:		Lab No:		Time delivered:	
SENDER	TITLE	INITIALS	PERSON/ORGANISATION RESPONSIBLE FOR THE ACCOUNT		TITLE	INITIALS	
Surname:			Name				
Postal address:			Postal address:				
Tel:			Fax:				
Tel:			Fax:		SIGNED		NAME PRINTED
Please note: DAFF will not consider payment for any controlled disease tests if this section is not completed in full and motivation is completed on back of form. Abuse of this constitutes fraud. State Veterinarian will be copied on the results.			OWNER (IF APPLICABLE)		TITLE	INITIALS	
STATE VETERINARIAN	TITLE	INITIALS		Surname:			
Surname:			Physical address:				
Postal address:							
			Province				
Province and State Vet Area:		Surveillance program:		Tel:		Fax:	
Tel:		Signature of State Vet: Form should also display official SV stamp		Registered farm name and number:			
Fax:							
Email:				Coordinates:	East:		South:
ANIMAL/S			TEST(S) REQUIRED				
Species:							
Number of animal sampled:							
Age::							
Sex: Male / Female (neutered/spayed)							
Type of specimen/s:							
			Purpose for testing: (Required field)				
Number of specimen/s:			Collection date:				
FOR OFFICE USE ONLY			Authorised for DAFF payment.				

CHECK LIST FOR RECEIVING SAMPLES : DATE :
(FOR OFFICE USE ONLY) TIME:
RECEIVED BY:


Sample type	Quantity
Total	

Remarks:

ANNEX F

Sample submission form for PCR testing.

Appendix 2: Procedure Manual for contagious equine metritis for the outbreak identified April 2011

	<p>DEPARTMENT OF AGRICULTURE, FORESTRY AND FISHERIES DIRECTORATE ANIMAL HEALTH</p>
	<p>PROCEDURE MANUAL FOR CONTAGIOUS EQUINE METRITIS FOR THE OUTBREAK IDENTIFIED APRIL 2011</p>
<p>COMPILED BY:</p>	<p>Dr Ayanda Mpehle, Prof Alan Guthrie, Prof Martin Shulman, Dr Cornelia Gerstenberg</p>
<p>FIRST DOCUMENT</p>	<p>13 April 2011</p>
<p>APPROVED BY:</p>	<p>Director Animal Health</p>
<p>APPROVAL DATE:</p>	
<p>EFFECTIVE DATE:</p>	<p>19 April 2011</p>
<p>NO OF PAGES:</p>	<p>18</p>

F. PURPOSE OF THIS PROCEDURE MANUAL

To lay down the procedures to be followed for the control of the Contagious Equine Metritis (CEM) outbreak diagnosed in April 2011 with the aim of :

- A.1. Preventing the disease from becoming endemic within South Africa
- A.2. Eradicating the disease (South Africa was considered free of CEM prior to an outbreak diagnosed in three stallions and one mare in April 2011).

G. SCOPE

This Procedure Manual is applicable to any **suspect** or **confirmed** CEM cases.

Of major concern is:

- B.1. The risk of CEM becoming endemic within South Africa and its detrimental effect on equine breeding.
- B.2. The potential negative impact of the disease on the equine industry.

H. LEGISLATION

- C.1 CEM is a controlled animal disease in terms of the Animal Diseases Act, 1984 (Act No. 35 of 1984).
- C.2. Any suspect or confirmed CEM cases must be reported to the Director in terms of the Animal Diseases Act, 1984 (Act No. 35 of 1984):
 - (i) Section 11(1)(b)(ii) of the Animal Diseases Act states: “Any owner or manager of land on which there are animals, and any owner in respect of animals, shall, whether or not such owner or manager has obtained advice regarding the health, or any certificate of fitness or health of the animals in terms of section 13(1)(c), from the director, whenever such animals have become or can reasonably be suspected of having become infected with any controlled animal disease, immediately report such incidence in the prescribed manner to the director.”
 - (ii) Section 11(2) of the Animal Diseases Act as amended states: “A veterinarian or any other person who finds the incidence or suspected incidence of any controlled animal disease in any animal or progeny or product thereof, shall immediately report such incidence to the director.”
- C.3. In terms of Table 2 of the Animal Disease Regulations the following control measures for CEM are described.
 - (i) In respect of susceptible animals: “Serving of infected mares or by infected stallions shall be prevented.”
 - (ii) In respect of contact animals: “Contact animals shall be isolated and tested under the supervision of or by an officer or authorized person.”
 - (iii) In respect of infected animals: “Infected animals shall be isolated, and in the case of (a) mares, destroyed under the supervision of or by an officer or authorized person; and (b) stallions, castrated or destroyed under the supervision of or by an officer or authorized person.”
- C.4. The Director: Animal Health will make derogation (exemption) in terms of Regulation 11(2) (b) to be issued to each owner/manager by means of an official order under the condition that the measures as stipulated in this Procedural Manual for Contagious Equine Metritis (CEM) for the Outbreak Identified April 2011, will be complied with in full.

I. CONTAGIOUS EQUINE METRITIS PRESENTATION IN EQUINES AND ZEBRAS

CEM is an acute, highly contagious and venereal disease of equines and zebras caused by gram negative bacteria, *Taylorella equigenitalis*. It is characterised by mucopurulent vaginal discharges and early return to oestrus in most affected mares. Infected stallions and chronically infected mares may not show clinical signs.

J. TRANSMISSION OF CONTAGIOUS EQUINE METRITIS

Infection can be transmitted to equines and zebras in any of the following ways:

- E.1 Direct transmission primarily during mating;
- E.2 Indirect transmission via fomites, including hands, clothing, artificial vaginas, vaginal speculums, other instrumentation and breeding dummies or phantoms used for semen collection
- E.3 Transmission to mares by means of the artificial insemination of semen obtained from a CEM infected stallion, or semen contaminated by CEM bacteria during semen collection or processing;
- E.4 Direct or indirect transmission during teasing. Direct transmission when an infected teaser transmits disease to mares through contact with the teaser's genitalia. Indirect transmission by a teaser after contact with infected vulval discharge and subsequent contact with mares through genital or naso-genital contact;

F. PROCEDURES TO BE FOLLOWED IN ALL SUSPECT OR CONFIRMED CASES OF CEM

F.1. NOTIFICATIONS

Any owner, laboratory or veterinarian who is aware of a suspect or confirmed positive test result is obliged to report this immediately to the local Provincial State Veterinarian or Provincial Veterinary Director. The Provincial Veterinary Authorities have to notify the Directorate Animal Health, Department of Agriculture, Forestry and Fisheries (DAFF) of the **suspect** or **confirmed** CEM infected animals via an immediate notification (SR1 report) and also include the incidence in monthly animal disease reporting. These reports must be sent to:

- (a) Sub-Directorate: Epidemiology
Dr Hannes Pienaar
Fax: 012 319 7470
Email: HannesP@daff.gov.za For more information, please contact:
- (b) Dr Romona Naidoo
Tel: 012 319 7630
Fax: 012 329 0499
Email: RomonaN@daff.gov.za

F.2. QUARANTINE

The responsible state veterinarian shall place the land on which the suspect CEM infected animals are located under quarantine by serving a quarantine

notice\official order on the responsible person in terms of Section 15 (Orders) of the Animal Diseases Act, 1984 (Act No. 35 of 1984) (attached hereto as Annex A), with due reference to Regulation 28 (Serving of documents) (attached hereto as Annex B) as well as the provisions of the Animal Diseases Act Regulations Table 2 pertaining to CEM (attached hereto as Annex C) and this procedure manual.

- (p) As from the date on which the land is placed under quarantine, no animal shall, without the written permission of the responsible state veterinarian, be moved from, to or through the land on which the equines (horses, donkeys mules) and zebras concerned are quarantined, except the movement of suspect CEM equines under red cross permit to either an isolation facility approved by the Director: Animal Health or by red cross permit for temporary or permanent relocation to a property recommended by the responsible state veterinarian and approved by the Provincial Director Veterinary services. No natural mating, artificial insemination, embryo transfer or veterinary examination of equine genitalia or treatment for CEM may be undertaken while the land is under quarantine, except by a veterinarian authorised by the Director: Animal Health.
- (q) When the order is served, it is recommended that two copies are signed.
- (r) It is recommended that the responsible state veterinarian personally serve the order on the owner or manager of the relevant equines and zebras.
- (s) One signed copy of the order should be retained by the owner and the other signed copy kept by the responsible state veterinarian.
- (t) A responsible person shall without delay identify all the susceptible equines and zebras by means of individual equine passports or equivalent documents.
 - Such responsible person shall keep a register in respect of all the Identified equines and zebras
- (u) The owner or manager shall:
 - Declare all natural breeding activities and all veterinary activities such as semen collection, semen freezing, artificial insemination, teasing, embryo collection and transfers, vaginal examinations at any date past or present.
 - Disclose all information on other equines and zebras that were on the premises at any date past or present.
 - In each case the responsible state veterinarian will make a decision as to which equine and zebras are considered suspect for CEM.
- (v) CEM confirmed equines and zebras shall be relocated immediately to an approved isolation facility as determined by the Director: Animal Health or put into an isolation facility approved by the Director: Animal Health on the premises for the purpose of further diagnostic testing and treatment if and as required.
- (w) The stay of the equines and zebras in isolation and all treatment and other related veterinary costs must be borne by the owner.

- (x) The equines and zebras will be released from the isolation facilities **ONLY** after certified CEM negative by the Director: Animal Health.
- (y) No semen or embryos may be collected from any equines and zebras that do not have a valid CEM Clearance Certificate
- (z) Possible in contact equines and zebras (direct or indirect contact) as decided by the state veterinarian may not necessarily be put under full quarantine but shall undergo diagnostic testing as described in point 3 of this document.
- (aa) All surfaces and equipment that may have been in contact with CEM suspect equines or zebras must be properly cleaned and then disinfected with F10 or Vircon.
- (bb) After all suspect CEM infected equines or zebras have been removed to an isolation facility approved by the Director: Animal Health and all surfaces properly disinfected, quarantine may be lifted.
- (cc) ALL test swabbing, treatments and test matings of suspect or in contact horses **MUST** be witnessed by a State Veterinary official/Para-Veterinary official.

G. APPROVAL OF ISOLATION FACILITIES

The Director: Animal Health will only consider the approval of an isolation facility following the receipt of an application letter (Annex D) with a detailed Standard Operating Procedure (SOP) for the isolation facility. The following must be included in the SOP:

- (k) The name of the isolation facility
- (l) A map detailing the layout of the isolation facility including all access points
- (m) A description of how the CEM confirmed equines and zebras will be housed and managed to prevent further transmission of CEM.
- (n) A statement confirming that no teasing, mating (except mating for CEM diagnostic purposes), artificial insemination, embryo collection or transfer will be undertaken within the isolation facility.
- (o) A description of the biosecurity measures that will be applied to all equines and zebras within the isolation facility.
- (p) A description of the biosecurity measures that will be applied for people that have access to the isolation facility.
- (q) The name and contact details of the private veterinarian who is proposed to be authorised to collect all samples, treat all horses and test mate mares at the isolation facility
- (r) A letter from the private veterinarian proposed in (g) above confirming that they are prepared to be authorised to perform the duties outlined in the SOP.
- (s) A name and contact details of a local SV official/Para-veterinary official prepared to be present to observe /monitor the CEM sample collection, CEM treatments and CEM test mating of mares.
- (t) Each and every isolation facility should be approved by the DAH at DAFF prior to testing and treatment procedures commencing. The completed application forms (Annex D) should be submitted by the PDVS to the DAH for this purpose.

H. DIAGNOSTIC PROCEDURES FOR SUSPECT CEM INFECTED EQUINES AND ZEBRAS

Only a veterinarian authorised by the Director: Animal Health shall test CEM suspect or infected equines and zebras **in the presence of a local State Veterinary/Para-**

veterinary official. The samples shall be collected only at an approved isolation facility (Annex D).

- (j) Serology is not considered a useful test for CEM diagnostics.
- (k) Two sets of swabs (duplicate swabs of each site) for CEM diagnostics shall be taken in the manner prescribed below.
- (l) One set of swabs taken in the prescribed manner below shall be placed in Amies charcoal transport medium. The other set of swabs must be kept dry and must be replaced in their plastic sleeves for PCR testing.
- (m) All swabs must be clearly labelled to show the date and time of collection, the identification of the equine or zebra as well as the site of swabbing.
- (n) For endometrial swabs, guarded swabs must be used.
- (o) For the clitoral fossa standard swabs may be used. However for clitoral sinus swabs, paediatric swabs **MUST** be used.
- (p) All swabs must reach the Onderstepoort Veterinary Institute (OVI) within 24 hours of collection, and must be transported on ice or at 4°C.
- (q) The sample submission form (Annex E) must clearly indicate that the swabs are submitted for CEM culture and PCR.
- (r) All samples taken should be coordinated with the OVI in advance:
Dr Laura Lopez at OVI
Tel: 012 529 9272
- (s) Duplicate swabs must be packed separately as separate sets.

H.1 Stallions, colts and geldings

- (c) Duplicate (two of each at the same site taken at the same time) swabs of each site must be taken
- (d) In stallions, colts and geldings, swabs must be taken from the following sites:
 - Urethra
 - Urethral fossa including Urethral sinus
 - Penile sheath (Lamina interna) with an extended penis (it is important to choose a site with an accumulation of smegma)
- (e) Swabs must be collected twice at intervals of at least seven days.

H.2 Mares and fillies

- (a) Duplicate (two of each at the same site taken at the same time) swabs of each site must be taken.
- (b) In the case of mares and fillies, swabs must be taken more than ten days after initial suspect contact from the following sites:
 - Clitoral fossa
 - Clitoral sinus using paediatric swabs
 - Endometrium using guarded swabs.
- (c) Swabs from all three sites must be collected twice at intervals of at least seven days.
- (d) In the case of pregnant mares, clitoral fossa and clitoral sinus swabs only must be taken twice at intervals of at least seven days.
- (e) Suspect pregnant mares must foal in isolation and testing must be repeated after foaling as described in point H.2.b) above.

- (f) For in contact pregnant mares, the Director: Animal Health will decide whether to declare these animals negative based only on the clitoral swab results.

I. TREATMENT OF CONFIRMED CEM INFECTED EQUINES AND ZEBRAS

Only a veterinarian authorised by the Director: Animal Health shall treat CEM infected equines and zebras **in the presence of a local State Veterinary/Para-veterinary official. The treatment shall only be conducted at an approved isolation facility (Annex D).**

I.1 Stallions, colts and geldings

- (a) The fully erect or extended penis shall be thoroughly cleaned with 2% chlorhexidine surgical scrub followed by the application of nitrofurazone ointment or silver sulfadiazine once a day for at least 5 days.
- (b) The stallion, colt or gelding shall be retested as prescribed in point H to H.1 above (i.e. Two sets of swabs must be taken 7 days apart) at least 21 days after completion of treatment.
- (c) If treatment was unsuccessful and the stallion, colt or gelding tests positive for CEM upon retesting, treatment may be repeated or the stallion, colt or gelding destroyed at the discretion of the Director: Animal Health.
- (d) If treatment was successful, the stallion or colt shall be test mated to two mares as described in (J) below.

I.2 Mares and fillies

- (j) The clitoral fossa and clitoral sinuses shall be flushed and cleaned of smegma with an appropriate agent and rinsed with warm Saline
- (k) Following the above flushing, the clitoral fossa and clitoral sinuses shall be thoroughly cleaned with 2% chlorhexidine surgical scrub followed by the application of nitrofurazone ointment or silver sulfadiazine on a daily basis for 5 days, or any other treatment as prescribed by the Director: Animal Health. This treatment shall be repeated daily for 5 days.
- (l) The mare shall be retested at least 21 days after completion of the 5 days treatment with full diagnostic protocol as in H and H2 above.
- (m) If treatment was unsuccessful and the mare tests positive for CEM upon retesting, treatment may be repeated or the mare destroyed at the discretion of the Director: Animal Health.

J. TEST MATING

If treatment was successful, the stallion or colt shall be test mated with two CEM negative mares in the presence of a local State Veterinary/Para-veterinary Official at an approved isolation facility (Annex D) under quarantine conditions for the stallion and the test mares. The test matings must be conducted within a three month period after the second set of negative swabs post-treatment unless otherwise authorised by the Director: Animal Health

The stallion or colt and the test mares must be swabbed after entry into the test breeding programme but prior to test mating and at least 7 days following any previous

swabbing for CEM. Test mating can only proceed following confirmation that these swabs have tested negative.

- h) Do not scrub or use disinfectant or detergent cleaners on the test mare prior to breeding.
- i) Test breeding consists of live cover of two qualified mares. At a minimum each test mare must experience at least two (2) complete penetrations from the stallion, one of which must result in ejaculation.
- j) Endometrial, clitoral fossa and clitoral sinus swabs shall be taken from these mares that were test mated 3, 6 and 9 days after test mating.
- k) Collect a serum sample from each mare 15 days after test mating and subject this to a Complement Fixation (CF) test for CEM.
- l) The stallion or colt and the mares used for test mating must remain in the isolation facility until such time as negative results have been returned on all samples collected.
- m) Quarantine will be lifted from those stallions or colts that have been through a test mating programme with negative results and a CEM Clearance Certificate will be issued. At this stage the quarantine will also be lifted for the test mares
- n) An individual owner has to apply in writing to the Director: Animal Health if he/she wants a particular stallion to be exempted from test breeding, with detailed reasons for why the exemption is requested. Such an application should be accompanied by a suggested appropriate alternative testing procedure recommended by the local state veterinarian and endorsed by the Provincial Director of Veterinary Services.

K. HANDLING AND TESTING OF FROZEN SEMEN.

Frozen semen from all suspect or confirmed cases of CEM should be placed under quarantine by the local state veterinarian until either the suspicion has been removed or the semen has been tested as described below.

- d) If the stallion is still alive, the stallion should be tested for CEM according to the procedure outlined on (H) above.
 - At least one (1) straw per batch of the frozen semen should be tested using qPCR.
- e) With Imported frozen semen, the import documents should be checked and evaluated for CEM tests done.
 - At least one (1) straw per batch of frozen semen must be tested by qPCR.
- f) With Dead stallion, records of breeding (semen collection facility etc.) should be evaluated.
 - At least two (2) straws per batch of frozen semen should be tested by qPCR.

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ANNEX A

15. (1) The director may, in order to achieve a controlled purpose, serve an order in the prescribed manner on any owner of animals or things, or any owner or manager of land, wherein he is directed, in respect of— Orders.

- 5 (a) any specified controlled animal or thing; or
 (b) land defined therein; or
 (c) any such animal or thing and such land,

to comply with a specified provision of this Act, or to perform, or abstain from performing, any other defined act.

10 (2) An order shall, subject to subsections (3) and (4), be binding on the person concerned and any other person who is his legal successor in respect of the relevant animals, things or land.

 (3) Notwithstanding any provision to the contrary in any order regarding the movement or removal of any controlled animal or
15 thing, the director may at any time on written application of a person concerned grant him written authority to move or remove any such animal or thing in accordance with the conditions of the authority (if any).

 (4) The director may by written notice served on an owner or
20 a manager, or his legal successor, in the prescribed manner—

- (a) amend any order; or
 (b) if the director is satisfied after such inspections or
25 examinations as he may deem necessary that the provisions of the order have been properly complied with and that the objects thereof have been achieved, withdraw any order.

 (5) A document purporting to have been signed by the director and framed in a manner which has in respect of the serving of orders and notices been prescribed for purposes of this subsection, shall be conclusive proof that an order under subsection
30 (1), or a notice under subsection (4), has been served on a person concerned.

ANNEX B

Serving of documents [14, 15]

28. (1) A written notification in terms of section 14 or 15 (4) of the Act, and an order refer[sic] to in section 15 (1) of the Act shall be served by-

- (a) forwarding it by registered post to the person concerned;
- (b) delivering it to the person concerned personally or to his authorised representative;
- (c) delivering it at the regular or most recently known residence or place of business of the person concerned, to some person who is apparently not younger than 16 years of age and apparently resides or is employed there; or
- (d) in the case of a juristic person, delivering it at the registered office of that juristic person.

(2) When such notification or order is served in terms of subregulation (1) (a) the director shall-

- (a) place the direction in an envelope addressed to the person concerned at his last known postal address and forward it by pre-paid registered post; and
- (b) at the time of registration thereof make application to be provided with an acknowledgement by the addressee of the receipt thereof as provided in regulation 44 (5) of the Post Regulations published by Government Notice R.550 of 14 April 1960: Provided that-
 - (i) a receipt form completed as provided in regulation 44 (8) of the said regulations shall be sufficient acknowledgement of receipt for the purposes hereof; and
 - (ii) if no such acknowledgement is received, this fact shall be recorded by the director on a copy of the notification or order concerned.

(3) When a notification or order has been served in terms of subregulation (1) (b), (c) or (d), the person by whom it was delivered shall immediately after delivery thereof, make an entry on a copy of that notification or order to indicate the manner in which, the person to whom, the place at which, and the date on and approximate time to which it was thus delivered.

(4) The entries referred to in subregulation (3) shall immediately after being made, be signed by the person by whom the notification or order was delivered.

(5) If the same land or animals are owned by two or more responsible persons, a notification or order referred to in regulation (1) may be served to any one of them.

ANNEX C

Animal disease	Nature, causal organism and symptoms	Susceptible animals	Controlled veterinary act to be performed in respect of—		
			susceptible animals	contact animals	infected animals
1 Contagious equine metritis ...	2 Contagious venereal disease caused by the bacterium <i>Haemophilus equigenitalium</i> , transmitted by coitus and characterised in mares by metritis, abortions, and low fertility	3 Equines and Zebras	4 Serving of infected mares or by infected stallions shall be prevented	5 Contact animals shall be isolated and tested under the supervision of or by an officer or authorised person	6 Infected animals shall be isolated and, in the case of— (a) mares, destroyed under the supervision of or by an officer or authorised person; and (b) stallions, castrated or destroyed under the supervision of or by an officer or authorised person.
Contagious haematopoietic necrosis	A contagious viral disease of fish, characterised by sudden deaths, dark discolouration, anaemia, eye-bulging with distention of the abdomen and hard faeces	Trout	—	All fish in infected dams shall be destroyed under the supervision of or by an officer or authorised person	All fish in infected dams shall be destroyed under the supervision of or by an officer or authorised person.
Contagious pancreatic necrosis	A contagious viral disease of fish, characterised by a sudden high mortality rate especially under young fish, rotating swimming movements, dark discolouration, distention of the abdomen with milky slime in the stomach and intestine	Trout	—	All fish in infected dams shall be destroyed under supervision of or by an officer or authorised person	All fish in infected dams shall be destroyed under supervision of or by an officer or authorised person.
Corridor or buffalo disease ...	Acute communicable animal disease caused by the protozoan <i>Theileria parva lawrencei</i> , transmitted by the brown ear tick (<i>Rhipicephalus appendiculatus</i>), occurring after contact with African buffaloes, and characterised by respiratory distress, enlarged lymph nodes, occasional blindness in one or both eyes, and marked oedema and sometimes emphysema in the lungs.	Cattle and African buffaloes	1. Contact between cattle and African buffaloes shall be prevented 2. All cattle in a controlled area shall be dipped or sprayed regularly by the responsible person with an efficient remedy 3. No animal shall be chemotherapeutically treated without the written authorisation of the director	Contact animals shall be isolated and dipped or sprayed with an efficient remedy under the supervision of an officer or an authorised person in the manner and at the intervals determined by the responsible State Veterinarian	Infected animals shall be isolated and dipped or sprayed with an efficient remedy under the supervision of an officer or an authorised person in the manner and at the intervals determined by the responsible State Veterinarian.
Dourine	Chronic contagious venereal disease caused by the protozoan <i>Trypanosoma equiperdum</i> , transmitted by coitus and characterised by swelling of the genitals, legs and abdomen and sometimes by nervous symptoms	Equines and Zebras	1. Serving of infected mares or by infected stallions shall be prevented 2. Susceptible animals may be tested by an officer, an authorised person or a veterinarian	Contact animals shall be isolated and tested by an officer or authorised person	Infected animals shall be isolated and, in the case of— (a) mares, sterilised, slaughtered or destroyed under the supervision of or by an officer or authorised person; and (b) stallions, castrated, slaughtered or destroyed under the supervision of or by an officer or authorised person.

Annex D

Application for approval of an isolation facility for the testing, treatment and test mating of CEM suspect and infected equines

Identification of property, isolation facility and biosecurity measures:

Agreement to do CEM sample collection, treatment and test mating

I, the undersigned veterinary official authorised thereto by the farm owner/manager,, agree to :

1. Collect all CEM samples as described in the **Procedure Manual for CEM Outbreak Identified during April 2011.**
2. Treat all horses as described in the **Procedure Manual for CEM Outbreak Identified during April 2011.**
3. Test mate mares as described in the **Procedure Manual for CEM Outbreak Identified during April 2011.**

- I (Private Veterinarian),..... hereby confirm that :
- a. I will comply with and uphold the requirement and the standards of the Isolation facility as stipulated/outlined in the D: AH approved CEM isolation facility SOP.
 - b. I will not come into contact with any horse involved in breeding activities for a period of at least 24 hours following any visit to the isolation facility.
 - c. All the activity stated on point 1; 2 and 3 above will be done under the monitoring/observation of the local State Veterinary/Para veterinary Official.

Signature.....
Date.....

PRIVATE VETERINARIAN

Name in print: **Official Stamp**

Address:
.....
.....

Signature..... Date.....

STATE VETERINARIAN

Name in print: **Official Stamp**

Address:
.....
.....

Description:

RECOMMENDED

Signature.....
Provincial Director of Veterinary Services

Date.....

Name in print:

Official Stamp

Address:
.....
.....

APPROVED

Signature.....
Director: Animal Health

Date.....

Name in print:

Official Stamp

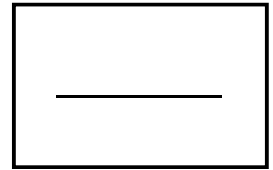
Address:
.....
.....

ANNEX E



ONDERSTEPSOORT VETERINARY INSTITUTE

100 Old Soutpan Road, ONDERSTEPSOORT, 0110, SOUTH AFRICA
Private Bag X5, ONDERSTEPSOORT, 0110 RSA
Tel (012) 529 9272
Fax (012) 529 9275
E-mail: diagreg@arc.agric.za



SUBMISSION FORM FOR SAMPLE TESTING

Sender Ref:		Date:		Lab No:		Time delivered:	
SENDER	TITLE	INITIALS	PERSON/ORGANISATION RESPONSIBLE FOR THE ACCOUNT		TITLE	INITIALS	
Surname:			Name				
Postal address:			Postal address:				
Tel:			Fax:				
Tel:			Fax:				
			SIGNED		NAME PRINTED		
Please note: DAFF will not consider payment for any controlled disease tests if this section is not completed in full and motivation is completed on back of form. Abuse of this constitutes fraud. State Veterinarian will be copied on the results.			OWNER (IF APPLICABLE)		TITLE		INITIALS
STATE VETERINARIAN	TITLE	INITIALS	Surname:				
Surname:			Physical address:				
Postal address:							
			Province				
Province and State Vet Area:		Surveillance program:		Tel:		Fax:	
Tel:		Signature of State Vet:		Registered farm name and number:			
Fax:		Form should also display official SV stamp		Coordinates:		East:	
Email:						South:	
ANIMAL/S			TEST(S) REQUIRED				
Species:							
Number of animal sampled:							
Age::							
Sex: Male / Female (neutered/spayed)							
Type of specimen/s:							
			Purpose for testing: (Required field)				
Number of specimen/s:			Collection date:				
FOR OFFICE USE ONLY Authorised for DAFF payment.							

CHECK LIST FOR RECEIVING SAMPLES : DATE :
(FOR OFFICE USE ONLY) TIME:
RECEIVED BY:

Sample type	Quantity
Total	

Remarks:

Appendix 3: Results of initial RT-qPCR testing of all resident stallions at the South African Lipizzaner Centre

Animal identity	RT-qPCR (Δ CT values)	Swab site
1	4.882645	Urethra
2	16.09037	Urethral fossa
3	17.31891	Urethral fossa
4	6.954865	Urethral fossa
5	14.0379	Distal urethra
6	10.40822	<i>Lamina interna</i>
7	2.730988	Distal urethra
8	6.650162	Distal urethra
9	7.150757	Urethral fossa
10	10.46843	Urethral fossa
11	6.172787	<i>Lamina interna</i>
12	14.33241	Urethral fossa
13	22.40339	Urethral fossa
14	7.408276	Distal urethra
15	18.59398	Distal urethra
16	15.4736	Urethral fossa
17	17.1962	Urethral fossa
18	17.78119	Urethral fossa
19	18.44573	Urethral fossa
20	19.00688	Distal urethra
21	18.11334	Urethral fossa
22	12.65981	Distal urethra
23	20.18825	Urethral fossa
24	18.47821	Distal urethra
25	0	
26	0	
27	0	
28	0	
29	0	
30	0	
31	0	
32	0	
33	0	