DIAGNOSIS OF *TRITRICHOMONAS FOETUS* IN BULLS BY CULTURE AND PCR METHODS

By

PETER CHARLES IRONS

Submitted in partial fulfilment of the requirements for the degree of MMedVet (Gyn) in the Department of Production Animal Studies in the Faculty of Veterinary Science, University of Pretoria

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SUMMARY

DIAGNOSIS OF *TRITRICHOMONAS FOETUS* IN BULLS BY CULTURE AND PCR METHODS

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PETER CHARLES IRONS

Promoter: Prof. Henk Bertschinger

Department: Production Animal Studies

Degree: MMedVet (Gyn)

The aim of this work was to examine the effects of sampling method on accuracy of culture for *Tritrichomonas foetus*; and the effects of sampling method, time delay, and addition of a DNA preservative on the accuracy of a PCR test.

Samples from two different sources were used for Experiment 1. Preputial scrapings were collected from one group of three infected and one uninfected bull 10 times. Secondly, samples were collected from 5 infected bulls by both sheath washing and scraping on 6 occasions, while 8 uninfected animals were sampled three or more times.

Twenty nine out of 30 samples from the first sample set were found to be positive, and 83 % of samples collected by both methods for the second sample set tested positive. No samples from the control bulls were found to be positive. Scraping was found to offer significant practical advantages over washing. It may be subject to greater operator variability than sheath washing.

The second experiment utilised the same samples as were used for the second data set under Experiment 1. Guanidinium thiocyanate (GuSCN) was added to half of each sample. Each sample was cultured, while all samples were subjected to DNA extraction within 6 and 30 hours and after 5 days of storage at 4 °C. PCR and agarose gel electrophoresis was performed.

No samples from the control animals tested positive on PCR. The sensitivity of the PCR on samples from infected bulls ranged from 0,9 in samples extracted within 6 hours to 0,31 in samples extracted after 5 days. Sampling method had no effect with the exception of samples held for 5 days with GuSCN, where sheath washing was superior to scraping. The addition of

GuSCN had no effect. Holding time reduced sensitivity at 5 days, but the effect was not significant at 30 hours.

It is concluded that preputial scraping is equal in sensitivity to washing for culture of *Tritrichomonas foetus*. Preputial samples for PCR testing should be submitted as soon as possible after collection, and the addition of GuSCN has no effect. Samples collected by sheath washing may be superior to those collected by scraping for PCR testing. The requirement for a test with sufficient sensitivity to allow reliable identification of infected bulls based on one sample has not been met with the described method.

Key words: *Tritrichomonas foetus,* venereal disease, cattle, bull, diagnosis, PCR,
                preputial wash, preputial scraping

OPSOMMING

DIAGNOSE VAN *TRITRICHOMONAS FOETUS* IN BULLE DEUR MIDDEL VAN
KWEKING EN PCR METODES

Deur

PETER CHARLES IRONS

Promoter: Prof. Henk Bertschinger

Departement: Produksiedierstudies

Graad: MMedVet (Gyn)

Die doel van die werk was om die effek van die monsternemingsmetode op die akkuraatheid van kweking vir *Tritrichomonas foetus* te bepaal; en om die effek van die metode van monsterneming, tydsverloop en byvoeging van 'n DNA preserveermiddel op die akkuraatheid van 'n PCR toets te bepaal.

Monsters vanaf twee verskillende bronne is vir Eksperiment 1 gebruik. Preputiale skrape is tien maal van een groep, wat uit drie besmette en een onbesmette bul bestaan het, geneem. Tweedens is monsters ses keer deur middel van beide skedewasse en skedeskrape van vyf besmette bulle en drie- of meer keer van agt onbesmette diere geneem.

Nege-en-twintig uit 30 monsters in die eerste groep is positief gevind en 83 % van die monsters wat met beide metodes geneem is was ook positief. Geen monsters van die onbesmette diere het positief getoets nie. Noemenswaardige praktiese voordele is met die skrapingsmetode gevind. Dit mag meer vatbaar vir variasie tussen operateurs wees.

Die monsters wat vir die tweede groep van Eksperiment 1 gebruik was is ook vir die Eksperiment 2 benut. Guanidinium thiosianaat (GuSCN) is by die helfte van elke monster gevoeg. Elke monster is gekweek en DNA ekstraksie is binne 6 ure, na 30 ure en na 5 dae gedoen. Monsters is teen 4 °C geberg. PCR en agarose gel-elektroforese is uitgevoer.

Geen monsters van die onbesmette diere het met PCR positief getoets nie. Die sensitiwiteit van die PCR op monsters van besmette bulle was van 0,9 in monsters wat binne 6 ure verwerk is tot 0,31 in monsters wat na 5 dae verwerk is. Monsternemingsmetode het geen effek gehad met die uitsondering van die monsters met GuSCN wat na 5 dae verwerk is. In laasgenoemde monsters het skedewasmonsters beter as skedeskraapmonsters vertoon. GuSCN het geen

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uitwerking gehad nie. Sensitiwiteit het na 5 dae se berging afgeneem, maar geen noemenswaardige effek is na 30 ure waargeneem nie.

Die volgende gevolgtrekkings is gemaak: Skedeskraping is gelyk aan skedewasse in sensitiwiteit met die kweking van *Tritrichomonas foetus*. Monsters vir PCR toetsing moet so gou as moonltik na versameling ingedien word. GuSCN het geen positiewe uitwerking op sensitiwiteit nie. Monsters wat deur middel van skedewasse versamel is mag beter wees as monsters wat deur middel van skedeskraping vir toetsing deur PCR versamel is. Die toetsmetode wat beskryf is voldoen nie aan die vereiste vir 'n toets met sodanige sensitiwiteit om besmette bulle op 'n enkele monster uit te ken nie.

Sleutelwoorde: *Tritrichomonas foetus,* veneriese siekte, beeste, bul, diagnose, PCR, skedewas, skedeskraap

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1. Introduction

At the time of writing one manuscript emanating from this work has been accepted for publication. This manuscript is reproduced in Chapter 3.1. The remainder of the dissertation complements the manuscript, in that information contained in the manuscript is not repeated in the body of the dissertation.

In accordance with the recommendations of Kassai *et al.* the term Tritrichomonosis will be used to refer to the disease caused by *Tritrichomonas foetus* (30). The term Trichomonosis is still in general usage.

This well-known and important disease has been well reviewed elsewhere (2,6,8,48,60,63). The reader is referred to these publications for broad discussions of the parasite and the disease. Only aspects relevant to the diagnosis of the carrier status in the bull will be discussed in depth here.

Control and prevention of the disease relies on implementation of managemental measures and identification and elimination of infected animals. The majority of animals show no clinical manifestations of infection, forcing almost complete reliance on assumptions based on epidemiological findings and diagnostic testing to identify infected individuals.

The objectives of this work were twofold, namely:

- To investigate the effect of sampling method on diagnostic sensitivity of cultural diagnosis; and
- To investigate the effect of sampling method, the addition of GuSCN, and sample storage on the diagnostic sensitivity and specificity of a PCR diagnostic test for *Tritrichomonas foetus*.

2. Literature Review

2.1 Aetiology

Tritrichomonas foetus is a motile flagellated parasitic protozoon inhabiting the preputial cavity of bulls and vagina, cervix and uterus of heifers and cows. It is transmitted venereally and causes embryonic or foetal death resulting in delayed returns to service, abortion, mummification or pyometra. The organism is indistinguishable from *T. suis*, with recent opinion calling for a revision of the distinct species status of the two species (8).

Notable biological characteristics of the organism include its characteristic rolling motion ascribed to the presence of a longitudinal undulating membrane, three anterior flagellae, and the caudal axostyle and posterior flagellum. It is an aerotolerant anaerobe, and does not possess mitochondria nor a functional Krebs cycle. Instead it utilises small dense organelles known as hydrogenosomes for oxidative degradation of carbohydrates. It also synthesises and secretes an array of proteases, and utilises so-called "salvage enzymes" to provide nucleotides, which it is unable to synthesise *de novo* (8).
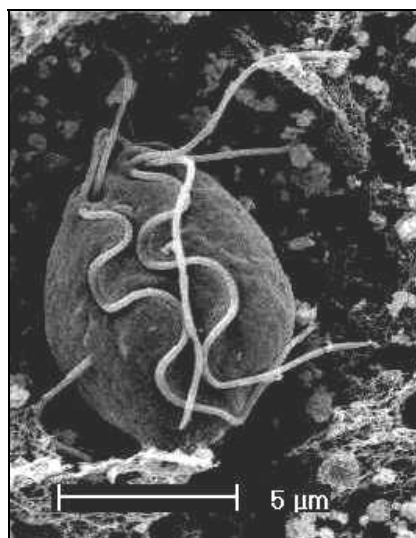
FIGURE 2.1: SCANNING ELECTRON MICROGRAPH OF TRITRICHOMONAS FOETUS UNDERGOING LENGTHWISE CELL DIVISION

2.2 Transmission

Tritrichomonosis is a venereally-transmitted disease. Although both males and females can harbour the infection, bulls are regarded as the maintenance hosts due to the fact that they generally remain lifelong carriers once infection has become established. Transmission by means of artificial insemination with contaminated semen and by mechanical means during sequential examination of animals also pose significant risks (25,40). Other means which are possible but require confirmation are the transmission by vectors such as flies and transmission within bull groups by means of mounting of one bull by an infected and uninfected animal in short succession (31,63).

2.3 Economic importance

Despite increased awareness and proven control programs, Tritrichomonosis continues to be a major source of economic losses in southern Africa, North, Central and South America, Australia, and Asia (3,13,33,38,39,47,65). In southern Africa the prevalence, as judged by

recovery rates submitted to regional veterinary laboratories and published works, is given in Table 2.1.

TABLE 2.1: PREVALENCE OF *T. foetus* IN SOUTHERN AFRICA

Location	Number of samples	Years	Samples positive (%)	Herds positive (%)	Reference
Free State	2012	1995- 98	0 – 4,5	0 – 12,6	(5)
Free State, Mphumalanga	2437	*	7,1	*	(14)
Kwa-Zulu Natal	*	*	1,8 – 25	12- 46	(33)
Northern Province	2949	1985- 98	4 – 22,4	0,8 – 16,9	(36)
Free State	*	1989 – 98	2,6 – 11	3 – 11	(45)
Transkei	*	*	23	*	(46)
Onderstepoort	832 sheath washes	*	13,8	*	(55)
Onderstepoort	182 vaginal mucus	*	61	*	(55)

* Data not available

The data in Table 2.1 is not drawn from randomised samplings and cannot be used to determine the true prevalence of the disease in southern African herds. However, it supports the view of the veterinary profession and livestock industry that the disease is of major economic importance in this country.

The economic impact of the disease can be ascribed to the increased days open, reduced calf crop, reduced average weaning weight as a result of delayed conception, culling of infected animals, and the costs of control and preventive measures (25,39,50). Typical calving percentages in chronically infected herds are between 70 and 85 %, while percentages as low as 20 % can be expected in naïve herds (2). Owners and herdsmen are often unaware of the presence of this disease in their herds due to the insidious nature of the disease once the chronic state is reached.

Rae *et al.* modelled the extent of losses for two prevalence rates, his model indicating an estimated loss of 5 to 35 % in the return per cow confined with a fertile bull (50). These estimations proved to be accurate in a subsequent case study (51). While no accurate estimates of economic losses are available for southern African conditions, Kitching estimated the application of stringent control measures in a beef suckler herd to result in an increase in gross income of 40 % in the third year of the program (33).

2.4 Diagnosis: Sample collection

As the bull is the most important maintenance host (11), and the organisms are found almost exclusively in the superficial layers of the preputial mucosa (28,43,54), preputial specimens are the sample of choice for confirmation of the presence of Tritrichomonosis in a herd.

Preputial material is generally collected by sheath washing or sheath scraping combined with aspiration, which are both well described (2,6,60). Sheath washing is favoured in Britain and Europe whereas scraping and aspiration is favoured in the Americas and Australia.

The relative merits of sheath washing versus sheath scraping and aspiration have been the subject of some research. Early work favoured washing over scraping (Johnson and Marquez *et al,* cited by Schönmann *et al.*) (20,59). In contrast, one more recent paper favoured scraping over washing (Mickelsen, cited by Schönmann *et al.*), while another found them to be similar in diagnostic sensitivity (59).

Sheath washing has the advantage of recovering material from the whole preputial cavity, which is then concentrated by centrifugation. In contrast, sheath scraping collects material from a limited portion of the caudal reaches of the preputial cavity and the free portion of the penis. While this material is more concentrated by virtue of the sampling method and is suspended in a smaller volume of fluid, it is not normally subjected to further concentration. It does however represent material collected from the portion of the cavity which has the highest concentration of organisms, providing that material is also collected from the penis during sampling (20,28,54).

The extent of cellular binding may also favour a collection technique which takes a deeper, more cellular sample. Early workers described *T. foetus* organisms as being limited to the secretions on the surface of the penis and preputial cavity (43). The presence of a number of cell adherence mechanisms and the demonstration of adherence to some cell types *in vitro* suggests that the organisms may be more cell-associated in the host than these earlier reports would suggest (8,17). In contrast to the earlier work, organisms were recently demonstrated in

tissue sections, specifically in cellular detritus and within the stratified squamous epithelium in the penile and preputial crypts (54). The precise extent of cellular binding *in vivo* has not been reported. However, the numbers of organisms in positive samples was found to be similar in samples collected by washing and scraping, indicating that cellular binding is unlikely to account for differences in sensitivity between the collection methods (20).

2.5 Diagnosis by culture and direct examination

Organisms are demonstrated by direct examination of preputial material and, if organisms are not observed, culture followed by direct examination. The size and shape and pattern of movement of the organism are used during screening, while closer examination of the morphology of the organism is required for confirmation of its identity. Examination is done using dark field or phase contrast microscopy. Isolation can also be attempted from material collected from the female genital tract and aborted material.

Direct examination and culture suffers from various deficiencies. Firstly, to ensure survival of the organisms samples need to be placed in suitable transport media and must reach the laboratory within a limited time. While immediate culture is the ideal, a delay of 24 hours may result in a loss of approximately 10 % in diagnostic sensitivity, with longer delays causing more drastic declines (34,52,62,67,70). This has lead to the general recommendation amongst South African laboratories of overnight shipping of samples to the laboratory, and the utilisation of enriched transport media where longer delays are unavoidable. Specific transport media or combined transport and culture kits are commercially available for *T. foetus*[1], making field handling of samples more practical and extending the time for which organisms remain viable. Even with enriched transport media some loss of sensitivity may be experienced when incubation is delayed by 24 hours (34). The above requirements place severe practical limitations on the field diagnosis of the disease, especially in areas far removed from diagnostic laboratories.

Another problem is the tendency of cultures to be overgrown with contaminants, destroying the culture for diagnostic purposes. Despite the presence of powerful antimicrobials in selective media this remains a problem (12,19,60).

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[1] In-Pouch[TM] TF, Fort Dodge; Trichtube, Vrede Veterinary Laboratory; Steve's TM, Vrede Veterinary Laboratory

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The limited sensitivity of these methods is further exacerbated by the tendency for some isolates of *T. foetus* to adapt to culture media better than others (19).

Direct examination and culture of organisms is a time-consuming, labour-intensive process, requiring highly skilled personnel. This places constraints on the numbers of samples which can be handled by a laboratory. The time taken for culture of organisms is a further disadvantage, most laboratories waiting for 5 days before making a final decision on the status of the animal.

The final problem with standard diagnostic procedures is a lack of specificity of the tests. Most media are not highly specific, and other protozoa which may grow in the culture media may be mistaken for *Tritrichomonas foetus*, especially where the numbers of organisms are low (7,19,60,66). Even careful morphologic examination may fail to distinguish between *T. foetus* and enteric tetratrichomonads (7).

Despite these considerations, sensitivities of over 90 % are attainable under optimal conditions (12,32,42,59,68,70). However, sensitivities of 70 - 90 % are more representative of samples collected or processed under sub-optimal conditions as is often the case in the field (23,49,51,59,62,70).These findings have lead to the generally accepted recommendation to test bulls on three successive occasions before they can be certified free of the disease (6,42,48). Three repeats of a test with a sensitivity of 70 % renders a combined sensitivity of 97 %. The practical and cost implications of repeated testing has resulted in non-compliance and a failure to ensure the negative status of many breeding bulls in South Africa.

2.6   *Diagnosis by molecular methods*

The advent of molecular methodologies to detect specific DNA sequences has opened up a whole new range of possibilities for the diagnosis of infectious diseases. Amplification of genetic material using the polymerase chain reaction (PCR) increases the potential sensitivity of these techniques to a single DNA molecule. This technology has the further advantages of speed, versatility, high specificity, ability to analyse large numbers of samples simultaneously, and its amenability to automation (19,41).

PCR technology has been shown to be effective in the diagnosis of *Trichomonas vaginalis* in humans, with a sensitivity of 95 % and a specificity of 98 % (44).

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Early attempts at diagnosis of *T. foetus* using a DNA-probe method lacked the sensitivity required for accurate diagnosis (1). The incorporation of amplification of the DNA using PCR techniques has lead to the development of more effective tests (18,19,29,53,56).

Ho *et al.* used oligonucleotide primers TF1 and TF2 to amplify a 162-bp product from *T. foetus* DNA, and hybridised a chemiluminescent internal *T. foetus* sequence probe to Southern blots of the amplification product (29). These authors were able to detect one organism from culture material and ten organisms in samples containing preputial smegma. They correctly identified 39 of 44 clinical samples from infected bulls, and no false positives from 8 uninfected bulls. Using the same method other authors found false positives due to the amplification of non-specific amplicons, as well as some degree of cross-reactivity with other Trichomonads (19).

Riley *et al.* used the TF1 and TF2 primers of Ho *et al.* and hybridisations to the probe TF3 to confirm the identity of 16 out of 17 field *T. foetus* isolates conclusively, the 17th giving only weak amplification (56). These authors also reported on the use of T17 PCR, which renders multiple product bands with isolate-specific patterns, RAPD PCR using the primer set TAP5 and TAP6, and TCO-1 PCR. These procedures identified different isolates of *T. foetus,* different species of Trichomonad and other protozoa, and several species of bacteria. They also demonstrated marked heterogeneity amongst the isolates studied, although some amplification products were conserved across isolates, namely at 220 and 330 bp. The results supported the identity of the isolate yielding inconclusive results on single band amplification as *T. foetus*.

Felleisen performed PCR with primers TFR1 and TFR2 followed by subcloning and sequencing to characterise the 5.8S rRNA gene region of the three species of Tritrichomonas and four other Trichomonads (15). While the Tritrichomonads were almost identical, other Trichomonads showed marked variations. The same author used RAPD PCR to demonstrate the identical genomic fingerprints of *T. foetus* and *T. suis*, as distinct from that of *T. mobilensis* (16). Using primes TFR3 and TFR4, Felleisen *et al.* used PCR, incorporating the uracil DNA glycosylase system to prevent carryover contamination, followed by gel electrophoresis or DNA enzyme immunoassay (DEIA) to amplify the entire 5.8S rRNA gene plus the flanking ITS1 and ITS2 regions (18). This resulted in an assay that was specific to *Tritrichomonas spp,* rendering an amplification product of 347 bp from all of eight strains. They were able to detect the amount of DNA equivalent to one organism from culture material, but in the presence of preputial smegma the sensitivity was reduced significantly. In addition, they were able to differentiate a morphologically similar organism from a preputial

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wash from a bull thought to be infected with *T. foetus*. The latter distinction was facilitated by the addition of DEIA to the procedure. No false positives were found and the assay was not affected by bacterial or fungal contamination, although 1,5 % of preputial samples did suffer from inhibition of the PCR. This test was potentially more sensitive and specific than culture. These authors suggest the combination of culture followed by PCR to increase diagnostic sensitivity to very high levels. This test was not found to suffer from the problems of unspecific amplicons as described for the TF-PCR method of Ho *et al.* (19).

Marked variation exists between different isolates in the impact on herd fertility, with some herds suffering disastrous consequences while in others the impact is barely discernable. Factors which can be expected to account for this variability include animal susceptibility, managemental factors, and variations in virulence between isolates. It may therefore be of some clinical significance to be able to characterise isolates, in order to predict more accurately the economic benefits of eradication of the disease. PCR procedures offer a potentially valuable tool in this regard.

In summary, PCR is a highly sensitive diagnostic method which has the ability to accurately distinguish between *Tritrichomonas sp.* and other similar organisms, and also to identify isolates of *T. foetus*. Whereas false positives were problematic with early applications, the development of primers of higher specificity has decreased the risk of false positives. Another potential problem is polymerase inhibition by substances found in smegma and vaginal mucous samples.

Published data on the use of PCR and allied molecular methods on field samples is limited to what has been reviewed above. As can be seen, the emphasis has fallen on primer selection and the optimization of laboratory protocols to render satisfactory results. Little attention has been paid to factors such as sample selection, sample collection technique, different transport media, time delay before DNA extraction and holding temperature during transport, all of which can be expected to affect test outcome (21).

Of the sampling methods, sheath scraping is the more traumatic of the two procedures, samples often containing blood and other products of superficial epithelial damage in addition to thick, mucoid smegma. Components of blood inhibit the polymerase reaction (57). Smegma originating from sheath scraping has been shown to reduce the sensitivity of PCR procedures (29). On the other hand, sheath washes often contain substantial amounts of urine, which also has inhibitory properties (24,57). Scraping also renders a more concentrated

sample of smaller volume than washing. The effects of these factors on PCR diagnosis of *T. foetus* have not been reported.

Studies on the effect of transport time on test accuracy have not been reported for PCR-based diagnostic tests for *T. foetus*. Time delay may lead to a decline in sensitivity of PCR testing due to degradation of the nucleic acids (21). *T. foetus* is known to secrete a range of hydrolytic enzymes (8,69), some of which cause rapid DNA breakdown following cell lysis (71,72). Other compounds either from the organisms themselves, from other organisms which are present in the preputial cavity, from the preputial membrane or the upper urogenital tract may also affect the integrity of nucleic acids over time. The addition of preservatives to prevent degradation of the nucleic acid component of stored samples may improve the sensitivity of PCR testing. The chaotropic agent guanidinium thiocyanate (GuSCN) causes cell lysis, inactivates nucleases, binds DNA and preserves its helical structure, enabling its purification and detection (9,72).

2.7   Control measures

Excellent work has been done to describe the epidemiology of *Tritrichomonas foetus* infection in the herd situation. This has enabled the development of managemental tools which are relatively effective in control of the disease (2). Despite this, the disease persists, particularly in extensively-farmed areas. Factors which can explain this anomalous situation include the following:

- Impracticality of breeding beef herds by artificial insemination,

- Inability to apply basic managemental practices of strict herd segregation and maintenance of fences consistently (4),

- Persistence of infection in some female animals for protracted periods, and even through normal pregnancies (37,61,64), and

- Presence of infection in some young bulls contrary to the widely held belief that these bulls play no role in perpetuating the disease.

A vaccine prepared from whole *T. foetus* organisms is commercially available[2]. It is not used in bulls. While it does not offer complete protection from infection in females, it reduces the duration of infection thereby mitigating the reproductive wastage caused by the organism

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[2] Trichguard, Fort Dodge

(22,27,35,58). Despite this it is not universally used, possibly due to perceptions of moderate efficacy and high cost (4). While work towards vaccine production is ongoing, no highly effective vaccines are yet available.

3. Experiment 1: Collection of preputial material by scraping and aspiration for the diagnosis of Tritrichomonas foetus in bulls

A copy of the publication in the Journal of the South African Veterinary Association follows.

4. Experiment 2: Effect of sample collection method, storage and transport medium on a PCR test for the diagnosis of Tritrichomonas foetus infection in bulls

4.1 Materials and methods

4.1.1 Animals and housing

Five bulls infected with *Tritrichomonas foetus* and 8 uninfected control bulls were used in this study. The infected bulls were housed in groups under extensive management systems on two commercial beef farms. Four of the control bulls were also pastured together on one of these farms, while four were housed semi-intensively in the Onderstepoort Teaching Animal Unit. None of the bulls were used for breeding shortly before or during the test period. These were the same bulls which were used in the second part of Experiment 1.

4.1.2 Sampling methods

The two sampling methods used were sheath washing and sheath scraping, with washing being done first. Washing was carried out by introducing 50 ml of phosphate-buffered saline (Dulbeco's Phosphate Buffered Saline (PBS), Onderstepoort Biological Products) into the preputial cavity through a latex tube, massaging the preputium vigorously for approximately 100 strokes, and then draining the fluid back into the sample bottle through the same tube.

A new hypodermic syringe attached by means of a short piece of silicon tubing to a rigid perspex pipette with an outer diameter of 5 mm and length of 450 mm (AI pipettes, Kyron Laboratories) was used for each sheath scraping. The syringe was held in one hand, the tip of the pipette was guided into the caudal reaches of the preputial cavity and manipulated vigorously with an in-and-out movement under guidance of the other hand while suction was applied with the syringe. The pipette was removed and the content flushed into a vial containing 5 ml PBS by repeated aspiration and expulsion of the medium. A new collection apparatus was used for each sampling procedure and disposable latex gloves were worn, which were changed between bulls.

Sampling was carried out by one of two operators on each occasion. The positive bulls were sampled on six occasions over a period of 18 days. Control bulls were sampled three times (n = 6), four times (n = 1) or six times (n=1).

4.1.3 Sample treatments

Once all samples had been collected for the day half of each sample was decanted into a second vial, to which a GuSCN stock solution was added. The final GuSCN concentration was 200 mmol/l. Higher concentrations of this compound cause lysis of organisms and interfere with extraction of DNA. Samples were chilled to 4 °C and transported to the laboratory within 3 – 6 h of collection.

4.1.4 *T. foetus* culture

A portion of the GuSCN-free sample was cultured on arrival at the laboratory or the following morning. Sheath wash samples were centrifuged and the pellet resuspended in 1 ml PBS prior to inoculation of a culture medium consisting of Trichomonas medium (CM161, Oxoid Limited), horse serum, distilled water and antibiotics. Sheath scrapings were not centrifuged prior to inoculation. Cultures were incubated at 32°C and a drop was removed and examined microscopically for the presence of organisms after 48, 96 and 144 h.

4.1.5 PCR procedure

Both the GuSCN-treated and GuSCN-free samples were subjected to DNA extraction within 6 h, after 30 h and after 5 days of storage at 4 °C. Extraction was done using the GuSCN and silica method described by Boom et al. (9). Sheath wash samples were first concentrated by centrifugation for 10 minutes at 800 x g, pellets were resuspended in 400 μl of sterile PBS (pH 7,4), and 200 μl from each sample was used for extraction. Sheath scrape samples were used as collected. The sample was transferred into sterile prelabelled micro-fuge tubes (Plastpro, Edenvale, South Africa) and incubated for 5 minutes with GuSCN lysis buffer and size-fractionated silica particles, which act as a nucleic acid carrier. The resultant nucleic acid-carrier complexes were sedimented by centrifugation. Complexes were washed twice with 70 % ethanol and once with 100 % acetone, dried, and the nucleic acids eluted in a Tris-EDTA buffer (pH 8).

The primers used for PCR were those of Felleisen and coworkers, namely TFR3 and TFR4 (18). Primer sequences for TFR3 and TFR4, given from 5' to 3', were CGGGTCTTCCTATATGAGACAGAACC and CCTGCCGTTGGATCAGTTTCGTTAA respectively. The primers were obtained from Integrated DNA Technologies, Inc. (Whitehead Scientific). The PCR reaction was carried out in a 25 μl mixture containing KCl, Tris-HCl, $MgCl_2$, 200 mmol/l each of the dNTPs (dATP, dGTP, dCTP, dTTP), primers TFR3 and TFR4, Takara Taq Polymerase and 4 μl of the isolated DNA. The thermocycling was performed in an Eppendorf Mastercycler thermocycler (Merck) using a program of 35 cycles

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with the following parameters: Initial denaturation 95 °C for 15 s, denaturation 94 °C for 30 s, annealing 60 °C for 30 s, extension 72 °C for 30 s, and final extension 72 °C for 5 minutes. PCR products were detected by electrophoresis on a 1,5 % agarose gel containing 0,5 µg/ml ethidium bromide and visualised under UV light.

4.1.6 Data analysis

Sensitivity of each treatment was expressed as the number of samples which tested positive of the total number tested, whereas specificity was expressed as the number of samples from known negative bulls which tested positive. A two-tailed Chi square test was used to test for differences between treatments.

4.2 Results

4.2.1 Cultures

Sampling proceeded smoothly with the exception of one occasion when one of the infected bulls was not available for sampling. All but one of the infected bulls tested positive on either sheath washing or scraping and culture throughout the sampling period, one bull testing negative to both sampling methods on the last day of testing. The percentage of sheath wash and sheath scraping samples testing positive on culture was 83 % for both sampling methods. When the results were combined all but one bull on one occasion tested positive.

No samples from the uninfected bulls were found positive on culture.

4.2.2 PCR results

No samples from the eight control animals subjected to any of the twelve treatments gave a positive PCR result.

The culture and PCR results of the infected bulls are shown in Table 4.1. There was no difference between the sensitivity of culture of fresh samples and PCR testing at any interval with the exception of the sheath scraping samples extracted after a delay of 5 days, where culture was more sensitive than PCR testing.

TABLE 4.1: CULTURE RESULTS COMPARED TO PCR RESULTS OF ALL SAMPLES SHOWING COLLECTION METHOD, STORAGE TIME AND PRESENCE OF GuSCN

Bull ID	Culture*	Time to DNA extraction											
		6 hours				30 hours				5 days			
		Wash		Scrape		Wash		Scrape		Wash		Scrape	
		GuSCN -	GuSCN +	GuSCN -	GuSCN +	GuSCN -	GuSCN +	GuSCN -	GuSCN +	GuSCN -	GuSCN +	GuSCN -	GuSCN +
9763	1	0	0	1	0	1	0	1	0	0	0	1	0
	1	0	1	1	1	1	1	1	1	1	1	1	1
	1	1	1	1	0	1	1	1	1	1	1	1	0
	1	1	1	1	1	0	1	1	1	0	0	0	0
	1	1	1	1	1	1	1	1	1	0	1	0	0
	0	1	1	1	0	0	0	0	0	0	0	0	0
9924	1	1	1	1	0	0	0	0	0	0	0	0	0
	1	1	1	1	1	1	1	0	0	1	1	0	0
	1	1	1	1	0	1	1	1	1	1	0	0	1
	1	1	0	1	1	1	0	1	1	1	0	1	0
	1	0	1	1	1	1	1	1	1	1	1	0	0
	1	1	1	1	1	0	0	0	0	0	0	0	0
9968	1	1	1	1	1	1	1	1	1	0	1	1	1
	1	1	1	1	1	1	1	1	1	1	1	1	1
	1	1	1	1	1	0	1	0	1	1	1	0	1
	1	1	1	1	1	0	1	1	1	1	1	0	0
	1	1	1	1	1	1	1	0	1	1	1	0	0
99001	1	1	1	1	1	0	1	1	0	0	1	1	0
	1	1	1	1	1	1	1	1	1	1	1	1	1
	1	1	1	0	1	0	0	0	0	1	0	0	0
	1	1	1	1	0	1	1	1	0	1	1	0	0
	1	1	1	0	1	1	1	0	1	0	0	1	0
	1	1	1	1	0	0	0	0	1	0	1	0	0
BIG	1	1	1	1	1	1	0	1	1	1	1	1	1
	1	1	1	1	1	1	1	0	0	1	1	0	0
	1	1	0	0	0	1	1	1	1	1	1	1	0
	1	1	1	0	1	1	1	1	0	1	1	0	1
	1	1	1	1	1	1	1	1	0	1	0	1	1
	1	1	1	0	1	1	1	0	1	0	0	0	0
Total positives		26	26	24	21	20	21	18	18	18	18	12	9
Total number tested		29	29	29	29	29	29	29	29	29	29	29	29
Sensitivity		0.9	0.9	0.83	0.72	0.69	0.72	0.62	0.62	0.62	0.62	0.41	0.31
95% Confidence Interval		0,73–0,98	0,73–0,98	0,64–0,94	0,53–0,87	0,49–0,85	0,53–0,87	0,42–0,79	0,42–0,79	0,42–0,79	0,42–0,79	0,24–0,61	0,15–0,51
Significance[#]		a	a	b	b	c	b	a', c	a', c	a', c	a', c	a', b'	a', b', c'

* Culture results given as positive if either of the sheath wash or scrape sample tested positive

[#] Treatments marked a and a', b and b', and c and c' differ in sensitivity P < 0,05

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The addition of GuSCN had no effect on test sensitivity. The results for the samples without GuSCN are therefore considered for the remainder of the report.

Sampling method had no effect with the exception of samples held for 5 days with GuSCN, where sheath washing gave higher sensitivity than sheath scraping.

A reduction in sensitivity was seen for samples collected by both methods at 5 days, but there was no significant effect at 30 hours. This loss of sensitivity is shown in the bar graph in Figure 4.1. A combination of sampling method and holding time did render significant differences, with samples collected by sheath washing and tested after 6 hours giving significantly better sensitivity than those collected by scraping and held for 30 hours.
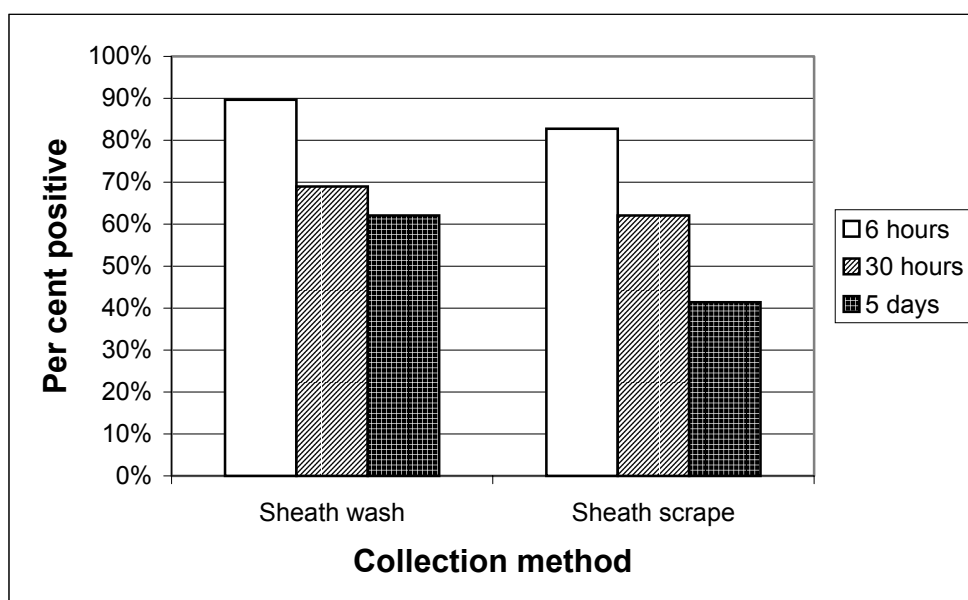

FIGURE 4.1: EFFECTS OF SAMPLING METHOD AND HOLDING TIME ON SENSITIVITY OF THE PCR TEST

5. Discussion

5.1 Experiment 1: Sheath scraping vs. sheath washing for cultural diagnosis

Our results of equal sensitivity of the two collection methods in a culture system agrees with Schönmann *et al,* while it disagrees with the findings of other authors who demonstrated differences in diagnostic sensitivity between the two methods (Johnson, Marquez *et al,* and Mickelsen, cited by Schönmann *et al.*) (20,59). The only advantages we found were qualitative rather than quantitative, involving practical considerations such as ease of

handling, speed and labour-intensiveness of sampling, and use of disposable equipment. In this respect our findings agree with the general opinion that sheath scraping has practical advantages over washing (20,59,63).

Observing the general recommendation to test bulls three times, all five of the infected bulls would have been found to be positive on culture of any three of the six sheath scraping samples. However, one of the infected bulls may have been found negative based on the results of culture of any three of four negative sheath washing samples. Possible explanations for the observation include low numbers of organisms in this animal, or the fact that only a small volume of each sample was available for culture. Alternatively, it may have been due to some difficulty in sample collection from this specific animal, such as a highly fractious temperament, repeated contamination of the sample with urine, or the presence of excessive contaminants in samples from this animal.

None of the published work examines the difference in susceptibility to operator effects between the different sampling methods, a significant factor, particularly in research and epidemiological work. Our trial was not designed to demonstrate differences between operators in collection of samples. Sample size is insufficient and randomisation and blinding procedures were not followed, which makes it impossible to draw any firm conclusions. The data does, however, indicate a possible difference in operator effect between sample collection methods, with sheath scraping being more prone to operator variability than sheath washing. If this were true, it would be imperative to ensure that operators have adequate training when this method is utilised in order to minimise variability in test outcome.

5.2 Experiment 2: Effect of sample collection method, storage and transport medium on a PCR test for the diagnosis of Tritrichomonas foetus infection in bulls

The effect of sampling method on molecular tests for *T. foetus* has not been reported elsewhere.

Sheath washing was always done before sheath scraping in order to avoid contamination of the sheath wash samples with blood or other products of mild tissue trauma associated with sheath scraping. This may have lead to some reduction in sensitivity of the sheath scrapings.

Sample collection and handling had no effect on test specificity, with no false positive results from any of the control bulls with any of the treatments. We did not see the unspecific amplification products leading to difficulty in interpretation of results, as has been described

elsewhere (18,19). However, as this trial did not constitute a diagnostic test validation protocol, test sensitivity and specificity based on this data are likely to be overestimated (26).

The main feature of our findings was the decline in sensitivity of PCR testing of stored samples with time. This effect had not reached significance at 30 hours, possibly due to sample size, but was significant or highly significant in all treatments after 5 days of storage. The loss of sensitivity with time was not prevented by the addition of GuSCN. This may have been due to the choice of DNA preservative or the concentration that was used.

Besides DNA degradation, other causes for a decline in sensitivity with storage time are accumulation of inhibitory compounds or contamination of the sample (21). We cannot exclude either of these possibilities on the basis of the available data.

Evidence of the superiority of sheath washing over sheath scraping only approaches significance after sample storage for 5 days. Such a difference may be caused by less DNA being present in the sheath scraping samples initially, more inhibitory compounds in the sheath scraping samples, more rapid DNA deterioration in the sheath scrape sample, or a combination of these factors.

The loss of test sensitivity with increasing holding time was particularly noticeable on the last day of sampling where results for culture are also indicative of low numbers of organisms in the fresh samples (data not shown). As mentioned earlier, it is known that the numbers of organisms in the preputial cavity decline with breeding activity (10). Our observation of a reduction in numbers at the end of a period of intensive sampling may emulate the effect of mating activity. This supports the commonly-applied practice of spacing sample collections at weekly intervals for diagnostic purposes.

5.3 Trial design

While a 'gold standard' test for Tritrichomonosis utilising a single test does not exist, repeated cultures read by one of two different, experienced laboratory technicians were regarded as sufficiently strong evidence of the bulls' true status. This was the basis on which bulls were classified as disease-positive or disease-negative for the purposes of comparison with the results of the PCR test. Since the results of the PCR confirmed this status in all cases we are confident that it was a valid assumption.

The trial reported in Chapter 3.2 was not designed to fulfil the requirements of validation of a new diagnostic test, which have been reviewed by Greiner and Gardner (26). Rather, this

work is preparatory in that we sought to define how best to collect and handle field samples for submission for testing by the PCR method. Test validation will follow, utilising diagnostic samples submitted to the laboratory.

5.4   Other possible avenues of investigation

The sensitivity of the current PCR test requires further optimisation. Only when samples were collected by sheath wash and processed within 6 hours did the sensitivity reach 0,9. This is no better than direct examination and culture, and is inadequate as a single sample test.

The following are possible ways of increasing the sensitivity of the current test:

1.   Further optimisation of DNA extraction methods,

2.   Further optimisation of PCR,

3.   Improved methods of detecting amplification products,

4.   Addition of GuSCN at a higher concentration or use of an alternative DNA preservative, and

5.   Preventing death of organisms in the sample by suspension in a transport medium.

Considering the lack of any effect of the addition of GuSCN in our current work, the short delay with which our samples were extracted and the success which other laboratories have had with PCR without any special preservatives or transport media (A Reinisch, personal communication; R H BonDurant, personal communication), it seems unlikely that the poor sensitivity is caused by sample taking and transport factors.

New methods combining DNA preservation and extraction are worth investigating. One example is the treated paper technology, which allows a sample to be adsorbed onto a card where the DNA is extracted and preserved for prolonged periods[3]. This is a robust storage method, provided that cards are kept in a dry environment. A small piece of the card is used for the PCR reaction.

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[3] FTA Classic Card, Whatman BioScience

Based on the 8 uninfected animals used in this trial, the specificity of the PCR assay appears to be excellent. This requires confirmation on a greater number of animals from different herds. Specificity is also not altered by sample collection or storage conditions.

As both Tritrichomonosis and genital Campylobacteriosis are prevalent in South Africa and the same sample, namely preputial material, is preferred for both organisms, a single test which could detect the presence of both organisms would be highly advantageous. A multiplex PCR test has the potential to fulfil this requirement. Work is currently underway at the Agricultural Research Council's Onderstepoort Veterinary Institute to develop such a test.

5.5 Conclusions

Measures to improve the sensitivity and specificity of culture methods or a new diagnostic method which is highly specific and sensitive utilising a single sample would represent a significant improvement in the ability of the veterinary profession and the livestock industry to curb Tritrichomonosis. The current work on comparison of sheath wash and scraping methods for direct examination and culture supports the practical advantages of sheath scraping but does not confirm any increase in diagnostic accuracy. From this work it would appear that increased diagnostic accuracy will have to be sought amongst newer diagnostic methodologies, such as PCR and allied molecular techniques.

Samples for PCR testing may be collected by sheath washing or scraping, although samples collected by sheath washing may be marginally superior to those collected by scraping when samples are stored prior to testing.

The addition of the chaotropic agent guanidinium thiocyanate at the concentration tested had no effect on test sensitivity.

DNA extraction must be done on the same day to attain high diagnostic rates from positive samples.

The sensitivity of the current PCR test used for this work is inadequate to fulfil the expectations of a once-off test. Additional optimisation is therefore required.

Should further test validation confirm the high specificity of the current PCR test, it will be a valuable adjunct to culture methods, assisting in eliminating false positive results.

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Article — Artikel

Collection of preputial material by scraping and aspiration for the diagnosis of *Tritrichomonas foetus* in bulls

P C Irons[a], M M Henton* and H J Bertschinger[b]

ABSTRACT

Two trials were carried out to assess the diagnostic sensitivity and practicability of preputial scraping as a method of collecting preputial material from bulls infected with *Tritrichomonas foetus*. In the 1st trial, preputial material was collected by simultaneous scraping and aspiration from 3 infected and 1 uninfected bull 10 times over a 5-week period. In the 2nd trial, samples from 5 infected bulls were collected by both sheath washing and scraping on 6 occasions, while 8 uninfected animals were sampled 3 times. Samples were cultured using a modified Trichomonas culture medium (Oxoid). In the first trial, 29 of 30 samples from infected bulls were found to be positive. In the second trial, 83 % of samples collected by both methods tested positive. In neither trial were any samples from the control bulls found to be positive. Scraping was found to be quick and safe, and offered advantages over preputial washing in that urine contamination was easily avoided, samples were smaller and more concentrated and contamination was reduced. It may, however, be subject to greater operator variability than sheath washing. It is concluded that preputial scraping is as effective as washing and represents a suitable alternative for the collection of material for direct examination and culture of *Tritrichomonas foetus.*

Key words: bull, diagnosis, preputial scraping, preputial wash, *Tritrichomonas foetus, venereal disease.*

Irons P C, Henton M M, Bertschinger H J **Collection of preputial material by scraping and aspiration for the diagnosis of *Tritrichomonas foetus* in bulls**. *Journal of the South African Veterinary Association* (2002) 73(2): 66–69 (En.). Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110 South Africa.

INTRODUCTION

Tritrichomonosis is a major source of economic losses for the beef industry in South Africa. The disease is characterised by embryonal and foetal death resulting in lowered calving percentages, prolonged intercalving periods, heifers failing to conceive, sporadic abortions, aberrant oestrus cycles, and the presence of post-coital pyometra in some animals. Prevalence of the disease in herds in various regions of southern Africa range from 0 to 46 %[6,13,17,22] (Bloemfontein Veterinary Laboratory, pers. comm., 1999; Louis Trichardt Veterinary Laboratory, pers. comm., 1999; S M Pefanis, Vrede Veterinary Laboratory, pers. comm., 1999).

Despite awareness of the disease and proven control programmes based on well-researched epidemiological principles, the disease remains problematic. One factor contributing to this situation is the lack of a highly sensitive and specific test for carrier animals. Culture of preputial material from bulls is the most commonly-used test. While this technique yields sensitivities of almost 100 % in some instances, diagnostic rates in the 70–90 % range are more commonly reported[1,3,5,9,12,14,18–21,23,25,27,28]. Factors contributing to reduced sensitivity include remoteness of farms, fractious animals, sample and animal identification errors, collection of large numbers of samples at the same time, sample contamination and overgrowth, and inconsistent laboratory techniques[7,20]. It is therefore necessary to test a bull repeatedly to obtain a reliable result, a requirement which is not universally adhered to due to cost and inconvenience.

Sheath washing and scraping are the 2 most widely used methods for the collection of preputial material. Material may also be collected by rinsing the liner of an artificial vagina after semen collection[11]. Scraping is most commonly performed with a dry Perspex artificial insemination (AI) pipette attached to a rubber bulb or syringe, which enables aspiration of preputial smegma as the preputial lining is scraped. Custom-made instruments for the collection of preputial scrapings have not shown to have any advantage[15]. Although both sheath washing and sheath scraping have been well described[2,3,24,27] and do not differ in effectiveness[23], sheath washing remains the predominant technique used by veterinary practitioners and animal health technicians in South Africa.

The aim of this trial was to show that scraping and aspiration is a practical method of collecting preputial material for testing bulls for the presence of *Tritrichomonas foetus* infection, and that samples collected by this method achieve high diagnostic sensitivity when subjected to culture.

MATERIALS AND METHODS

In Trial 1, 3 adult Bonsmara bulls, which were known to be *Tritrichomonas foetus* carriers, and 1 uninfected 2-year-old Jersey bull were included. Preputial material was collected on 10 occasions over a 5-week period, with a mean interval of 3.2 days between collections. Collection was by means of scraping and simultaneous aspiration using a dry perspex AI pipette (AI pipettes, Kyron Laboratories) connected to a sterile disposable 20 ml hypodermic syringe with a silicon-rubber tube. For collection, bulls were restrained in a sturdy crush with a neck clamp while an assistant applied a tail-grip. Additional restraint consisted of tying one back leg or the application of low-level electrical stimulation delivered by an electro-ejaculator probe placed in the rectum. This was only necessary in a few instances when the reaction of the bull placed the operator at risk.

The technique of collection was as follows: the collection apparatus was held in one hand by grasping the syringe. The tip of the pipette was guided into the caudal reaches of the preputial cavity and manipulated vigorously with an in-and-out movement while suction was applied with the syringe. The tip of the pipette was guided to different areas of the preputial membrane and glans penis using the other hand (Fig. 1). After an average of approximately twenty strokes

[a]Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110 South Africa.
E-mail: pirons@op.up.ac.za
[b]Veterinary Wildlife Unit, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110 South Africa.
*Present address: Golden Vetlab, PO Box 1537, Alberton, 1450 South Africa.

Fig. 1: **Collection apparatus in the preputial cavity of a bull.**

the pipette was withdrawn and the contents inspected. If insufficient cloudy material was present the procedure was repeated for a longer period.

The material was then transferred to a plastic tube containing approximately 4 mℓ phosphate-buffered saline (PBS Dulbeco, Onderstepoort Biological Products) (Fig. 2). The material in the pipette and syringe was flushed into the PBS by repeatedly aspirating the medium into the pipette. New collection equipment and disposable latex gloves were used for each bull.

Tubes were marked with a sample number only, placed in a polystyrene cool box with a frozen ice pack and transported to the ARC-Onderstepoort Veterinary Institute within 2 hours of collection. Laboratory staff were unaware of the origin of each sample. Samples were cultured in a medium consisting of Trichomonas medium (CM161, Oxoid Limited), horse serum, distilled water and antibiotics. Cultures were examined by direct microscopy.

In Trial 2, 5 positive bulls on 2 large commercial farms were sampled by sheath washing and scraping on 6 occasions over a period of 18 days. The washing was done first by instilling 50 mℓ PBS into the preputial cavity through a sterile latex tube, massaging the preputium vigorously for approximately 100 strokes and then siphoning the fluid back into the
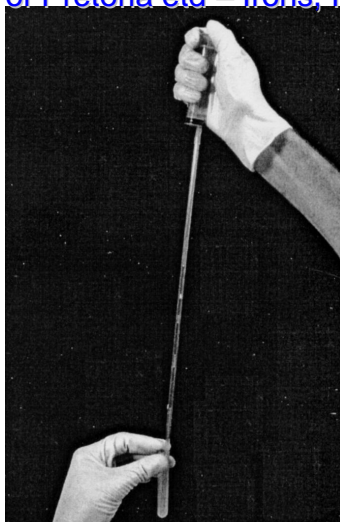

Fig. 2: **Transferring the contents of the pipette and syringe to a tube containing phosphate-buffered saline.**

sample bottle through the tube. This was followed by sheath-scraping samples, which were collected as described in Trial 1. Eight uninfected bulls were sampled 3 or more times each. Four of the control bulls were on one of the farms mentioned above, while 4 were housed semi-intensively in the Faculty of Veterinary Science's teaching animal unit. Samples were collected by 1 of 2 operators on each occasion. Samples were chilled and transported to the laboratory within 6 or 24 hours. Only a small portion of each sample was used for culturing and the remainder for molecular diagnostic techniques.

A two-tailed Chi-square test was used to test for differences between the samples collected using the 2 sampling methods.

RESULTS

Besides some mild discomfort during collection, no bull showed any adverse reaction to the repeated collection of material at short intervals.

In all cases, sufficient opaque whitish

or bloody, mucoid material could be obtained using scraping. Collection of the sample could generally be accomplished on the 1st attempt without additional restraint of the bull. Adequate samples could usually be obtained without the use of the free hand to guide the tip of the pipette, which enhanced operator safety. As the trial progressed the time required for the collection of sheath scrapings reduced.

In Trial 1, 29 of 30 samples collected from the infected bulls were found to be positive on laboratory examination, giving a sensitivity of 0.96 with a 95 % confidence interval of 0.83–0.99. The 1 negative result was obtained on the 8th collection. None of the 10 samples from the uninfected control bull was positive.

The results of tests on infected bulls in Trial 2 are summarised in Table 1. Twenty-four of the 29 samples collected by both methods tested positive (0.83). Twenty-one of the 29 samples were in agreement. One bull was unavailable for testing on one occasion. No positive results were obtained for the control animals.

There was a significant difference in sensitivity between operators in the sheath-scraping samples in Trial 2 (9/14 compared to 15/15, $P < 0.05$).

DISCUSSION

The collection of preputial material by scraping with simultaneous aspiration has several practical advantages over preputial washing. Speed of collection, the ability to collect the sample without an assistant, and the fact that contamination of the sample by urine is easily avoided, are significant advantages. Although *T. foetus* organisms do survive in urine[16], dilution of the cellular content of the sample is undesirable. The use of disposable collection equipment eliminates the possibility of cross-contamination of samples or of the transmission of

Table 1: **Culture results for 5 infected bulls collected by sheath washing and sheath scraping on 6 successive occasions.**

Bull	Sample: Operator: Transport time:	1 A 6 hours	2 A 24 hours	3 B 6 hours	4 A 24 hours	5 B 6 hours	6 B 24 hours
9763	Wash	−	−	+	−	+	−
	Scrape	+	+	+	+	−	−
9924	Wash	+	+	+	+	+	+
	Scrape	+	+	−	+	+	+
9968	Wash	−	+	n/a	+	+	+
	Scrape	+	+	n/a	+	+	−
99001	Wash	+	+	+	+	+	+
	Scrape	+	+	+	+	+	−
BIG	Wash	+	+	+	+	+	+
	Scrape	+	+	+	+	+	+

n/a: not available for sampling.

organisms between successive animals. Mechanical transmission of *T. foetus* is a potential hazard whenever infected animals are examined[10]. Special receptacles for the larger volume of PBS need not be ordered beforehand, and the smaller volume of the sample obtained by scraping facilitates sample transport and laboratory processing. Lastly, as the sample is collected primarily from the caudal reaches of the preputial cavity there is less likelihood of contamination from the environment, particularly in bulls that have gross contamination of the anterior portion of the preputial cavity caused by habitual eversion of the lamina interna. This is in agreement with findings of other workers sampling for *Campylobacter fetus*[26].

The presence of blood in the sample did not constitute a problem, which confirms the findings of other authors[2]. Roughening the tip of the pipette, as is commonly advocated, is not necessary to obtain a satisfactory sample. The fact that scraping was done after washing on all occasions may have biased the results in Trial 2 by reducing the number of organisms in the preputial cavity.

The high diagnostic sensitivity attained in Trial 1 is ascribed to sampling technique, close proximity to the laboratory facilitating rapid delivery of samples, optimal handling facilities, experienced staff and the small number of bulls sampled on any one occasion.

The lower diagnostic sensitivity attained in Trial 2 for both methods is ascribed to less optimal collection conditions and to the fact that only a small volume of each sample was available for direct examination and culture. While some authors have found decreased sensitivity with a 24 h delay in processing of preputial samples[15,25], we could not demonstrate any effect.

There was a tendency for more false negative tests towards the end of the sampling period in Trial 2 but not in Trial 1. This has also been observed by other authors, who ascribed it to an increase in bacterial contamination in the sheath after repeated sampling[8]. It is known that more false negative cultures are obtained from bulls during periods of active breeding, presumably due to a reduction in numbers of organisms in the preputial cavity[4]. A similar reduction in the number of organisms due to frequent sheath washing is one plausible explanation for our observation. Alternative explanations include the increased exposure of organisms to blood containing antibodies and other serum factors by virtue of repeated scrapings and an increase in bacterial contamination of the preputial cavity.

The difference between operators in the sensitivity obtained by sheath scraping suggests that this technique is more prone to operator variability than sheath washing, although larger sample sizes may have demonstrated differences in the latter method as well. This warrants further investigation. If this is the case, thorough training of operators would be necessary to attain consistent diagnostic accuracy.

Whether sheath scraping or washing are equally suited to the collection of samples for molecular diagnostic techniques requires further investigation.

It is concluded that preputial scraping is a suitable alternative to preputial washing for the collection of material for culture of *Tritrichomonas foetus*, offering several important practical advantages over the latter method. Routine use of this technique by competent operators is expected to render at least diagnostic rates equal to preputial washing.

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