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Faculty of Veterinary Science
University of Pretoria, South Africa

Drs. Sebastian F. A. Claessens

Supervisor:
Prof. Pete C. Irons
Ass. Prof. Peter L. A. M. Vos



[Prevalence of Tritrichomonosis in bulls in a communal farming system in Mpumalanga Province, RSA: A pilot study]

Culture and microscopic diagnosis at the Hans Hoheisen Wildlife Research Station.

Prevalence of Tritrichomonosis in bulls in a communal farming system in Mpumalanga Province, RSA: A pilot study

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ABSTRACT

This pilot study in the Mnisi area (Mpumalanga) estimates the individual prevalence rate of *Tritrichomonas foetus* in bulls. Samples were collected using the scraping technique, and were cultured for 5 days at an average of 36,9 °C. Every day, a droplet from the bottom of the tube was examined using light microscopy.

89 bulls, from 11 dip tanks, were included in the study, with an average age of 3 years and 4 months (SD ± 1 year and 4 months). No positive results were found at microscopic examination, resulting in a possible prevalence of 0,0 to 4,5 % (CI 95%, Mid-P value).

Different factors could have influenced this result, and the prevalence might be underestimated. Possible causes of a low prevalence rate of *T. foetus* in this communal farming system were mentioned but need further investigation to be confirmed.

SAMENVATTING

Deze studie in het Mnisi onderzoeksgebied (Mpumalanga) heeft als doel een schatting te maken over de individuele prevalentie van *Tritrichomonas foetus* in stieren. De monsters zijn verzameld door middel van de 'scraping'-techniek, en vervolgens gekweekt voor 5 dagen met een gemiddelde temperatuur van 36,9 °C. Dagelijks werd een druppel van de bodem van deze cultuur onderzocht met behulp van licht microscopie.

89 stieren, van 11 dip tanks, werden betrokken bij deze studie. De gemiddelde leeftijd was 3 jaar en 4 maanden (SD ± 1 jaar en 4 maanden). Er werden geen positieve resultaten gevonden bij het microscopisch onderzoek. De mogelijke prevalentie wordt geschat op 0,0 tot 4,5 % (BI 95 %, Mid-P waarde).

Verschillende factoren kunnen dit resultaat hebben beïnvloed, en het is mogelijk dat de prevalentie wordt onderschat. Er worden mogelijke oorzaken voor een lage prevalentie van *T. foetus* in dit gebied genoemd, maar extra onderzoek is noodzakelijk om deze veronderstellingen te bewijzen.

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INTRODUCTION

A. TRITRICHOMONAS FOETUS

INTRODUCTION

Tritrichomonas foetus remains, a major cause of infertility in cows, despite the introduction of artificial insemination. In regions in which natural service is the predominant means of mating, the prevalence can be high. *T. foetus* is a venereal protozoal agent, and is transmitted by bulls or AI (contaminated semen) (Noakes, Parkinson, & England, 2009).

The causative organism has been variously named, the more common names being *Trichomonas foetus*, *Trichomonas bovis*, *Trichomonas genitalis* and *Trichomonas bovines* (Schutte, Herr, & Kitching, 1994). The existence of different serotypes within *T. foetus* was established by agglutination, passive hemagglutination and skin tests, and the three most studied would be Manley, Belfast and Brisbane strains (BonDurant & Honigberg, 1994).

PATHOGENESIS

Trichomonads are aerotolerant anaerobes and generally inhabit predominantly anaerobic environments; they lack mitochondria, a functioning tricarboxylic acid (Krebs) cycle and a cytochrome system which make them sensitive to high levels of oxygen (BonDurant & Honigberg, 1994) (Lindmark & Müller, 1973).

T. foetus is generally invasive and has potential to directly damage bovine tissue. The presence of surface antigens was demonstrated by Burgess in 1986 and 1988 (Burgess, 1986) (Burgess, 1988). These might play a role in the immunity against the agglutination of *T. foetus*, or cell-parasite interaction. Adherence is an important step in the infection. It uses its flagellae to adhere to the epithelial cells of the host. Adherence can be inhibited in vitro by antiserum fractions enriched for IgG₁. Results from other studies indicate that adhesion of *T. foetus* to mammalian cells is an important step in

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cytotoxic damage of these targets and that a surface adhesin on the parasite is involved in the adhesion mechanism (Burgess & McDonald, 1992). Several authors have shown that experimentally infected or immunized and challenged females generate parasite-specific immunoglobulins of several isotypes, but especially of the types IgG₁ and IgA (BonDurant & Honigberg, 1994). The source of the antibodies found in the reproductive tract is not absolutely certain, although the IgA is presumably secreted locally (BonDurant & Honigberg, 1994).

In vaginal discharges from infected cows, the presence of leukocytes predominates, suggesting that macrophages may play a role in *T. foetus* infection (Eaglesome & Garcia, 1992).

Mammalian cells can be destroyed by the secretion of hydrolytic enzymes. These enzymes include acid hydrolases, acid phosphatase and beta-N-acetyl-glucosaminidase, which originate from the lysosomes. These lysosomal extracts also contain neuraminidase, which is cytotoxic to epithelial monolayer cells. They have hydrogenosomal enzymes which produces hydrogen and acetate, energy by phosphorylation and which process a portion of the cells carbon flow. Extracellular proteinases secreted by *T. foetus* can cleave host macromolecules in the bovine reproductive tract. The protection by the host immune response against *T. foetus* is achieved by complement-mediated killing of the trichomonads (Eaglesome & Garcia, 1992).

B. CLINICAL SIGNS

In bulls, *T. foetus* often causes an asymptomatic infection. It resides in the preputial mucosa and the crypts of the penile integument. The bull's age can affect the course of the infection. Bulls older than three years are more likely to remain life-long carriers, because of the well developed crypts (Figure 1). Younger bulls have less developed mucosa and crypts, and generally have short-term transient infections (Figure 2) (Noakes, Parkinson, & England, 2009), or seldom become infected (Noakes, Parkinson, & England, 2009; Schutte, Herr, & Kitching, 1994).

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Transmission can occur by direct contact (coitus) or through infected semen collection equipment. If a bull is closely observed following infection, a swelling of the prepuce

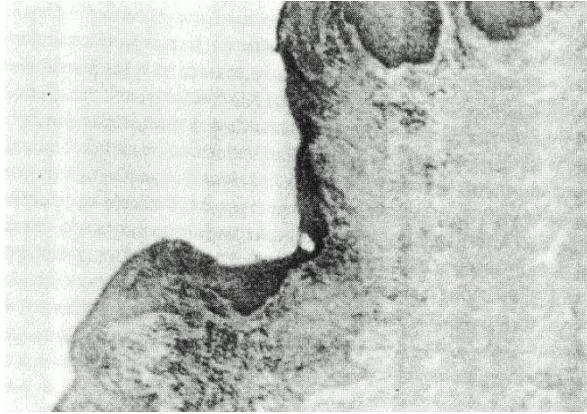


FIGURE 1: EPITHELIAL CRYPTS OF A YOUNG BULL (< 2 YEARS) (BONDURANT & HONIGBERG, 1994)

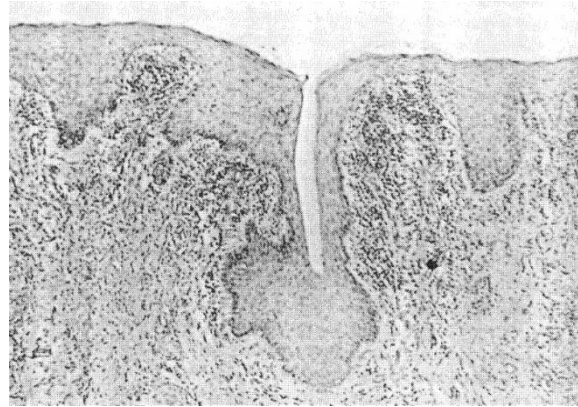


FIGURE 2: EPITHELIAL CRYPTS OF AN OLDER BULL (> 4 YEARS) (BONDURANT & HONIGBERG, 1994)

with accumulation of a mucopurulent exudate may be seen, but this does not last beyond two weeks (Schutte, Herr, & Kitching, 1994; Bondurant & Honigberg, 1994). Older bulls remain chronically infected, although recovery has been recorded (Clark, White, & Banfield, 1971).

In cows that are infected by persistently infected (older) bulls, *T. foetus* colonizes the uterus, cervix and vagina. It causes endometritis and vaginitis. The number of trichomonads needed to establish infection is large, but transmission rates are high (Noakes, Parkinson, & England, 2009).

The disease causes infertility by means of embryonic death, which results in irregular returns to estrus. Infected cows are still fertile, but most pregnancies fail at 30 to 50 days of gestation (Noakes, Parkinson, & England, 2009). Sometimes a pyometra can develop, being filled with large quantities of greyish-white pus and trichomonads, accompanied with vaginal discharge (Noakes, Parkinson, & England, 2009). In the female, infection does not prevent conception but may prevent implementation or cause abortion or death of the fetus followed by a pyometra (10 %). These abortions usually occur between 1 and 16 weeks of pregnancy. The placental tissues are invaded by

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trichomonads. *T. foetus* can be detected in reproductive tract secretions of infected heifers for 13 to 28 weeks (Skirrow & BonDurant, 1990).

After a few returns to estrus antibody-mediated immunity develops in cows, so they can maintain a pregnancy (Skirrow & BonDurant, 1990). This immunity lasts no more than 15 months, so cows are again fully susceptible in successive seasons (Noakes, Parkinson, & England, 2009).

The economic losses from this disease in a beef cow-calf operation can be related to costs of treatment, culling and replacement of infected cattle, and, most importantly, the loss of calf crop because of delayed establishment of a viable full-term pregnancy (Clark, Dufty, & Parsonson, 1983) (Rodning, et al., 2008). It is difficult to measure these losses, but there is an article from 1989 that describes two simulations, where either one or two out of five bulls were infected (Rae, 1989). The calf crop that can be expected in a *T. foetus* infected herd on an annual basis is 14.1% less, if one out of five bulls is infected, and 49,7 % less, if two out of five bulls are infected, than that expected in a non infected herd. The average suckling/growing periods were shortened. With a shorter growing time, calves are smaller and less market ready. It is noteworthy that doubling the prevalence rate among the bull population causes not a simple half-again reduction in calf crop revenues, but an exponential reduction, a compounding effect of fewer and younger/lighter calves (Rae, 1989). Although cattle owners are not producing for commercial purposes, their herds still represent their savings accounts which should increase on a long term base.

C. DIAGNOSIS

HISTORY & CLINICAL EXAMINATION

Bovine tritrichomonosis can be suspected by field history: increasing numbers of failure of conception, prolonged periods after service before the next estrus and cows coming back into estrus after several months. Occasionally infection can result in abortion or pyometra.

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In recently infected herds, most cows and heifers are susceptible. In chronically infected herds, most cows have an acquired immunity but virgin heifers are still susceptible and they will exhibit the typical clinical signs of infection, as described in the previous chapter. Bulls may develop a mild posthitis and balanitis, but these are subclinically (Noakes, Parkinson, & England, 2009).

Diagnosis of tritrichomonosis is confirmed when *T. foetus* organisms can be demonstrated in specimens obtained from the genital tract of female cattle, preputial material of bulls or aborted fetal and placental tissues. Failure to demonstrate these organisms does not imply its absence. Because other trichomonad-like organisms are present in faecal material, contamination has to be prevented. In the bull the infection is permanent, but the pathogen is present in small numbers, so definite diagnosis depends on the examination of a series of preputial samples (Noakes, Parkinson, & England, 2009) (Eaglesome & Garcia, 1992).

SAMPLE COLLECTION

In bulls, sampling can be done by preputial scrapes or preputial washes. Before sampling, the bull should not be used for mating for a period of 5 to 10 days, so trichomonads can increase (Noakes, Parkinson, & England, 2009).

The traditional method of collection, scraping of the preputial mucosa was used by Clark et al. in 1971. They collected preputial secretions using plastic pipettes fitted with firm 90ml rubber bulbs. The pipette was introduced to the full extent of the preputial sac and moved back and forth over the surfaces of the glans penis and surrounding preputial mucosa always keeping the beveled surface of the pipette against the mucosal surface. The orientation of the pipette was controlled through the wall of the sheath by one hand and the pipette was moved by the other hand. During collection, which occupied approximately one minute, the bulb was compressed and relaxed from 15 to 20 times as recommended by Todorovic and McNutt, and shown in Figure 3 (Todorovic & McNutt, 1967).

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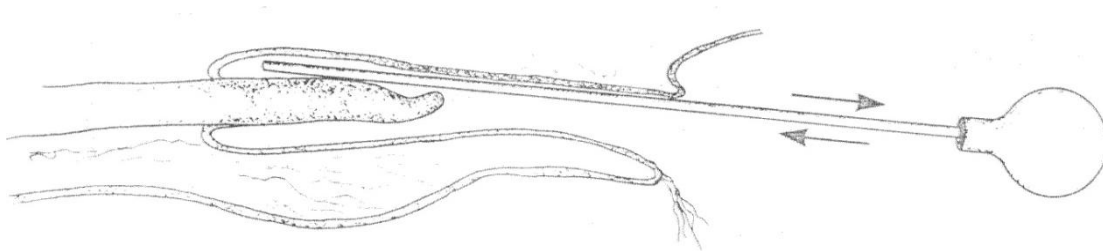


FIGURE 3: SCRAPING (TODOROVIC AND MCNUTT, 1967)

The tubes were shielded from direct sunlight and brought to a laboratory within 3 to 4 hours of collection. *T. foetus* was cultured from 139 of 143 preputial samples collected from 13 of the 15 bulls. Failure to isolate *T. foetus* from two samples from one bull and from one sample from another bull was attributed to gross bacterial contamination. The rapid growth of *T. foetus* in the culture medium when incubated at 37 °C usually ensured that enormous numbers of organisms were present after four days, which greatly facilitated the microscopic examination (Clark, White, & Banfield, 1971).

Irons confirmed in 2002 that the scraping method has several practical advantages over preputial washing (Irons, Henton, & Bertschinger, 2002). Oosthuisen, however, reported in 1999 that washing the preputial with 50 ml of saline solution or Ringer's lactate is a very reliable method (Noakes, Parkinson, & England, 2009). Scraping is a technique which can easily be performed by one person, and it is certainly quicker than washing. Under field conditions, where speed and practicality are crucial factors, scraping should be the method used (Schonmann, BonDurant, Gardner, Van Hoosear, Baltzer, & Kachulis, 1994). The percentage of sheath wash and sheath scraping testing positive on culture is 83 % for both sampling methods (Irons, 2005). The sensitivity of the test increases with the length of culturing. After one week of culture, the sensitivity can reach 67,8 %, and after repeated cultures done weakly, sensitivity can increase up to 76,0 % (two weeks), and 80,0 % (three weeks)(Cobo, et al., 2007). Vigorous scraping is highly recommended to obtain as much smegma as possible (Wikse, Barrett, Mickelson, BonDurant, Mortimer, & Kwasnicka, 1991). It is important to avoid fecal contamination, as this may introduce

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intestinal trichomonads that may be confused with *T. foetus* (Noakes, Parkinson, & England, 2009).

DIRECT EXAMINATION

For direct examination of the samples, drops are mounted on microscopic slides without cover slips and under low power. If protozoa are observed, cover slips are placed and samples are examined under high power to determine morphology (Figure 4). If direct examination of a sample is negative, they should be processed by culture (Eaglesome & Garcia, 1992).

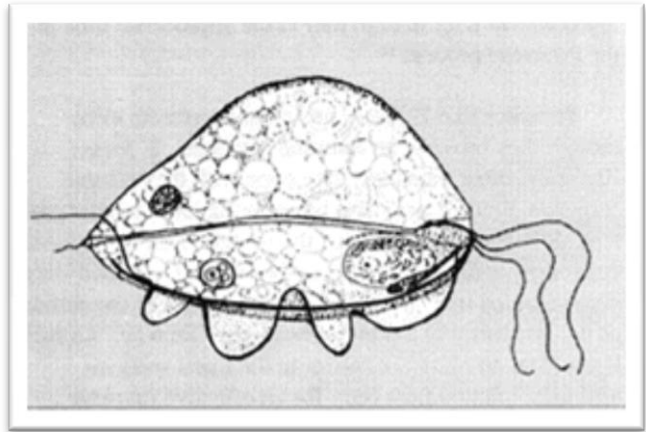


FIGURE 4: TRITRICHOMONAS FOETUS
([HTTP://CATI.CSUFRESNO.EDU/SJER/RESE/94/940301/](http://CATI.CSUFRESNO.EDU/SJER/RESE/94/940301/))

Still the organisms are sensitive to high levels of oxygen, meaning that microscopic examination in an aerobic environment can result in a lot of false negative test results. For this reason medium that will be examined microscopically should be taken from the bottom of the tube (Todorovic & McNutt, 1967) (Oxoid, 2010).

TRANSPORT

Where samples must be submitted to a laboratory and cannot be delivered within 24 hours, a transport medium should be used (Trichomonosis, 2004). During transportation, the organisms should be protected from exposure to daylight and extremes of temperature, which should remain above 5°C and below 38°C (Trichomonosis, 2004) (Oxoid, 2010). Samples should be kept in a dark environment during transport. Saline solutions, with fetal bovine serum, or Ringer's lactate may be effective if the samples are in transit for up to 48 hours (Noakes, Parkinson, & England, 2009).

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CULTIVATION

For the cultivation, the CM0161 Dehydrated Trichomonas Medium from Oxoid[®] was used. This medium has a pH of $6,4 \pm 0.2$ at 25°C (Oxoid, 2010).

Typical formula	gm/litre
Liver digest	25.0
Glucose	5.0
Sodium chloride	6.5
Agar	1.0

TABLE 1: TYPICAL FORMULA OF CM1061 (OXOID, 2010)

The inoculated media should be incubated at 37°C and examined microscopically at 400x magnification on several occasions for up to 3 to 5 days (Oxoid, 2010). The medium should be taken from the bottom of the tube (Todorovic & McNutt, 1967) (Oxoid, 2010). The temperature should be checked and registered at least once a day.

PCR

Definite diagnosis can be made on recovering *T. foetus* or by using PCR, which is of particular value, if the numbers of organisms present in culture remains low (Parker, Campbell, McIntosh, & Gajadhar, 2003). The specificity of PCR assay is 98 % and the sensitivity declines with storage time from 90 % at 6 hours after collection to 31 % at 5 days (Mukhufhi, Irons, Michel, & Peta, 2003).

VIRGIN HEIFER TEST

A virgin heifer test is described by Ball et al.: ten to twenty days after deposition of infected preputial material into the vagina, cervical mucus samples are collected and cultured (Ball, Ott, Mortimer, & Simons, 1983). The cervix is the preferred site for recovering *T. foetus* from females (Eaglesome & Garcia, 1992).

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OIE

The OIE recommends the InPouch TF-test from Biomed Diagnostics to examine the presence of *T. foetus* in bulls in veterinary practices (Trichomonosis, 2004). Compared to the Diamond's medium, samples tested with this commercial available kit are 6,95 times more likely to be positive ($P < 0.001$) (Parker, Campbell, & Gajadhar, 2003).

D. TREATMENT

Curative treatment of *T. foetus* has been successfully achieved by a number of workers. The methods included the administration of potassium iodide orally, sodium iodide intravenously, nitrofurazone locally, iodide preparations locally and hydrogen peroxide locally (Schutte, Herr, & Kitching, 1994). Since Gasparini in 1963 reported the treatment of tritrichomonosis with metronidazole, this therapeutic agent and other imidazole compounds had been recommended (Gasparini, Vaghi, & Tardamo, 1963) (Schutte, Herr, & Kitching, 1994). The successful use of these agents requires simultaneous intrapreputal antibiotic therapy to reduce the number of resident bacteria which counteract the curative properties of the imidazole compounds (Schutte, Herr, & Kitching, 1994). Unfortunately, according to the U.S. Food and Drug Administration, the family of nitroimidazoles might have carcinogenic effects, resulting in a prohibition for their use in cattle (BonDurant & Honigberg, 1994).

E. CONTROL

Control measures include artificial insemination, separation of the infected from unexposed heifers and cows, sexual rest of females before rebreeding, selective culling and monitoring bulls and cows for infection prior to introduction in clean herds (Schutte, Herr, & Kitching, 1994).

Different articles mention the control of *T. foetus* by replacing bulls at a certain age. In 1974, Clark et al. describes the control of tritrichomonosis by using non-infected bulls aged from 1-3 years for service. Prior to the mating season, they culled all the bulls that were 4 or more years of age, except 26 stud bulls. These bulls, together with 69 samples

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of the culled bulls and 644 bulls that were 2 or 3 years of age, were examined once, and those found to be infected were slaughtered (Clark, Parsonson, White, Banfield, & Young, 1974). The method of examination was the same as described in Clark, 1971. *T. foetus* was demonstrated in 24 (3.25%) of the 739 bulls that were examined prior to the mating season, The incidence of infection in 644 bulls that were either 2 or 3 years of age was 5 (0.78 %) whereas the incidence in 95 older bulls was 19 (20 %) (Clark, Parsonson, White, Banfield, & Young, 1974). They used 515 yearling bulls, to replace these infected bulls, which never had mated, and were therefore presumed to be not infected by *T. foetus* (Clark, Parsonson, White, Banfield, & Young, 1974). *T. foetus* was not found in any of the 942 bulls examined after the end of mating in 1971 (Clark, Parsonson, White, Banfield, & Young, 1974). In the following season, a survey of 353 bulls failed to demonstrate *T. foetus* infection (Clark, Parsonson, White, Banfield, & Young, 1974). These results show the efficacy of replacing old and infected bulls by young 'virgin' bulls in order to prevent the spread of tritrichomonosis.

Tritrichomonosis has been controlled in a large Victorian herd by using 1- to 3- year old bulls during 2 consecutive annual mating seasons of 12 weeks. In this instance, infected bulls were removed prior to mating seasons and the majority of the non-pregnant cows were culled each year. 30 of the old bulls were examined for infection with *T. foetus* by collection and culture of preputial secretions (Clark, White, & Banfield, 1971). *T. foetus* was not isolated until December 1974 when 1 out of 99 bulls was found to be infected. At that collection, the bulls were 3.5 to 4 years old. In September 1975, when all replacement bulls were more than 4 years of age, 3 out of 80 were found to be infected (Christensen, Clark, & Parsonson, 1977).

Following this research, Christensen and Clark controlled the spread of *T. foetus* in an infected herd by replacing 300 8 year old Shorthorn and Santa Gertrudis bulls for 325 2 year old Brahman bulls. A survey of 30 of the old bulls at the time of removal from the herd in 1973 showed that 14 (47%) were infected with *T. foetus*. Two years after introduction, only 3 (4%) out of a sample of 80 replacement bulls were found to be infected although the prevalence of the infection in older bulls in other herds in the region remained high (Christensen, Clark, & Parsonson, 1977). They conclude that: bulls

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would need to be routinely culled at 4 years of age in order to minimize venereal transmission of *T. foetus* (Christensen, Clark, & Parsonson, 1977).

Under conditions where artificial insemination is highly impractical, one must expect that it may take 1-2 years for the combined effects of control measures and herd immunity to bring about a “normal” calf crop (BonDurant & Honigberg, 1994).

VACCINATION

In bulls, encouraging results have been reported regarding the development of a vaccine protecting them against infection with *T. foetus* (Schutte, Herr, & Kitching, 1994). After vaccinating bulls twice with 2 milliliters containing whole cell killed *T. foetus* in oil adjuvant, they resisted infection and developed IgG1 and IgG2 antibodies in preputial secretions and serum (Cobo, Corbeil, Gershwin, & BonDurant, 2010).

A clinical evaluation of the efficacy of a vaccine against *T. foetus* performed in 1992 showed a 97.1 % increase in maintenance of pregnancy in vaccinated heifers (Kvasnicka, et al., 1992). Although the conception rates were 89,2 % and 85,9 %, respectively for the vaccinated and control group, within the next 4 months, it decreased to approximately 30 % for the control group, compared to over 60 % for the vaccinated group (Kvasnicka, et al., 1992). It is possible that antibodies induced by systemic immunization may be effective in limiting *T. foetus* infection of the reproductive tract, by preventing colonization of the vaginal epithelium (Kvasnicka, et al., 1992). Results of a field trial performed by Hall et al. showed improved resistance and reduced fetal wastage associated with *T. foetus* infection in cows (Hall, Kvasnicka, Hanks, Chavez, & Sandblom, 1993). They suggest that control strategies should be combined with a vaccination program (Hall, Kvasnicka, Hanks, Chavez, & Sandblom, 1993). There is a commercial vaccine available named TrichGuard, to protect cows. This commercial immunogen contains antigens of *T. foetus*, *C. fetus venerealis*, and *Leptospira canicola*, *L. grippotyphosa*, *L. icterohaemorrhagiae* and *L. Pomona* (TrichGuard V5L, Fort Dodge, USA). Compared to a control group, heifers vaccinated with this vaccine, resisted or quickly cleared infection after naturally challenged (Cobo, Morsella, Cano, Cipolla, & Campero, 2004).

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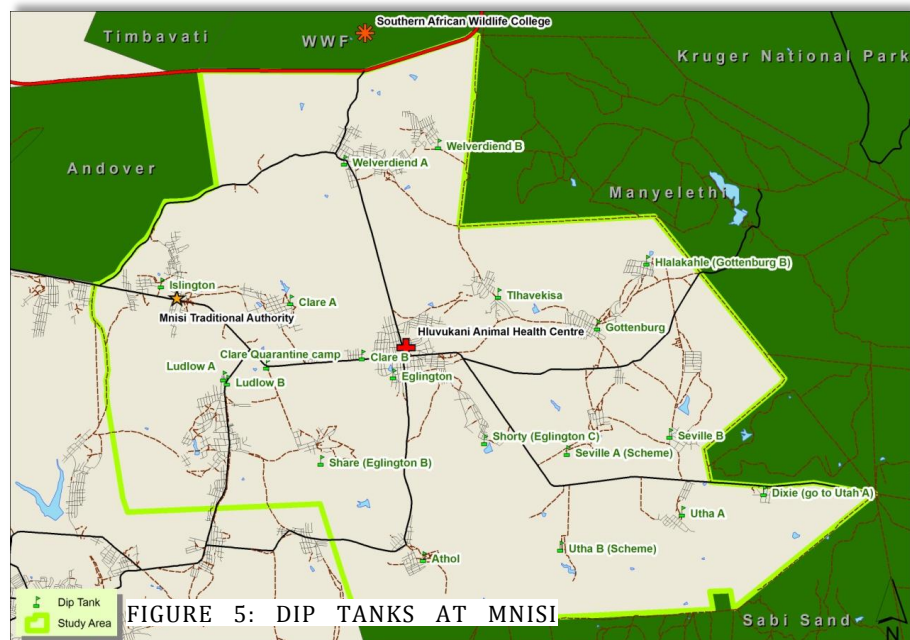
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F. MPUMALANGA PROVINCE: MNISI AREA

Available data of the individual prevalence rates from South Africa indicate a prevalence ranging from 2,7 % to 26.4 % (Retief & Schutte, 1975) (Kitching, 1988) (Erasmus, De Wet, Van der Merwe, & Pienaar, 1989) (Pefanis, Herr, Venter, Kruger, Queiroga, & Amaral, 1988).. These are all individual prevalence rates. Retief and Schutte found 2,7 % of the 95 bulls tested, in 32 South African beef herds, were positive for *T. foetus* (Retief & Schutte, 1975). Kitching found 3,7 % of bulls tested in Natal in 1988 were infected (Kitching, 1988) (Schutte, Herr, & Kitching, 1994). Other articles even mention prevalence rates from 0 % to 46 % of herds were infected with *T.foetus*. (Irons, Henton, & Bertschinger, 2002).

The Mnisi area is a study area located next the Kruger National Park in the Foot and Mouth Disease (FMD) buffer zone. The University of Pretoria is planning to do



research (animal and veterinary science) in this area for probably 15 years. Preliminary data of this area: approximately 1300 owners, and 2 bulls per owner (Van Rooyen & Vandamme, 2009) (Van Rooyen, 2010). These bulls will be used for the sample collection, if permission from the owner is obtained.

There are no data available from the Mnisi area or other areas in the Mpumalanga Province. The article from Pefanis et al. can be seen as a good reference for this pilot study, as it focused on the Republic of Transkei, a former homeland of South Africa, where communal grazing systems were used (Pefanis, Herr, Venter, Kruger, Queiroga, & Amaral, 1988).

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COMMUNAL FARMING SYSTEM

Cattle owners in the Mnisi area use their livestock for financial security, meat supplies and traditional ceremonies (Van Rooyen & Vandamme, 2009). Herd boys and owners release the cattle in the morning, so they can seek out grazing areas and water supplies. In the afternoon, the herd boys will go and look for them, and the animals are locked in kraals. Weekly, the herds go to dip tanks, to prevent damage caused by ticks.

These dip tanks are also designed for the control of FMD, because the Mnisi area is situated within the FMD buffer zone.

Since the herds are allowed to range freely, diseases are easily transmitted. It is

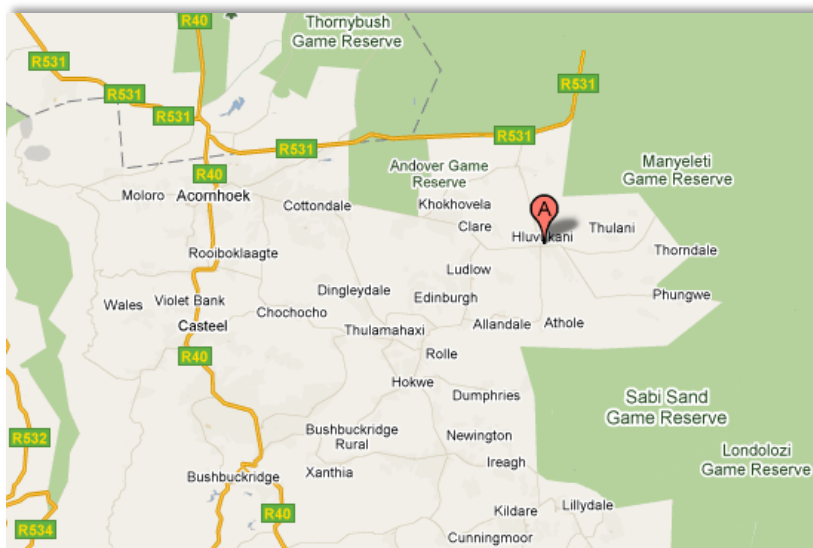


FIGURE 6: LOCATION OF MNISI AREA (GOOGLE 2010)

confirmed that commingled grazing and exposure to a greater number of other herds are risk factors regarding the prevalence of *T. foetus* (Gay, Ebel, & Kearley, 1996). Of the 65 test positive herds in this study, 27 (42 %) grazed with > 7 other herds

during the 1993 grazing season, and 18 (28 %) grazed with > 14. The odds ratio of 2.1 for herds that grazed with 7-13 other herds was not significantly ($p = 0.163$) different from 1, but the odds ratio of 9.0 for herds that grazed with > 14 other herds was significant ($p < 0.0001$). For herds that grazed with > 14 other herds, the estimated fraction in which infection was attributable to commingled grazing was 20 % for the small herds and 28 % for the large herds. For all herds, the estimated attributable fraction was 33 % (Gay, Ebel, & Kearley, 1996). This pilot study will focus on the population of bulls visiting the dip tanks around the Animal Clinic at Hluvukani. Districts, dip tanks and sites have to be within easy travelling distance of the Animal

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Clinic or the Hans Hoheisen Research Station to ensure the survival of the organisms during transport to the laboratory.

G. SAMPLE SIZE DETERMINATION

The sample size has been determined according to the software of EpiTools (Ausvet Animal Health Service, 2009). Although Pefanis et al. found a prevalence of 26,4 % in 1988, no research has been done before in the Mnisi area. That is why a prevalence of 50 % is assumed, in order to obtain the largest possible sample size according to the formula (1) (Fosgate, 2009).

$$n = \frac{P*(1-P)*(Z_{1-\alpha/2})^2}{e^2} \quad (1)$$

There are approximately 1300 herd owners in the area, with an estimate of two bulls per owner (Van Rooyen & Vandamme, 2009). The population size is estimated at 2600 bulls (Van Rooyen, 2010). With a confidence level 95 % and a desired precision of 10 %, we have to collect at least 94 samples (Ausvet Animal Health Service, 2009). If we add 10 % to this number, as a margin for possible mistakes at laboratories, contamination or missing data, the goal is to collect 104 samples.

H. HYPOTHESIS

The hypothesis is that trichomonosis is present in the Mnisi area at a similar prevalence rate to other communal grazing areas (Pefanis, Herr, Venter, Kruger, Queiroga, & Amaral, 1988).

Using the results of this pilot study, the faculty can estimate the need of risk assessment studies in the Mnisi area and adjacent communal areas in the Mpumalanga Province. Of course the prevalence data are academically very useful, because the Faculty of Veterinary Science can form a picture of what diseases (and their consequences) occur in the area. Local farmers can be informed about the necessary measures they should take to optimize the fertility rates of their livestock.

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Economically the efficiency of the reproduction of cattle herds can be optimized when the owners know 1) the causes of infertility in their herds, 2) which bulls infect their herds and 3) how they can prevent this disease from spreading to other animals and/or herds. Intercalving periods can be shortened, and with the use of fenced camps, calves are born before the summer, when grazing area's and water is easy accessible. Since the farmers use their livestock as a savings account, they do not have the financial capacity to invest in all sorts of possible solutions without getting the guarantee that their situation will improve. So this is the main reason why research (followed by feedback and risk assessment studies) is very important for the local people. Veterinary and animal science can improve the economical strength, resulting in a better social environment for the people living in these rural areas.

MATERIAL AND METHODS

A. SAMPLE COLLECTION

Once a week, every herd has to come to their respective dip tank, as a part of the Foot and Mouth Disease monitoring and the control of ticks, tick derived lesions and tick borne diseases. This is the ideal occasion to do research, to take samples, to examine animals or to question the farmers. Twelve dip tanks from the Mnisi area were included in the study. A part of them were randomly selected, the others were part of the schedule of the Hluvukani Animal Clinic, of whom we depend for transportation.

Date:	18th October	19th October	20th October	21th October	22th October
Dip Tank:	Gottenburg	Clare A	Utah Scheme	Wolverdiend B	Thlavekisa
# Samples	9	9	9	10	10

Date:	26th October	27th October	28th October	29th October	1st November	2nd November
Dip Tank:	Utha A	Wolverdiend A	Burlington	Halakahle	Clare B	Share
# Samples	3	10	9	6	4	10

TABLE 2: DIP TANKS INCLUDED IN STUDY

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To inform the owners about the study, the Animal Health Technicians were informed about the objectives and the sample collection one week before we would visit the area. Before a sample could be collected from a bull, the owner or herdsman had to give his or her permission.

As *T. foetus* is a venereal pathogen, transmitted by direct sexual contact, only bulls that were able to service cows were included in the study. This was obtained by either looking at their behavior, the age or the information from the owner. From this group of bulls, a sample was collected from every second or third animal, depending on the total

amount of animals that was presented at the dip tank.



FIGURE 7: RESTRAINING OF BULL

The animals were blocked while they were walking through the dip tank. Using other animals from the herd, the bulls were put into a crush. The hind leg, closest to the side from where the sheath scrape was performed, was restrained against the pen, as shown in Figure 7.

An electro-ejaculator was available to immobilize the animals, but was found to be too big to use since most of the bulls are Nguni-type breeds. Because not all the dip tanks are well maintained, it was sometime difficult to restrain the animals. An elektro-ejaculator was used only three times to immobilize the animals. Four bulls were able to escape, and were not included in the study. This situation slightly influenced the random selection of animals.

The sheath scrape was performed using a Perspex AI pipette, connected with 2 cm of silicon tubing, to a 20 ml sterile syringe. To prevent contamination, a cover was placed

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over the AI pipette. After restraining the bull, the pipette was inserted into the preputium and the cover was broken. Applying negative pressure with the syringe, the pipette was scraped at least 20 times vigorously against the preputial mucosa, while controlling the movement of the pipette with the other hand. If enough preputial material was collected, the pipette was flushed using 4.5ml of PBS (Dulbeco). The vial was protected from direct sunlight, kept cool and transported to the Hans Hoheisen Wildlife Research Station within

10 hours. Image 8 shows the materials used at the dip tank: rope, consumables, polystyrene cool box, labels, data forms and the elektro-ejaculator. After sample collection, the information from the owner and



the age of the bulls was collected using the bull data form (Attachment 1).

B. MICROSCOPIC DIAGNOSIS

At the microbiology lab in Hans Hoheisen Wildlife Research Station, all the samples were examined by the same person. This was performed according to the standard operating procedure (SOP, Attachment 2) of the Onderstepoort Veterinary Institute (OVI). A light microscope (Olympus BH-2) was used, and the cultures were incubated at approximately 37 °C for 5 days. The culture medium was produced by IDEXX- Vet Path, using a Dehydrated Trichomonas Medium®(CM0161) from Oxoid®, with chloramphenicol (SR 0078) (Oxoid, 2010).

The temperature of the incubator was checked at least once a day, using an electronic thermometer (Microlife ® Vet-Temp).

The samples were examined before inoculation (direct) and 24, 48, 72, 96 and 120 hours after collection. A droplet from the bottom of the sample or culture medium was placed on a microscopic slide, and examined at 200x on low power. If there was a sign of

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movement, or a shape similar to *T. foetus*, the power and magnification was increased, up to 400x.

C. STATISTICAL ANALYSIS

The apparent prevalence of *T. foetus* was estimated using Epi Info version 6.04d applying the option to calculate proportions of simple random sampling and calculating the Mid-p value (Centers for Disease Control & Prevention (CDC), USA, 2001). The prevalence is estimated with a confidence interval of 95%. The Mid-p value has an average of 0.5 and so is more appropriate particularly when combining results from several studies (Berry & Armitage, 1995). The Mid-p value is recommended for tests and confidence intervals with highly discrete distributions (Agresti, 2002).

$$P_m(i) = \frac{1}{2} \{P(i) + P(i + 1)\} = \frac{1}{2} \{P(i) + P'(i)\} \text{ (Lancaster, 1961)}$$

For this binomial study, with a proportion with discrete distributions, the Mid-P value is more exact. (Fosgate, 2010).

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RESULTS

In 89 samples from 89 bulls collected at the dip tanks, none showed any signs of *T. foetus* at microscopic examination. According to Epi Info the true prevalence (Mid-P value) ranges from 0,0000 % to 4,4902 % (CI 95 %). This value is corrected for the sensitivity of 67 % (Cobo, et al., 2007). This is not corrected for the specificity, because there were no false positive results.

All samples were incubated for 5 days at an average temperature of 36,9 °C, ranging from 36,2 °C to 39,2 °C (SD: ± 0,6 °C). The registration of the daily temperature (n=33) is shown in Figure 9.

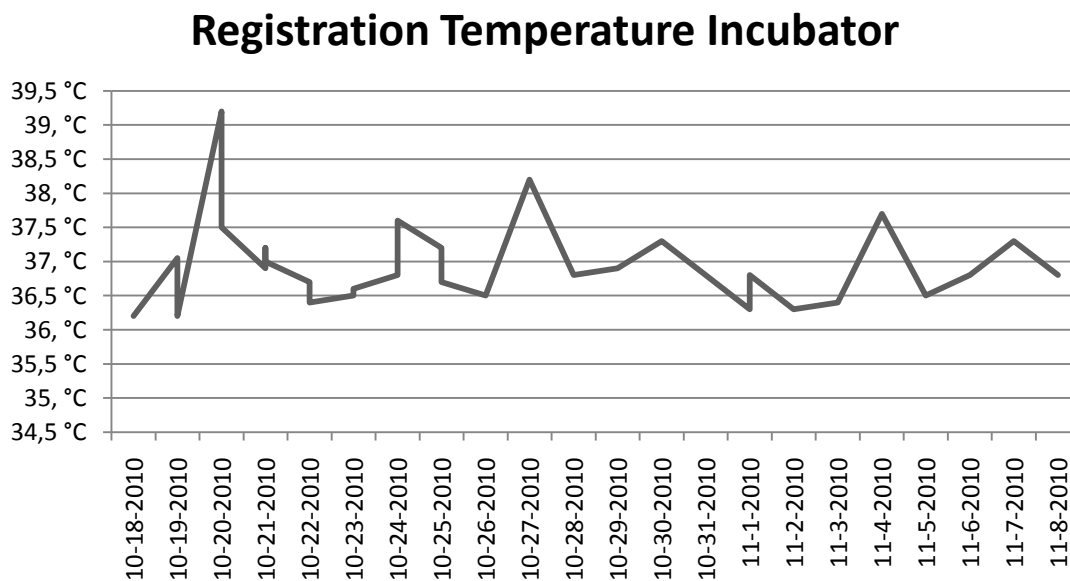


FIGURE 9: REGISTRATION TEMPERATURE INCUBATOR

The average age of the bulls included in the study was 3 years and 4 months, ranging from 1 to 9 years, and with a SD of ± 1 year and 4 months. 27 of 89 bulls (30,3 %) were older than three years. The majority of the bulls were Nguni type or Brahmenn type breeds, 40 and 39 out of 89 bulls respectively. The others, 10 out of 89, were mixed, consisting Afrikaner, Bonsmara or other indigenous breeds.

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DISCUSSION

Microscopical examination was carried out at the microbiology laboratory at Hans Hoheisen Wildlife Research Station. This pilot study was the first research project in this facility since it was rebuilt (Van Schalkwyk, 2010). The person responsible for the microscopical examination was trained at the Onderstepoort Veterinary Institute, but did not have previous experience with examining wet slide mounts for *T. foetus*. This situation may have influenced the sensitivity of the test. Moreover, a positive control to check the testing procedure was not available on site and could not be included in this study.

Although a culture media with antibiotics was used, there still was a lot of bacterial growth, influencing the microscopic examination. 37 % of the samples had bacterial growth in the culture medium, causing movement in the droplets. Bacteria have been described to influence the growth of *T. foetus* in vitro (Johansson, Morgan, & Winkler, 1947). Especially the enteric group of bacteria, *Escherichia coli*, *Aerobacter aerogenes*, *Shigella dysenteriae*, *Shigella paradysenteriae* and *Staphylococcus aureus* and *S. albus* substantially inhibit the growth of *T. foetus* (Johansson, Morgan, & Winkler, 1947). The factors contributing to this inhibition are not clearly understood. Although two factors seem to play a role in the inhibition of the growth of *T. foetus*: a change in the hydrogen concentration and toxin production (Johansson, Morgan, & Winkler, 1947). Morphologically the bacteria found in the cultures looked like Enterobacteriaceae and Actinomyces species (Quinn, Markey, Carter, Donnelly, & Leonard, 2002). However, no further diagnostic procedure was done to confirm this observation.

The samples were transported in PBS Dulbecco and inoculated in the culture media, within 24 hours, being within the standards from the World Organization for Animal Health (Trichomonosis, 2004). It is unlikely that transporting the samples to Hans Hoheisen has influenced the survival and growth of *T. foetus*.

The average age of the bulls was 3 years and 4 months, an age which was comparable with other studies (Mokantla, McCrindle, Sebei, & Owen, 2004). Although sperm abnormalities are higher in yearling bulls, and hence seminal quality improves with age,

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there are no significant differences in sex drive or breeding soundness examinations among different age groups (Chenoweth, Farin, Mateos, Rupp, & Pexton, 1984). Brahman bulls have a higher age of puberty compared to *Bos taurus* bulls, the mean age is 17 ± 0.4 months (Silva-Mena, 1997). This implies that bulls younger than 3 years should be able to service the cows, and spread the infection. The low average age possibly plays a role in the control of *T. foetus* in the herds in the Mnisi area. Research showed that if bulls are replaced at the age of 3 years, one is able to control the spread of the disease (Christensen, Clark, & Parsonson, 1977) (Clark, Parsonson, White, Banfield, & Young, 1974). But still, 27 bulls, 30 %, were older than 3 years, and therefore were able to remain life-long carriers if infected with *T. foetus*. Some authors have suggested that *Bos taurus* is more susceptible than *Bos indicus*, up to 7 times the risk, but, according to the authors, this might be due to the increased number of matings accomplished by *Bos taurus* bulls in the same period of time (Perez, et al., 1992),

More than only one negative sample should be obtained before there is reasonable assurance that a bull is uninfected (Trichomonosis, 2004). The pathogen is present in small numbers, so the diagnosis depends on examination of a series of samples under optimal conditions with weekly intervals (Noakes, Parkinson, & England, 2009) (Schutte, Herr, & Kitching, 1994) (Eaglesome & Garcia, 1992). It has been reported that the test should be repeated 3 times at intervals of 7 days to increase its sensitivity (Schonmann, BonDurant, Gardner, Van Hoosear, Baltzer, & Kachulis, 1994). The OVI advises to check bulls three times in 21 days (with weekly intervals) before declaring them negative, regarding *T. foetus* infection. Because of practical restrictions within this pilot study, it was not possible to take three samples from the bulls with intervals of 7 days. Therefore some of the bulls tested in this study may have been positive, but the amount of *T. foetus* present in the sheath at the moment of sample collection was too low to be recognized after culturing.

Research in other communal areas in South Africa confirmed that infectious diseases like tritrichomonosis, campylobacteriosis and brucellosis played a less important role than predicted (Mokantla, McCrindle, Sebei, & Owen, 2004). Causes for low calving rates were: sub-fertility of bulls and poor body condition score of cows (Mokantla, McCrindle,

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Sebei, & Owen, 2004). Nevertheless one still has to consider that communal farming is an important risk factor for trichostrongylosis (Gay, Ebel, & Kearley, 1996).

Another cause of the high number of negative results of *T. foetus* found in this study may be the geographical location of the Mnisi area. Previous research in comparative rural areas in South Africa showed individual prevalence rates up to 26,4 % (Pefanis, Herr, Venter, Kruger, Queiroga, & Amaral, 1988). Situated in the Foot and Mouth Disease control zone, the import and export of cattle is limited in the Mnisi area (Figure 10) (Jori, Vosloo, Du Plessis, Brahmabhatt, Gummow, & Thomson, 2009).

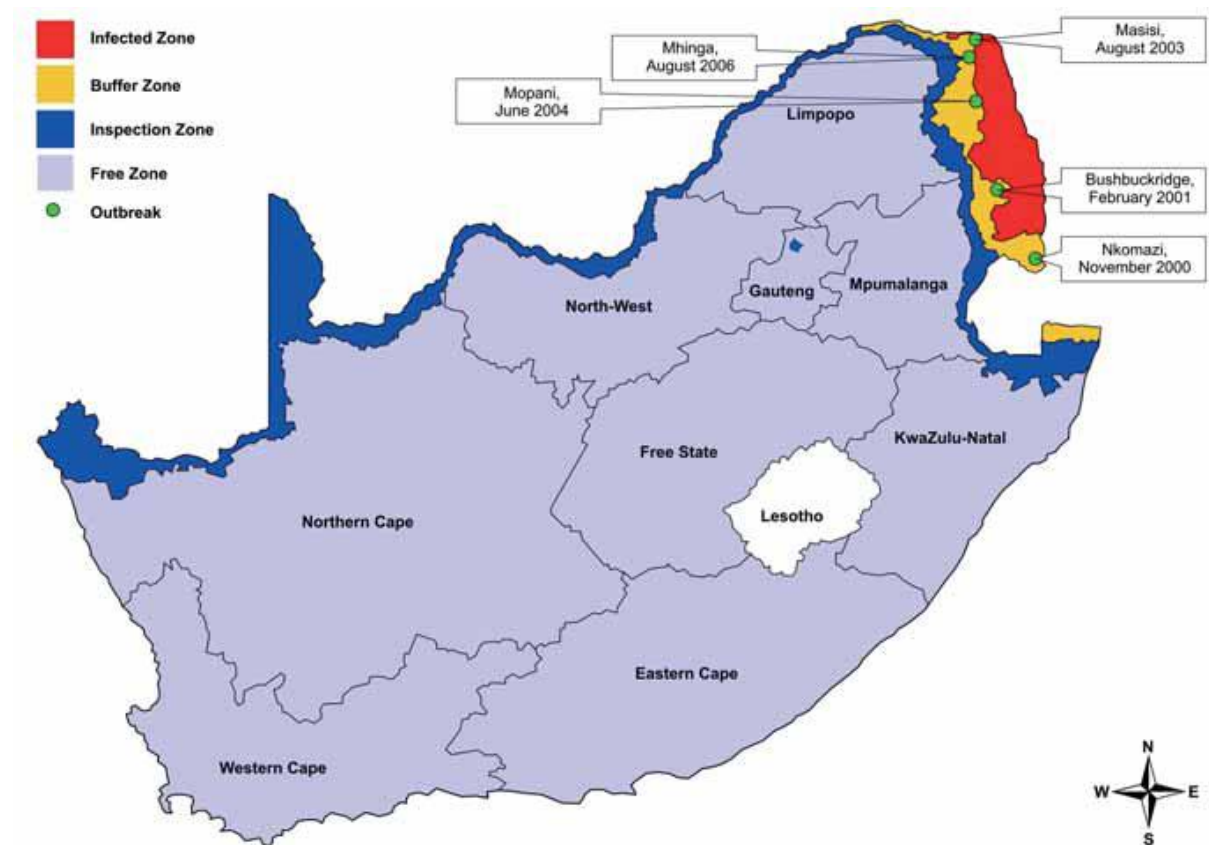


FIGURE 10: DIFFERENT FMD-ZONES IN SOUTH AFRICA (OIE.INT)

Data from the southern parts of the Foot and Mouth Disease control zone showed that the human density is high (5 people/ha), meaning the demand for local slaughter/sale is very high (Van Schalkwyk, 2010). 4.85 animals are slaughtered for every 1 animal that is sold or moved out of the area. In other areas, with lower human density (0.25 people/ha), the amount of animals slaughtered is lower than those sold or moved out of

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the study area (Van Schalkwyk, 2010). Selling cattle directly to the consumer on the farm offers the greatest profit margin on live animals, because all middlemen and their fees are eliminated (Nkhori, 2004) (Musemwa, Mushunje, Chimonyo, Fraser, Mapiye, & Muchunje, 2008). The average price a farmer will receive when selling a bull to a butcher is 3,686.00 SAR, compared to 4,000.00 SAR when selling to other farmers. (Van Rooyen, 2010) Divided by 400 kg (broad estimation of carcass weight), it gives an estimate 9,215 SAR/kg carcass weight. So the value is very low compared to 19,44 SAR/kg carcass weight at abattoirs elsewhere in South Africa (for class C2/C3) (Agrimark Tendense (ATM), 2010). For cattle owners it is quite expensive to buy special breeding bulls. They seem to prefer their own bulls for breeding purposes. These factors can result in a low risk of introducing *T. foetus* in the area. Further research regarding this matter in Foot and Mouth Disease buffer zones and the transmission of venereal diseases should give clarity about this possibility. Informing the cattle owners about the risks of introducing venereal diseases into their herds by means of buying breeding bulls, may reduce the risk even more in the future.

The probability of the outcome of this pilot study in an area with a certain prevalence can be calculated using Survey Toolbox. According to literature, the prevalence of *T. foetus* varies from 2.7 % and 3.7 %, up to 7.1 % or even 26.4 % (Retief & Schutte, 1975) (Kitching, 1988) (Pefanis, Herr, Venter, Kruger, Queiroga, & Amaral, 1988) (Erasmus, De Wet, Van der Merwe, & Pienaar, 1989). The probability of finding not one positive result in populations with these individual prevalence rates is shown in Table 3 and shown in Figure 11. If the prevalence is higher than 5 %, the chances of finding zero positive results with 89 samples are extremely low.

Prevalence	2.7 %	3,7 %	4,94 %	7.1 %	26.4 %
P-value	0.08445	0.033125	0.010328	0.001246	0.000000

TABLE 3: PROBABILITY OF ZERO POSITIVE RESULTS AT CERTAIN PREVALENCE

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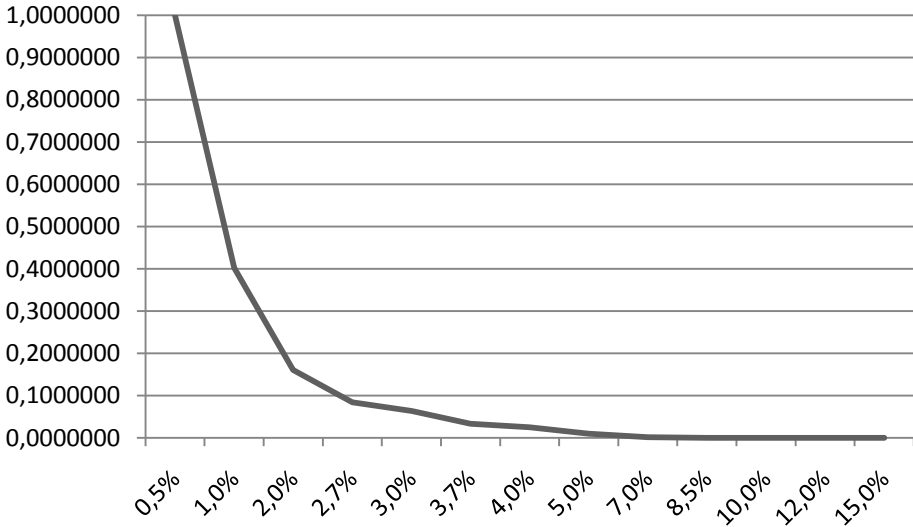


FIGURE 11: P-VALUE OF PROBABILITY OF FINDING ZERO POSITIVE RESULTS

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CONCLUSION

This pilot study unveiled that *Tritrichomonas foetus* plays a less important role in fertility problems in the Mnisi area. There are different factors and possible causes contributing to this result. The microscopic examination, microbial growth in culture medium and the average age of bulls may have directly influenced the results. Moreover the geographical location, the value of cattle and the human density possibly do minimize the risk of introducing *T. foetus* in this area. Further research has to provide give more information about the importance of these factors.

The prevalence calculated in this pilot study may be underestimated, and research performed in a validated lab, by more experienced diagnosticians may have resulted in more reliable prevalence rates. Finally one has to consider that low calving rates and hence fertility problems may be caused by other, probably non infectious, factors. Research in the future has to focus also on these topics.

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ATTACHMENTS

BULL DATA FORM

DATE: ___/___/___ (dd/mm/yyyy) DIP TANK: _____

BULL: _____ (# _____) OWNER: _____

1. HERD

- SINGLE / MULTISIRE

- COMMINGLES? _____

2. BREEDING SEASON: _____

3. AGE: _____ (if not known, findings teeth eruption in box below)

4. BREED: BRAHMANN / NGUNI / BONSMARA / AFRIKANER / SIMMENTAL / MIXED / INDIGENOUS

5. SAMPLE: # _____ (COLLECTED BY: _____)

REMARKS:

For example: discomfort, amount and aspect of preputial material, sampling issues, ...

6. MICROSCOPIC EXAMINATION

Time	Direct	24h	48h	72h	96h	120h
Result						

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STANDARD OPERATING PROCEDURE (OVI)

1. Take Culture Medium out fridge, to warm it before inoculation.
2. Take Polystyrene cool box with ice packs, PBS and plastic tubes.
3. Collect samples, add 4.5ml of PBS and store in cool box.
4. Transport to lab within 5-6 hours. (OVI: The specimen should arrive at the lab within 24 hours of collection and inoculation into transport medium. Older samples are not suitable for the detection of *Tritrichomonas foetus*)
5. Process samples in lab:
6. Centrifuge 10 min on 2000 rpm.
7. Discard supernatant.
8. Re-suspend the pellet with rest of fluid using a plastic pipette.
9. (Add antibiotics with Fungizone* (1 ml) to culture medium).
10. Inoculate 4 droplets of sample to 1 culture medium, mix gently.
11. Incubate in air at 37 °C for 5 days. (OVI: The specimen should be maintained between 15-37 °C after collection and in transit)
12. Use 1 droplet for direct microscopic examination of smear.
13. Examine 1 droplet daily, for 5 consecutive days after inoculation.
 1. If protozoa with typical *Tritrichomonas* size, shape (axostyle, 3 flagellae) and motility (jerky motion, undulating membrane) are observed, the sample is reported as "*Tritrichomonas foetus*".
 2. If no trichomonads are observed during the incubation period, the sample is reported as "Negative Culture *T. foetus*".

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I would like to say thank you to the Mnisi community, all the farmers who gave their permission to include their bulls in this study, who were very patient even in these periods of draught and who were very interested in our activities and objectives.

I really appreciate the help from the Animal Health Technicians, Gypsey Mathumbu, Solly Mokone, Jerry Mabunda and PB, and their assistants. Without them, I would never have been able to collect all the samples.

I would like to thank everybody at the Department of Production Animal Studies, I really enjoyed working and celebrating my birthday at Onderstepoort. And Johannes Kekana and Dr. Awoke for the bench training at the Onderstepoort Veterinary Institute.

Special thanks for Stephanie Malan from IDEXX/VetPath, for producing the culture media, and giving me the opportunity to look at every Trich that went through VetPath during September, October and November 2010.

Thank you, Emma, I've had a wonderful three months in South Africa, and I will never forget the pleasure and fun we had here.

And last but not least, the people at home: my parents, brothers and Julie for supporting me, especially with the preparations of this trip, and I'm really looking forward to show you all the pictures and tell you the stories when I return in January.

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