A voluntary trichomonosis inter-laboratory comparison study in South Africa

By

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Declaration

I, Tinashe Alan Zangure, hereby declare that the research study presented in this dissertation is original and my own work except for the professional guidance and inputs provided by my supervisors.

Neither the material, nor any part of this dissertation has to my knowledge been submitted in the past, nor will it be submitted to any other University for degree purposes.

This dissertation is presented in partial fulfillment of the requirements for the degree

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Summary

Trichomonosis is currently the most important venereal disease of cattle in South Africa with adverse economic implications to the beef production industry due to cow abortions, infertility and culling of carrier bulls. Once diagnosed in a herd, eradication is difficult due to financial and biological implications. Bulls are asymptomatic carriers and susceptibility increases with age. In infected females, clinical signs include embryonal death, abortion, pyometra, foetal maceration and uterine discharge.

Diagnostic accuracy is one of the major clinical problems preventing easy eradication of trichomonosis from a herd and can be influenced by biological variance in the occurrence of the organism, sampling errors, sample degradation during sample transport and diagnostic laboratory inaccuracies.

This study aimed to validate the accuracy of voluntarily enrolled private (n = 8) and state-owned (n = 5) laboratories that perform trichomonosis diagnostic tests by estimating the sensitivity (Se) and specificity (Sp) per laboratory. It was hypothesized that diagnostic laboratories in South Africa play an insignificant role in the inaccuracy of the diagnosis of trichomonosis.

Laboratories performed either the culture method (n = 5), polymerase chain reaction (PCR) (n = 6) or a combination of culture and PCR (n= 2). Fresh preputial scrapings from four bulls with known negative status for trichomonosis were pooled in 200ml of phosphate buffered saline (PBS) to form the sample base for 12 subsamples of 13ml each. Duplicate subsamples were then contaminated with 2ml originating from four different laboratory cultures of *Tritrichomonas foetus* or 2ml of culture medium for four negative samples. Aliquots of the subsamples were transferred to an anaerobic transport medium, and the final concentration reached in these samples submitted to the laboratories,

were categorised as follows: weak (<10 organisms/µl), moderate (10 – 30 organisms/µl) or strong (>30 organisms/µl). A total of 312 samples were sent by courier in two separate rounds: eight (4 duplicates) positive and four negative samples per round. Multiple logistic regression was performed on sensitivity, using sampling round, laboratory sector, diagnostic test type and sample concentration as independent variables, and removing variables in a stepwise manner based on the highest P-value.

Two public laboratories only reported on one round of sampling, and one batch of 12 samples was severely delayed in reaching another public laboratory. The sample identifications of a further two batches were not recorded by the respective private laboratories. The results from these 60 unreported samples were not included in the analysis. Laboratories that performed the PCR assay (solely, or in addition to culture) were grouped for data analysis. The overall specificity (Sp) was 100% and the sensitivity was 88.7% (95% CI 83.9% - 93.5%). Laboratories using PCR recorded higher sensitivity than those using the culture method (95.5%; 95% CI 91.0% – 99.9% and 81.3%; 95% CI 72.5% - 90.0% respectively, $P < 0.01$), and private laboratories recorded higher Se than public laboratories (96.4%; 95% CI 92.9% - 99.9% and 73.2%; 95% CI 61.2% - 85.2%, $P < 0.01$). For laboratories using PCR, weak positive samples recorded a lower sensitivity than strong positive samples (86.4%; 95% CI 70.8% - 101.9% and 100%; 95% CI 100% - 100%, respectively, $P < 0.01$). One public and six private laboratories obtained 100% accuracy during the two sampling rounds.

In the logistic regression model, private sector (compared to public), an increasing concentration of organisms in the sample and the second round of sampling (compared to the first round) were independent predictors of laboratory sensitivity for the detection of *Tritrichomonas foetus*.

It is concluded that inaccuracies in the diagnostic laboratory contributes to the deficiencies in diagnostic sensitivity for trichomonosis in South Africa, but does not influence diagnostic specificity. It is further concluded that diagnostic sensitivity was independently influenced by the sector in which

the laboratory operates (private vs public) and the concentration of *Tritrichomonas foetus* organisms in the sample.

Keyword: Bovine, Infertility, Tritrichomonas foetus, Specificity, Sensitivity

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Abbreviations

RMRDSA	Red Meat Research and Development South Africa
RuVASA	Ruminant Veterinary Association of South Africa
DNA	Deoxyribonucleic acid
Se	Sensitivity
Sp	Specificity
PCR	Polymerase Chain Reaction
Ig	Immunoglobulin
USA	United States of America

1. Introduction

Trichomonosis is a venereal disease of cattle caused by the flagellated protozoan parasite *Tritrichomonas foetus (T. foetus)* (Schutte, A.P., Herr, S., Kitching, 1994)(Clavijo, Erol, Sneed, Sun, & Swinford, 2011). The extracellular organism is pyriform in shape, with three anterior and one posterior flagellum. It also possesses an undulating membrane which spans the length of the organism giving it a characteristic, rolling, jerky motility (Adeyeye, Ate, Bale, & Lawal, 2012). Sexual reproduction has not been demonstrated to date and the organism multiplies asexually by longitudinal binary fission (BonDurant, 2005). Under adverse environmental conditions, the pseudocyst form is predominant. This is a defensive mechanism to survive harsh conditions such as presence of drugs, decrease in nutrients and sudden changes in temperature. The pseudocyst form may revert back to the trophozoite form when conditions are favourable (Pereira-Neves, Ribeiro, & Benchimol, 2003).

Trichomonosis is currently the most important venereal disease of cattle in South Africa due to its economic importance and challenges associated with its control given its widespread distribution in the country (Madoroba, Gelaw, Hlokwe, & Mnisi, 2011). Trichomonosis is prevalent mostly in beef herds where natural service is practiced. Once diagnosed in a herd, eradication is difficult due to financial and biological constraints (Holm, Tshuma, & Irons, 2012)(Rae, Crews, Greiner, & Donovan, 2004). Financial constraints in eradication include the cost of making a diagnosis and control practices, which include sample collection, laboratory tests, culling of positive animals and the cost of replacement. *T. foetus* also causes production losses in cows due to embryonal deaths, abortions, pyometra, foetal macerations and infertility (Ondrak et al., 2010). Biological constraints include a lack of 100 percent sensitivity and specificity of the diagnostic test and relatively low success of treatment and vaccination (BonDurant, 1985).

Although both males and females can harbour and establish infection, bulls are regarded as maintenance hosts because once infected, they usually become lifelong carriers (Irons & others, 2006) On the contrary, cows and heifers usually clear the infection when allowed to have three oestrous cycles without conception (Parsonson, Clark, & Dufty, 1976). The oestrogenic phase of the oestrous cycle is defined by ovarian release of oestrogen which enhances local immunity. It also opens up the cervix and aids in cleaning infection from the uterus. Heifers however, have a higher incidence of disease compared to cows as previous exposure leaves cows immune for up to three years post infection (Adeyeye et al., 2012; Bartlett, 1949).

Diagnostic accuracy is one of the major clinical problems preventing easy eradication of trichomonosis from a herd, and may be influenced by biological variance in the occurrence of the organism, sampling errors, sample transport factors and diagnostic laboratory inaccuracies. This study investigated the role that South African veterinary laboratories play in the overall accuracy of trichomonosis diagnosis.

2. Literature review

2.1 Epidemiology

Trichomonosis is a venereally transmitted disease of cattle caused by the protozoon parasite *Tritrichomonas foetus* (Schutte, A.P., Herr, S., Kitching, 1994; Taylor, M A, Coop R.L.,Wall, 2015). Although both males and females can harbour and establish the infection, bulls are regarded as maintenance hosts because once infected, they may become lifelong asymptomatic carriers (Irons & others, 2006) The disease has a worldwide distribution and has been reported in countries in Africa, South and North America, Europe, Asia and Australia (Taylor, Marshall, & Stack, 1994)(Jin, Schumaker, Logan, & Yao, 2014). Due to the nature of transmission, the disease is mostly confined to beef herds relying on natural service and extensive herd management (McMillen & Lew, 2006). Agglutination studies have demonstrated three serotypes of *T. foetus* namely the Belfast strain, predominant in Europe, Africa and the United States of America, the Manley strain reported in a few outbreaks and the Brisbane strain reported to occur in Australia. All three serotypes have been demonstrated to be equally pathogenic (Christensen, Clark, & Parsonson, 1977).

1.1.1. Transmission

Transmission is usually during coitus, however iatrogenic transmission may occur if basic hygienic practices are not adhered to during collection of preputial material for diagnostic purposes and when an improperly sanitized vaginal speculum is used in gynaecological examinations of cows or heifers (Goodger & Skirrow, 1986). Artificial insemination does not in itself limit or stop the spread of disease as the protozoon survives the extremely low temperatures in liquid nitrogen used for freezing semen (Mendoza-Ibarra et al., 2012). The strict screening processes of semen donors prior to semen collection in developed countries limit the spread of the disease via artificial insemination through

contaminated semen (Irons, Henton, and Bertschinger, 2002) Infection in bulls is usually asymptomatic, however, a preputial discharge associated with minute preputial nodules has been reported to present soon after infection (BonDurant, 2005). Chronically infected bulls do not develop any gross lesions and often remain asymptomatic while harbouring the organisms in the prepuce, around the glans penis and in the fornix (BonDurant, 1997).

2.1.1 Risk factors

Concurrent infection with *Campylobacter fetus* subspecies *venerealis* is not uncommon although there is no evidence to suggest infection with one increases the chances of being infected with the other (Campero, Ballabene, Cipolla, & Zamora, 1987). Susceptible females are infected by positive bulls through coitus. Studies have shown that infection rates of trichomonosis tend to increase with age in bulls. Young bulls exhibit a greater resistance to the infection or are able to eliminate the infection more efficiently. For this reason, bulls older than five years of age are considered to be the primary carriers of the disease. As bulls get older, the preputial and penile epithelia change and develop deeper crypts and more mucosal folds, thereby creating an environment with a decreased oxygen tension ideal for the survival and proliferation of the anaerobic *T. foetus,* and the chronic carrier status persists even in the presence of a considerable quantity of specific immunoglobulins within the preputial environment (Rae & Crews, 2006). Bulls older than five years rarely recover spontaneously making them a permanent source of infection within the herd while those younger than three to four years may be infected transiently (BonDurant, 1997).

Older bulls are also more promiscuous leading to them being more exposed to cows and heifers compared to their younger inexperienced counterparts. This might be due to them being ranked higher in the multi bull herd hierarchy (Van Eenennaam et al., 2007). The influence of age as a risk factor was demonstrated in an experiment wherein 12 of 13 bulls in the age group of three to

seven years became infected after exposure to *T. foetus* compared to three bulls out of 19 which became infected in the age group of 1 to 2 years (Christensen et al., 1977). Another study investigated the outcome of equal exposures by exposing two groups of bulls, those younger than three years and the other bulls older than three years to *T. foetus* infected females. They concluded that older bulls were more likely to contract trichomonosis. The exclusive use of young bulls in the breeding season may be beneficial in controlling trichomonosis (Christensen et al., 1977).

Trichomonosis is more prevalent in beef herds where bulls are shared between farms. Lack of proper demarcations and fencing between farms may also lead to the spread of the disease to uninfected herds in close proximity. A complete lack of trichomonosis testing within a herd or an incomplete testing where bulls are tested less than three consecutive times also predisposes a herd to *T. foetus* infection. It is more likely to find positive trichomonosis results on farms where there is an incomplete testing of the disease (Irons, Nöthling, & Bertschinger, 2007) .

Cows may remain infected for up to 22 months after initial infection and hence maintain infection in a herd by infecting susceptible negative bulls in subsequent breeding seasons (Corney, 2013). In extremely rare circumstances, infected cows have been reported to carry pregnancies to term and still remain infected post-partum. This type of prolonged carrier status has not been reported many times. In a case study in a California beef herd, a group of 280 cows was examined from a herd of 3000 that had been exposed to *T. foetus* positive bulls. Five of these cows were found to be positive 5 months after exposure. Two of these five cows were 5 and 7 months pregnant. The cow that was 7 months pregnant was tested again 6 weeks post- partum and was still *T. foetus* positive and no further tests were done after this. The cow that was 5 months pregnant was tested 5 months post- partum and found to be *T. foetus* negative (Skirrow, 1987).

2.1.2 Clinical signs

In bulls, the protozoon does not invade the epithelium and is superficially located within the mucosae whereas in females, *T. foetus* colonizes the stratified squamous epithelial surfaces of the vagina and the mucosal surfaces of the uterus, resulting in vaginitis, cervicitis, endometritis and abortion (Ondrak, 2016). In bulls, *T. foetus* infection is therefore usually asymptomatic, although in a few cases a preputial discharge is seen together with development of minute nodules on the penile and preputial membranes (BonDurant, 1985).

Adverse effects are observed in female cattle. The protozoan parasite invades the stratified squamous epithelial vaginal surfaces and the mucosal surfaces of the uterus. Genital infections characterized by an initial vaginitis are observed in infected females. In animals that become pregnant, invasion of the uterus and cervix follows. Various consequences can occur from this point, including a placentitis resulting in early abortion (less than 16 weeks), pyometra and uterine discharge. Abortion before four months of pregnancy is the most common course of disease progression (BonDurant, 2005). In some cases, the developing foetal membranes are retained resulting in a purulent endometritis, a persistent uterine discharge and anoestrus (Ball, Dargatz, Cheney, & Mortimer, 1987). The corpus luteum may, in a few cases, be retained leaving the cervical seal closed, resulting in pyometra (Rae, 1989).

2.1.3 Treatment

Antiprotozoals such as metronidazole, dimetridazole and ipronidazole have been used to good effect in treating trichomonosis (Yule, Skirrow, & Bondurant, 1989). In the USA, the use of imidazole compounds to treat trichomonosis is banned. Concurrent intrapreputial or systemic antibiotic therapy with a penicillin is also required to reduce resident bacteria which metabolize imidazole compounds. Ipranidazole is not readily available in South Africa while metronidazole has cost and residue concerns,

leaving dimetridazole as the only drug available to practitioners. Neither of these drugs is however registered for the treatment of cattle. Dimetridazole is available as a powder in South Africa and its use is considered extra-label. Intensive treatment of infected bulls with a five day oral dimetridazole course at 50mg/kg is a regimen that has been instituted in South Africa. Dimetridazole is a water soluble powder and is mixed with 5 to 10 litres of water and is administered intraruminally via a stomach tube. It is advised to give good quality roughage and rumenotorics to limit gastrointestinal side effects of therapy (Campero et al., 1987).

Treatment has been reported to be moderately successful, time consuming and difficult to administer as bulls have to be adequately restrained and tend to become more difficult as days pass into the treatment regimen. Aqueous solutions have also been shown not to penetrate the preputial membrane effectively and this has been a factor in treatment failure. Treatment is also discouraged as some bulls subsequently tested positive for *T. foetus* after initially testing negative post treatment. This then led to a recommendation of waiting for at least six weeks between treatment and follow up testing. A possible explanation of treatment failure would be pseudocyst formation due to the presence of drugs. After treatment the pseudocysts would then revert back to the trophozoite form. (Pereira-Neves et al., 2003).

In a study by (Fitzgerald, Johnson, & Hammond, 1963) involving 30 bulls, 22 out of the 30 bulls were successfully treated using either dimetronidazole or diminazene giving an overall treatment success of 73.3%. . For all bulls treated with dimetridazole (dimetridazole alone and in combination with other drugs), 11 out of 12 (91.7%) were treated successfully. For bulls treated with topical diminazene alone, 11 out of 18 (61.1%) were treated successfully, including 1 of 5 bulls treated with the paste formulation (Irons, n.d.). Disadvantages of dimetridazole use include intractability of bulls due to discomfort, injection site abscesses, inappetence, indigestion, respiratory difficulty, rumen

stasis, decreased rumen motility, ataxia and rapid resistance to the drug by the organism (Ball et al., 1987; BonDurant, 1997).

Due to the problems stated above, bulls which test positive for trichomonosis are normally culled (Cobo et al., 2007). Cows and heifers usually clear the infection when allowed to have three oestrous cycles without conception. The oestrogenic phase of the oestrous cycle is defined by release of oestrogen which enhances local immunity. It also opens up the cervix and aids in cleaning infection from the uterus. Heifers however have a higher incidence of disease compared to cows as previous exposure leaves cows immune for up to 3 years post infection (BonDurant, 2005; Campero et al., 1987; Collantes-Fernández et al., 2019).

2.1.4 Immunology and control

Convalescent and acquired immune responses to *T. foetus* infection have shown that bovines have an ability to mount innate responses at a local and systemic level. Upon invasion of the genital epithelia, presence of antigen sets into motion formation of mucosal associated lymphoid tissue and local IgA and IgG1 response. Infected heifers mount minimal systemic antibody responses but strong vaginal, cervical and uterine IgA and IgG1 responses 7 to 12 weeks after infection. In infected bulls significantly increased levels of IgG1, IgA, IgM and IgG2 antibodies to *T. foetus* have been observed (Chapwanya, Usman, & Irons, 2016).

Prevention and control of trichomonosis is hinged on a good herd health regimen (Rae & Crews, 2006). A number of strategies may be implemented in the prevention and control of trichomonosis. Vaccination of cows with TrichGuard® (Zoetis, South Africa) 4 to 6 weeks prior to the start of the breeding season has been shown to have a marked positive impact on reproductive performance. Vaccination does not prevent disease transmission but reduces the severity and duration of clinical signs (Cobo et al., 2007)(Rae & Crews, 2006). In bulls, use of the vaccine has not been

approved. Anecdotal reports have suggested that vaccination of bulls against trichomonosis does not clear infection and may additionally confound diagnostic sensitivity by decreasing parasite burdens below detectable limits. In a study by (Alling et al., 2018) in the USA, 20 *T. foetus* positive beef bulls were divided into a control and a test group. The test group received 2 Trichguard® (Boehringer, Ingelheim Vetmedica Inc, 2018) vaccines 2 weeks apart and antibody response was then measured then and at 4 monthly intervals. Although the vaccinated group evoked a statistically significant humoral immune response, there was no improvement in infection relative to the control group that had only received vaccine adjuvant despite the robust humoral response to vaccine.

Keeping a closed herd or control of movement of animals in and out of a herd should be instituted to prevent introduction of trichomonosis (BonDurant, 1985). All newly purchased cows and heifers should be separated during breeding to decrease risk of exposure from affected cows. Cows that abort within a herd should be separated and given sexual rest as during this period they undergo oestrus and develop temporary immunity. All newly introduced bulls should have undergone a bull breeding soundness exam and should have been tested for trichomonosis. Use of young bulls for breeding in herds is also a control strategy since age (>3 years old) has been listed as a risk factor (Rae, 1989; Yule et al., 1989). Use of artificial insemination over natural breeding is also an option though it may not be practically feasible in large beef herds under extensive ranching (Irons et al., 2007).

2.1.5 Diagnostic Accuracy

Laboratory validation refers to a series of interrelated procedures in an experimental process which aid to document that a particular protocol used by the laboratory has a guaranteed performance for its intended use and will accurately predict the infection status of animals within a particular population of interest. Validation assesses a test for its suitability for a given use. It involves analysis of reagents to be used in the different stages of sample processing together with protocols to be

followed in the scientific assay (Greiner & Gardner, 2000). The validation process ensures assay confidence as its repeated use affirms that it is a robust method and this is affirmed by accuracy and precision of results obtained (Jacobson, 1998). Test validation consists of five stages as summarized below.

1. Feasibility studies
2. Assay development and standardization
    - Optimisation of reagents, protocols and equipment
    - Estimate of repeatability
    - Determination of critical control parameters
    - Determination of analytical sensitivity and specificity
    - Range
3. Determination of assay performance characteristics
    - Diagnostic sensitivity and specificity
    - Repeatability and reproducibility
4. Monitoring the validity of assay performance
5. Maintenance and enhancement of validation criteria

Although tests are optimized during validation, the majority of tests will still possess a certain proportion of false-negative or false-positive results resulting in a wrong classification of diseased/infected or non-diseased/uninfected animals. These anomalies may be quantified by comparing test results to a nearly perfect test referred to as the 'gold standard' (Conraths & Schares, 2006). Validation is an integral part of quality management and quality assurance. It plays a pivotal role in assuring that test results portray the true status of the samples. The process of validation is vital in determining the extent to which a test result may vary from the true status due to imperfect

reliability as a result of systemic errors, validity and chance. If a diagnostic test measures what it purports to measure, it is valid (Dohoo, I., Martin, W., Stryn, 2003).

Basic aspects of the validation process include analytical sensitivity and analytical specificity. Analytical sensitivity may be defined as the lowest concentration of analyte that is detectable by a test. In PCR techniques, this may refer to the lowest number of genome copies that can be detected by a specific PCR protocol. Analytical specificity refers to the capacity of a test to react to only one analyte (Thrusfield, 2005). The validation process also includes diagnostic sensitivity (DSe) and diagnostic specificity (DSp). The DSe is defined as the proportion of known infected samples that test positive in the assay with the remainder of infected animals that test negative considered to be false negative. The DSp is the proportion of uninfected animals that test negative in an assay with the remainder of uninfected animals testing positive being considered to be false positive. Decision fractions refer to the observed frequencies of true negatives, false negatives, true positives and false positives and these are recorded in a 2x2 contingency table where the diagnostic specificity, diagnostic sensitivity, positive predictive value and negative predictive values may be calculated from (Conraths & Schares, 2006).

2.2 Factors affecting diagnostic tests for trichomonosis

2.2.1 Biological factors

T. foetus organisms are not always obtainable from the preputium of infected bulls using standardised sampling methods for reasons which have not yet been clearly explained. It has been shown that frequent testing may decrease the ability to obtain organisms from infected bulls (Yao, 2013). For this reason, three consecutive scrapes 7 to 10 days apart are recommended for trichomonosis diagnosis to allow accumulation of organisms (Yao, 2013).

T. foetus is a pleomorphic organism. It can either exist in the trophozoite or pseudocyst forms. The more commonly referred to form is the trophozoite form which is the organism's typical

microscopic appearance under favourable environmental conditions. As a trophozoite, the organism appears as a pyriform, mononucleate with three anterior flagella and a trailing fourth one. When viewed under a light microscope, a characteristic wave like motion of the undulating membrane is observed (Yao, 2013).

Under adverse environmental or stressful conditions, the protozoan adopts the pseudocyst form. Stressfull conditions include sudden temperature changes, a decrease in nutrient availability and presence of drugs. The pseudocyst form is a non-motile, spherical organism with internalized flagella. It is currently assumed that the pseudocyst form is reversible. (Pereira-Neves, Campero, Martinez, & Benchimol, 2011) found that culturing fresh preputial samples before microscopic examination greatly decreased the pseudocyst percentage further supporting the theory that culture media provides favourable nutritional conditions which result in the pseudocyst transformation to the trophozoite form.

2.2.2 Sampling techniques

Preputial samples are the most ideal specimen for collection to confirm trichomonosis in a herd since the bull is the most important maintenance host. Preputial material can be collected by sheath scraping in combination with aspiration, sheath washing (Schonmann et al., 1994) , by use of a gauze sponge, or by use of specialized scraping devices such as the Tricamper. The tricamper is a 60 cm long polyethylene tube with a 75mm long, 8mm diameter corrugated scraper head with a 1.5mm collection bore attached to a 6mm diameter tubing with a 1.5mm internal diameter. It is not necessary to aspirate and scraping is done over the surface of the glans penis and preputial mucosa (Corney, 2013). When scraping a bull ensure proper restraint in a crush and immobilisation by use of an electroejaculator to ensure safety of the operator and improve the operator's ability to collect a satisfactory sample. A sterile artificial insemination pipette connected to a 20ml syringe by way of a

silicone rubber tube are used. During sample collection 2ml of PBS are aspirated into the pipette for lubrication following which the pipette tip is inserted into the preputial cavity all the way back to the fornix. Negative pressure is then applied by pulling up the syringe plunger and the pipette scraped back and forth 15 to 20 times while maintaining the suction (Mendoza-Ibarra et al., 2012). On withdrawal of the apparatus, a small amount of thick, opaque, blood tinged liquid should be seen in the pipette. Sheath scraping collects material from a limited portion of the extremities of the preputial cavity and free portion of the penis. This collected material is more concentrated than that of the wash and has less volume and it is not routinely further concentrated by centrifugation (Kennedy, Pearl, Tomky, & Carman, 2008).

Sheath washing recovers material from the entire preputial cavity in a larger volume and can be concentrated by centrifugation. If collecting a sample by washing, a 50ml solution of PBS is instilled into the preputial cavity via a funnel and a flexible tube. The preputial cavity is massaged vigorously 10 to 100 times and the fluid collected thereafter (McMillen & Lew, 2006). A good sample appears opaque and contains visible flecks of cellular debris. It is important when using either of the methods to avoid contamination of samples by removing debris and soiled hair from around the preputial orifice by trimming of the hairs. Cleaning of the area with disinfectant is not advised as this may decrease the diagnostic sensitivity of the test. No superiority in sensitivity of washing to scraping was found upon conducting gel-PCR (Mukhufhi, Irons, Michel, & Peta, 2003).

Sheath scraping or washing are not perfect collection techniques. It may be quite difficult to gauge that an adequate sample has been collected. A previous study also demonstrated that for right handed veterinarians, samples were most likely to be positive after culture when the operator scraped while on the right side of the crush pen compared to when they scraped from the left (Parker, Campbell, McIntosh, & Gajadhar, 2003).

During sample collection using a gauze sponge, the penis is extended by electrostimulation with a rectal probe. A 16 ply gauze sponge is used to wipe around the glans of the penis and down the penile shaft and exposed preputial mucosae 2 to 3 times. In a study to compare the Se of sheath scraping to gauze sponge method, 111 samples from bulls of unknown status from high risk areas were used. Samples were placed in the In-Pouch™ media and after PCR analysis 37 bulls (33%) tested positive after scraping while 39 bulls (35%) tested positive after the gauze sponge method of collection. Se of scraping was 92% while that of the gauze sponge method of collection was 95% showing that the penile sponging technique is a reliable alternative (Dewell, Phillips, Dohlman, Harmon, & Gauger, 2016).

2.2.3 Sample transport

Under the most favourable of conditions in South Africa, sample shipping and delivery to diagnostic laboratories takes approximately 24 to 48 hours. A delay may result in a loss of approximately 10 % in diagnostic sensitivity with longer delays causing more drastic decreases (Kittel, Campero, Van Hoosear, Rhyan, & BonDurant, 1998; Tedesco, Errico, & Baglivi, 1979). Factors which may affect the test result in this case are the medium in which the samples are stored, the time elapsed between sample collection and delivery and the temperature at which the sample is kept. In South Africa, Steve's transport medium (Onderstepoort Bilogical Products, Pretoria, South Africa) is used while an In-Pouch™ TF (BioMed Diagnostics, San Jose, California) transport medium is used in the USA. The use of PBS as a transport medium has yielded conflicting results. In one study, no cultivability was observed for samples held at 4˚C for 48 hours or at 37˚C for 24 hours when PBS was used as a transport medium (Reece, Dennett, & Johnson, 1983).

2.2.4 Laboratory factors

The diagnosis of trichomonosis is complex as a myriad of problems may compromise sensitivity and specificity. Factors relating to sample collection, shipping and testing may influence results. With reference to the laboratory, time delay between receiving the samples and start of processing may have a detrimental effect especially if diagnosis is by culture methods which rely on the organism being alive (Davidson, Ondrak, Anderson, Swinford, & Erol, 2011). The personnel at the labs also play a role especially when culture and microscopic identification are used as even experienced personnel may mistake other trichomonads for *T. foetus* resulting in false positive diagnoses (Corbeil, Campero, Van Hoosear, & BonDurant, 2008). It has also been demonstrated that most clinical microscopes lack the resolution to clearly identify the number of anterior flagella (Ondrak, 2010).

The type of PCR method to be used by the laboratory (i.e. conventional versus real time PCR) may also have a bearing on results obtained. In conventional PCR, the nucleic acid DNA or RNA is measured at the plateau or end point while real time PCR measures the nucleic acid material as the reaction occurs. Real time PCR therefore has a higher accuracy than end point PCR detection which compares the test band to a *T. foetus* positive control band at 347 base pairs and has a primer that is specific for a sequence in the 5.8 s ribosomal unit (Campero, Rodriguez, Dubra et al, 2003).

Nutrient media commonly used for collection of preputial samples include modified Diamond's medium. Diamond's medium comes as a screw cap culture. Inoculation may be done whilst still in the field or upon delivery to the laboratory in a transport medium such as Steve's transport medium. Agar is added into the medium to ensure confinement of contaminating organisms largely in the upper portion of the culture medium while maintaining the microaerophilic conditions on the lower portion of the medium which are ideal for supporting trichomonad viability and reproduction. The sensitivity of Diamond's medium has been said to be between 81.6 to 93.2% (Rae & Crews, 2006).

An In-Pouch™ TF (BioMed Diagnostics, San Jose, California) medium is available on the market and facilitates examination even in situations where the least sophisticated laboratory facilities are used. It is both a transport and a growth medium and consists of two chambers with the upper chamber containing 1ml of an enriched peptone medium while the lower chamber has 3ml of the same medium. *T. foetus* organisms concentrate in the bottom of the pouch in an anaerobic environment because the pouch is kept in a vertical position. The In-Pouch™ TF (BioMed Diagnostics, San Jose, California) comes with a reusable plastic viewer which fits on the microscope stage and allows samples to be viewed without having to reopen the pouch. Samples collected in the pouch can also be stored for up to one year at room temperature while maintaining their effectiveness (Rodning, 2007; Thomas, Harmon, White, & others, 1990). Other transport media used include Trichtube and Steve's transport medium which is commonly used in South Africa. Samples which can be directly inoculated using the In- pouch test kit are those collected via preputial scraping. Those collected by washing need to be centrifuged first before the In-pouch test kit can be used. Culture samples are incubated at 37˚C for up to 120 hours and the live protozoa are checked microscopically on a daily basis.

2.2.5 Laboratory processing techniques

2.2.5.1 Culture

Numerous methods, spanning from culture, direct microscopy to molecular methods are available to aid in diagnosis of bovine trichomonosis. The reference test for diagnosis of *T. foetus* infection is culture of a bull's preputial smegma (BonDurant, Campero, Anderson, & Van Hoosear, 2003). The median sensitivity and specificity of culture in the field ranges from 72 to 92% and from 95 to 100% respectively. As a result of the above, it is generally sufficient to consider a herd as being *T. foetus* positive after one positive result, however at least three consecutive results are necessary to demonstrate freedom from *T. foetus* infection (Mardones, Perez, Martinez, & Carpenter, 2008; Villarroel, Carpenter, & BonDurant, 2004).

Identification of the protozoa microscopically is by observation of the characteristic three anterior flagella, an undulating membrane, a posterior flagellum and the characteristic rolling movement of the live organisms (Yao, 2013) .The advantage of culture is its simplicity. Culture is also used as an amplification method where organisms collected are too few to make a positive diagnosis by direct microscopic examination. Demerits of culture on the other hand include the fact that culture is time consuming and may take several days for a diagnosis to be made. Viable organisms are required for the culture technique to be effective. The specificity of culture is also low as it is impossible to precisely differentiate contamination by related trichomonad species even if personnel are highly experienced (Ondrak et al., 2010).

Nonsexually transmitted trichomonads such as *Tetratichomonas* species and *Pentatrichomonas hominis* may be difficult to distinguish microscopically from *T. foetus*. Young bulls sometimes mount each other in what is known as herdmate sodomy (Walker et al., 2003). This results in faecal contamination of the prepuce thereby disseminating the lower bowel commensals into the bull's reproductive tract. The presence of these trichomonads results in false positive diagnoses when culture and microscopic analysis are used thereby lowering specificity (Corbeil et al., 2008).

2.2.5.2 Polymerase Chain Reaction (PCR)

As an aid to culture and microscopic examination, PCR methods have been developed to diagnose bovine trichomonosis (Felleisen, Schimid-Lambelet, & Walubengo, 1997). Using culture alone as a diagnostic tool is limited by Se which ranges from 72 to 92%. PCR testing is superior to culturing samples if collection was done in remote areas where a delay to the processing laboratory is anticipated. The aim of a polymerase chain reaction is DNA or RNA detection. The primers used in the PCR techniques specifically amplify the DNA fragments and precisely differentiate between *T. foetus* and the other trichomonad species therefore making up for

limitations of culture and microscopic examination. False positive diagnoses are also reduced or completely eliminated resulting in increased specificity (Morgan & Thompson, 1999). In addition to its specificity, PCR is very sensitive and it can detect as few as one organism (McMillen & Lew, 2006).

3. Research questions

Do the individual laboratories play a major role in the accuracy of *T. foetus* diagnosis?

What factors influence the accuracy of the individual laboratories?

What is the influence of inter-laboratory comparison feedback on the accuracy of laboratories?

What other factors besides laboratory factors influence diagnostic sensitivity and specifity of individual laboratories?

What recommendations can be given to improve on factors that cannot be controlled by the individual laboratories but have a negative bearing on their accuracy?

4. Hypotheses

- H0: The Sensitivity of individual laboratories taking part in the study for the detection of *Tritrichomonas foetus* organisms in preputial samples is similar.
- H0: The Specificity of individual laboratories taking part in the study for the detection of *Tritrichomonas foetus* organisms in preputial samples is similar.
- H1: The Sensitivity of individual laboratories taking part in the study for the detection of *Tritrichomonas foetus* organisms in preputial samples is different.
- H1: The Specificity of individual laboratories taking part in the study for the detection of *Tritrichomonas foetus* organisms in preputial samples is different.
- H0: Factors influencing laboratory accuracy for the detection of *Tritrichomonas foetus* cannot be determined in South Africa

5. Objectives

The aim of the project was to validate the accuracy of private and state owned laboratories that perform diagnostic tests for trichomonosis

The objectives of the project included:

- To estimate the sensitivity of individual private and state owned laboratories that perform diagnostic tests for trichomonosis.
- To estimate the specificity of individual private and state owned laboratories that perform diagnostic tests for trichomonosis.
- To gather data on existing state and privately owned diagnostic laboratories that provide diagnostic tests for trichomonosis.
- To publish a list of validated laboratories on the website of the Ruminant Veterinary Association of South Africa (RuVASA).

6. Materials and Methods

6.1 Model system and justification

A laboratory experimental study design was used. Live animals were only used initially to provide negative preputial samples while *T. foetus* cultures were used to infect some of the negative samples collected in the laboratory.

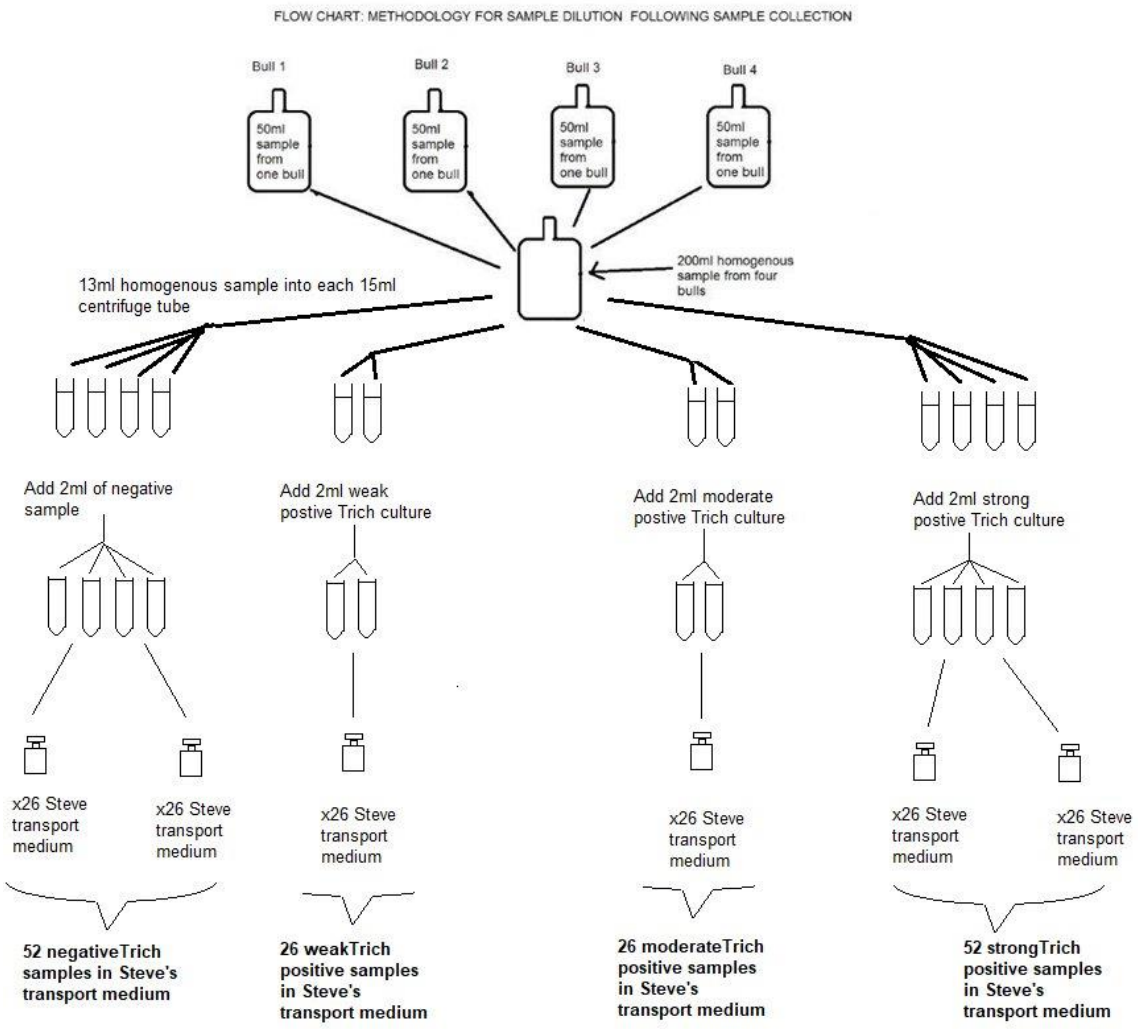
6.2 Experimental design

One laboratory was conveniently nominated from the list of participating laboratories to provide samples containing live cultures of *Tritrichomonas foetus*. The nominated laboratory submitted the cultures in a suitable transport medium to the researchers at the Faculty of Veterinary Science, University of Pretoria, Onderstepoort. The live *T. foetus* cultures were maintained at the research laboratory using the standard operating procedures provided by the laboratory submitting the cultures. The concentration of each received culture was determined using a haemocytometer. After determining the concentration per sample, samples were grouped into three categories of *T. foetus* concentration for the analysis: weak (<10 organisms/µl), moderate (10 – 30 organisms/µl) and strong (>30 organisms/µl). Four bulls were tested for bovine trichomonosis prior to the start of the research project by way of three consecutive preputial scrapings collected at weekly intervals. Preputial scrapings from these four bulls were collected for every repeat of the study. Each of these four preputial samples were diluted in 50ml phosphate buffered saline (PBS) and were pooled to create a homogenous sample of 200ml.

Figure 2 provides a diagrammatic representation of the procedure applied to create the test samples: Twelve 15ml centrifuge tubes were used to create four different concentrations of samples namely negative (4 samples), weak positive (1 x duplicate sample), moderate positive (1

x duplicate sample) and strong positive (2 x duplicate samples). From the 200ml pooled homogenous sample, 13 ml were transferred to each centrifuge tube and the remainder thereof discarded. Two millilitres of the three concentrations namely weak positive, moderate positive and strong positive were added to the centrifuge tubes in duplicate to come up to the 15ml mark. To the remaining four centrifuge tubes, 2ml of the transport medium without any positive culture were added and these became the negative culture. One millilitre of each of the four duplicate aliquots mentioned above was transferred into Steve's transport medium (Onderstepoort Biological products, Onderstepoort, South Africa). Each participating laboratory then received 12 samples in Steves transport medium consisting of four negative samples and eight positive samples of different concentrations. The laboratories were blinded to the identity of the samples and a unique sample identification code was allocated per bottle sent to each laboratory. Records of the samples were kept by the researchers until reporting.

Figure 1: Diagrammatic representation of the method used to create the 12 test samples for every participating laboratory.



6.3 Experimental procedures

Preputial sample collection from bulls:

Preputial scrapings were collected by restraining the bull in a crush pen and inserting a lubricated electro-ejaculator into the rectum to immobilise the bull using electro-immobilisation in order to ensure the operator's safety. Hairs on the preputial opening were clipped and the area

cleaned using a dry paper towel. A sterile artificial insemination pipette was connected to a 20ml syringe by way of a silicone rubber connector. The 20ml syringe provided negative pressure ensuring ease of collection of the sample. Two millilitres of PBS were aspirated into the artificial insemination pipette in order to lubricate it and the pipette was inserted through the preputial opening all the way up to the fornix of the preputium. Negative pressure was applied by pulling up the syringe plunger while simultaneously scraping back and forth about 15 to 20 times against the preputial mucosa. On withdrawal of the apparatus a small amount of thick, blood tinged smegma was visible within the pipette. This smegma was rinsed into the 50ml PBS in the glass container.

Procedure for determining the concentration in the final sample sent to the laboratories:

The concentration of the final test samples containing *T. foetus* cultures was obtained by systematically counting the organisms using a haemocytometer. Two counts of the cells measuring 1/25 square millimeters divided into a further 16 small squares were done at 100X magnification and an average of the two obtained. *T. foetus* organisms that overlapped a ruling were counted as belonging to the top or right small square. The result was then recorded as number of *T. foetus* organisms per 0.04 cubic millilitres. This figure was then converted to organisms per microliter by dividing by 9 and multiplying by 10.

Sample dispatch:

The 12 uniquely identified samples prepared for every laboratory were cling wrapped and packaged into styrofoam packaging on ice. These were then sent via overnight courier to the participating laboratories for diagnostic analysis by the respective laboratories.

6.4 Observations

Information was obtained from every participating laboratory: contact information, province, sector, laboratory techniques used.

Concentration of received *T. foetus* positive samples was recorded in number of organisms per µl of each of the final duplicate test samples.

Participating laboratories submitted diagnostic test results according to each laboratory's standard operating procedure, which included the results for every sample and the date (and in some cases the time) of sample receipt.

6.5 Data analysis

Data were cleaned according to the following procedure: in cases where laboratories provided results without sample identification, or where no results were provided, these were treated as missing values, and were not included in further analysis. In cases where laboratories provided no positive test results, all the data from that laboratory in that round, was treated as missing values and not included in further analysis. This was distinct from false negatives in that they were known positives even before the samples had been dispatched to the different participating laboratories.

Laboratories that included the PCR technique in their analysis (as the only procedure, or in combination with an initial culture phase) were classified as PCR laboratories, and laboratories that only performed the culture method were classified as culture laboratories.

Outcomes determined included the proportion of true positives, false positives, true negatives and false negatives per laboratory, the sensitivity (the ability of the laboratory to detect the presence of *T. foetus* in a diagnostic sample) and the specificity (the ability of the laboratory to detect the absence of *T. foetus* in a diagnostic sample).

Initially raw data were summarised in a table where paired samples were placed in rows and the different laboratories were placed in columns grouped by sector and laboratory procedure used. Using this information the sensitivity and specificity were calculated for each laboratory using MS Excel®. This feedback was sent to every participating laboratory in an official letter.

An analysis of diagnostic sensitivity followed, using each sample delivered to every participating laboratory as the experimental unit. Sensitivities were compared between the different laboratory sectors, the two different sampling rounds, the different diagnostic tests and the different concentrations of *T. foetus* organisms in the sample using a T-test.

Multiple logistic regression was performed on the individual sample sensitivity outcome using the sampling round (first vs second), the laboratory sector (public vs private), the laboratory test (PCR vs culture) and the concentration of organisms in the sample as covariates. Covariates were removed in a step-wise manner based on the highest wald P-value, and remained in the final model if the P-value was < 0.10. Confounding was considered if the coefficient of any of the remaining covariates changed by >15% when another covariate was added to the model.

Statistical analyses were performed using NCSS 2007 (Kaysville, Utah, USA) and STATA 14 (Stata Corp, Texas, USA).

6.6 Experimental animals

Use of animals for this study was approved by the University of Pretoria's Animal Ethics Committee (AEC protocol V17-041). Four bulls were used in the study. The bulls acted as a source of trichomonosis negative preputial samples and were therefore not infected with *T. foetus.* Live cultures of *Tritrichomonas foetus* were sourced from participating laboratories, and were used to contaminate some of the negative samples at known concentrations.

7. Results

Laboratories performed either the culture method (n = 5), polymerase chain reaction (PCR) (n = 6) or a combination of culture and PCR (n = 2). A total of 312 samples were sent by courier in two separate rounds: eight (4 duplicates) positive and four negative samples per round. The results of the two rounds are summarised in Table 1. One public and six private laboratories obtained 100% accuracy during the 2 sampling rounds.

Table 1: Raw data Summary

	Sample submitted to laboratories					Private lab 1	Private lab 2	Private lab 3	Private lab 4	Private lab 5	Private lab 7	Private lab 6	Private lab 8	Public lab 1	Public lab 2	Public lab 3	Public lab 4	Public lab 5
				Concentration														
Round	Identification	Duplicate of	Status	(organisms/µl)	Category	PCR	PCR	PCR	PCR	Combi	Combi	Culture	Culture	PCR	PCR	Culture	Culture	Culture
1	ACB1	ACB7	positive	21	moderate	1	1	1	1	Sample identification not captured	1	1	0	No results received	0	1	0	0
1	ACB7	ACB1	positive	21	moderate	1	1	1	1		1	1	1		1	1	1	0
1	ACB2	ACB6	negative		negative	0	0	0	0		0	0	0		0	0	0	0
1	ACB6	ACB2	negative		negative	0	0	0	0		0	0	0		0	0	0	0
1	ACB3	ACB12	positive	9	weak	1	1	1	1		1	1	0		0	1	0	0
1	ACB12	ACB3	positive	9	weak	1	1	1	1		1	1	0		0	1	0	0
1	ACB4	ACB31	negative		negative	0	0	0	0		0	0	0		0	0	0	0
1	ACB31	ACB4	negative		negative	0	0	0	0		0	0	0		0	0	0	0
1	ACB5	ACB8	positive	38	strong	1	1	1	1		1	1	1		1	1	1	0
1	ACB8	ACB5	positive	38	strong	1	1	1	1		1	1	1		1	1	1	0
1	ACB9	ACB11	positive	32	strong	1	1	1	1		1	1	1		1	1	1	0
1	ACB11	ACB9	positive	32	strong	1	1	1	1		1	1	1		1	1	1	0
2	X1	X7	negative		negative	0	0	0	Sample identification not captured	0	0	0	0	0	0	0	Samples received 30 days after dispatch	No culture media available
2	X7	X1	negative		negative	0	0	0		0	0	0	0	0	0	0		
2	X2	X6	positive	10	weak	1	1	1		1	1	1	1	0	1	1		
2	X6	X2	positive	10	weak	1	1	1		1	1	1	1	1	1	1		
2	X3	X12	negative		negative	0	0	0		0	0	0	0	0	0	0		
2	X12	X3	negative		negative	0	0	0		0	0	0	0	0	0	0		
2	X4	X9	positive	29	moderate	1	1	1		1	1	1	1	1	1	1		
2	X9	X4	positive	29	moderate	1	1	1		1	1	0	1	1	1	1		
2	X5	X8	positive	39	strong	1	1	1		1	1	1	1	1	1	1		
2	X8	X5	positive	39	strong	1	1	1		1	1	1	1	1	1	1		
2	X10	X11	positive	32	strong	1	1	1		1	1	1	1	1	1	1		
2	X11	X10	positive	32	strong	1	1	1		1	1	1	1	1	1	1		
					True positive	16	16	16	8	8	16	15	13	7	13	16	5	0
					False positive	0	0	0	0	0	0	0	0	0	0	0	0	0
					True negative	8	8	8	4	4	8	8	8	4	8	8	4	4
					False negative	0	0	0	0	0	0	1	3	1	3	0	3	8
					Sensitivity	100%	100%	100%	100%	100%	100%	94%	81%	88%	81%	100%	63%	0%
					Specificity	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%

Two public laboratories only reported on one round of sampling, and one batch of 12 samples was severely delayed in reaching another public laboratory. The sample identifications of a further two batches were not recorded by the receiving private laboratory (Table 1). The results from these 60 unreported samples were treated as missing values and not considered in the analysis of sensitivity. Laboratories that included the PCR process were grouped for further data analysis. The overall sensitivity (Se) was 0.89 and the specificity (Sp) was 1 (95% CI 0.84 – 0.94). Laboratories using PCR recorded higher sensitivity than those using the culture method (0.96; 95% CI 0.91 – 1.00 and 0.81; 95% CI 0.73 – 0.90 respectively, $P < 0.01$), and private laboratories recorded higher Se than public laboratories (0.96; 95% CI 0.93 – 1.00 and 0.73; 95% CI 0.61 – 0.85, $P < 0.01$).

In the case of samples with a concentration of <10 organisms/µl, there were three laboratories in the first round of sampling that reported false negative results for both of the duplicate samples. One of these three laboratories included the PCR method. The same three laboratories reported one false negative result for one of the two duplicate samples in the case where the sample concentration was between 10 and 30 organisms/µl. In the second round, this pattern was less evident, when two laboratories recorded one of two duplicate samples as a false negative result: for one laboratory in the case of a sample concentration <10 organism/µl, and in the other case a sample with a concentration between 10 and 30 organisms/µl. The latter two laboratories used the PCR and culture methods respectively.

Public laboratories recorded a lower sensitivity in the first round of sampling when compared to the second round, and also lower than private laboratories in both rounds (Table 2).

Table 2: The interaction of the effects of sample round and laboratory sector on the sensitivity of trichomonosis diagnostic tests.

Laboratory sector	Diagnostic sensitivity by sampling round (95% CI)	
	First round	Second round
Public	0.75[a] (0.56 – 0.94)	0.96[b] (0.87 – 1.04)
Private	0.95[b] (0.89 – 1.01)	0.98[b] (0.95 – 1.02)

[a,b]Values with differing superscripts differ significantly (P < 0.05)

For laboratories using PCR, weak positive samples recorded a lower sensitivity than strong positive samples, whereas this difference was not evident in the case of laboratories using only culture (Table 3).

Table 3: The interaction of the effects of sample concentration and diagnostic test type on the sensitivity of trichomonosis diagnostic tests.

	Diagnostic sensitivity by sample concentration (95% CI)		
Test type	Weak (<10 organisms/µl)	Moderate (10 - 30 organisms/µl)	Strong (>30 organisms/µl)
Includes PCR	0.86[a,b] (0.71 - 1.02)	0.96[b,c] (0.86 – 1.05)	1.00[c] (1.00 – 1.00)
Culture only	0.70[a] (0.48 – 0.92)	0.75[a,b] (0.54 – 0.96)	0.90[a,b] (0.80 – 1.00)

[a,b,c]Values with differing superscripts differ significantly (P < 0.05)

In the multiple regression model, sampling round, laboratory sector and concentration were the remaining independent predictors of diagnostic sensitivity. The odds of a private laboratory correctly identifying the presence of *T. foetus* in a sample was 6.60 times higher than that of a public

laboratory, after adjusting for the effect of the sampling round and the concentration of *T. foetus* organisms in the sample, and for every one additional organism per μl the sensitivity is estimated to increase by 0.13, adjusted for the effects of sampling round and laboratory sector (Table 4).

Table 4: Logistic regression model of sensitivity.

Predictor	**Level**	Regression coefficient (Beta) (95% CI)	**Odds Ratio (95% CI)**	**P-value**
Sample concentration (organisms/µl)		0.13 (0.05 – 0.20)	1.14 (1.05 – 1.23)	<0.01
Laboratory sector	Public	0.00	0.00	-
	Private	1.86 (0.41 – 3.31)	6.44 (1.51 – 27.5)	0.01
Round of sampling	First	0.00	0.00	-
	Second	1.65 (-0.05 – 3.36)	5.23 (0.95 – 28.67)	0.06

8. Discussion

8.1 Contribution of veterinary laboratories to diagnostic accuracy of trichomonosis

The results of this study indicate that South African laboratories contribute significantly to the lack of diagnostic sensitivity for trichomonosis. This is contrary to our expectations as we anticipated that sensitivity and specificity of the individual laboratories taking part in the study for the detection of *T. foetus* organisms in preputial samples would be similar. The range of sensitivity in laboratories that used either culture or PCR in the study was quite wide as it ranged from 63% to 100% and 81% to 100% respectively as an average for both sampling rounds. Literature reports average sensitivity of culture at between 72 to 92% (Mardones et al., 2008; Villarroel et al., 2004)(Irons, Henton and Bertschinger, 2002) and the sensitivity of PCR between 76.8 to 100% (Kennedy et al., 2008).

This wider than average range of sensitivity therefore implies that some laboratories testing for *T. foetus* in South Africa are very accurate while others are less accurate. Implications of this are quite worrying as often veterinarians will submit samples to the laboratory due to cost considerations and also a laboratory closest to them as they would want to avoid delaying sample processing. If the most conveniently located laboratory has a low sensitivity for the diagnostic technique employed, the prevalence of *T. foetus* might be understated resulting in further spread of the disease within that particular geographical location.

The least sensitive laboratory had a sensitivity of 63%. If, for example, that laboratory was processing 500 bull samples, which were serving a total of about 12000 cows and the true prevalence of *T. foetus* in that area was 10%, this would translate to 50 true positive bulls. The laboratory would therefore only be able to identify 32 positive bulls out of the 50 total positive bulls leaving 468 bulls

that would have tested negative. This would actually be an error as 450 bulls were supposed to have tested negative and this would then translate into a negative predictive value of 96%. This means that one in every 25 bulls that tests negative is a false negative and is in fact positive and has the potential to spread the disease to susceptible females.

On a positive note, overall specificity in this study was 100% for all the participating laboratories meaning all negative bulls were correctly identified as such. This was a good thing as no unnecessary culling of false positives would be done resulting in financial losses to the farmer through culling and replacement costs.

8.2 Effect of laboratory technique on diagnostic sensitivity

Sensitivity refers to the ability of a test to identify *T. foetus* positives in the presence of disease (Mardones et al., 2008). Laboratories using PCR method recorded higher sensitivity than those using the culture method (0.96; 95% CI 0.91 – 1.00 and 0.81; 95% CI 0.73 – 0.90 respectively, $P < 0.01$). Literature has reported that the reference test is culture and microscopic identification of the *T. foetus* organism by its characteristic, rolling, jerky movement and confirmed by its morphologic characteristics (BonDurant et al., 2003) . Results from the logistic regression model in this study neither confirms nor disputes this previous finding, seeing that the effect of laboratory technique appears to have been confounded by the laboratory sector: laboratories preforming only culture were over-represented amongst public laboratories, and laboratories performing PCR were over-represented amongst private laboratories. It was the laboratory sector, and not the laboratory technique used, that independently predicts diagnostic sensitivity in this study.

Furthermore, it may be in this case, that due to the fact that laboratory cultures of *T. foetus* appear to be less resilient than cultures obtained from bulls, the difference observed between culture and PCR laboratories was exaggerated in this study because in the same sample, *T. foetus* DNA might

have been preserved after the organisms were no longer viable explaining the positive PCR result and a negative culture result. It is even possible that the dilution effect on sensitivity observed in this study in the case of PCR laboratories may have been affected by sample inoculated with *T. foetus* derived from culture that had died before being processed in the laboratory: if the sample contained fewer organisms, it is possible that the particular *T. foetus* study sample was less viable and therefore died off earlier, or, it is possible that the protozoa in a low concentration test sample died.

When giving feedback of results, some laboratories made a comment that samples had been grossly contaminated with bacteria leading to a degraded sample quality. This may have been a consequence of using Steve's transport media which does not contain any antibiotics. To further support this, in a study by (Johansson et al, 1947), it was observed that certain bacteria inhibit the growth of *T. foetus*. These include, but are not limited to, *Corynebacterium* species, *Staphylococcus aureus, Streptococcus bovis and Escerichia coli*. None of the laboratories that reported bacterial contamination went ahead to culture and identify the bacteria.

8.3 Effect of laboratory sector on diagnostic sensitivity

In this study, private laboratories recorded higher diagnostic sensitivity than public laboratories, independently of the other predictors of diagnostic sensitivity. We speculate that this finding could have been due to a more organized system and better work ethic by private laboratories compared to the public laboratories, although one of the public laboratories achieved 100% Se and Sp. One of the public laboratories received samples on Friday and only processed them the following Monday using culture. The organisms had probably died hence their result of all 12 samples being negative. This applies to both laboratories using culture as a delay in processing results in a decreased sensitivity (Perez, Cobo, Martinez, Campero, & Späth, 2006) and for the PCR technique, a delay in processing would have resulted in *T. foetus* releasing hydrolytic enzymes which denatures genetic

material (BonDurant & Honigberg, 1994) . The same laboratory did not have media in stock, to culture when the second batch of cultures was sent out.

8.4 Effect of sample concentration on diagnostic sensitivity

Sample concentration was of significance in laboratories that used PCR but did not have an effect in laboratories that did culture. This was probably as a result of intense amplification of genetic material in PCR which did not happen in culture samples, some of which could have not been viable at the time of reception. In the case of PCR laboratories, if a *T. foetus* inoculated sample had died off before it reached the laboratory, and had a low concentration, it is possible that the saprophytic contaminant organisms in the culture media had already denatured the DNA (Johansson et al., 1947) whereas this may have taken longer in the case of a more concentrated sample.

8.5 Effect of inter-laboratory comparison feedback on laboratory accuracy

The inter-laboratory comparison feedback enabled the researchers to identify laboratory errors and make suggestions on how the laboratories could rectify these errors during the second round of testing. The second round was better than the first round especially for most of the public sector laboratories that had made errors during the first round of sampling resulting in very poor sensitivity. The logistic regression model indicated a significant independent effect of the round on the sensitivity, meaning that the feedback had a significant effect in that laboratories realized their shortcomings and were more accurate in their processes during the second round. Of course it is possible that they then anticipated that there should be 8 positive samples as well, which could be seen as a weakness in the study design.

8.6 Potential weaknesses of the study

Potential weaknesses of the study include sample size. A total of 12 samples was sent to each participating laboratory after considering cost implications to the laboratories. Perhaps if a larger sample size had been used, the results may have differed, in particular in the logistic regression model laboratory technique may have remained as a significant independent predictor. Efficiency of the courier was also another weakness as sample dispatch was delayed during both rounds. Sample delay in delivery to the laboratories for processing was however not unique to some laboratories while excluding others as all the participating laboratories were affected and received samples on the same day hence the effect of transit time on diagnostic accuracy was not investigated. For laboratories employing the culture method, a delay in delivery is a delay in processing and diagnostic sensitivity declines by about 10% with a 24 hour delay with increasing delays causing a greater loss in sensitivity (Perez, Cobo, Martinez, Campero, & Späth, 2006) .

In the case of laboratories using PCR, a delay in processing samples results in cleavage of nucleic acids and this may give false negative results (Mukhufhi et al., 2003). *T. foetus* secretes a range of hydrolytic enzymes that cause injury to the DNA since the cells are lysed (BonDurant & Honigberg, 1994). Samples to the laboratories were sent in Steve's transport medium. Samples could have been sent in a variety of media including PBS and the In-pouch TF to see if the type of media also has an effect on test result.

Cultured samples rather than samples obtained from bulls were used in the study. These were however mixed with fresh preputial washes from the negative controls. Biological interactions between preputial sample material and *Tritrichomonas foetus* exist, which may affect the ability of the laboratories to detect the organisms and for this reason fresh preputial samples were needed to simulate practical reality. The viability of these samples differs from samples obtained from bulls,

which may have affected the results of this study. Furthermore, the concentration of these cultured samples did not necessarily reflect the typical concentration of samples.

The strains of *T. foetus* cultures used in this study were not typed. Had the samples been typed, we could have possibly seen if there was a difference in sensitivity or specificity of the different serotypes although this is purely speculative. Addition of the non–pathogenic trichomonad *Pentatrichomonas* or *Tetratrichomonas* species would have been ideal to test the efficiency of the laboratories that use culture methods as false positive results have been recorded in literature even when done by experienced personnel.

9. Conclusions

It is concluded that inaccuracies in the diagnostic laboratory contributes to the deficiencies in diagnostic sensitivity for trichomonosis in South Africa, but does not influence diagnostic specificity. It is further concluded that diagnostic sensitivity was independently influenced by the sector in which the laboratory operated (private vs public), the concentration of *Tritrichomonas foetus* organisms in the sample and the feedback provided by this inter-laboratory comparison study to individual laboratories.

10. Implications

- For veterinarians that do sample collections at the farm, the ideal day for collection should be adhered to and samples must never be collected closer to the weekend unless they will personally deliver samples to the laboratory to ensure that the processing is initiated timeously.
- Courier companies should also be educated on live sample handling and the importance of swift delivery to laboratories.
- Participating laboratories should also be educated on the importance of following standard operating procedures.
- Things that are usually overlooked such as sample identification should be strictly monitored as some laboratories have made mistakes of not taking note of sample identification during processing. This is rather worrisome and may result in severe economic implications on the farmer's part as they may end up culling the wrong bulls and retaining infected ones thereby defeating the whole purpose of trichomonosis testing before breeding.
- It should also be taken into account that errors in this testing may result in lawsuits and that systems must be put in place to minimize errors and increase efficiency. Two of the laboratories that took part in this study used culture but went ahead and did PCR whenever suspicious organisms were seen even in the absence of growth on culture medium. This is highly recommended to all laboratories that use culture whenever they are in doubt that a sample might be positive even in the absence of growth.

- Sensitivity of diagnostic laboratories significantly influences the overall accuracy of *T. foetus* diagnostics and therefore the effectiveness of trichomonosis control, it is essential that a long-term sustainable national inter-laboratory validation project be implemented representing all laboratories to which veterinarians submit samples, in order to have any chance to improve our control of the disease in South Africa.

11.Further research questions resulting from this work

Results from this study have shown that several factors may influence the diagnostic accuracy of individual laboratories. Some of these factors are beyond the laboratory's control while others may be controlled by the laboratory. The whole process from sample collection right up to sample processing has a number of steps that must be optimised to ensure that errors are minimized. Further research questions emanating from this work include the following:

- Influence of the collector and their contribution to diagnostic accuracy. How best may variables that may occur during sample collection to sample processing be controlled to result in uniformity?
- One finding from this study is that samples should ideally be collected on a Monday or Tuesday morning to allow time for the courier to collect them and ensure that they are delivered to laboratories for processing before the start of the weekend as samples that are received late on Friday will most probably only be processed on Monday morning.
- The effect of false negative results to the farmer must not be taken lightly as it negates the whole point of testing bulls for trichomonosis before the start of the breeding season. The potential economic losses to the farmer in such cases should also be investigated.
- It is therefore essential that critical control points be devised from sample collection right up to sample processing at the laboratory to avoid false results whether positive or negative which are detrimental to the farmers' business enterprise.

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Appendix

Appendix 1

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**TrichLabcheck-A voluntary Trichomonosis inter laboratory comparison project in South Africa**
PROJECT NUMBER	**V041-17**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. TA Zangure**

STUDENT NUMBER (where applicable)	**U_10214209**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES	**OTAU Bulls**	
NUMBER OF SAMPLES	**4**	
Approval period to use animals for research/testing purposes		**May 2017-May 2018**
SUPERVISOR	**Prof. D Holm**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 29 May 2017
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15

Appendix 2

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

Extension No. 1

PROJECT TITLE	**TrichLabcheck-A voluntary Trichomonosis inter laboratory comparison project in South Africa**
PROJECT NUMBER	**V041-17**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. TA Zangure**

STUDENT NUMBER (where applicable)	**U_10214209**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES	**OTAU Bulls**
NUMBER OF SAMPLES	**4 used and returned**
Approval period to use animals for research/testing purposes	**April 2018 – April 2019**
SUPERVISOR	**Prof. D Holm**

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 23 April 2018
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15

Appendix 3

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/8

Dr Tinashe Alan Zangure
Department of Production Animal Studies
Faculty of Veterinary Science
University of pretoria

Dear Dr Zangure,

RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Your fax / memo / letter/ Email dated 10 July 2017, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
3. The only field samples that may be collected are preputial scrapings from bulls that are part of the Onderstepoort Teaching Animal Unit (OTAU);
4. Live cultures of *Tritrichomonas foetus* may only be obtained from the ARC-OVI;
5. Red cross permits are not required for the movement of samples, but samples to be transported must be packaged in compliance with the Regulations of the National Road Traffic Act, 1996 (Act No 93 of 1996) and/or IATA requirements;
6. Wastech must be used as accredited waste management company;
7. This section 20 approval is valid until 30 November 2018.

Title of research/study: TrichLabCheck- A voluntary Trichomonosis inter laboratory comparison project in South Africa.
Researcher (s): Dr Tinashe Zangure
Institution: Faculty of Veterinary Science, University of Pretoria.
Your Ref./ Project Number: V041-17
Our ref Number: 12/11/1/1/8

Kind regards,

Maja

DR. MPHO MAJA

DIRECTOR OF ANIMAL HEALTH

Date: 2017 -07- 21