Dissertations & Theses in Veterinary and Biomedical Science

Veterinary and Biomedical Sciences, Department of

5-2010

Tactics for Identifying and Eliminating Tritrichomonas foetus from Infected Beef Herds

Jeff D. Ondrak
University of Nebraska at Lincoln, jondrak@gpvec.unl.edu

TACTICS FOR IDENTIFYING AND ELIMINATING *TRITRICHOMONAS FOETUS*

FROM INFECTED BEEF HERDS

by

Jeff D. Ondrak

A THESIS

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Master of Science

Major: Veterinary Science

Under the Supervision of Professor James E. Keen

Lincoln, Nebraska

May, 2010

TACTICS FOR IDENTIFYING AND ELIMINATING *TRITRICHOMONAS FOETUS*

FROM INFECTED BEEF HERDS

Jeff D. Ondrak, M.S.

University of Nebraska, 2010

Advisor: James E. Keen

The protozoan, *Tritrichomonas foetus* (TF), has been recognized as a cause of bovine infertility for more than 100 years (Skirrow and BonDurant, 1988). As an obligate parasite of the bovine reproductive tract its control and eradication seems achievable (Harding, 1950). However, this disease continues to trouble US cattle producers and a recent epidemic in the Western US has lead to increased interest in research and regulatory efforts (Cima, 2009).

Outbreak investigations were carried out on three Nebraska ranches to assess the efficiency of currently available diagnostic tests, culture, gel polymerase chain reaction (PCR), and real time PCR (rtPCR), in identifying TF infected bulls in known TF infected herds with the following objectives:
 (1) to compare the agreement of the three assays for classifying the status of individual preputial specimens.
(2) to compare the agreement of the three assays in identifying TF infected bulls based on three sequential samples.
 (3) to correlate cow herd pregnancy percentages with TF herd bull prevalence.

Comparisons of diagnostic tests were conducted using Cohen's Kappa statistic and McNemar's paired sample Chi square test p values. Simple linear regression was

used to assess the relationship between non-pregnancy percentages and prevalence of TF positive bulls.

No significant differences between culture and gel PCR for individual specimen and bull TF classification were found. Real time PCR had a high rate of apparent false positives relative to culture and gel PCR for individual specimen and bull TF classification. However, all assays required multiple, sequential specimens to adequately identify all TF infected bulls in the study herds. Cow non-pregnancy rates correlated linearly with TF positive bull prevalence.

These studies indicate similar diagnostic assay performance for culture, gel PCR, and real time PCR which suggests opportunities for improved TF control may be found by focusing on pre-analytical aspects of diagnostic TF detection such as consistent bull identification, optimization of specimen collection techniques, and pre-incubation specimen handling factors.

Table of Contents

List of Tables

List of Figures

Glossary of Terms

AV – Artificial vagina
BGPS – Beef extract-glucose-peptone-serum medium
bp – base pair
CI – Confidence interval
cm – Centimeter
CPI – Consumer Price Index
CPLM – Cysteine-peptone-liver extract-maltose-serum medium
DEIA – DNA enzyme immunoassay
DNA – Deoxyribonucleic acid
EU – European Union
ITSR – Internal transcribed spacer region
KH_2PO_4 – Monopotassium phosphate
K_2HPO_4 – Dipotassium phosphate
mDM – Modified Diamond's medium
mL – Milliliter
mm – Millimeter
PCR – Polymerase chain reaction
PBS – Phosphate buffered saline
rRNA – Ribosomal riboneucleic acid
rt PCR – Real time PCR
TF – Tritrichomonas foetus
US – United States of America
WADDL – Washington Animal Disease Diagnostic Laboratory

Chapter 1

Overview of Trichomoniasis in Cattle

Introduction

Trichomoniasis is a venereal disease of cattle caused by the protozoan *Tritrichomonas foetus* (TF). Many well-written reviews of bovine trichomoniasis can be found in veterinary literature, and readers should refer to these publications for complete, in-depth discussions of the disease (BonDurant and Honiberg, 1994; Skirrow and BonDurant, 1988; Rae and Crews, 2006). The intent of this thesis is to discuss optimization of tactics for identifying and eliminating trichomoniasis from extensively managed TF infected beef herds. Only those aspects relevant to these tactics will be discussed fully in this manuscript while emphasizing the importance of accurate diagnosis of infection in bulls.

Historical perspective

History - Trichomonads were first reported as a cause of bovine infertility by Kunstler in France in 1888 (Skirrow and BonDurant, 1988). In 1900 Mazzanti of Italy reported isolating protozoa consistent with TF from three female bovine uteri after they were slaughtered for chronic reproductive failure and concluded the organism which he named *Trichomonas utero-vaginalis vitalae* was the cause of the infertility (Skirrow and BonDurant, 1988). However, not until the mid-1920's and later were trichomonads again linked to bovine reproductive failure; this time by multiple investigators around the world (Dikmans and Poelma, 1938) with a consensus naming the parasite *Tritrichomonas foetus* (Schmidt, 1937).

The first report of trichomoniasis in the US was in 1932 by Emmerson (1932) in Pennsylvania dairy cows, but not until 1958 was TF reported in western US beef herds (Fitzgerald and Johnson, 1958). Currently TF has been practically eliminated from intensively managed cattle populations around the world where the management includes limited comingling of cattle and artificial insemination is commonly used for breeding while it remains endemic in herds managed under range conditions with natural service breeding as found in the western US (Skirrow and BonDurant, 1988).

Prevalence –A range of prevalence estimates for US geographic locations and for infected bulls within TF infected herds have been reported in the literature and are summarized in Table 1.1. All prevalence estimates are based on bull specimen culture results, and the protocols for obtaining, maintaining, and examining these cultured specimens if reported at all leave the accuracy of many estimates in question.

Hall et al. (1993) reported the data from the state of Idaho TF regulatory bull testing program from 1989 through 1991. For the 1989-90 bull testing season 123 of 2,794 (4.4%) herds held at least one TF infected bull. The following testing season, 1990-91, 67 of 2,226 (3.0%) herds were found with at least one TF infected bull. Unfortunately the author did not report the sample collection and handling protocol for this program which may have affected the accuracy of the prevalence estimate by under detecting TF positive bulls and therefore TF positive herds.

Two studies which appear to provide reliable estimates of prevalence of TF infected herds in specific US geographic locations were conducted at the state-wide level

Table 1.1 –*Tritrichomonas foetus* US Prevalence Summary. N/R = not reported; N/A = not applicable.

Study Period	Testing Locale	Sampling Site	Bull Age	Herd n	Bull n	Samples/ Bull	Premises Prevalence	Bull Prevalence	Sample Method	Test Method	Reference
Fall 1954-Spring 1956	Utah Idaho Colorado	Grazing Association	N/R	8	383	N/R[a]	4(50%)	23(6.0%) Range: 8/142(5.6%)-8/44(18.2%)	Saline douche	Culture; modified Plastridge media; examined day 4 or 5	Fitzgerald et al., 1958
1956-1963	7 Western US states[b]	Grazing Association	N/R	34	828	N/R[a]	9(26%)	62(7.5%)	Modified douche	Culture; modified Plastridge media; examined day 3 or 4	Johnson, 1964
Nov 1977-Jun 1978	Oklahoma	Central Oklahoma auction markets	>2 yrs	N/A	280	1[c]	N/A	22(7.8%)	Cotton swab of prepuce	Culture; thyio-glycollate broth; examined at 24 hrs	Wilson et al., 1979
5 days in 1979	South Florida	Florida abattoir	<3->5 years	N/A	109	1[c]	N/A	8(7.3%)	Dry pipette aspiration	Culture; modified Plastridge media; examined days 1&2	Abbitt and Meyerholz, 1979
1984-1987	Nevada	Abattoir & private ranches	N/R	78	2,389	1[c]	26.7-44.1% by year	4.7%; range 2.7-13.5% by year	N/R	N/R	Kvasnicka et al.,1989
N/R; Publish Date: Aug 1985	California	Private ranches	≥3 years	3	195	3	100%[d]	total 75 (38.5%); 52/149(34.9%) 7/18(38.9%) 16/28(57.1%)	Dry pipette aspiration	Culture; Diamond's media; examined days 2,4, & 7	Skirrow et al., 1985

[a] N/R indicates mostly likely a single sample was collected from each bull, but multiple samples may have been taken. The actual number of samples per bull was not reported by the source.
[b] Utah, Idaho, Wyoming, Nevada, Colorado, Arizona, and New Mexico.
[c] Although the actual number of samples per bull was not explicitly stated in the source, the study protocol suggests a single sampling event per bull.
[d] These 3 ranches were initially investigated because of reproductive failure believed to be due to *Tritrichomonas foetus*.

Table 1.1 (cont'd) – *Tritrichomonas foetu*s US Prevalence Summary. N/R = not reported; N/A = not applicable.

Study Period	Testing Locale	Sampling Site	Bull Age	Herd n	Bull n	Samples/ Bull	Premises Prevalence	Bull Prevalence	Sample Method	Test Method	Reference
Apr 1988-Jul 1989	California	Private ranches	<2 to >6 years	57	729	1[c]	9(15.8%)	39(4.1%); range in TF + herds: 4.0% - 38.5%	Dry pipette aspiration	Culture; modified Diamond's media; examined alternate days through day 9	BonDurant et al., 1990
Fall 1988-winter 1990	14 states[e] and Canada	Nebraska and Colorado abattoirs	N/R	N/A	2,909	1[c]	N/A	5(0.172%)	Dry pipette aspiration	Culture; modified Diamond's media; examined days 1,2, & 3.	Grote-lueschen et al., 1994
Oct 1989-Jun 1990	Idaho	Private ranches	N/R	2,794	20,375	N/R[a]	123(4.4%)	332(1.63%); in TF+ herds 10.4% + bulls	N/R	N/R	Hall et al., 1993
Sep 1990-Jun 1991	Idaho	Private ranches	N/R	2,226	17,757	N/R[a]	67(3.0%)	131(0.74); in TF+ herds 9.1% + bulls	N/R	N/R	Hall et al., 1993
Jun 1995-Jan 1996	Florida	Private ranch	1-10 years	11	1,383	≥1[f]	9(81.8%)	165(11.9%) in TF+ herds: 0.9-35.9%	Dry pipette aspiration	Culture; commercial trans-port and culture media; examined days 1,2, & 4or 5	Rae et al., 1999
Nov 1997-Oct 1999	Florida	Private ranches	2-15 years	59	1,984	1[c]	17(28.8%)	119(6.0%) range in TF+ herds:1.8-27.0% (mean =11.9%)	Dry pipette aspiration	Culture; modified Diamond's media; examined days 1,2, & 5	Rae et al., 2004

[a] N/R indicates mostly likely a single sample was collected from each bull, but multiple samples may have been taken. The actual number of samples per bull was not reported by the source.

[c] Although the actual number of samples per bull was not explicitly stated in the source, the study protocol suggests a single sampling event per bull.

[e] Colorado, Idaho, Iowa, Kansas, Missouri, Montana, Nebraska, New Mexico, North Dakota, Oklahoma, South Dakota, Texas, Utah, Wyoming.

[f] Bulls were repeatedly tested until no new positive bulls were found in each management group with TF positive bulls being removed from the herd after their initial positive test. Therefore some bulls were sampled only once while other bulls were sample multiple times.

in California (BonDurnant et al., 1990) and Florida (36 Rae et al., 2004). Herds were invited to participate voluntarily based on stratification by herd size which may have introduced selection bias into the study. However, the culture collection, incubation, and examination protocols suggest these studies may provide the best estimates of TF infected herd prevalence to date. In 1988 and 1989 investigators found 9 of 57 (15.8%) sampled herds in California with at least one TF positive bull. A survey of Florida cattle operations from 1997 through 1999 found 17 of 59 (28.8%) herds with at least one infected bull.

These studies also report prevalence of TF infected bulls in TF infected herds and across populations of bulls in general. BonDurant et al. (1990) found a mean prevalence of TF positive bulls across all bulls sampled in the California project of 4.1%, but a range of prevalence in TF infected herds of 4.0 to 38.5%. In Florida the prevalence of TF positive bulls across all bulls tested was 6.0%, but the mean prevalence of TF positive bulls in TF infected herds was 11.9% with a range of 1.8 to 27.0% (Rae et al., 2004). It is interesting to note the overall TF positive bull prevalence of this study was similar to the prevalence reported from a survey of 109 bulls sampled at a Florida abattoir in 1979 of 7.3% (Wilson et al., 1979). Both Florida prevalence estimates were considerable higher than a study conducted in Colorado and Nebraska abattoirs (Grotelueschen et al., 1994) which found only 0.172% of bulls positive for TF when cultured. The lower prevalence in this study may represent a regional difference in TF prevalence due to differences in herd management between regions, varying levels of regulatory TF control programs, and fluctuations in TF prevalence related to the cyclic nature of the disease. All four studies

relied on single bull samples which may lead to under detection of TF positive bulls (Kimsey et al., 1980).

A report (Skirrow et al., 1985) of an investigation into reproductive failure ascribed to TF on three extensively managed California ranches identified 52, 7, and 16 TF infected bulls of 149, 18, and 28 total bulls on each respective ranch for a TF positive prevalence of 34.9, 38.9, and 57.1% respectively. A separate investigation (Rae et al., 1999) into reproductive failure on a large Florida ranch (1383 bulls) found a mean TF positive bull prevalence for the eleven distinct management units on the ranch of 11.9%, but a range of TF positive bull prevalence on the TF infected units of 0.9 to 35.9%.

These prevalence estimates give some indication of the level of TF which may be currently present in the US cattle population at the herd level. However, an accurate determination of current TF prevalence in the US has not been reported to the author's knowledge. The reported within-herd TF positive bull prevalence for TF infected herds provides an estimate of expected TF positive bull prevalence when investigating naturally occurring TF outbreaks.

Economics – Wilson et al. (1979) attempted to estimate the cost of trichomoniasis to the Oklahoma cattle industry in 1979. Using TF prevalence data from a survey of bulls passing through an Oklahoma auction market to estimate reproductive loss in virgin heifers being bred to produce their first calf he concluded the cost to the Oklahoma cattle industry was $2.5 million dollars per year. This report also estimated additional loses to the industry through other direct and indirect costs could lead to an overall cost in excess of $7 million per year. Consumer Price Index (CPI) adjustment of these amounts to the

current dollar value produced modern day values of $7.4 to 20.8 million per year
(Williamson, 2009).

In 1958 Fitzgerald et al. (1958) attempted to quantify the economic impact of TF
on the cattle operations using results from surveillance testing of beef herds in western
US states and assumptions based on knowledge of the impact of TF on the reproductive
rates in cattle. He concluded each TF infected bull in a herd cost the herd owner
approximately $800.00. Consumer Price Index adjustment of this amount to the current
dollar value produced a modern day value of $5955.82 per bull (Williamson, 2009). This
amount is likely an underestimate of the true cost as it only accounted for lost calf
production due to cows failing to produce a live calf or reduced weaning weights for
transiently TF infected cows that produced live calves but later in the calving season due
to delayed breeding. Other factors not accounted for in this estimate which would have
increased the cost of infection per bull include feed and other maintenance costs for non-
productive cows, replacement costs of TF infected bulls and non-productive females, and
treatment/testing costs to control TF in the herd.

Rae (1989) utilized a computer spreadsheet simulation model to assess the
financial impact of TF on individual cattle operations. The model predicted a 14 to 50%
reduction in calf crop, a 12 to 30 day longer breeding season, a 5 to 12% reduction in the
suckling period, a reduction of 4 to 10% in monetary return per calf born, and a 5 to 35%
reduction in the return per cow confined with a fertile bull. Because production costs
vary greatly between cattle operations a single dollar value cannot be placed on overall

reduction in the return per cow. However, a 5 to 35% reduction in return is significant in light of the typically small profit margins found on most cattle operations.

A computer spreadsheet simulation model was used by Villarroel et al. (2004) to evaluate the effect of TF vaccination on reproductive efficiency in beef herds. The model estimated a reduced income of up to 23% for a 300 cow herd when TF was left uncontrolled in the herd which agrees closely with Rae's findings shown previously. Using this estimate Villarroel et al. conservatively estimated an $11.6 million loss to the California cattle industry.

Etiology

Tritrichomonas foetus is a spindle- to pear-shaped single-celled protozoa with three anterior flagella, an undulating membrane along the length of its body containing an accessory filament at its margin, and a single posterior flagellum (Figure 1.1).
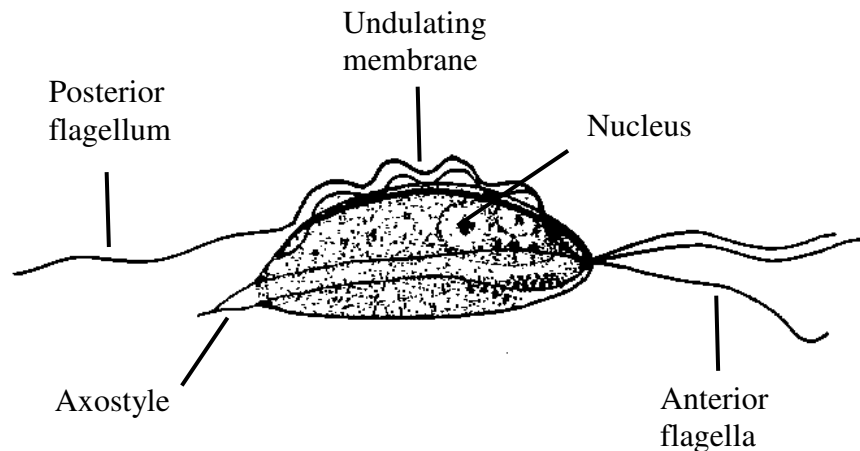
Figure 1.1 – Diagrammatic Representation of TF. (Adapted from Levine, 1985)

Individual cells range in size from 9-25 micrometers (μm) long and 3-15 μm wide (BonDurant and Honiberg, 1994) and exhibit a jerky, rolling motility (BonDurant, 1985).

Although morphologic features are not easily or typically seen under standard light microscopy, glimpses of any distinctive structures coupled with the characteristic erratic motility pattern and appropriate shape and size parameters are strong indicators for TF identification by compound microscopic examination.

Agglutination, passive hemagglutination, and skin tests have identified different serotypes of TF with the most commonly discussed serotypes being Brisbane, Manley, and Belfast (BonDurant and Honiberg, 1994). However, BonDurnant and Honiberg (1994) reported vaccination trials performed by Floret resulted in homologous and heterologous resistance to infection when a single serotype vaccine was given to heifers. This suggests different antigenic types do not play an important role in the development of immunity to this parasite (BonDurant and Honiberg, 1994) and no published evidence could be found suggesting variations in serotypes influences TF diagnostic tests.

Pathogenesis

Transmission – Natural transmission of TF is considered to be strictly venereal and occurs during coitus between infected and uninfected cattle (Bartlett, 1947; BonDurant and Honiberg, 1994). The rate of natural transmission is high with a majority of naïve females becoming infected after a single exposure through coitus with an infected bull (Hammond and Bartlett, 1945; Parsonson et al., 1976) with as few as 200 TF organisms reliably infecting susceptible cows (Clark et al., 1977). Clark et al. (1974b) found 10^4 TF organisms experimentally inoculated into the prepuce of bulls could reliable produce chronically infected bulls with some bulls becoming TF infected from inoculums containing as few as 10^2 TF.

Other means of transmission are possible as described by Goodger and Skirrow (1986) on a large California diary where vaginal examinations for estrus detection without proper sanitation between cows lead to transfer of TF from infected to non-infected cows. Murname (1959) used a glass rod to transfer vaginal mucus from infected to non-infected cows which resulted in 10 of 10 non-infected cows becoming infected with TF.

Clark et al. (1977) investigated possible transmission by flies, direct contact of a non-infected cow's vulva with an infected cow's vulva or tail, and passive transfer via a non-infected bull's penis. Results of this study indicated successful TF transmission through superficial contamination of cows' vulvas as expected by flies or incidental, direct contact was unlikely. Non-infected bulls were found to be capable of transferring the organism from infected to non-infected cows at a low rate of transmission and only when the time interval between coitus with the infected and non-infected cows was less than 20 minutes.

Tritrichomonas foetus is capable of surviving under conditions consistent with temperatures used to maintain frozen semen for artificial insemination (Clark et al., 1971; Levine and Marquardt, 1955; Blackshaw and Beattie, 1955). Jeffries and Harris (1967) reported freezing TF for 6 months did not reduce its virulence for producing lesions in mice when injected subcutaneously. This suggests frozen semen contaminated with TF at the time of cryopreservation could lead to transmission of the parasite through artificial insemination without direct bull to cow contact.

While non-venereal methods of transmission are possible as described above and should serve as a warning to veterinarians, livestock producers and others working with potentially TF infected animals, it appears non-venereal transmission is a rare event with transmission through natural breeding as the primary means of dissemination of TF.

Pathology in females – *Tritrichomonas foetus* can be isolated from the female bovine reproductive tract as soon as four days after introduction (Murname, 1959), but does not appear to interfere with conception or maternal recognition of pregnancy (BonDurant, 1985) or express any macro or microscopic lesions in the reproductive tract until after 50 days gestation (Parsonson et al., 1976). After day 50 Parsonson et al. (1976) were able to isolate TF from the surface secretions of the vagina and cervix and to a lesser degree uterus and oviducts of infected cows and began seeing mild inflammatory changes with eventual fetal loss in a majority of the infected females up to 95 days post exposure.

The exact mechanism for fetal loss is not clearly understood. Examination of aborted fetuses found fetal damage through TF penetration of pulmonary and gastrointestinal mucosal epithelium and underlying connective tissue and lymphatics (Rhyan et al., 1995b) which explains the necrotizing enteritis and pyogranulamatous bronchopneumonia found in another study (Rhyan et al., 1988) examining TF aborted fetuses. In the 1988 report Rhyan et al. also identified placentits associated with TF infection which was supported by a later study (Rhyan et al., 1995a) that found TF associated with placental tissue. These changes and ultimately fetal death may be the result of cytotoxic and hemolytic affects of TF as described by Burgess et al. (1990)

following adhesion to target cells in the fetus and reproductive tract (Burgess and McDonald, 1992).

Most fetal loss occurs within the first five months of gestation followed by a period of two to six months infertility as the immune system clears the parasite from the reproductive tract (BonDurant, 1985). The precise immunological mecahanism for TF clearance from the female bovine reproductive tract is not known. Aydinug and coworkers identified possible methods for the bovine immune response to TF in two studies which found antibodies and complement activated by TF surface antigens promoted protection from TF (Aydintug et al., 1990) and maximal killing of TF occurred when the trichomonads were opsonized with antibodies and complement before exposure to neutrophils (Aydintug et al., 1993).

The immune response appears to lead to an amnestic response to repeated TF infection as demonstrated in multiple studies. Clark et al. (1986) found the length of time cows remained infected decreased on subsequent exposures with mean infection lengths for first, second, and third exposures being 20.3, 9.8, and 11 weeks respectively. In another study (Skirrow and BonDurant, 1990) trichomonads were cleared from previously TF infected heifer's reproductive tracts within 3 weeks of reinfection. Murname (1959) reported six cows were resistant to reinfection when exposed to TF 4 months after recovery from a previous TF infection. This immunologic memory appears to be short lived as shown in a report by Clark et al. (1983) which estimated the length of partially protective immunity to be less than 15 months based on productivity of the cows in the face of TF exposure.

Complete clearance of TF from the female reproductive tract in a relatively short period of time, 5 to 20 weeks, is the typical outcome of infection although some exceptions occur (Murname, 1959). Pyometra, an accumulation of purulent debris in the uterus, and chronically infected cows are the most notable exceptions to this rule. Pyometra may be one of the earliest clinical signs of TF infection in the cow herd (Rae and Crews, 2006) and the purulent debris in the uterine lumen frequently contains TF (Emerson, 1932). Chronically TF infected cows have been reported to carry infections for as long as 300 days (Mancebo et al., 1995) and 22 months post breeding (Alexander, 1953). Chronic infections have been carried through normal pregnancy with TF isolated up to 9 weeks (Skirrow, 1987) and 63 to 97 days after delivering a normal appearing calf (22 Goodger JAVMA 1986).

Pathology in males –In 1943 Hammond and Bartlett (1943b) contradicted earlier work with *Tritrichomonas foetus* by suggesting the organism was primarily an inhabitant of the bull's preputial cavity and not routinely found in other locations of the male's reproductive tract. This was supported by Parsonson and colleagues (1974) through in-depth cultural, macro, and microscopic examinations of reproductive tracts from TF infected bulls from which they concluded TF was restricted to the secretions of the penis and prepuce, did not penetrate penile or preputial epithelium, and caused no detectable gross or microscopic pathological changes. Rhyan and associates (1999) found the organism in the superficial layers of the penile and preputial epithelium through histological examination of TF infected bull reproductive tracts, but failed to detect invasion of the basement membrane or dermis of the these structures.

The absence of tissue invasion may be the reason a limited immune response is seen in TF infected bulls. A study (Soto and Parma, 1989) involving artificially infected bulls found no agglutinating antibodies in the preputial cavity following exposure to TF and a response to intradermal injection of TF antigen three months post exposure similar to non-infected males suggesting limited systemic immune response to the organism. A more recent study (Rhyan et al., 1999) found significantly higher levels of specific anti-TF antibodies in preputial secretions of TF infected bulls than of non-infected bulls which were the result of local antigen uptake and processing and antibody deposition. This agrees with an earlier study (Bier et al., 1977) of general bull reproductive tract immune function whose findings were consistent with local synthesis and section of immunoglobulin in the prepuce. In spite of the elevated anti-TF antibodies present in preputial secretions in the Rhyan study (1999) nearly all infected bulls remained infected through post mortem examination suggesting the immune response was inadequate for TF elimination from the preputial cavity.

The lack of pathologic changes and failure of the immune response to eliminate TF from the preputial cavity leads to chronically infected bulls especially in older bulls. One factor which may lead to an increased risk for older bulls to become TF carriers is their greater opportunity to contract the infection through breeding activity based on their longevity in the herd and their hierarchical dominance. The disproportionate breeding activity between bulls was well described by Van Eenennaam et al. (2007) who found five of 27 sires in a single breeding unit produced over 50% of the 625 calves in the unit with 9 of the ten sires producing no offspring being yearling bulls.

Longevity and dominance do not completely explain the age related phenomenon as shown by reports from a large TF infected Australian herd in the 1970's (Christensen et al., 1977; Christensen and Clark, 1979). In an effort to control TF on this large beef cattle ranch whose bulls were all over 8 years of age, all bulls were removed from the herd prior to the breeding season and replaced by young, TF test negative bulls. A sample of these replacement bulls were TF tested at two and four years after introduction to the herd. All replacement bulls were the same age which alleviated age related dominance and were in the herd for the same length of time as many of the bulls they replaced and yet the prevalence of infected bulls remained significantly lower in the replacements than that of the older bulls at the time of their removal from the herd suggesting their youth may have been a factor in limiting prevalence of the carrier state.

Several studies have attempted to assess the correlation between bull age and risk of TF infection, and they are summarized in Table 1.2. All the studies indicate an increased risk for TF carrier bull status as the bull ages, but the validity of many of the studies are difficult to assess because of the small number of animals used and the potential in bias from uneven distribution of age groups or lack of control of other variables which could affect the risk of infection. Three well done studies attempted to account for these factors. BonDurant et al. (1990) found 2 % of bulls 3 years of age and younger infected with TF compared to 6.7% infected bulls in bulls 4 years of age and older which was significantly different (P<0.025). Rae and collegues conducted two large epidemiologic studies and found similar trends. In 1999 they reported the mean age of

Table 1.2 – Bull Age Susceptibility Summary. N/A = not applicable

Year	Study type	Infection mode	Age differentiation		Comments	Reference
			Bull age	Infected		
1973	Abbattoir survey	Natural	9 mos - 3 yrs 3yrs - 7 yrs > 7 yrs	0/33 (0%) 13/52 (25%) 67/180 (37.2%)		Ladds et al., 1973
1974	Outbreak investigation	Natural	2 – 3 yrs > 3 yrs	5/644 (0.78%) 19/95 (20%)		Clark et al., 1974c
1974	Laboratory trial	Experimental	1 yr 2 yrs 3 yrs 4 yrs 5 yrs 6 yrs	1/12 (8%) 1/6 (17%) 2/2 (100%) 1/1 (100%) 4/5 (80%) 1/1 (100%)	Yearling infected for 4 wks; 2 yr old infected for 3 months	Clark et al., 1974b
1974	Laboratory trial	Natural	3 yrs 4 yrs 5 yrs 6 yrs	1/2 (50%) 2/2 (100%) 2/2 (100%) 2/2 (100%)	9 services to infection for 3 yr old; 3 and 4 services to infection for 4 yr olds; 5 and 6 services to infection for 5 yr olds; 4 services to infection for both 6 yr olds	Clark et al., 1974a
1977	Outbreak investigation	Natural	N/A	N/A	30 of 300 bulls 8 yrs old sampled with 47% infected; all bulls removed from herd and replaced with 325 test negative bulls; 2 yrs later 80 replacement bulls sampled with 4% infected	Christen-sen et al., 1977
1979	Outbreak investigation	Natural	N/A	N/A	Follow-up of previous investigation; 112 replacement bulls sampled 4 yrs after introduction; % infected bulls significantly > at 2 yrs post-introduction, but significantly < % old bulls infected at time of introduction	Christen-sen and Clark, 1979
1979	Auction market survey	Natural	2 yrs 3 yrs 4 yrs 5 yrs 6 yrs ≥ 7 yrs	0% 2.3% 7.8% 11.6% 14.6% 9.10%	280 bulls sampled	Wilson et al., 1979
1985	Outbreak investigation	Natural	3 yrs 4 yrs > 4 yrs	5/23 (21.7%) 14/41(34.1%) 48/113 (43.4%)		Skirrow et al., 1985
1990	Statewide herd survey	Natural	< 2 yrs 2 yrs 3 yrs 4 yrs 5 yrs 6 yrs > 6yrs	0/38 (0%) 1/221 (0.5%) 7/137 (5.1%) 5/156 (3.2%) 8/86 (9.3%) 7/55 (12.7%) 2/31 (6.5%)		Bon-Durant et al., 1990
1999	Epidemiologic study	Natural	N/A	N/A	1,383 bulls sampled; mean age of infected bulls = 5.5+/-1.6 yrs; mean age of uninfected bulls = 3.9+/-2.3 yrs; significantly different with P<0.001	Rae et al., 1999
2003	Outbreak investigation	Natural	N/A	N/A	155 bulls sampled; majority of bulls sampled were > 7 yrs of age; odds of bulls ≥5 yrs of age being infected was 9 times that of younger bulls (OR=9.17, P<0.001)	Hoevers et al., 2003
2004	Epidemiologic study	Natural	N/A	N/A	1984 bulls sampled; bulls > 5 yrs of age were 2.2 times more likely to be TF positive than bulls ≤ 5 yrs of age (OR=2.2; 95% CI, 1.1-4.3; P=0.022) when all other factors were constant	Rae et al., 2004

infected bulls at 5.5 +/-1.6 years and mean age of uninfected bulls at 3.9 +/- 2.3 years

(P<0.001) (Rae et al., 1999), and in 2004 they found bulls greater than 5 years of age

were 2.2 (OR=2.2; 95% CI, 1.1-4.3; P=0.022) times more likely to be TF positive than

bulls 5 years of age or younger when all other factors were constant (Rae et al., 2004).

One explanation for the relationship between age and TF carrier bull status may

be the development of crypts in the epithelium of the penis and prepuce. Crypts are

microscopic invaginations of the penile and preputial epithelium as shown in Figure 1.2.

Figure 1.2 – Histologic View of a Crypt. (Source: Dr. Bruce Brodersen, University of
Nebraska-Lincoln Veterinary Diagnostic Laboratory)

Investigation of bovine vibriosis found bull susceptibility to the disease increased

significantly at or beyond five years of age and the increased susceptibility was

associated with and possibly linked to an increase in size and number of epithelial crypts

on the penis after that age (Samuelson and Winter, 1966). Investigators of TF have

likewise implicated the development of crypts in aged bulls as a cause for age related

susceptibility to TF (Ball et al., 1984; BonDurant and Honiberg, 1994).

Although older bulls appear to be more likely to become unapparent carriers of

TF young bulls can be infected. Kimsey et al. (1980) found four 2 year old bulls infected

with TF during an outbreak investigation and Skirrow et al. (1985) found 21.7% of three

year old bulls in a TF infected herd test positive for TF.

In a study by Rhyan et al. (1999) TF was most often found in the penile crypts of

the midshaft and caudal penis and to a lesser degree the crypts of the prepuce by

immunohistochemical staining of paraffin embedded sections of preputial tissues from

TF infected bulls. Predilection for these anatomical sites agree with early culture based

work of Hammond and Bartlett (1943b) which found the organism in highest number at

the midshaft and caudal penis followed by the prepuce adjacent to the penis and then the

remainder of the penile and preputial locations. A depiction of these locations can be

found in Figures 1.3 and 1.4.

Breed predisposition to TF infection has been proposed, and several studies have

reported TF prevalence by breed. (Abbitt and Meyerholz, 1979; BonDurant et al., 1990;

Rae et al., 1999; Rae et al., 2004; Skirrow et al., 1985) However, the presence of bias

through uneven breed distribution across herds or breeding groups which potentially

affects risk of exposure leaves the validity of the findings in question. Other unknown

individual specific factors may also play a role in the development of TF infection and

carrier bull status as suggested by Hammond and Bartlett (1943a), "The results indicate
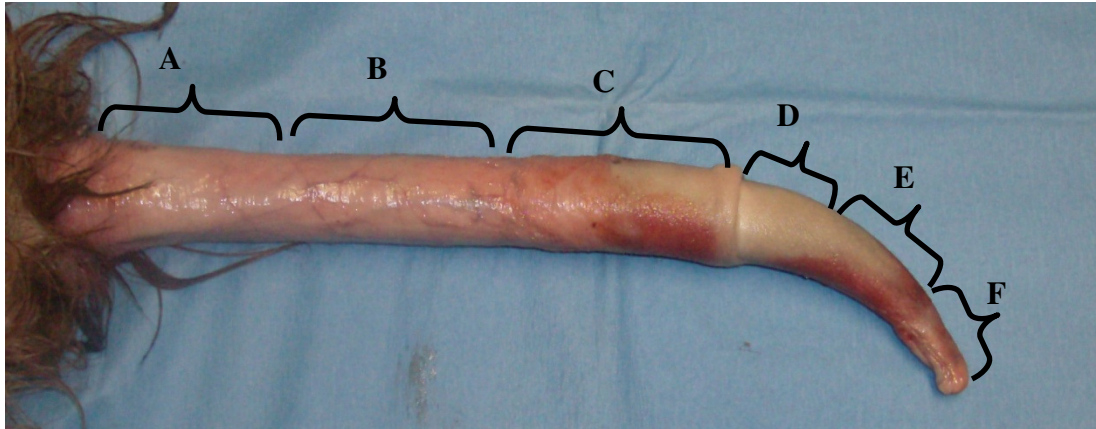
Figure 1.3 – Bull Penis in Full Extension. Key: A = distal prepuce; B = mid prepuce; C = prepuce adjacent to the glans penis; D = caudal penis; E = midshaft penis; F = glans penis or galea glandis.
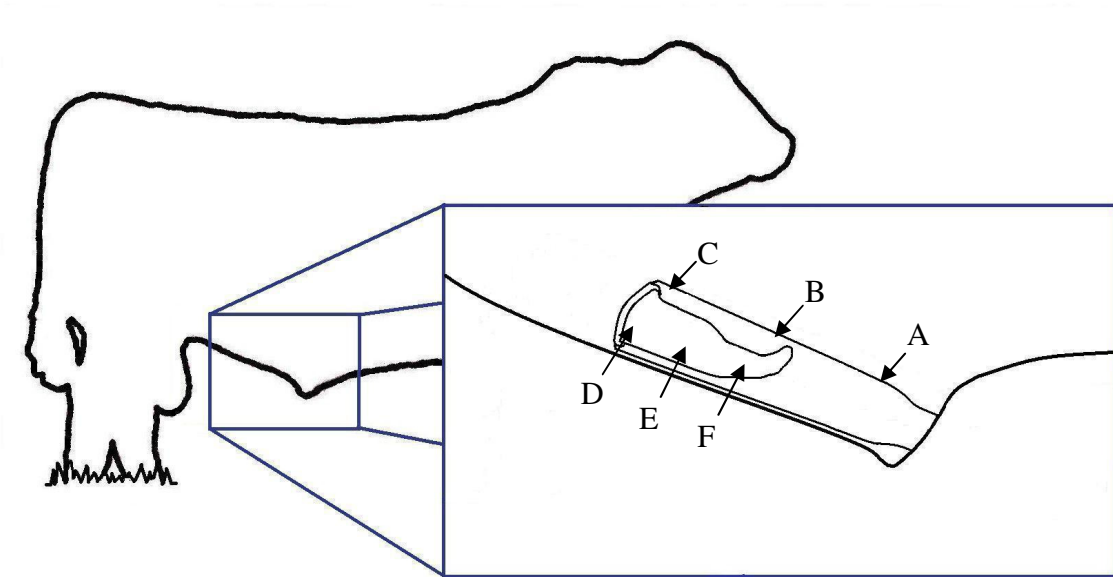
Figure 1.4 – Bull Penis Retracted. Key: A = distal prepuce; B = mid prepuce; C = prepuce adjacent to the glans penis; D = caudal penis; E = midshaft penis; F = glans penis or galea glandis.

that there are distinct individual differences in natural resistance of bulls to infection with

T. foetus."

Clinical signs

Cows –A list of probable outcomes of TF infection in bovine females and their expected incidence rate based on expert opinion and clinical experience applied to a computer model are shown in Table 1.3 and serve as a reference for the level of common and less common outcomes which might be expected during a natural TF outbreak investigation. Early embryonic death, abortion, and temporary infertility following TF infection clearance are expressed as early termination of pregnancy and an early return to estrus which is the most common clinical sign of TF infection in the bovine female (Bartlett, 1947).

Table 1.3 – Summary of Outcomes of TF Infection in Cows. (Rae, 1989)

Outcome	Incidence risk
Early embryonic death	13.1 – 50.2%
Abortions	3.1 – 14.1%
Fetal macerations	0.6 – 2.4%
Pyometras	2.1 – 8.0%
Pregnant carrier state	0.2 – 0.7%
Infertile, TF infection cleared	9.4 – 35.4%

Observant livestock owners may detect the early return to estrus as the first clinical sign of TF infection during the breeding season. Early estrus may lead to an infected cow found not pregnant at the end of the breeding season if a limited length breeding season is utilized as part of the herd management system or found pregnant, but bred later in the breeding season than normally expected if an extended breeding season is utilized. Although not visible, pyometra and fetal maceration can be detected through transrectal palpation of the reproductive tract by a skilled palpator and may indicate the presence of TF infection (Mickelson, 1983; Mickelson, 1984).

Bulls - The absence of macro and microscopic pathologic changes and a limited immunologic response to TF infection in bulls results in no visible clinical signs being exhibited by infected bulls and the development of unapparent chronically infected bulls.

Herd –Clinical signs on a herd basis are the culmination of clinical signs exhibited by individuals within the herd associated with the parasite's impact on female reproductive efficiency through increased numbers of non-pregnant cows, pyometras, abortions, and cows pregnant but with a later than normal expected calving date.

The number of non-pregnant cows found in a TF infected herd increases dramatically as demonstrated by Barling et al. (2005) and Alsted et al.(1984) who found non-pregnant cow rates of 57% and 45.3% respectively in TF infected herds. After implementing TF control and eradication measures non-pregnant rates were found to be 5% following the succeeding breeding season. The increased non-pregnant cow rate leads to decreased annual calf crop as predicted by Rae et al.'s (1989) computer model that indicated 20-40% bull TF infection prevalence in a herd would lead to a 14-50% reduction in annual calf crop.

Clark et al. (1983) also found decreased annual calf production due to increased non-pregnant cows in a four year trial comparing cows bred to TF infected bulls to cows bred to TF free bulls. Seventeen percent fewer calves were produced by the TF exposed cows with most of the losses occurring in the first two years of the trial. In addition a second more insidious yet common herd based clinical sign was noted in this study; an increase in the overall calving interval which is the average days between consecutive calvings. Cows exposed to TF infected bulls experienced calving intervals of 96.5 and

98.9 days longer than non-exposed cows during the first and second year of herd infection respectively. Herd records would specifically identify this herd based clinical sign or it may be recognized in general as an increased number of cows calving late in the calving season or lower weaning weights due to younger calves at weaning.

Control

H. P. Harding (1950) made the following statement regarding the control of trichomoniasis in a 1950 paper on the subject, "In conclusion, may I say that it is my opinion that if more care was taken in the sale and purchase of barren cows, in the purchase of bulls of breeding age, and if the farmers could be sufficiently educated to the unique opportunities for control that this disease offers, then its total elimination should be practicable in the quite near future." As indicated in this quote the distinctive features of this organism as described in the previous thesis pages, venereal transmission, typically transient infection in females, and predilection for the prepuce in chronically infected older bulls, seem to offer the opportunity for rapid control of TF in an infected herd and elimination from the herd.

Transmission - Because natural TF transmission is strictly venereal (Bartlett, 1947; BonDurant and Honiberg, 1994) control of this disease by breaking the transmission cycle requires preventing infected cows from infecting additional bulls and infected bulls from infecting additional breeding females (Bartlett and Dikmans, 1949). The most direct way to accomplish this is to eliminate sexual contact through the use of artificial insemination utilizing semen from a reputable source to insure the semen is TF free (Kvasnicka et al., 1999). However, large scale artificial insemination programs may

not be practical in extensively managed beef herds and natural service breeding must be used while identifying infected or potentially infected animals and managing them to prevent TF transmission as described in the following paragraphs.

Females – The typically transient nature of TF infection in females (Murname, 1959) aids identification of potentially infected females through assessment of time from possible exposure and stage of reproduction. In a TF infected herd non-pregnant females at the end of the breeding season, females with pyometras or other reproductive tract pathology suggestive of TF infection, and females which abort should be considered TF infected. Virgin heifers and females producing full-term calves are unlikely to be TF infected (Ball et al., 1987) while non-pregnant females with a minimum of 150 days sexual rest provide intermediate risk of TF infection (Mancebo et al., 1995; Alexander, 1953). Removal of all potentially infected females from the herd is the most certain method of preventing transmission from infected females to males (Clark et al., 1974c; Ball et al., 1987). However, segregating potentially TF infected females from those not likely to be infected and utilizing specific management tactics with them as a distinct, quarantined group within the TF infected herd to minimize risk of transmission has been successfully implemented as a potentially less severe control option for the cow herd (Barlett and Dikmans, 1949; Barling et al., 2005). The point of this section is not to describe specific management strategies for potentially TF infected cows, rather to make clear the typically transient nature of TF infection in females allows identification and management of potentially infected females through clinical signs without positively identifying truly infected females when controlling an outbreak in a beef cattle herd. A
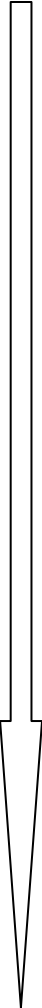
summary of management options for females in the herd and replacement females are listed by their relative risk in Table 1.4.

Bulls – There are currently no FDA approved medications for the treatment of TF infection in bulls available in the US (Rae and Crews, 2006), and bulls, especially those over 3 years of age, may become chronically TF infected (BonDurant et al., 1990; Rae et al., 1999; Rae et al., 2004). Utilizing young bulls, three years of age and younger, who are less likely to become chronically infected decreases the likelihood of transmission during natural mating, but does not necessarily eliminate transmission. (Christensen et al., 1977; Christensen and Clark, 1979) Complete depopulation of bulls in a TF infected herd is the most reliable method for eliminating the risk of transmission from infected bulls to females, but may not be agreeable to the herd owner. Therefore identifying and removing TF infected bulls from the herd is the only means of eliminating transmission from infected bulls to susceptible females. A summary of management options for bulls in the herd and replacement bulls are listed by their relative risk in Table 1.4.

Relevance

As illustrated in the preceding pages rapid control of trichomoniasis in a cattle operation requires several management tactics; the cornerstone of which is identification and removal of TF infected bulls. The following chapter will review diagnostic testing of bulls.

Table 1.4 – Relative Qualitative Threat for TF Maintenance.

THREAT	Management Group			
	BULLS	**BREEDING FEMALES**	**REPLACEMENT BULLS**	**REPLACEMENT FEMALES**
Lowest Threat	Sell all non-virgin bulls	Cull all open, aborted, or late calving females	Virgin bulls or semen from reputable source	Virgin females from reputable source
	Sell all non-virgin bulls >3 yrs of age; test all remaining non-virgin bulls 3 times and cull all positive bulls	Cull all open/aborted females; retain late calving females in herd without segregation	Non-virgin bulls from reputable source tested 3 times	Alleged virgin females with no history
	Sell all non-virgin bulls >3 yrs of age; test all remaining non-virgin bulls < 3 times; cull all positive bulls	Cull all open/aborted females and segregate late calving females from remaining herd	Non-virgin bulls from reputable source tested <3 times	Primiparous pregnant females from reputable source
	Test all non-virgin bulls 3 times and cull all positive bulls	Retain selected open and aborted females with segregation from the herd based on suspected *T. foetus* free status of the management group of origin until segregated females are culled or produce live calves	Non-virgin bulls from reputable source not tested	Multiparous, pregnant females from a reputable source
	Test all non-virgin bulls < 3 times and cull all positive bulls			Non-pregnant females with young calf (<2 months of age) at side from a reputable source
	Test all non-virgin bulls once then test all bulls in management groups with at least one positive bull twice more and cull all positive bulls		Alleged virgin bull with no history; tested 3 times	Non-pregnant females with older calf (>2 months of age) at side from a reputable source
		Segregate all open, aborted, and late calving females into a distinct management group from the remainder of the breeding females until segregated females are culled or produce live calves	Alleged virgin bulls with no history; tested <3 times	Primiparous pregnant females with no history
				Multiparous, pregnant females with no history
	Test all bulls in management groups suspected of T. foetus infection based on cow reproductive rates 3 times and cull all positive bulls		Alleged virgin bull with no history not tested	Non-pregnant females with young calf (<2 months of age) at side with no history
			Non-virgin bulls with no history tested 3 times	
	Test all bulls in management groups suspected of T. foetus infection based on cow reproductive rates < 3 times and cull all positive bulls	Retain selected open and aborted females without segregation based on suspected *T. foetus* free status of the management group of origin	Non-virgin bulls with no history tested < 3 times	Non-pregnant females with older calf (>2 months of age) at side with no history
Highest Threat	Selectively test bulls based on owner intuition and cull positive bulls	Retain open, aborted, and late calving females without segregation	Non-virgin bulls without history or testing prior to breeding herd introduction	Non-pregnant, non-virgin females with no calf or history

Chapter 2

Review of Trichomoniasis Diagnostic Testing

Introduction

As discussed in the previous chapter TF has a long history as a known reproductive pathogen in cattle yet continues to afflict the US cattle population. Current prevalence estimates are not available, but an apparent epidemic in western U.S. states has led to increased concern with trichomoniasis (Cima, 2009). Reports of the economic impact of TF on the cattle industry in general and individual producers specifically coupled with uncertain but potentially high prevalence indicate this disease is important to the financial sustainability of the cattle industry and deserves attention for optimizing control tactics to limit its impact.

The previous chapter showed cows mount an immune response to TF infection which typically leads to elimination of TF from their reproductive tract with demonstration of clinical signs of infection. Bulls frequently become unapparent, chronic carriers of the organism with no available, effective treatment to eliminate the carrier state. This has led to a focus on identifying and removing infected bulls as the primary means of preventing the spread of this disease which makes accurate diagnostic testing of bulls the cornerstone of TF control programs.

The purpose of this chapter is to examine the various pre-analytical and analytical aspects of TF diagnostic testing of bulls which potentially influence the outcome of the diagnostic test and resulting TF status classification of the specimen and ultimately the bull from which it came.

Pre-analytical considerations

Pre-analytical considerations of TF diagnostic testing include those factors related to diagnostic specimen collection and handling up to the point of initiating the actual testing of the specimen. As pointed out by Ball et al. while discussing TF testing pre-analytical factors affecting the quality of a diagnostic specimen may influence the outcome of a TF diagnostic test and the accuracy with which a bull's TF status is determined (Ball et al., 1984)

Specimen collection- Sexual rest of one to two weeks is commonly recommended prior to specimen collection to allow TF numbers to increase thereby improving the likelihood of TF identification in TF positive bulls' specimens (Peter, 1997) This practice is supported by an Australian study (Clark et al., 1983) which found two TF positive bulls consistently culture positive on weekly specimens except during an intense 10 week breeding season when only three and six specimens for the respective bulls were positive out of the 10 weekly specimens collected from each of the bulls.

Various TF specimen collection techniques have been proposed including artificial vagina washing (Gregory et al., 1990), preputial swabbing with a cotton swab (Fitzgerald et al., 1952), preputial scraping with a specially designed instrument (Sutka and Katai, 1969), preputial lavage (Fitzgerald et al., 1952) and preputial specimen aspiration via pipette (Hammond and Bartlett, 1943a).

Specimen collection by artificial vagina washing is described as flushing residues from an artificial vagina (AV) following routine collection of a bull's semen sample in the AV (Gregory et al., 1990). A single study involving two experimentally infected bulls found "artificial vagina washings are almost as reliable as preputial lavage for the

detection of T. foetus." (Gregory et al., 1990). The limited scope of this study (n=5 specimens per bull per collection method) leads to questions regarding the validity of the conclusion and the impracticality of artificial vagina use for field TF specimen collection makes the practice unusable in extensively managed beef herds.

The swab technique attributed to Morgan by Fitzgerald (Fitzgerald et al., 1952) and Hammond and Bartlett (1943a) consists of passing a cotton swab six to eight centimeters (cm) in length and one to two cm in diameter into the preputial cavity through a speculum in the preputial orifice after affixing it to a steel wire 60 cm in length and four mm in diameter. The swab is inserted into the fornix area, moved back and forth as well as rotated around the glans penis, and then removed from the prepuce. The sample is collected from the cotton swab by saturating it with saline and expressing the saline from the swab by rolling it along the inside surface of the specimen container.

Sukta and Katai (1969) described a metal device designed in the Soviet Union to scrape samples from the bovine preputial cavity which consisted of a long, thin rod with shallow grooves at one end perpendicular to the shaft. Exact dimensions of the metal device were not given, but a plastic version of this device is shown in Figure 2.1 and is commercially available as the Tricamper™[1] which is 61 centimeters long and 5 millimeters in diameter with a grooved end 7 cm in length containing 16 grooves. Tedesco et al. (1979) utilized a similar instrument that was a 70 cm long, three mm diameter metal rod with a 13 cm long, eight mm diameter metal cylinder engraved with 31 circular grooves soldered to one end. Specimen collection with these devices is

[1] Tricamper, Queensland Department of Primary Industries and Fisheries, Brisbane QLD
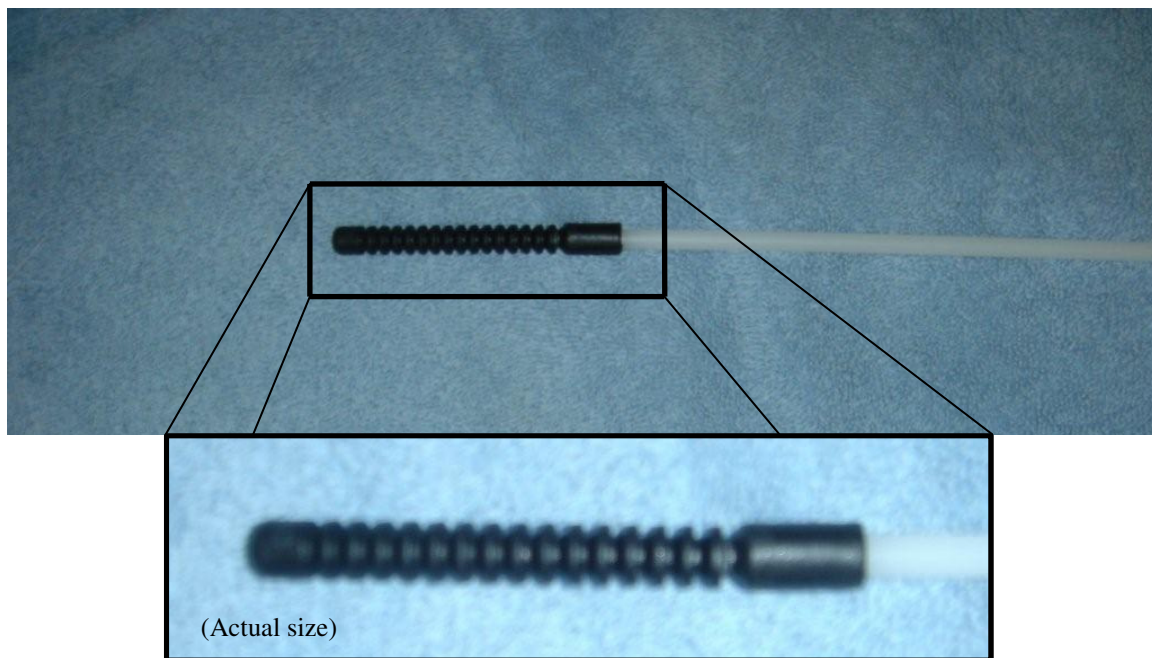
Figure 2.1 – Tricamper™.

achieved by passing the grooved end of the rod into the preputial cavity and thrusting the

head forward into the fornix area and drawing it back repeatedly for 20-30 cycles then

withdrawing it from the prepuce and flushing the specimen from the instrument.

The precise details for preputial lavage or douche technique vary between

investigators; however, the general concept of the technique is to instill a volume of

sterile normal or phosphate buffered saline into the preputial cavity with a syringe or

rubber bulb via a pipette whose free end is positioned in the fornix area, massage the

prepuce and penis while holding the preputial orifice closed to prevent leakage of the

saline, and collect the lavage fluid by aspirating with the instillation apparatus. Fluid

retrieved by this method is typically placed in a test tube and spun in a centrifuge or

allowed to sediment to concentrate the preputial debris in a pellet at the bottom of the

container after which the pellet is examined directly for TF or inoculated into media for enrichment and later TF analysis (Fitzgerald e al., 1952; Schonmann et al., 1994).

Hammond and Bartlett (1943a) modified a glass vaginal pipette and rubber bulb used used by earlier investigators to collect TF samples from female cattle and developed a technique for aspirating samples from bull preputial cavities. The free end of the pipette is passed into the preputial cavity to the fornix, aspiration is applied with the rubber bulb as the free end of the pipette is moved back and forth over the surface of the penis and prepuce multiple times, and then the pipette is removed from the preputial cavity after gently releasing the suction from the rubber bulb. Over the years this device has been adapted to utilize a plastic infusion pipette typically 45 to 53 cm in length and 0.497 to 0.571 cm in diameter and a 12 or 20 milliliter (mL) disposable syringe with the collection technique as previously described (Peter, 1997).

Sukta and Katai (1969) concluded their scraping device was superior to preputial lavage for identifying TF in preputial specimens although supporting data and its analysis were not clearly shown. Three experimentally TF infected bulls were sampled for 33 weeks by preputial scraping with a device similar to and used as the one described by Sukta and Katai and preputial aspiration via a pipette and syringe on alternating weeks (Tedesco et al., 1979) The conclusions of this study were scraping with the device was superior to pipette aspiration for direct examination of the specimen for TF identification, equal to pipette aspiration when the specimen was placed in culture media within two hours of collection, and resulted in a three times greater TF survival time in culture compared to specimens aspirated by pipette. Another study (Parker et al., 1999)

compared Tedesco et al.'s instrument to pipette aspiration with the collected specimens placed into a commercially available culturing system. Thirty naturally and experimentally TF infected bulls were sampled weekly for six weeks using both techniques and the investigators found no significant difference between the techniques with the sensitivity of pipette aspiration at 91.6% (95% CI, 84.3 to 95.7%) and the sensitivity of the scraping device at 93.3% (95% CI, 87.2 to 96.7%).

Two studies compared the number of TF organisms found on direct specimen examination for specimens collected by preputial swab compared to specimen aspiration via pipette. Hammond et al. (1950) found an average of 2,990 TF per mL and 190 TF per mL in specimens collected by pipette aspiration and preputial swab respectively when nine specimens per technique were collected from a single TF infected bull on alternate days. They concluded pipette aspiration may be more reliable for direct examination of specimens than swabs when TF is in low numbers in the prepuce.

Another study (Fitzgerald et al., 1952) found swabbing produced specimens with an average TF concentration of 4.2×10^3 per mL with a range of 0.8×10^3 to 7.8×10^3 per mL and pipette aspiration produced specimens with an average TF concentration of 20.6×10^3 per mL with a range of 4.2×10^3 to 74.6×10^3 per mL. Of the 63 pipetted samples and 62 swab samples from seven TF infected bulls in this study 6.3% and 8.1%, respectively were TF negative. While absolute TF numbers favored pipette aspiration over preputial swabs, the minimal difference in negative samples between methods suggests they are not significantly different in their ability to recover TF from the

preputial cavity, and the expected difference would be less if specimens were placed into enrichment media and incubated rather than examined directly.

Fitzgerald's group also compared the efficacy of preputial lavage and pipette aspiration for TF recovery by sampling 3 TF positive bulls with each technique on alternate days and directly examining the specimens for TF concentration (Fitzgerald et al., 1952). Pipette aspiration produced specimens with an average TF concentration of 7.1 x 10^3 per mL and preputial lavage produced specimens with an average TF concentration of 13.5 x 10^3 per mL with 34.4% and 18.5% TF negative specimens respectively out of 119 specimens per technique. The authors concluded lavage was the more efficacious method for recovery of TF at low preputial concentration levels for direct examination.

Preputial lavage and pipette aspiration were used on 6 alternate weekly collections from 14 TF infected bulls to obtain specimens for enrichment and incubation in a comparison of the two sampling methods (Schonmann et al., 1994). Pipette aspiration yielded 65 TF positive specimens of 83 total specimens and lavage produced 69 TF positive specimens from 84 total specimens for sensitivities of 78.3% (95% CI, 67.6 to 86.3%) and 82.1% (95% CI, 71.9 to 89.3%) respectively. The authors concluded the two collection methods were comparable because their sensitivities were not significantly different.

A South African study (Mukhufhi et al., 2003) compared preputial lavage and pipette aspiration collected specimens for culture and PCR analysis. Of 29 specimens collected by each method 24 were TF culture positive for a sensitivity of 83% (95% CI,

64 to 94%). Sensitivities for the two methods when the specimens were analyzed by

PCR were not significantly different although the difference approached significance

when DNA extraction from the specimen was delayed for 5 days as shown in Table 2.1

which lead the authors to conclude there was no difference between the two techniques

when culture or PCR were used to determine the TF status of a specimen.

Table 2.1 – Trial Sensitivity of Culture and PCR*. (Mukjufhi et al.,2003)

Collection method	Addition of GuSCN	Culture	PCR		
			Time delay before DNA extraction		
			6 hours	30 hours	5 days
Lavage	No	83% (64-94%)	90%[a] (73-98%)	69% (49-85%)	62%[b] (42-79%)
	Yes	N/A	90%[a] (73-98%)	72% (53-87%)	62%[b] (42-79%)
Pipette	No	83%[a] (64-94%)	83%[a] (64-94%)	62% (42-79%)	41%[b] (24-61%)
	Yes	N/A	72%[a] (53-72%)	62% (42-79%)	31%[b] (15-51%)

*Within lines, treatments with different superscripts (a,b) differ in sensitivity, P<0.05.

The data presented here does not indicate clear superiority of one TF specimen

collection technique over another. Use of an AV wash appears to be impractical in

extensively managed herds while preputial swabbing with a cotton swab, preputial

scraping with a specially designed instrument, preputial lavage, and preputial specimen

aspiration via pipette appear to be reasonable options. Because of the apparent lack of

difference the various collection techniques for TF recovery and the convenience of

specimen handling afforded by the pipette aspiration technique under field conditions

typically encountered in the US, pipette aspiration has become the method of choice for

TF preputial specimen collection in the US (Peter, 1997).

When the pipette method is used to collect specimens for TF testing Ball et al.

(1984) suggest the sample "be cloudy and blood tinged." The presence of turbidity and

blood in the specimen presumably indicates the scraping action of the pipette tip is

adequate to remove TF from the epithelial crypts which are assumed to harbor the

organism. No substantiating data was given by the authors to support this idea and no other references were found containing documentation to confirm or dispute this suggestion. Specimen volume would also appear to be a consideration when assessing the quality of a preputial sample collected by pipette aspiration. Hammond et al. (1950) found the average volume of specimen collected by the pipette method in their study to be 0.52 mL and they concluded there was no relationship between specimen volume and the number of TF present in the specimen.

Media – Maintenance of TF preputial specimens has been a significant pre-analytical concern for TF diagnostic testing since early work in this area. Specimens not subjected to direct examination were frequently collected and maintained in a solution whose purpose was to preserve TF viability until they could be inoculated into laboratory media for incubation or placed directly into the laboratory incubator. Peptone water (Tedesco et al., 1979), various formulations of cow's milk (Reece et al., 1983; Todorovic and McNutt, 1967), a range of saline solutions (Fitzgerald et al., 1954; Reece et al., 1983; Kimsey et al., 1980), lactated ringer's solution (Skirrow et al., 1985; Kimsey et al., 1980), forms of Kupferberg medium (Kimsey et al., 1980), and variants of Diamond's medium (Kimsey et al., 1980) are examples of media that have been used with varying degrees of success as transport media for TF. The use of solutions, broths and media strictly for TF specimen transport has been all but eliminated in the US due to direct inoculation of growth media at the collection location for use as transport and incubated enrichment medium of the specimen as described in the following pages.

A variety of growth media have been used to allow TF in preputial specimens to propagate under incubation in an effort to increase the likelihood of detection of the organism in the specimen by diagnostic testing including cysteine-peptone-liver extract-maltose-serum medium (CPLM), beef extract-glucose-peptone-serum (BGPS) or modified Plastridge's medium, Diamond's trypticase-yeast extract-maltose-cysteine-serum medium, thioglycollate broth plus 1% beef serum, skim milk containing antibiotics (Levine, 1973),Kupferberg medium and broth, Claussen's medium, Sutherland's medium, and most recently a proprietary media in a specially designed *in vitro* cultivation envelop called InPouch™TF[2] (Rodning, 2007). See Appendix A for complete details of the InPouch™TF system. By the late 1960's the most commonly used media for TF cultivation were based on Plastridge's and Diamond's media (Todorovic and McNutt, 1967); however, the European Union eventually adopted selective media such as Claussen's medium as the required medium for official TF testing (Schonmann et al., 1994) and the InPouch™TF and variants of Diamond's medium referred to as modified Diamond's medium became the most common media for *in vitro* TF cultivation in the US (Rodning, 2007).

The original medium described by Diamond (1957) consisted of trypticase, yeast extract, malstose, L-cysteine hydrochloride, L-ascorbic acid, agar, distilled water, sheep serum, potassium penicillin, and streptomycin sulfate. Modification to this original formulation proposed by Kimsey (1986) added dipotassium phosphate (K_2HPO_4), monopotassium phosphate (KH_2PO_4) to the recipe while replacing sheep serum with

[2] InPouch™TF, BioMed Diagnositcs Inc., San Jose, CA.

bovine serum. The formulas for these media are shown in Table 2.2. Examples of

further modifications of the Diamond's medium include substitution of newborn lamb

serum for sheep serum and inclusion of gentamicin with or without adding amphotericin

B (Bryan et al., 1999; Lun et al., 2000) and the exclusion of agar (Huang et al., 1989). As

pointed out by Parker et al. (2003) the actual formulation of Diamond's medium used by

different researchers and laboratories varies.

Table 2.2 – Ingredients for Diamond's (Diamond, 1957) and Modified Diamond's Medium (Kimsey, 1986).

Ingredient	Amount (in grams unless otherwise stated)	
	Diamond's medium	Modified Diamond's medium
Trypticase	2.0	2.0
Yeast extract	1.0	1.0
Maltose	0.5	0.5
L-cysteine	0.1	0.1
L-ascorbic acid	0.02	0.02
Agar	0.05	0.05
Distilled water – to make 90mL		
Sheep serum – inactivated	10 mL	Not used
Bovine serum – inactivated	Not used	10 mL
Dipotassium phosphate (K_2HPO_4)	Not used	0.08
Monopotassium phosphate (KH_2PO_4)	Not used	0.08
Potassium penicillin G	100,000 units	100,000 units
Streptomycin sulfate	0.1	0.1
Sodium hydroxide	As needed to adjust pH	As needed to adjust pH
Hydrochloric acid	As needed to adjust pH	As needed to adjust pH

The formula for the medium contained in the InPouch™TF is not public

knowledge, but is described on the package insert accompanying a shipment of pouches

as, "the proprietary medium is selective for the transport and growth of the

trichomonad,while inhibiting the growth of yeast, mold and bacteria which might

interfere with a reliable diagnosis"(Appendix A).

Antibiotics (Reece et al., 1983) and antifungal agents (Ribeiro, 1990) are commonly included in TF media to limit the growth of commensal preputial bacteria and fungi which contaminate TF specimens and if left unchecked may prohibit adequate TF proliferation and detection.

The earliest mention of InPouch™TF in the literature was a 1990 report comparing it to modified Diamond's medium (mDM) for TF detection which utilized single specimens from 83 bulls of unknown TF status across 5 different herds and multiple specimens from three TF infected bulls (Thomas et al., 1990) The authors concluded with limited data and analysis no difference existed between the two media for TF detection, but the pouches produced more positive results by 48 hours and no new positives after 72 hours compared to mDM which they speculated was due to optimized media, more anaerobic conditions, and larger volume of media examined of the InPouch™TF. Other suggested advantages were a more durable media container, storage at room temperature, a longer shelf life, and specimen examination without opening the container which introduces air and contaminants.

Borchardt et al. (1992) compared the performance of InPouch™TF and Kimsey's mDM by spiking both with various, known numbers of TF organisms and examining the incubated specimens for several days. No difference in sensitivity between the two media was mentioned; however, the authors concluded while mDM supported a more rapid initial TF growth rate the pouches required fewer inoculated TF for detection at earlier incubation times possibly due to the larger volume of media examined at each

examination. They also cited the elimination of pipettes, microscope slides, and cover slips for examination of pouches as an advantage for InPouch™TF use.

A study (Appell et al., 1993) comparing InPouch™TF, Diamond' medium prepared by Washington Animal Disease Diagnostic Laboratory (WADDL), and a commercially available Diamond's medium for *Trichomonas vaginalis* (TV) detection but frequently used for TF detection found InPouch™TF and WADDL medium in agreement on 147 of 150 individual bull specimens examined where the pouches identified two TF positive specimens not found by the WADDL medium and one TF positive specimen in the WADDL medium not found by the pouches. Of 50 bulls sampled one time and specimens placed in all three media 11 were positive by both InPouch™TF and WADDL medium with only one bull positive in the commercially available TV Diamond's medium in agreement with the other two media. The authors concluded no statistical difference in sensitivity of InPouch™TF and WADDL medium (P > 0.8), but the commercially available TV Diamond's medium was inferior to both media for TF detection. They speculated the pH of the commercially available TV medium (pH =6.0) versus WADDL Diamond's medium (pH =7.2 +/- 0.2) may have been the reason for the lack of agreement.

The previously discussed study by Schonmann et al. (1994) also compared Claussen's medium to InPouch™TF over a seven day examination period and found the sensitivity of Claussen's medium and InPouch™TF significantly different (P = 0.0003) on day two at 65.0% and 84.3% respectively. The difference was still detectable at day seven with sensititivities of 73.5% and 88.0% for Claussen's medium and InPouch™TF

respectively which lead to recommending InPouch™TF over Claussen's medium for its improved convenience and sensitivity.

Felleisen et al. (1997) compared Diamond's medium and InPouch™TF for TF cultivation and detection and found Diamond's media produced positive TF findings with fewer inoculated organisms than the pouches. However, they experienced frequent, 56.1% of Diamond's media specimens, bacterial or fungal overgrowth of the bull specimens inoculated into Diamond's media which prevented TF analysis and lead them to conclude InPouch™TF was the medium of choice because of its lack of bacterial or fungal contamination issues.

In a study (Bryan et al., 1999) examining the influence of time, temperature, TF isolate, and media on TF detection investigators compared the performance of a transport medium of thioglycollate broth, sterile distilled water, and inactivated newborn calf serum coupled with modified Diamond's medium as the incubation medium against InPouch™TF. While some minor difference between the two systems occurred for specific time, temperature, and TF isolate treatments the sensitivity across the entire project was not significantly different at 68% (307/449) for the transport/mDM technique and 72% (325/450) for the InPouch™TF.

As in the Bryan study Parker et al. (2003) compared the same transport/mDM combination to InPouch™TF for specimens collected from TF positive bulls with the added feature of the transport media was held at room temperature for 24 hours before transfer to mDM for incubation and InPouch™TF was likewise held at room temperature for 24 hours before incubation. InPouch™TF yielded 161 of 168 positive specimens for a

sensitivity of 95% (95% CI, 89.6 to 98.5%) and transport/mDM provided 129 of 168

positive results for a sensitivity of 76.8% (95% CI, 65.6 to 85.2%) with specimens

inoculated in the InPouch™TF being 6.95 times more likely to be positive than those in

the transport/mDM (P<0.001).

Sixteen isolates from around the world were inoculated into mDM, InPouch™TF,

and liver infusion broth medium and examined for growth characteristics (Lun et al.,

2000). All isolates exhibited significant growth in the three media with peak

concentrations occurring at days two to four, two to six, and two to seven for mDM,

InPouch™TF, and liver infusion broth medium respectively. The slight growth

characteristic differences between isolates and media included higher peak organism

concentrations in mDM and longer duration of TF detection in liver infusion broth

medium and InPouch™TF. The authors' conclusion was all three media could be used

successfully around the world.

Because Diamond's medium and InPouch™TF do not demonstrate consistent

differences in TF detection and InPouch™TF offers many advantages as described earlier

it has become the medium most commonly recommended for TF field diagnostic

purposes in the US (Rae and Crews, 2006)

Transit time and temperatures – Another pre-analytical consideration for TF

diagnosis is the influence of shipping conditions on the viability of TF in the specimen

from the time the specimen is collected until it reaches a laboratory incubator.

Tritrichomonas foetus investigators have long recognized exposure of TF specimens to

extreme temperatures ranges and time delays before incubation could affect diagnostic test sensitivity.

When examining the influence of time and temperature on microscopic examination results Fitzgerald et al. (1954) found specimens held in sterile, normal saline for 24 to 48 hours at room temperature before inoculation into modified Plastridge medium resulted in progressively fewer TF positive results while storage up to 48 hours at 3.9^o C had minimal affect on the number of positive tests. Todorovic and McNutt (1967) found the opposite effect of refrigeration to be true in their study when specimens from known TF infected bulls were inoculated into various milk media, refrigerated at 4° C for 24 hours, and incubated at 37° C resulted in a marked decrease in the percentage of TF positive samples. These findings were supported by an Australian study (Reece et al., 1983) that found modified Plastridge's medium held at 4°C for 24 and 48 hours prior to incubation resulted in reduced TF detection.

Two studies were found that examined the influence of time and temperature on PCR results. Mukjufhi et al. (2003) tested preputial lavage specimens transported to the laboratory in phosphate buffered saline (PBS) with or without guanidinium thiocyanate (GuSCN) as a preservative and held for 6 hours, 30 hours, and 5 days at 4° C before DNA extraction. They found a significant decline in PCR sensitivity at the five day holding time whether or not GuSCN was added to the specimen. See Table 2.1 for sensitivities at the various treatment levels. Another study (Mutto et al., 2006) involving preputial lavage specimens held at room or 4° C up to seven days in PBS prior to PCR assay. They found specimens stored at room temperature more than 72 hours produced

TF negative PCR results and the refrigerated specimens yielded positive PCR results through the entire seven day study period. The disagreement between these two studies may be due to the small sample sizes or differences in PCR technique.

While these studies give some indication of the importance of time and temperature exposure on TF specimens during transport, only two references could be found that examined the influence of these factors when specimens were subjected to media currently used in the US.

In Bryan et al.'s (1999) study examining the influence of time, temperature, TF isolate, and media on TF detection investigators compared the performance of a transport medium of thioglycollate broth, sterile distilled water, and inactivated newborn calf serum coupled with modified Diamond's medium as the incubation medium to InPouch™TF. In one portion of the study inoculated transport medium and InPouch™TF were held at 4°C, 22°C, and 37°C from three to seven days prior to incubation at 37° C with the incubation of the transport medium specimens occurring in mDM. They reported an overall agreement between the two media across all treatment groups, but significant differences between holding times, temperatures, and isolates where noted with fewer positive specimens found as time to incubation increased especially at the 4° C treatment level.

In another part of this study inoculated transport medium and InPouch™TF were held at 4°C from one to five days prior to incubation at 37° C as before. As specimens were held at 4° C from one to five days before incubation the number of positive specimens in both media decreased until day five when no positive specimens were found

in either. While there was no overall difference between media, differences in

sensitivities between holding times for the respective media was reported to be significant

with P ≤ 0.0001.

A third segment of this study held inoculated media at -20° C from 0.25 to 24

hours before incubation as described for the earlier portions and found differences

between holding times where no positive specimens were found in either media at

holding times of six hours or greater and between media with a sensitivity of 30 percent

(19/64) for the transport/mDM and 59 percent (38/64) for the InPouch™TF (P = 0.0007).

The previous study reported impacts of pre-analytical factors on microscopic

examination of specimens for TF detection. Cobo et al. (2007) examined the impact of

these factors on microscopic examination and PCR by inoculating two InPouch™TF from

each collected specimen, holding them at approximately 22° C for 4 or 24 hours followed

by incubation at 37° C, and microscopically examining the pouches on days 1, 3, 5 and 7

post-inoculation. Specimens for PCR analysis were collected from the same bulls and

split into two containers and held at 4° C for 4 or 24 hours before DNA extraction.

Microscopic examination found only one discrepant specimen which was positive at 4

hours but negative at 24 hours while PCR had no discrepancies between treatments.

The InPouch™TF package insert recommends maintaining inoculated pouches

between 15° C and 37° C during transport although no specific references were given to

support this recommendation (AppendixA).

Incubation conditions – Most references agree inoculated media should be

incubated at 35° to 37° C although no study showing this as the optimal temperature for

TF incubation could be found (Rae and Crews, 2006; Appendix A). Storing incubating medium in an upright position is also recommended as this causes the motile TF organisms to concentrate at the bottom of the media container which increases the likelihood of TF detection by microscopic examination and PCR (Kimsey, 1986).

It appears no single specimen collection technique or medium is superior for TF detection. The use of specimen aspiration via pipette and direct inoculation into InPouch™TF appears to offer the most convenient technique for collection and transportation of the specimen from the collection location to a laboratory for most field situations encountered in the US. Although more research is needed to clarify the impact of delayed incubation and various temperature exposures on the detection of TF it appears protecting inoculated media from freezing or prolonged refrigeration and upright placement into a 37° C incubator as soon as possible are practices which increase the likelihood of TF detection regardless of the media or diagnostic test utilized.

Analytical considerations

Analytical considerations of TF diagnostic testing refers to the factors related directly to the procedures of the test applied to the specimen. Early TF investigators relied on direct microscopic examination of preputial specimens as the primary TF diagnostic test (Bartlett et al., 1947; Hammond and Bartlett, 1943a; Fitzgerald et al., 1952) until the development of culture media that provided superior isolation and detection of TF in the specimen (Fitzgerald et al., 1954; Fitzgerald et al., 1958; Tedesco et al., 1979). Specimens are currently preserved in media as described in the previous section and submitted to microscopic examination following incubation which is

commonly referred to as culture or analyzed for the presence of TF specific DNA by PCR.

Culture – Table 2.3 summarizes several of the more recent, relevant studies of TF diagnosis by culture and gives an indication of the varied techniques for indentifying the organism and a sensitivity estimation of culture as a TF diagnostic test ranging from 67.7% (Cobo et al., 2007) to 98.4% (Appell et al., 1993). Future mention of sensitivity will be reference to diagnostic sensitivity; the percentage of TF infected bulls which are identified by the test as TF positive, rather than analytical sensitivity, the ability of a test to detect a small number of TF in a specimen, unless otherwise stated (Saah and Hoover, 1997) Because InPouch™TF is widely used throughout the US for TF cultural detection and was used in the studies to be described in later chapters the focus of discussion on analytical considerations for cultural TF detection will be on InPouch™TF use.

Examination of InPouch™TF may be done in wet mount fashion (Cobo et al., 2007), but is more practically carried out by fixing the lower portion of the pouch in a plastic clip approximately the size of a microscope slide provided by the manufacturer, placing the clip on a compound microscope for systematic scanning of the pouch for motile organisms morphologically consistent with TF, and repeating daily for six days as recommended by the manufacturer (Appendix A). Daily examination should last several minutes before declaring the specimen TF negative (Parker et al., 1999; Parker et al., 2003).

Lun et al. (200) found TF numbers peaked on days 2 through 4, 2 through 6, and 2 through 7 for Diamond's medium, InPouch™TF, and liver infusion broth medium

Table 2.3 –Culture Sensitivity Summary.

Year	Collection technique	Media	Incubation temp (in ° C)	Examination schedule (in hours or days post-inoculation)	Microscopic examination technique	Micro-scope magnific-ation	Sensitivity[a]	Author
1971	Pipette aspiration	Transport broth/ modified Sutherlands's	37	Once; day 4	Wet mount slide	80X	97%[b] (139/143)	Clark et al.
1985	Pipette aspiration	Diamond's	35	Days 2,4,& 7	Inverted microscope exam of tube bottom	400X	81.6%[b]	Skirrow et al.
1993	Pipette aspiration	InPouch™TF	37	24,48,72, & 120 hours	N/R	N/R	98.4%[c] (62/63)	Appell et al.
	Pipette aspiration	Modified Diamond's	37	24,72,96,& >102 hours	N/R	N/R	96.8%[c] (61/63)	
1994	Pipette aspiration	Diamond's	37	Daily for 7 days	Inverted microscopic exam of tube bottom	400X	93.2%[b] (517/555)	Schon-mann et al.
	Preputial lavage & pipette aspiration[d]	Claussen's	37	Days 2,4,&7	Wet mount slide	400X	73.5%[b] (61/83)	
	Preputial lavage & pipette aspiration[d]	InPouch™TF	37	Days 2,4,&7	in vitro direct exam	400X	88%[b] (73/83)	
1994	Pipette aspiration	Modified Diamond's	37	Daily for 7 days	Inverted microscopic exam of tube bottom	N/R	81.8%[b] (36/44)	Ho et al.
1995	Pipette aspiration	InPouch™TF	35	Daily for 7 days	N/R	N/R	70.4%[b,e] (38/54)	Peter et al.
1996	N/R	InPouch™TF/ modified Diamond's	N/R	N/R	N/R	N/R	81.0%[f] (138/171)	Gay et al.

[a] Sensitivity shown as a % with positive tests from total possible positive tests shown in parenthesis.

[b] Sensitivity based on number of positive specimens from total number of specimens from known TF positive bulls.

[c] Sensitivity based on number of positive specimens for medium from total number of specimens positive for both media.

[d] Equal numbers of samples were collected by each method, preputial lavage and pipette aspiration, and inoculated into medium.

[e] All bulls in herd were tested 3 times with sensitivity for second week of testing 44.4% and weeks 1 and 3 sensitivities 83.3%.

[f] Sensitivity based on Idaho Department of Agriculture trichomoniasis testing database and determined by number of bulls found positive on first test in a series of tests in positive herds.

Table 2.3 (cont'd) – Culture Sensitivity Summary.

Year	Collection technique	Media	Incubation temp (in ° C)	Examination schedule (in hours or days post-inoculation)	Microscopic examination technique	Micro-scope magnific-ation	Sensitivity[a]	Author
1999	Inoculation of 4-5,000 organisms[g]	Transport broth/ modified Diamond's	37	Days 0,3,6,7, & 10	Wet mount slide	100X	68%[h] (307/449)	Bryan et al.
	Inoculation of 4-5,000 organisms[g]	InPouch™TF	37	Days 0,3,6,7, & 10	*in vitro* direct exam	100X	72%[h] (325/450)	
1999	Pipette aspiration	InPouch™TF	37	Twice; 24-48 hrs and 4-5 days	*in vitro* direct exam	100X	73%[i] (120/165)	Rae et al.
1999	Scraping device	InPouch™TF	37	Days 1,3,&7	*in vitro* direct exam	100X	93.3%[b] (167/179)	Parker et al.
	Pipette aspiration	InPouch™TF	37	Days 1,3,&7	*in vitro* direct exam	100X	91.6%[b] (164/179)	
2003	Pipette aspiration	InPouch™TF	37	Days 0,3,&7[j]	*in vitro* direct exam	100X	95.8%[b] (161/168)	Parker et al.
	Pipette aspiration	Transport medium/ modified Diamond's	37	Days 0,3,&7[j]	Wet mount slide	100X	76.8%[b] (129/168)	
2003	Preputial lavage	Transport PBS/ trichomonad medium	N/R	N/R	N/R	N/R	83%[b]	Muk-hufhi et al.
	Pipette aspiration	Transport PBS/ trichomonad medium	N/R	N/R	N/R	N/R	83%[b]	
2006	Pipette aspiration/ scraping device	Modified Plastridge	37	Daily for 7 days	Wet mount slide	100X	72.04%[k]	Perez et al.
2007	Pipette aspiration	InPouch™TF	37	Days 1,3,5, &7	Wet mount slide	40-100X	67.8%[l]	Cobo et al.

[a] Sensitivity shown as a % with positive tests from total possible positive tests shown in parenthesis.

[b] Sensitivity based on number of positive specimens from total number of specimens from known TF positive bulls.

[g] All media were inoculated with standard number (4,000 to 5,000) of *Tritrichomonas foetus* organisms from two isolates maintained in laboratory

[h] Sensitivity based on number of positive specimens from total number of inoculated media representing sensitivity across all specimen treatment groups.

[i] Sensitivity based on number of positive specimens found on first test from total specimens collected from bulls TF positive after multiple tested specimens

[j] Day 0 represents the day specimens placed in incubator following 24 hours at room temperature post-inoculation

[k] Sensitivity based on testing field samples of bulls of unknown *Tritrichomonas foetus* infection status and evaluating the test using Bayesian techniques

[l] Sensitivity based on number of positive specimens from total number of specimens from experimentally infected bulls. Four bulls did not produce any positive specimens during the study, but samples from these bulls were included in the analysis as samples from infected bulls

respectively. Knowledge of peak TF concentrations in cultures combined with balancing

efficiency of microscope technician time with concerns over low analytical sensitivity of

culture has led to the common recommendation of microscopic examination of TF

cultures every other day for seven days before declaring the specimen negative

(BonDurant, 1985). Figure 2.2 shows the appearance of a pure culture of TF in an

InPouch™TF at 100X magnification with examples of selected organisms indicated by

white arrows.
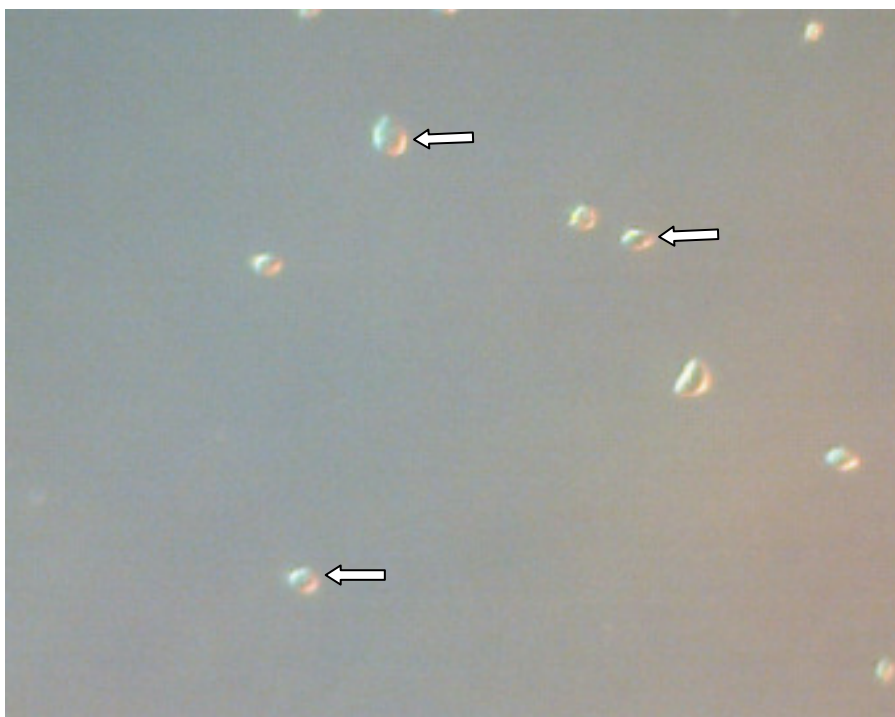
Figure 2.2. *Tritrichomonas foetus* in Pure Culture. (Courtesy of Dr. Joe Wright, Genetic Management Services, San Antonio, TX)

Appell et al.'s (1993) study found the sensitivity of InPouch™TF cultures to be

98.4% when a onetime specimen was collected from 150 bulls and examined once daily

on days one, two, three, and five post-inoculation while incubated at 37° C. The

sensitivity was calculated by dividing the number of InPouch™TF positive specimens by

the total number of positive InPouch™TF and modified Diamond's medium specimens concurrently tested from each bull. This may be an overestimation of the sensitivity of InPouch™TF as subsequent specimen collection from the original 150 bulls may have identified additional TF positive bulls as Kimsey et al. (1980) demonstrated in their study where three weekly sampling events were necessary to identify all TF infected bulls in a herd with a greater than 99% probability.

Parker and colleagues reported TF culture sensitivities of 91.6% (95 CI, 84.3 to 95.7%) (Parker et al., 1999) and 95.8% (95% CI, 89.6 to 98.5%) (Parker et al., 2003) in two similar studies examining pre-analytical factors of TF detection where sensitivity was calculated as the number of positive specimens detected from the total number of specimens collected from naturally and artificially infected bulls. During both studies pouches were transported to the laboratory within three hours of inoculation under guarded conditions with specimens from the first trial placed directly into a 37° incubator while specimens in the second trial were held at room temperature for 24 hours prior to incubation. The first trial represent near optimal conditions for InPouch™TF handling while the second trial represents ideal conditions for specimens shipped overnight to a laboratory for incubation with the sensitivities representing would should be expected when specimens are handled impeccably.

In part of their study on the influence of various pre-analytical factors on TF detection Schonmann et al. (1994) inoculated InPouch™TF with equal numbers of preputial lavage and pipette aspirated specimens from know naturally TF infected bulls, incubated samples at 37° C, and examined pouches once daily on days two, four, and

seven post-inoculation using the manufacturer supplied plastic clip and 400 times

magnification on an inverterd microscope. The sensitivities for preputial lavage and

pipette aspiration were not statistically different so their results were combined and the

total number of positive specimens by both collection methods in InPouch™TF (n=73)

was divided by the total number of specimens collected from known positive bulls (n=83)

for an overall sensitivity of 88%. Specimens were transported to the laboratory within

two hours of collection in a protective container which provided near optimal pre-

analytical conditions for the InPouch™TF culture which makes this sensitivity estimate a

realistic expectation for properly handled field specimens examined with proper

analytical methods.

Rae et al. (1999) also investigated infertility in a large beef cattle herd and found

TF contributing to the infertility when they collected specimens from all herd bulls

multiple times until no new TF infected bulls were detected with some bulls tested six or

seven times. Specimen collection was by pipette aspiration with incubation of the

inoculated InPouch™TF at 37⁰ C for five days and examination on days one or two and

four or five. Culture sensitivity for this study based on the number of positive specimens

from the first sampling event divided by the total number of specimens collected from

bulls determined to be TF infected after all sampling events was 73%. The authors

concluded the lower than other previously reported TF culture sensitivities was likely due

to pre-analyitical conditions affecting specimen quality such as extensive and remote

cattle working facilities which made handling and transport of large numbers of

specimens difficult, fractious bulls, harsh environmental conditions, inconsistent bull

identification, and contamination of specimens with dirt and feces, and analytical factors

related to examining large number of specimens following each sampling event such as

750 specimens collected at the first sampling event. Examining each specimen twice

may have contributed to reduced detection of TF positive specimens after the first

sampling event.

During an investigation of infertility in a beef cattle herd Peter et al. (1995)

collected specimens from all herd bulls three times at weekly intervals using pipette

aspiration and InPouch™TF with pouches examined daily for seven days during

incubation at 35° C. The overall sensitivity of TF culture for the study was 70.4%;

however, sensitivity for specimens collected during weeks one and three was 83.3%

while week two sensitivity was 44.4%. Investigators concluded pre-analytical factors

such as variations in collection, handling, culture techniques, and fluctuating preputial TF

populations may have resulted in the decreased week two sensitivity. This study

highlights the significance of pre-analytical factors over analytical factors when

reasonable analytical techniques are used.

The lowest reported sensitivity for TF culture was from Cobo et al.'s (2007) study

of experimentally infected bulls sampled weekly for six weeks by pipette aspiration with

the InPouch™TF incubated at 37° C for 7 days and examined on days one, three, five, and

seven post-inoculation for an average sensitivity of 67.8% (95% CI, 51.1 to 84.1). The

sensitivity was calculated as the number of culture positive samples out of the total

number of samples from experimentally infected bulls which is probably an

underestimation of sensitivity as four bulls produced no TF positive specimens by culture over the six week study and were most likely not TF infected.

While the sensitivity of TF culture has a wide range due to pre-analytical and analytical factors which vary between studies the diagnostic specificity of TF culture, percentage of TF free bulls identified as uninfected by the test (Saah and Hoover, 1997), has been assumed to be nearly 100% until recent years (Rodning, 2007).

BonDurant et al. (1999) identified trichomonads in specimens maintained in InPouch™TF from virgin bulls with morphologic and motility characteristics as seen under 100 to 400 magnification brightfield microscopy consistent with TF. Further testing of the trichomonads through staining, scanning electron microscopy, and PCR revealed the organisms possessed four anterior flagellae which led the authors to speculate their identity as lower bowel commensal trichomonads, possibly *Tetratrichomonas pavlovi* or *Tetratrichomonas buttreyi,* which were transferred to the prepuce in feces during sodomy of herdmates. Several subsequent investigations of non-TF preputial trichomonads supported these findings (Campero et al., 2003; Cobo et al., 2003) and identified additional non-TF preputial trichomonads such as *Pentatrichomonas hominis* (Walker et al., 2003; Corbeil et al., 2008) and *Pseudotrichomonas* sp. (Dufernez et al., 2007).

Young bulls tend to mount each other frequently which make them more likely to experience fecal contamination of the prepuce (BonDurant et al., 1999) which is indicated by Campero et al. (2003) findings of 8.4% of 567 virgin bull preputial specimens culture positive with non-TF trichomonads. However, mature bulls may also

produce non-TF culture positive specimens as shown by Corbeil et al.'s (2008) report of 14 virgin bull specimens positive mostly for *Tetratrichomonas* sp. and 31 breeding age bull specimens positive for mostly *Pentatrichomonas hominis*.

Cobo et al. (2004) inoculated cultures a *Tetratrichomonas* sp. isolated from a virgin bull into the prepuces of four 6 year old bulls but failed to generate a single positive culture from subsequent specimens collected from the bulls suggesting the *Tetratrichomonas* sp. did not colonize the preputial cavity and serve only as confounders for TF diagnosis when present in the prepuce. In a separate study Cobo et al. (2007) inoculated mature bulls with *Tetratrichomonas* sp., *Campylobacter fetus venerealis*, TF, and both *Campylobacter fetus venerealis* and TF to examine sensitivity and specificity of TF diagnostic tests and found *Tetratrichomonas* sp. was only sporadically detected by culture resulting in a specificity of 99%. Because of the experimental nature of this study it may not reflect the true specificity of field acquired specimens.

Pre-analytical factors for cultural TF detection make it difficult to assess the impact of analytical aspects of TF culture on the test's sensitivity and specificity, but the studies discussed in this section illustrate specimens handled under ideal pre-analytical conditions with appropriate analytical conditions yield TF culture sensitivities above 90% while specimens handled under less than ideal pre-analytical conditions or with subpar analytical techniques produce sensitivities below 80%.

Although TF culture specificity is no longer assumed to be 100% only the single reference discussed earlier could be found that gave a data based estimated specificity of 99% which is influenced by pre-analytical factors such as bull age and fecal

contamination and the analytical factor of microscope technician skill in differentiating between TF and other trichomonads under standard microscopy.

Polymerase chain reaction – To overcome concerns with TF culture sensitivity and specificity investigators have examined the value of PCR as a TF diagnostic assay based on the assumptions of amplification of DNA segments specific to TF would reduce or eliminate false positives thereby increasing testing specificity and would allow identification of TF positive specimens without the presence of living TF or when specimens contained few TF which would increase testing sensitivity by decreasing false negatives (Morgan et al., 1998).

In general PCR involves subjecting DNA of interest to a series of alternating temperatures in the presence of polymerase, primers, and other reaction components which leads to the replication of specific segments of the DNA called amplicons (BonDurant et al., 2003). Table 2.4 provides specific details of PCR techniques used for TF detection. Methods for detection of the amplicon varies between gel and real time PCR. As suggested by its name gel PCR relies on electrophoresis of the PCR product on an agarose (BonDurant et al., 2003) or polyacrimide (Felleisen et al., 1998) gel for detection of the amplicon after the amplification process is completed. Figure 2.3 demonstrates the appearance of a TF positive gel PCR with the expected 347 base pair amplicon indicated for lanes 3, 4, 5, and 6 while lanes 1 and 2 are from non-infected control animals and lane 7 is an empty control. Real time PCR is conducted under the same general concepts but with slightly different techniques that allow the amplicon to be detected through fluorescence as the amplification process proceeds through the cycle of

Table 2.4 – PCR Techniques.

Author	Year	Target DNA/ Gene	Primer	Temperature Profile					Cycles	Amplicon (in base pairs)	Amplicon Detection
				Initial denature	Denature	Annealing	Extension	Final extension			
Ho et al.	1994	N/R	TF1 & TF2	94°;4 min	94°;1 min	45°;1 min	72°;2 min	72°;7 min	41	162	Chemilminescent internal probe
Riley et al.	1995	N/R	TF1 & TF2	94°;5 min	94°;1 min	45°;1 min	72°;2 min	72°;7 min	41	162	Chemilminescent internal probe
Felleisen	1997	5.8S rRNA, ITS1, ITS2	TFR1 & TFR2	N/R	94°;30 sec	66°;30 sec	72°;90 sec	72°;15 min	40	372	10% polyacrylamide gel
Felleisen et al.	1998	5.8S rRNA, ITS1, ITS2	TFR3 & TFR4	N/R	94°;30 sec	67°;30 sec	72°;90 sec	72°;15 min	40	347	10% polyacrylamide gel or 2% agar gel/ ethidium bromide or silver stain or DEIA[a]
Felleisen et al.	1997	N/R	TF1 & TF2	94°;4 min	94°;1 min	45°;1 min	72°;2 min	72°;7 min	41	162	Chemilminescent internal probe
		5.8S rRNA, ITS1, ITS2	TFR3 & TFR4	N/R	94°;30 sec	67°;30 sec	72°;90 sec	72°;15 min	40	347	10% polyacrylamide gel or 2% agar gel/ DEIA
Parker et al.	2001	5.8S rRNA, ITS1, ITS2	TFR3 & TFR4	N/R	94°;30 sec	67°;30 sec	72°;90 sec	72°;15 min	40	347	1.5% agar gel/ ethidium bromide
Nickel et al.	2002	18S rRNA, ITS1, 5.8S rRNA	TF211A & TF211B	N/R	94°;30 sec or 60 sec	67°;30 sec or 60 sec	72°;30 sec or 60 sec	N/R	35	211	1.5% agar gel/ ethidium bromide
Mukhufi et al.	2003	5.8S rRNA, ITS1, ITS2	TFR3 & TFR4	N/R	94°;30 sec	67°;30 sec	72°;90 sec	72°;15 min	40	347	1.5% agar gel/ ethidium bromide
Hoevers et al.	2003	5.8S rRNA, ITS1, ITS2	TFR3 & TFR4	N/R	94°;30 sec	67°;30 sec	72°;90 sec	72°;15 min	40	347	DEIA

[a] DNA enzyme immunoassay

Table 2.4 (Cont'd) – PCR Techniques.

Author	Year	Target DNA/ Gene	Primer	Temperature Profile					Cycles	Amplicon (in base pairs)	Amplicon Detection
				Initial denature	Denature	Annealing	Extension	Final extension			
Campero et al.	2003	5.8S rRNA, ITS1, ITS2	TFR1 & TFR2/ TFR3 & TFR4	N/R	N/R	N/R	N/R	N/R	40	372/347	2% agar gel/ ethidium bromide
BonDur-ant et al.	2003	5.8S rRNA, ITS1, ITS2	TFR3 & TFR4	N/R	94°;30 sec	67°;30 sec	72°;90 sec	72°;15 min	40	347	2% agar gel/ ethidium bromide
Grahn et al.	2005	ITS1 between 18S & 5.8S rRNA	ITS1 primers	94°;3 min	94°;30 sec	58°;20 sec	72°;30 sec	72°;20 min	30	157	6% polyacrylamide & 2.5% agar gels
McMil-len and Lew[b]	2006	5.8S rRNA, ITS1, ITS2	TFR3 & TFR4	94°;90 sec	94°;30 sec	67°;30 sec	72°;90 sec	72°;15 min	40	347	2% agar gel/ ethidium bromide
		ITS1	TFF2 & TFR2	50°;2 min/95°.; 2 min	95°;20 sec	60°;45 sec		N/R	40	N/R	Fluoresence
Mutto et al.	2006	5.8S rRNA, ITS1, ITS2	TFR3 & TFR4	N/R	94°;30 sec	67°;30 sec	72°;60 sec	N/R	30	N/R	2% agar gel/ ethidium bromide
Cobo et al.	2007	5.8S rRNA, ITS1, ITS2	TFR1 & TFR2/ TFR3 & TFR4	95°;10 min	95°;30 sec	60°;30 sec	72°;60 sec	72°;7 min	35	372/347	1.5% agar gel/ ethidium bromide
Huby-Chilton et al.	2009	5.8S rRNA, ITS1, ITS2	TFR1 & TFR2	95°;10 min	95°;30 sec	60°;30 sec	72°;60 sec	72°;7 min	30	425	1.5% agar-TBE gel/ SYBR gold stain
		16S rRNA	NTAC1 & NTAC2	95°;5 min	95°;60 sec	55°;60 sec	72°;60 sec	72°;7 min	30	360-400	1.5% agar-TBE gel/ SYBR gold stain

[b]First reference found discussing the use of real time PCR for *T. foetus* detection

temperature changes(McMillen and Lew, 2006). The fluorescence is recorded and charted on a graph while the reaction continues with those specimens whose fluorescence exceeds the predetermined threshold prior to completion of the reaction being declared positive (Figure 2.4).
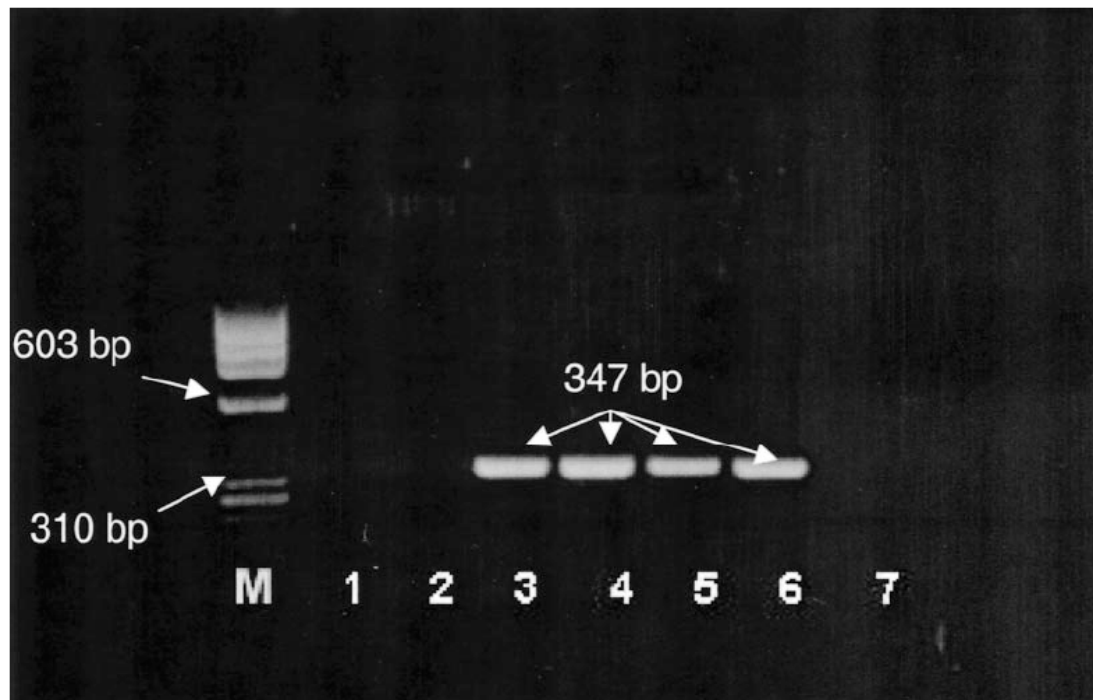
Figure 2.3. PCR Gel Electrophoresis (BonDurant et al., 2003).

Ho et al. (1994) developed a PCR assay utilizing primers TF1 and TF2 to amplify a 162 base pair (bp) product from an unspecified region of TF DNA. The oligonucleotide sequence of these primers and other primers used for TF PCR are shown in Table 2.5. The assay's sensitivity of 88.6%, 39 positive specimens out of 44 specimens from known positive bulls, was comparable to traditional culture in spite of its ability to detect as few as one TF in pure medium and 10 TF in medium with smegma. No false positives were detected from eight TF negative bulls. Detection limit (analytical sensitivity), diagnostic
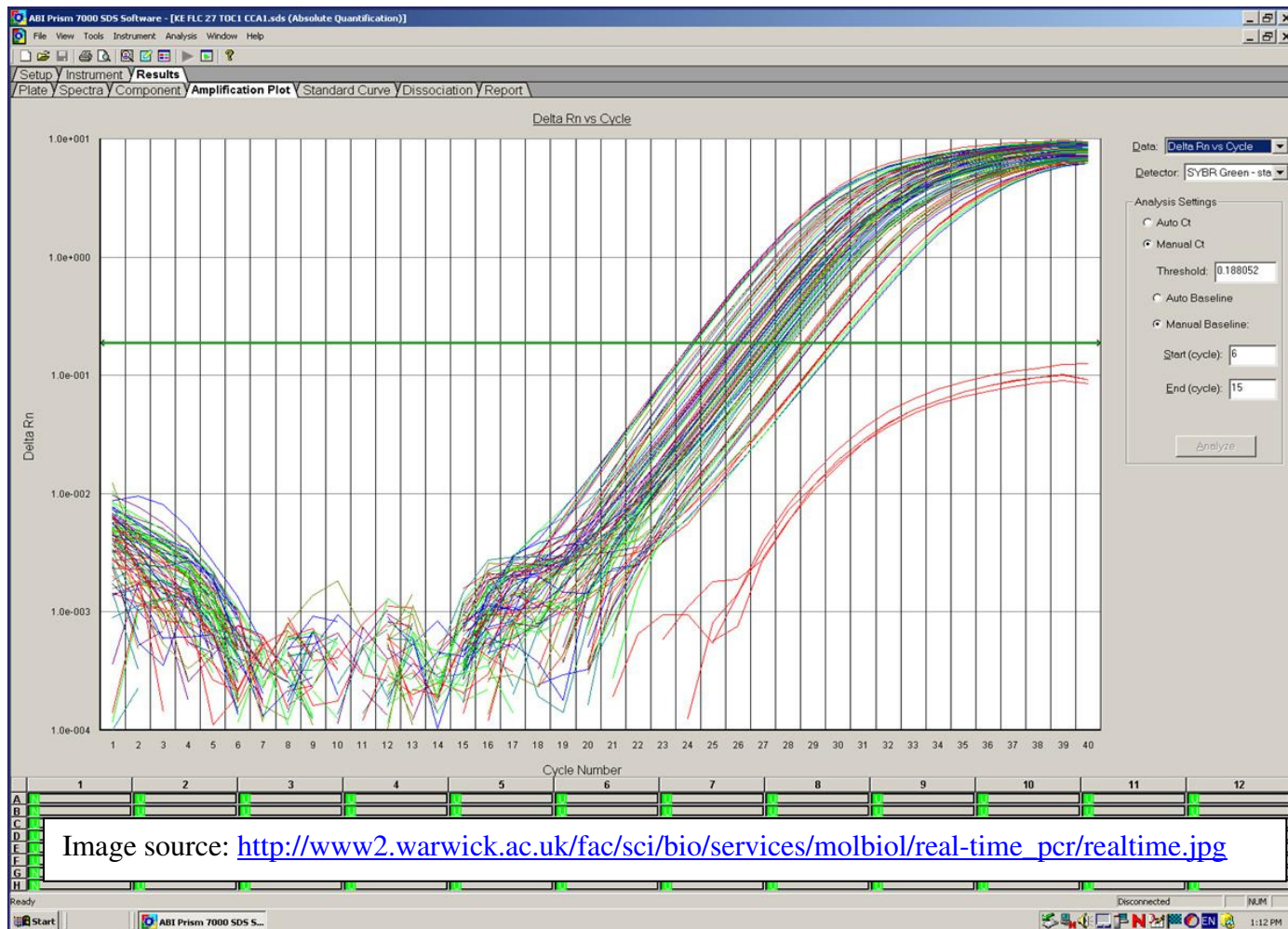
Figure 2.4. Real time PCR Output Screen.

Table 2.5 – PCR Primer Sequences. Key: C = cytosine;   A = adenine; T = thymine; G = guanine.

Primer	Sequence (5' to 3')	Reference
TF1	CATTATCCCAAATGGTATAAC	Ho et al., 1994
TF2	GTCATTAAGTACATAAATTC	
TFR1	TGCTTCAGTTCAGCGGGTCTTCC	Felleisen, 1997
TFR2	CGGTAGGTGAACCTGCCGTTGG	
TFR3	CGGGTCTTCCTATATGAGACAGAACC	Felleisen et al., 1998
TFR4	CCTGCCGTTGGATCAGTTTCGTTAA	
TFR3pK	CGGGTCTTCCTATATGAGACAGAACCGGAGCTGAATG	
TFR4pK	CCTGCCGTTGGATCAGTTTCGTTAAGGGATTTTGGT	
TF211A	CCTGCCGTTGGATCAGTTTCGTTA	Nickel et al., 2002
TF211B	GCGCAATGTGCATTCAAAGATTCG	
Forward	GTAGGTGAACCTGCCGTTG	Grahn et al., 2005
Reverse	ATGCAACGTTCTTCATCGTG	
TFF2	GCGGCTGGATTAGCTTTCTTT	McMillen & Lew, 2006
TFR2	GGCGCGCAATGTGCAT	
NTAC1	CTCCAGAAGTGAATTATG	Huby-Chilton et al., 2009
NTAC2	TCTAGATAACGTGATTTAATCAC	

sensitivity, and diagnostic specificity reported for selected references are summarized in Table 2.6.

Riley et al. (1995) conducted further evaluation of the TF1-2 primers PCR assay and found 16 of 17 specimens produced strong amplification of the expected 162 bp PCR product while one specimen produced a weak amplicon which was attributed to genetic variation at the TF1 and TF 2 primer amplification site or to the isolate not being TF. Felleisen et al's. (1997) evaluation of Ho's PCR assay found approximately one third of control specimens produced an amplification product which was slightly larger than the expected 162 bp diagnostic band and assumed to be the result of nonspecific amplification.

Felleisen (1997) used primers TFR1 and TFR2 in a PCR assay to sequence the 5.8S ribosomal riboneucleic acid (rRNA) and adjacent internal transcribed spacer regions (ITS) 1 and 2 and found TF, *Tritrichomonas suis*, and *Tritrichomonas mobilensis*

Table 2.6 – PCR Detection Limit, Sensitivity (Se), and Specificity (Sp).

Author	Year	Medium	Specimen	Detection limit	Se[a]	Sp[b]
Ho et al.	1994	Modified Diamond's	Reference & field	1 TF in pure medium; 10 TF in smegma	88.6% (39/44)	100% (8/8)
Felleisen et al.	1998	Modified Diamond's/ InPouch™TF/ other media	Reference & field	1 TF in pure medium; 50 TF in field specimens	N/R	N/R[c]
Parker et al.	2001	Modified Diamond's	Reference & field	50 TF/mL or 5 TF/ extracted specimen	N/R	N/R
Nickel et al.	2002	Diamond's	Reference & field	1 TF in pure medium	N/R	N/R
Mukhufhi et al.	2003	PBS w/ or w/o GuSCN/ commercial medium	Reference & field	100 TF / specimen; 2 TF/mL of specimen	31-90%[d]	98%
Mutto et al.	2006	Diamond's	Reference & field	5 TF/ specimen	98.3%[e]	93.75%[e]
Cobo et al.	2007	InPouch™TF	Reference	N/R	66.1%[f] (119/180)	98%

[a] Se shown as % with number positive from total possible positive tests in parenthesis.
[b] Sp shown as % with number of negative tests from total possible negative tests in parenthesis.
[c] No data given, but no false positives were detected so specificity was reported as "very high".
[d] Se varied due to pre-analytical treatment effects.
[e] PCR Se and Sp calculated with specimen culture results as the reference TF status.
f Sensitivity based on number of positive specimens from total number of specimens from experimentally infected bulls. Three bulls did not produce any positive specimens during the study, but samples from these bulls were included in the analysis as samples from infected bulls.

displayed a high degree of similarity at this region while other trichomonads were more diverse suggesting this region which is duplicated 12 times in the TF genome (Chakrabarti et al., 1992) as a highly suitable target for DNA amplification.

Primers TFR3 and TFR4 were developed by Felleisen et al. (1998) to target the 5.8S rRNA, ITS1, and ITS2 regions for PCR amplification while incorporating a uracil DNA glycosylase system to prevent DNA carryover from previous reactions and a DNA enzyme immunoassay (DEIA) for the detection of the amplicon. The 347 bp amplification product was obtained from eight strains of TF, *Tritrichomonas suis*, and *Tritrichomonas mobilensis*, but no amplification product was produced from PCR assays of specimens containing other trichomonads, bacterial DNA, or bovine DNA. The assay

was able to detect quantities of DNA equivalent to a single TF organism in pure media and as few as 50 organisms per mL in specimens containing smegma, bacteria, and other debris. False positive specimens which were an issue with the TF1-2 PCR were not found with the TFR3-4 assay indicating higher test specificity. Several subsequent studies of TFR3-4 PCR supported these findings with similar results (Felleisen et al., 1997; Parker et al., 2001; Hoevers et al., 2003; BonDurant et al., 2003).

As shown in Table 2.6 multiple studies have reported high analytical sensitivity and high diagnostic specificity for PCR regardless of the primers used in the assay. However, few have examined the diagnostic sensitivity of the test. Mukhufhi et al.'s (2003) evaluation of PCR was discussed earlier under pre-analytical considerations and indicated sensitivity for TFR3-4 PCR of 31 to 90% depending on the pre-analytical conditions applied to the specimens in spite of a reported detection limit of two TF organisms per mL of specimen. See Table 2.1 for details of sensitivity ranges. Diagnostic specificity for TFR3-4 PCR from this study was 98%.

Mutto et al. (2006) utilized TFR3-4 PCR to test 203 specimens collected from bulls of unknown TF status and found a detection limit of five TF per specimen, diagnostic sensitivity of 98.3%, and diagnostic specificity of 93.7% with culture results as the reference for calculating the sensitivity and specificity (Table 2.7). Three culture negative, PCR positive specimens came from bulls which were culture positive on second

Table 2.7 –Trial 2X2 Culture/Gel PCR Comparison.

		Culture		
		Positive	Negative	Total
TFR3-4 PCR	Positive	58	9	67
	Negative	1	135	136
	Total	59	144	203

or third sampling events suggesting PCR is more sensitive than culture and the single culture positive, PCR negative specimen may have been due to the presence of a non-TF trichomonad resulting in a false positive culture.

In 2003 Campero et al. (2003) suggested a two step TF diagnostic approach utilizing both TFR1-2 and TFR3-4 PCR assays. The first step would be the relatively inexpensive culture with culture positive specimens confirmed by a second step involving two separate PCR assays where the presence of a 372 bp amplicon in the TFR1-2 PCR indicates trichomonad DNA in the specimen and the presence of a 347 bp amplicon in the TFR3-4 PCR reaction indicates the presence of TF. Production of a 372 bp amplicon without the 347 bp amplicon from a culture positive specimen indicates adequate DNA for analysis, but not TF DNA, suggesting a false positive culture result.

In the most thorough investigation of PCR sensitivity and specificity Cobo et al. (2007) applied Campero et al.'s (2003) TFR1-2 and TFR3-4 PCR tandem to specimens collected from artificially TF infected bulls and found sensitivity of 66.1% and specificity of 98% for single specimen testing. The sensitivity is considerably lower than expected and is likely due to the sensitivity being calculated as the number of PCR positive samples out of the total number of samples from experimentally infected bulls which is probably an underestimation of sensitivity as three bulls produced no TF positive specimens by PCR over the six week study and were most likely not TF infected.

McMillen and Lew (2006) examined the use of rtPCR for TF diagnosis using primers TFF2 and TFR2 and a fluorescent probe after employing a heat lysis method for crude cell lysate preparation. The sequence of McMillen and Lew's TFR2 is different

from Felleisen's (1997) TFR2 as shown in Table 2.3. The detection limit for rtPCR was reported to be a single cell equivalent for laboratory spiked preputial smegma specimens with less than a cell equivalent per assay reliably detected from several heat-lysed specimens which was a 2500-fold greater analytical sensitivity than culture and similar to TFR3-4 PCR in analytical sensitivity. A field based comparison of culture and rtPCR utilizing specimens from 159 animals in known TF infected herds found 3 TF culture positive specimens and 14 TF rtPCR positive specimens which the authors concluded demonstrated the superior sensitivity of rtPCR over culture. However, no repeat testing of test positive animals was undertaken to confirm their TF status leaving open the possibility of rtPCR false positive results. No rtPCR diagnostic sensitivity or specificity estimates were given in this reference.

Other primers and PCR techniques have been investigated for TF diagnostic utility with the purpose of differentiating between TF and non-TF trichomonads, but will not be discussed as the primers were not utilized by the diagnostic laboratories cooperating with the investigations described later in this thesis. See Tables 2.4, 2.5, and 2.6 for other PCR techniques, primer sequences, and performance.

Statement of problem

Chapter 1 demonstrated the importance of accurately identifying TF infected bulls for a successful TF prevention or control program. Early investigators (Bartlett et al., 1947; Fitzgerald et al., 1952) recognized inconsistent positivity of preputial specimens from known TF positive bulls which could be ascribed to primitive pre-analytical and analytical practices except examples of this continued to appear in more recent studies

utilizing modern sampling and cultural techniques (Clark et al., 1971; Skirrow et al., 1985; Peter et al., 1995; Parker et al., 1999). To account for the reduced sensitivity of TF culture resulting from inconsistent positivity the standard procedure was six consecutive cultured specimens collected at weekly or greater intervals. More recently three sequentially collected and cultured specimens were shown to approach 100% probability of detecting all infected bulls tested (Kimsey et al., 1980). However, repeated testing is difficult under field conditions because of time, cost, and management constraints as well as safety concerns for the bulls and handlers.

The development of PCR as a TF diagnostic test offered hope of a more sensitive and specific test that may reduce the number of specimens required before confidently declaring a bull TF positive or negative. However, studies by Mukhufhi et al. (2003) and Hoevers et al. (2003) demonstrated similar diagnostic sensitivities between culture and TFR3-4 PCR when applied to field specimens in spite of superior PCR analytical sensitivity. Based on the findings of their study in experimentally infected bulls Cobo et al. (2007) proposed an alternative TF testing strategy that involved combinations of tests and fewer sampling events, but still required multiple sampling events to adequately detect all TF infected bulls tested.

McMillen and Lew's (2006) application of rtPCR to TF diagnostic testing appears to have a lower detection limit than culture or conventional PCR, but limited data is available to assess whether this will overcome pre-analytical and analytical concerns associated with the other TF diagnostic tests.

The purpose of the studies described in the following chapters is to examine the ability of culture, conventional gel-based PCR, and rtPCR to detect TF in field collected specimens and develop tactics for efficiently identifying TF infected bulls in beef cattle herds using one or more of these tests while reducing the number of sampling events per bull.

Chapter 3

Spring 2008 Outbreak Investigation

Introduction

As discussed in the previous chapter PCR has the potential of rapid, accurate detection of TF positive bulls. The low theoretical detection limit of PCR (2 parasites/ml) (Mukjufhi et al., 2003) suggests use of PCR may require fewer sampling and testing events than the currently recommended three sequential cultures for identifying all infected bulls in an infected herd (Kimsey et al., 1980) assuming consistent colonization of sampled sites. Our study was conducted to assess this assumption under field conditions.

In the present study, a prospective cohort design, sequential bull preputial sampling and three TF diagnostic assays (culture, gel PCR and rtPCR) were used to investigate and control a TF outbreak. The study sites were two adjacent beef cattle ranches on semi-arid rangeland in the central Nebraska Sandhills which utilize grazing allotments on public land. Ranches A and B were commercial Angus herds with approximately 1,500 cows and 3,000 cows respectively. Although results from three diagnostic tests were compared, the purpose of this study was not to validate or justify TF assays. Rather we wanted to utilize TF tests currently offered to veterinarians and livestock producers from accredited veterinary diagnostic laboratories during an outbreak investigation to investigate optimum TF testing strategies. The specific study objectives were threefold: (1) to compare the agreement of culture with gel and rtPCR assays in identifying TF positive preputial specimens; (2) to compare the agreement of culture with

gel and rtPCR assays in identifying TF infected bulls based on three sequential specimens; (3) to examine the agreement between single and tri-sequential specimen testing in classifying bull TF status.

Materials and methods

Bull preputial specimen collection **–** A census cohort of breeding bulls (n=125 Angus bulls, 2 to 8 years of age) on both ranches were identified and enrolled with owners' consent. Initial diagnosis of TF in the respective herds by the herd health veterinarian occurred during an investigation of recent, increased cow reproductive failure. Both ranches utilized the same herd health veterinarian. Bulls were isolated from cows following the previous breeding season which ended July 2007 and remained isolated from cows throughout the sequential bull samplings in order to prevent new bull infections. Bull preputial specimen collection began on April 7, 2008 and concluded on May 3, 2008 and May 20, 2008 for Ranches A and B respectively. Days between specimen collections were 8 to 27. Multiple bulls were lost to follow up primarily due to misidentification of bulls from repetitive identifiers, inadequate record keeping, and rapid processing and failure of owner compliance to the study protocol (Figures 3.7 and 3.8).

Sampling events 1, 2, and 3 – The technique utilized for collection of preputial specimens was one commonly used in the United States (Peter, 1997) and employed a plastic disposable uterine infusion pipette (0.50 cm outside diameter x 53.34 cm) and a sterile 12 ml plastic disposable syringe. A new pipette, syringe, and latex examination gloves were used by investigators when sampling each bull. The free end of the uterine infusion pipette was introduced into the prepuce to the level of the fornix with the syringe

attached to the opposite end (Figure 3.1). The free tip of the pipette was moved back and

forth to scrape the surface of the prepuce and penis while suction was applied with

Figure 3.1 –Specimen Collection Location.

the syringe. After approximately 20 scraping cycles the suction in the syringe was gently

released and the pipette was removed from the prepuce and examined for specimen

adequacy. An adequate specimen was defined as slightly blood-tinged preputial mucus

filling at least 1.3 cm of the lumen of the pipette. If an inadequate specimen was

obtained the same pipette was reintroduced and a second collection was attempted. The

preputial specimen was inoculated into the upper chamber of an InPouch™TF (Figure

3.2) by tearing off the upper plastic portion of the upper chamber at the notch and

repeatedly drawing media from the upper chamber into the pipette and flushing it back

into the upper chamber until the preputial material was sufficiently transferred to the

pouch. Closure of the pouch according to manufacturer's directions consisted of

expressing as much air as possible from the upper chamber, rolling the open end of the

pouch down to the level of the top of the label, and folding each end of the wire tab over

Figure 3.2 - InPouch™TF.

the pouch to prevent unrolling (Appendix A). Inoculated pouches were placed upright in

an insulated container with hot water bottles which provided an ambient temperature of

approximately 20^0 C until it could be transferred to an incubator. The environmental

temperature across all sampling events ranged from 5.6^o to 20.0^o C. Inoculated pouches

were placed in an incubator at 37° C within 6 hours of field collection.

Sampling event 4 – Due to loss of follow-up of a significant number of positive bulls, bulls from both ranches sold for slaughter and purchased by a local abattoir were sampled for trichomoniasis. The external reproductive tracts ("pizzles") were retrieved from the abattoir after removal from the carcass during routine slaughter processing and transported to the Great Plains Veterinary Educational Center laboratory for sampling (Figure 3.3). Specimen collection was carried out in a manner that closely duplicated the ante mortem sampling technique. Preputial specimens were collected with a uterine infusion pipette (0.50 cm outside diameter x 53.34 cm) and a sterile 12 ml syringe within 3 hours of the bulls' death. A new pipette, syringe, and latex examination gloves were used by investigators when sampling each pizzle.

Figure 3.3 – Pizzle.

The exposed edge of the remnant of the prepuce was held with gloved fingers and the free end of the uterine infusion pipette was introduced into the prepuce to the level of the fornix with the syringe attached to the opposite end (Figure 3.4).

Figure 3.4 – Post Mortem Specimen Collection

The free tip of the pipette was moved back and forth to scrape the surface of the
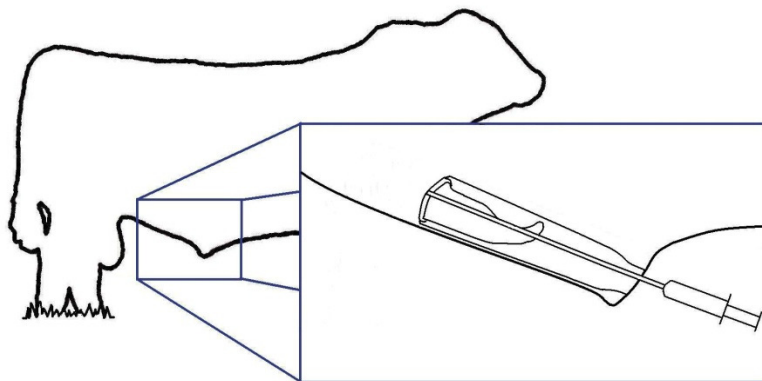
prepuce and penis while suction was applied with the syringe. After approximately 20

scraping cycles the suction in the syringe was gently released and the pipette was

removed from the prepuce and examined for specimen adequacy. An adequate specimen

was defined as preputial mucus filling at least 1.3 cm of the lumen of the pipette. If an

inadequate specimen was obtained the same pipette was reintroduced and a second

collection was attempted. The preputial specimen was inoculated into the upper chamber

of an InPouch™TF by tearing off the upper plastic portion of the upper chamber at the

notch and repeatedly drawing media from the upper chamber into the pipette and flushing

it back into the upper chamber until the preputial material was sufficiently transferred to

the pouch. Closure of the pouch according to manufacturer's directions consisted of expressing as much air as possible from the upper chamber, rolling the open end of the pouch down to the level of the top of the label, and foldi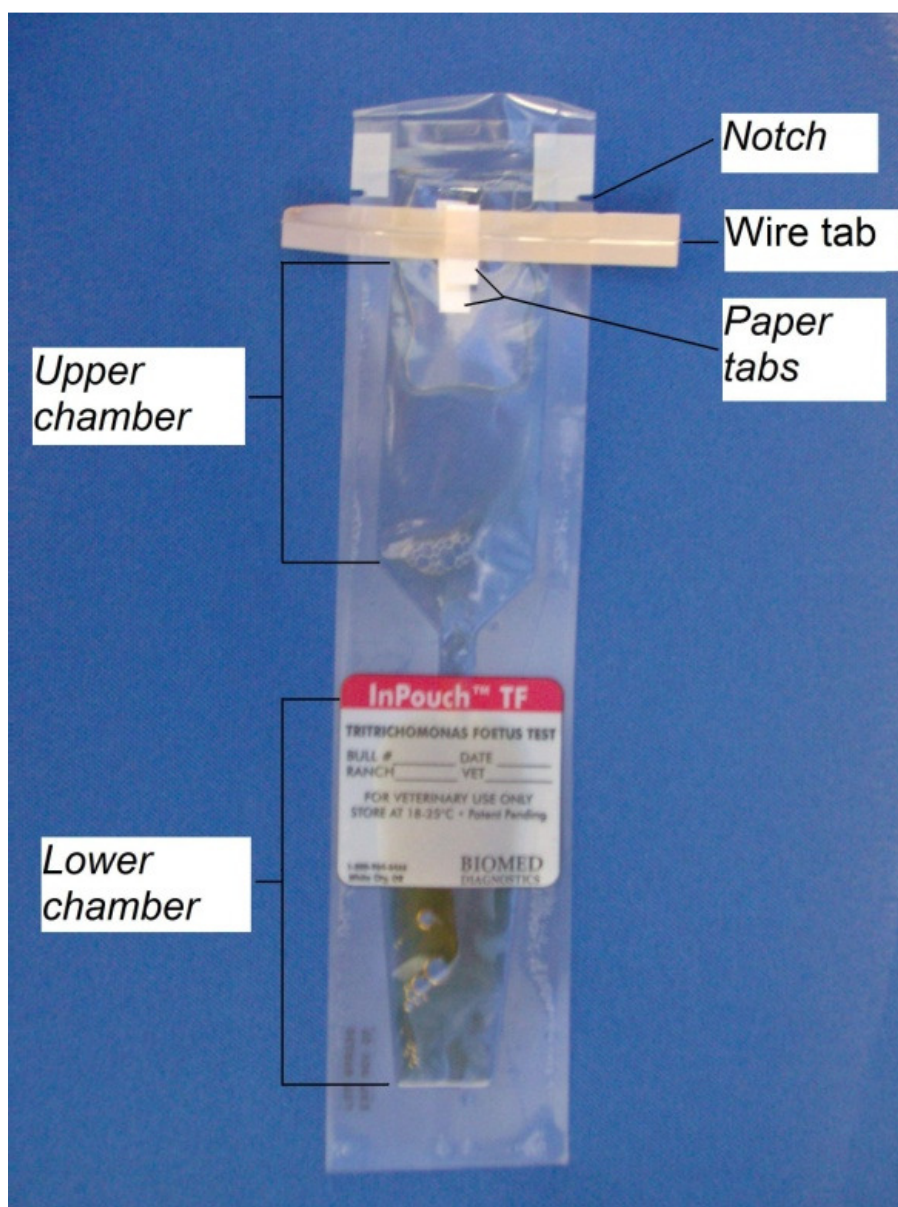ng each end of the wire tab over the pouch to prevent unrolling. Inoculated pouches were placed in the investigator's shirt pocket until transfer to an incubator after all specimens were collected. Inoculated pouches were placed in an incubator at 37° C within 5 hours of the bulls' death and within 2 hours of inoculation into an InPouch.

Culture - Sampling event 1 – Inoculated pouches were incubated upright at 37° C for six days. Microscopic examination of pouches started 24 hours after collection and continued on days 2, 4, and 6 post collection. All pouches were read by the local herd health veterinarian at his clinic under a compound light microscope at 100X magnification by fixing the pouch in a plastic clip provided by the manufacturer (Figures 3.5 and 3.6) The lower chamber of the pouch was systematically scanned along its seam edges starting approximately 1 cm from the bottom on one side, down to and along the bottom, continuing up the other side approximately 1 cm, and then directly across the lower compartment to the original starting point. Approximately 2-3 minutes were required to complete the examination of each negative pouch. Pouches were classified presumptive positive based on visualization of live protozoa with size, morphology and motility patterns consistent with TF on one or more of the examinations.

After day six microscopic examinations, the pouches were submitted to an American Association of Veterinary Laboratory Diagnosticians (AAVLD) accredited veterinary diagnostic laboratories by overnight delivery. Real time PCR was conducted
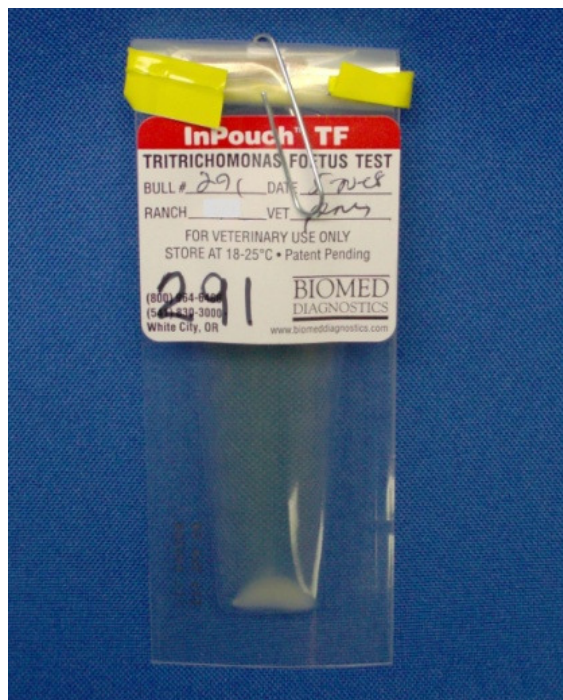
Figure 3.5 – Inoculated and Incubated InPouch™TF.
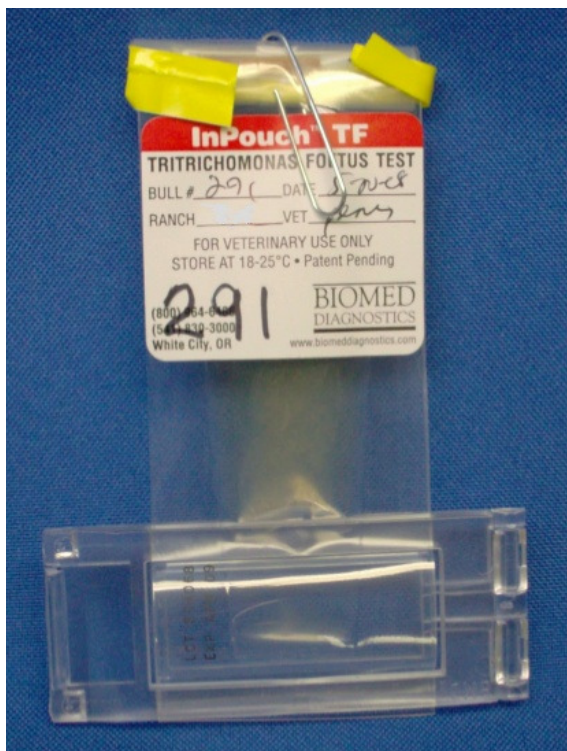
Figure 3.6 - InPouch™TF Prepared for Microscopic Examination.

using standard laboratory protocols at the University of Nebraska-Lincoln Veterinary Diagnostic Laboratory. Specimen aliquots unused for rt PCR were frozen at -20° C and held at the laboratory for 5 weeks after which time they were shipped to another AAVLD accredited veterinary diagnostic laboratory by overnight delivery. Gel PCR was conducted using standard laboratory protocols at the Colorado State University Veterinary Diagnostic Laboratory at Rocky Ford.

Culture - sampling events 2, 3, and 4 -Inoculated pouches were incubated upright at 37° C for four days. Daily microscopic examination of pouches started 24 hours after collection and continued through day four post collection. A single experienced veterinarian blinded to previous daily examination results and bull identification numbers examined all pouches. A clip provided by the manufacturer fixed the pouch for examination under a compound light microscope at 100X magnification. The lower chamber of the pouch was systematically scanned along its seam edges starting approximately 1 cm from the bottom on one side, down to and along the bottom, continuing up the other side approximately 1 cm, and then directly across the lower compartment to the original starting point. Approximately 2-3 minutes were required to complete the examination of each negative pouch. Pouches were classified presumptive positive based on visualization of live protozoa with size, morphology and motility patterns consistent with TF on one or more of the five days of examination.

After day four microscopic examinations, the pouch sediment was suspended in the media by gently pulling the pouch up and down across the edge of a counter 3-4 times. Two equal aliquots of approximately 2 ml were then aseptically pipetted into

sterile cryogenic vials for submission to two separate AAVLD accredited veterinary

diagnostic laboratories by overnight delivery. Gel PCR and rt PCR were conducted using

standard laboratory protocols at the Colorado State University Veterinary Diagnostic

Laboratory at Rocky Ford and the University of Nebraska-Lincoln Veterinary Diagnostic

Laboratory, respectively.

<u>Polymerase Chain Reaction</u> - Gel-based and rt PCR assays used specific primers

targeting the TF 5.8S ribosomal RNA gene and the flanking internal transcribed spacer

regions ITS1 and ITS2. For gel PCR, DNA was extracted by a commercial kit[3] per

manufacturer's protocol and the assay was performed using primers TFR3 and TFR4

(Kennedy et al., 2008) Specimens were considered gel PCR positive if a 347 base pair

amplicon was visualized following electrophoresis on an ethidium bromide stained

agarose gel (Appendix B). Culture-positive, TF gel PCR-negative samples were retested

by gel PCR with pan-trichomonal primers TFR1 and TFR2 and were considered gel PCR

positive for non-TF trichomonads if a 372 base pair amplicon was visualized following

electrophoresis. Specimens that tested pan trichomonad PCR positive but TF PCR

negative were considered non-TF trichomonad positive (Campero et al., 2003)

For rt PCR, DNA was extracted using the heat lysis method (McMillen and Lew,

2006) and the assay was performed utilizing commercially available primers TFF2 and

TFR2[4] and probe 6FAM[5]. The analysis was carried out in a commercial rt PCR detection

and analysis system[6] (Appendices C and D). Specimens were defined as rt PCR positive,

[3] DNeasy Blood and Tissue Kit, Qiagen Inc., Valencia, CA.
[4] TaqMan Gene Expression Master Mix, Applied Biosystems, Carlsbad, CA.
[5] TaqMan Probe, Applied Biosystems, Carlsbad, CA.
[6] 7500 Fast PCR System version 2.0.1, Applied Biosystems, Carlsbad, CA.

suspect, and negative when cycle threshold values were less than 34.00, 34.01 to 40.00, and greater than 40.00, respectively.

Data analysis - Comparative pouch culture, gel PCR, and rt PCR results were analyzed by a 2x2 contingency table spreadsheet (Mackinnon, 2000) in order to estimate agreement between methods in determining TF status of individual pouches and tri-sequential bull infection status. Cohen's Kappa statistic and McNemar's paired sample Chi square test p values calculated from the 2x2 tables were used to assess agreement and statistical difference between test results or bull tri-sequential infection status results. Individual specimens were declared positive by each test if they met the criteria for positivity for that test as described in the previous section. Real time PCR laboratory reports included a "suspect" category which was intended to signal a retest of the bull due to an inconclusive test. After the end of this study the laboratory adjusted the cutoff value for positive samples and eliminated the "suspect" category by defining specimens as positive when cycle threshold values were less than 38.25 and negative when cycle threshold values were greater than 38.25. The original cycle threshold values were used to make decisions regarding bull disposal by the owner and herd veterinarian, but the new definition for positive rt PCR specimens was used in data analysis to avoid challenges in data analysis presented by the 'suspect' category. The individual test results were used to compare culture results to gel and rt PCR results. All specimens with a culture, gel PCR and rt PCR test result were used in the comparison of individual culture results to gel and rt PCR results except for 31 specimens collected from 31 young, once sampled bulls on Ranch B. Across all sampling events a bull was classified as TF infected by each test if

one, two, or three of its preputial specimens were TF positive (parallel test interpretation) by that test. Parallel interpretation of tri-sequential culture bull results were compared to gel and rt PCR defined bull TF infection status. Bull TF infection status based on initial specimen results by gel and rt PCR were compared to bull TF infection status as defined by parallel interpretation of tri-sequential results for the respective tests.   Bull TF classification comparisons utilized only those bulls with three or four sequential specimens with the first three specimens used for classification purposes when four specimens were collected.

Results

A total of 274 preputial samples were collected from 125 bulls on two participating ranches. Six post mortem samples from Ranch A were not included in the count of bulls sampled as abattoir and ranch records confirmed they were previously sampled bulls but they could not be correlated to ante mortem bull identification. However all post mortem specimens were included in the comparison of individual specimen classification by the three tests. Thirty-one one and two year old bulls from ranch B were presented once for sampling without owner disclosure of their circumstances. They were managed as an exclusive group at a location isolated from the main breeding herd and were not considered at risk for TF infection. The uniqueness of their age profile, management attributes, and TF infection risk did not qualify them as cohorts in this herd and therefore excluded them from our study.

The net total bull preputial samples for the two herds were 243 samples from 94

bulls. A total of 39 bulls were sampled at least one time on Ranch A (Table 3.1 and

Figure 3.7).

Table 3.1 – Ranch A Bull Sampling Pattern. Key: 0 = no specimen collected at sampling
event; 1 = specimen collected at sampling event; n = number of bulls represented by the
sampling pattern; column totals = number of bulls sampled at each sampling event

Sampling Event				
First	Second	Third	Fourth	n
1	1	1	0	20
1	1	0	0	9
1	1	0	1	3
1	0	0	0	3
0	1	1	0	4
35	36	24	3	39

Twelve positive bulls from the first two sampling events were not presented for testing at

the third sampling event because they were being held at an alternate location for

shipment to slaughter the following day. In an attempt to complete three serial samplings

of these bulls pizzles from all Ranch A bulls sold to a local abattoir (n=9) were collected

and sampled. Three pizzles were correlated to ante mortem bull identifications.

Eighty six bulls were sampled at least one time on Ranch B (Table 3.2 and Figure

3.8). Eight positive bulls from the first sampling were not presented for testing at the

second sampling event at the owner's discretion, but were offered for testing at the third

sampling event. Pizzles from all Ranch B bulls sold to a local abattoir (n=11) were

collected and sampled in an effort to complete three serial samplings of the bulls. All 11

First sampling event
April 7, 2008 at ranch
35 bulls; 11 positive specimens

4 bulls added:
• 4 bulls unavailable at first sampling
event for unknown reasons

3 bulls lost:
• 3 bulls without matching
identification to subsequent sampling
events

Second sampling event
April 23, 2008 at ranch
36 bulls; 12 positive specimens-11 previously positive bulls, 1 newly positive bull

12 bulls lost:
• 12 previously positive bulls held at
shipping facility and unavailable for
sampling

Third sampling event
May 1, 2008 at ranch
24 bulls; 0 positive specimens

Fourth sampling event
May 3, 2008 at slaughter facility
9 bulls; 3 specimens successfully matched to ante mortem bull identifications – all 3 positive. 6
specimens not matched to ante mortem bull identifications – 4 of 6 unmatched specimens positive

Figure 3.7 – Ranch A Bull Sampling Flow Chart

Table 3.2 – Ranch B Bull Sampling Pattern. Key: 0 = no specimen collected at sampling
event; 1 = specimen collected at sampling event; n = number of bulls represented by the
sampling pattern; column totals = number of bulls sampled at each sampling event

Sampling Event				
First	Second	Third	Fourth	n
1	1	1	1	3
1	1	1	0	26
1	1	0	1	1
1	1	0	0	8
1	0	1	1	5
1	0	1	0	3
1	0	0	0	2
0	1	1	0	1
0	1	0	0	1
0	0	1	0	3
0	0	0	1	2
48	72	41	11	55

First sampling event
April 7, 2008 at ranch
48 bulls; 9 positive specimens

33 bulls added:
• 31 young bulls for first and only sampling
•1 bull unavailable at first sampling event, reason unknown
•1 bull without matching identification to previous sampling event

9 bulls lost:
• 8 previously positive bulls not sampled at owner's discretion
• 1 bull without matching identification to subsequent sampling events

Second sampling event
April 23, 2008 at ranch
72 bulls; 4 positive specimens from 4 newly positive bulls

11 bulls added:
• 1 bull without matching identification to previous sampling event
•2 bulls owner opted to sample only once
•8 previously positive bulls sampled for second time

41 bulls lost:
• 31 young bulls owner opted to sample only once
• 7 older bulls owner opted to sample only twice
•1 bull without matching identification to subsequent sampling event
•1 bull not sampled due to severe preputial cellulitis
•1 previously positive bull not sampled; reason unknown

Third sampling event
May 1, 2008 at ranch
41 bulls; 10 positive specimens - 9 previously positive bulls, 1 newly positive bull

Fourth sampling event
May 20, 2008 at slaughter facility
11 bulls; 11 specimens successfully matched to ante mortem bull identifications
•8 of 9 specimens from previously positive bulls were positive
•2 specimens from ante mortem negative bulls were negative

Figure 3.8 – Ranch B Bull Sampling Flow Chart

pizzles were correlated to ante mortem bull identification.

Table 3.3 summarizes by ranch and overall the net total number of bulls sampled after excluding 31 young, once sampled bulls from Ranch B. Fifty-eight of 94 bulls (61.7%) across both ranches were sampled three or four times and used to compare tests for classifying TF status of bulls. The first three specimens were used for bull

Table 3.3 – Sampling Events per Bull

Specimens per bull	Ranch A (n)	Ranch B (n)	Total
1	3	8	11
2	13 [a]	12 [b]	25
3	23	32	55
4	0	3 [c]	3
Total	39	55	94

[a] Six bulls at slaughter would have matched these bulls to give 3 samplings, but we could not confirm their identification. However, all slaughter samples are included in the individual sample analysis
[b] Bull SP36 was sampled twice, but the first sample did not have a gel PCR result so only the second sample was included in the individual sample analysis
[c] Only the first 3 sampling event results were used to classify these bulls, but all 4 samples were used in the individual sample analysis

classification when four specimens were collected. All four specimens were used to compare individual specimen classification by the three tests. The high loss to follow-up, 36 out of 94 (38.3%), was due to lack of consistent bull identification (n=7), failure of owner compliance with the study protocol (n=28), and bull injuries (n=1). Attrition for the outcome groups was 13 of 28 test positive bulls (46%) and 23 of 66 test negative bulls (35%).

Two hundred forty-three specimens were used to compare tests for classifying the TF status of individual specimens. Ninety-four bulls produced 238 individual samples. One ante mortem specimen was not used in the analysis due to sample loss during shipment between diagnostic laboratories resulting in no gel PCR test result. Six additional post mortem samples were used for the analysis of individual samples in spite of a lack of correlation to ante mortem bull identification.

Tritrichomonas foetus infection status determined by culture for 58 bulls sampled three or four times, 25 bulls sampled twice, and 11 bulls sampled one time are shown in Table 3.4. Among the 58 bulls sampled three or four times culture declared 47 bulls test negative, 1 positive on a single specimen, 3 positive on two specimens, and 7 positive on all three specimens. Of the 47 culture negative bulls 43 were negative by all three tests, culture, gel PCR, and rt PCR, at all samplings. Two bulls were negative on all samplings by culture and gel PCR, but one time positive by rt PCR. The remaining two culture negative bulls were inconsistently positive by the PCR assays with one bull determined to be positive by culture on a fourth sampling. Eleven bulls were positive by culture on one, two, or three samplings. Of these eleven bulls five were positive by all three tests on all three specimens with the remaining six bulls inconsistently positive across tests and across specimens by test.

Among the 25 bulls sampled twice culture declared 13 bulls non-TF infected, one bull positive on a single sample, and 11 bulls positive on both samples. The 13 culture negative bulls were also negative by gel and rt PCR on both sampling events. The single one time culture positive bull was positive by gel and rt PCR on both specimens. Nine bulls culture positive on both specimens were also positive by gel and rt PCR on both specimens with the remaining two bulls inconsistently positive between assays and sampling events. Eleven bulls were sampled one time with ten bulls negative by all three tests and one bull negative by culture and gel PCR, but positive by rt PCR.

From 243 samples representing 94 bulls, culture identified 57 positive specimens from 23 different bulls, rt PCR identified 55 positive specimens from 23 different bulls,

Table 3.4 – Testing Summary[a]. Key: 0 = test negative; 1= test positive.

Status [b]	N[c]	Culture	Gel PCR	rt PCR
A. Bulls sampled three or four times, n = 58				
Negative	43	0-0-0	0-0-0	0-0-0
	1	0-0-0	0-0-0	1-0-0
	1	0-0-0	0-0-0	0-1-0
	1	0-0-0-0	0-1-0-0	0-0-0-0[d]
	1	0-0-0-1	0-1-0-1	0-0-0-1
Positive once	1	0-0-1	0-0-1	0-0-0
Positive twice	1	0-1-1	0-1-1	1-1-1
	1	1-0-1	1-1-1	1-0-1
	1	0-1-1-1	0-1-1-1	1-1-0-0
Positive thrice	2	1-1-1	1-1-1	1-0-1
	5	1-1-1	1-1-1	1-1-1
B. Bulls sampled twice, n = 25				
Negative	13	0-0	0-0	0-0
Positive once	1	1-0	1-1	1-1
Positive twice	1	1-1	1-1[e]	0-1
	9	1-1	1-1	1-1
	1	[f]	-99-1[e]	0-0
C. Bulls sampled once, n = 11[f]				
Negative	10	0	0	0
	1	0	0	1

[a] This table represents 238 individual specimens. Six additional specimens were collected at slaughter, but could not be connected with any ante mortem bull identification. They were not counted as unique bull sampling events, but their specimens were utilized in the comparison of tests for classifying individual specimens. The total number of individual specimens used in the analysis was 243(see note 43 for further clarification)

[b] In order to organize this table status was based on the bull *T. foetus* infection status as determined by culture. Bull status was defined as *T. foetus* negative if the first three specimens from a bull were negative. Positive status bulls had one, two, or three culture positive test results

[c] Number of bulls with a given set of test results across all three tests

[d] The fourth sample was the result of previously thrice sampled bulls being sampled at slaughter. The individual specimen results from all four sampling events were used in the comparison of tests for classifying individual specimens. However, only the results of the first three specimens were used for comparison of tests for classifying bull infection status

Cross classified culture and rt PCR results found 49 specimens positive and 182 specimens negative by both tests. Seven specimens were culture positive and rt PCR negative. Five specimens were culture negative and rt PCR positive (Table 3.5). This cross classification generated a kappa of 0.86 (95% CI 0.78 to 0.94) and Yates corrected

Table 3.5 – 2X2 Culture/rtPCR for Individual Specimens.

rt PCR

		Positive	Negative	
	Positive	49	7	56
Culture	Negative	5	182	187
		54	189	243

McNemar's p = 0.77. Cross classified culture and gel PCR results found 56 specimens positive and 183 specimens negative by both tests. No specimens were culture positive and gel PCR negative. Four specimens were culture negative and gel PCR positive (Table 3.6). This cross classification generated a kappa of 0.89 (0.75 to 1.0) and Yates corrected McNemar's p = 0.48.

Using the herd owners' definition of a positive bull, any bull with a positive specimen by any test, a total of 28 bulls declared TF positive were removed from the two

Table 3.6 - 2X2 Culture/gel PCR for Individual Specimens.

gel PCR

		Positive	Negative	
Culture	Positive	56	0	56
	Negative	4	183	187
		60	183	243

herds and slaughtered (Table 3.7). Three bulls were declared positive only by rt PCR and each was positive on a single specimen by this test including one bull sampled a single time. Two bulls were declared positive only by gel PCR. One was positive on a single specimen by this test. However the other uniquely positive gel PCR was positive by all three tests on the post mortem specimen. Two bulls were declared positive by culture and gel PCR and negative by rt PCR with one being positive by both tests on the third specimen out of three and the other positive on the second specimen from two sampling events. Twenty-one of the 28 positive bulls were positive by all three tests with 14 of these bulls positive on all specimens collected.

In terms of classifying 58 bulls as TF infected or not based on three sequential specimens, culture, rt PCR, and gel PCR identified 11, 12, and 13 infected bulls respectively. Cross classification of culture and rt PCR bull infection status resulted in

Table 3.7 – Bulls with One or More Positive Tests. (n = number of specimens tested by all three assays for each bull) Key: 0 = test negative; 1 = test positive.

Bull ID	Culture	rt PCR	Gel PCR	n
4MID	0-0-0-0 [a]	0-0-0-0	0-1-0-0	4
SP13	0-1-1-1	1-1-0-0	0-1-1-1	4
Y57	0-0-0-1	0-0-0-1	0-1-0-1	4
11N2	0-1-1	1-1-1	0-1-1	3
3P93	1-1-1	1-1-1	1-1-1	3
95AMB	0-0-1	0-0-0	0-0-1	3
Leon	0-0-0	0-1-0	0-0-0	3
NITE	1-1-1	1-0-1	1-1-1	3
R1	1-1-1	1-0-1	1-1-1	3
R49	1-0-1	1-0-1	1-1-1	3
R56	1-1-1	1-1-1	1-1-1	3
R7	1-1-1	1-1-1	1-1-1	3
T364	1-1-1	1-1-1	1-1-1	3
W119	0-0-0	1-0-0	0-0-0	3
White 406	1-1-1	1-1-1	1-1-1	3
O1R4	1-1	1-1	1-1	2
O2K2	1-1	0-1	1-1	2
OL65	1-1	1-1	1-1	2
OP34	1-1	1-1	1-1	2
399N	1-1	1-1	1-1	2
3NO8	1-1	1-1	1-1	2
LO63	1-1	1-1	1-1	2
MO97	1-1	1-1	1-1	2
PACK 26	1-1	1-1	1-1	2
R68	1-1	1-1	1-1	2
SP36	1-1	0-0	-99-1	1
White 107	1-0	1-1	1-1	2
Tag 56	0	1	0	1
UNK1 [c]	1	1	1	1
UNK3 [c]	1	1	1	1
UNK4 [c]	1	1	1	1
UNK6 [c]	1	1	1	$\underline{1}$
				76

[a] All specimens used for comparison of classification of individual specimen TF classification of individual specimen TF status by testing method. Only first three specimens were used in classifying the TF status of the bull

[b] -99 indicates this specimen was not tested by gel PCR and therefore not used in the comparison of test for classification of individual specimens

[c] Post mortem sample that could not be correlated to ante mortem bull identification. Specimens were used in the comparison of classification of individual specimen TF status, but not counted as a new bull in the total number of bulls sampled

11 concordant positive and 46 concordant negative bulls. No bulls were culture positive

and rt PCR negative. One bull was culture negative and rt PCR positive (Table 3.8).

This cross classified data generated a kappa of 0.95 (95% CI 0.84 to 1.0) and a Yates

Table 3.8 – 2X2 Culture/rtPCR for Bulls.

rt PCR

		Positive	Negative	
	Positive	11	0	11
Culture	Negative	1	46	47
		12	46	58

corrected McNemar's p value of 0.06. Cross classification of culture and gel PCR bull

infection status resulted in 11 concordant positive bulls and 45 concordant negative bulls.

No bulls were culture positive and gel PCR negative. Two bulls were culture negative,

but gel PCR negative (Table 3.9). This cross classified data generated a kappa of 0.89

(95% CI 0.75 to 1.0) and a Yates corrected McNemar's p value of 0.48.

When comparing the efficiency of the initial sample to three serial samples for the

detection of TF infected bulls from 58 tri-serially sampled bulls, culture found 8 positive

bulls on the initial sampling event out of a total of 11culture positive bulls for a first test

efficiency of 0.72. Real time PCR found 9 positive bulls on the initial sampling event out

of a total of 12 rt PCR positive bulls for a first test efficiency of 0.75. Gel PCR found 8

Table 3.9 – 2X2 Culture/gelPCR for Bulls.

gel PCR

		Positive	Negative	
Culture	Positive	11	0	11
	Negative	2	45	47
		13	45	58

positive bulls on the initial sampling event out of a total of 13 gel PCR positive bulls for a first test efficiency of 0.62 (Table 3.10).

Table 3.10 – Test Efficiency.

	Sampling Event				
	1st	2nd	3rd	Total	Efficiency
Culture	8	2	1	11	0.72
rt PCR	9	3	0	12	0.75
Gel PCR	8	4	1	13	0.62

Discussion

This study examined the agreement of culture with gel and rt PCR assays in identifying TF positive bull preputial specimens and designating bull TF status based on three sequential samples and the agreement between single and tri-sequential specimen

testing in classifying bull TF status. The overall goal was to identify the most efficient method of identifying TF infected bulls for removal from a TF infected herd.

To assess agreement between tests and within tests this study required sampling all cohort bulls three times in accordance with current recommendations for culture based TF detection. Fifty-eight of 94 bulls across both ranches were sampled three times or more for a successful follow-up of 62 %. In this study the main causes for loss to follow-up were inadequate bull identification (7 misidentified bulls/36 total lost) and owner non-compliance with the study protocol (28 withdrawn bulls/36 total lost) while one bull was lost to injury. All bulls across both ranches had at least one and usually multiple identifiers in the form of a visual ear tag, an electronic ear tag, a freeze brand, and/or a hot iron brand. However, inconsistent reporting and recording of these identifiers led to the inability to link bull specimens across sampling events to complete a triad of specimens for each bull.

A much larger cause for bull attrition was owner compliance to the study protocol. Both ranchers withheld previously test positive bulls from sampling because they failed to appreciate the necessity of including them in the tested group at each sampling event. This led to incomplete tri-serial sampling of 13 out of 28 test positive bulls. Sample collection took place near the beginning of breeding season which prompted the owner of Ranch B to begin selectively sampling only those bulls he felt were high risk for TF infection or bulls not needed immediately in the breeding pastures. The low risk bulls removed from the study by the owner were likely TF negative as subsequent TF surveillance testing at the end of the breeding season by the local herd

health veterinarian found no TF test positive bulls, but they contributed greatly to the loss

to follow-up. A portion of the bulls incompletely sampled for what appeared to be owner

noncompliance may have been the result of inaccurate identification, but our inability to

consistently identify bulls did not allow us to determine the exact reason for incomplete

sampling.

Attrition in cohort studies may lead to selection bias with potentially adverse

affects on the validity of the study even when the loss to follow-up is evenly distributed

across exposure or outcome categories (Greenland, 1977) or when a substantial number

of subjects are lost to follow-up. Suggested minimum follow-up is 80% of subjects to

provide sufficient assurance against bias while studies that trace less than 60% of subjects

are generally regarded with skepticism (Rothman et al., 2008). In our study the attrition

was not evenly distributed between outcome groups and the complete trace of 62% of the

cohort was well below the desired minimum of 80%. While these numbers suggest a

subsequent study with improved cohort bull follow-up is necessary to confirm our

findings the current study's results provide interesting and potentially useful insights into

TF testing.

Bulls in these two herds had not been exposed to cows since the previous

breeding season which ended at least 8 months before the beginning of TF sampling.

This would preclude new bull infections from developing during the sampling period, yet

not all bulls declared positive were positive on their first specimen nor were they positive

on each specimen tested. The inconsistent test positivity between specimens for a each

test were most likely due to pre-analytical specimen collection and handling issues

including suboptimal collection tools, insufficient specimen volumes, fast bull throughput during collection, and inadequate transport conditions of inoculated pouches in addition to biological factors related to the potentially uneven distribution and fluctuating population of TF in the preputial cavity.   Other specimen related factors which may have lead to inconsistent positivity include the presence of manure, blood, urine, and semen in the specimens. While the affect of the presence of manure and semen in specimens can only be speculated on, blood components and urine are potentially inhibitory to PCR (Mukhufhi et al., 2003). These specimen related concerns are poorly understood and warrant further investigation to clarify their role in TF diagnostic testing.

In spite of the inconsistent positivity between specimens from the same bull TF categorization of individual specimens and bull status between tests did not appear to be greatly affected. Real time PCR was numerically different from culture when comparing the two tests for individual specimen classification, but statistically both rt and gel PCR categorized individual samples and bull TF stats nearly identically to culture as indicated by the high Kappa and low McNemar's p values for these comparisons. This agrees with earlier studies (Mukjufhi et al., 2003; Cobo et al., 2007) that found similar TF detection capabilities for culture and gel PCR, and suggests culture and rt PCR are also functionally equivalent for determining individual specimen and bull TF status.

The inconsistent positivity prevented any of the three tests from accurately identifying all the positive bulls on a single, initial specimen. Culture, rt PCR, and gel PCR detected less than 75% positive bulls on the first test out of the total number of bulls each test declared positive based on three serial specimens. Culture and gel PCR

required three specimens to find all their respective positive bulls while rt PCR found all positive bulls with two specimens. This indicates no single test can be relied on to identify all TF positive bulls in an infected herd based on testing of a single specimen and suggests three specimens should be collected from each bull in a TF infected herd to insure all TF positive bulls are identified.

In conclusion inconsistent test positivity was the major finding of this study. It affected all three assays and prevented any one assay from successfully identifying all TF infected bulls with a single specimen. At the same time it did not greatly diminish the agreement between tests for classifying individual specimens or tri-sequentially sampled bulls which suggests pre-analytical factors may play a more pivotal role in improving TF detection than enhancement of tests currently offered through veterinary diagnostic laboratories. Unfortunately losses to follow-up due to failure to adequately capture bull identification and owner non-compliance issues raise validity concerns with these conclusions. We recommend additional outbreak investigations with higher standards for follow-up to confirm this study's findings.

Chapter 4

Fall 2008 Outbreak Investigation[7]

Introduction

The study findings presented in the previous chapter indicate a high level of

agreement between culture, gel PCR and rt PCR to determine individual specimen or bull

TF status. The inconsistent positivity of specimens collected from test positive bulls

suggest single specimen testing to determine bull status in infected herds appears to be

inadequate for complete removal of all TF infected bulls. Because of the degree of bull

attrition in the previous study this study was conducted to confirm the previous studies

findings and further investigate the testing strategies and potential tactics for eliminating

T. foetus from an infected herd.

In the present study, a prospective cohort design, sequential bull preputial

sampling and three TF diagnostic assays (culture, gel PCR and rt PCR) were used to

investigate and control a TF outbreak. The study site was a 16,000 hectare, 3,000 cow

beef cattle ranch on semi-arid rangeland in the western Nebraska Sandhills. Although

results from three diagnostic tests were compared, the purpose of this study was not to

validate or justify TF assays. Rather we wanted to utilize TF tests currently offered to

veterinarians and livestock producers from accredited veterinary diagnostic laboratories

during an actual outbreak investigation in an attempt to optimize TF control strategies.

The specific study objectives were threefold: (1) to compare the agreement of three

matched sequential (tri-sequential) culture and gel PCR assays in identifying TF-positive preputial specimens and in classifying bulls as infected or not under natural field conditions; (2) to examine the agreement between the established TF diagnostic tests, culture and gel PCR, with the recently available rt PCR, and (3) to correlate cow herd pregnancy percentages with TF herd bull prevalence.

Materials and methods

Bull specimen collection - A census cohort of ranch breeding bulls (n=120 Angus bulls and 1 Horned Hereford bull, 1½ to 6 years of age) was identified and enrolled with owner's consent following initial diagnosis of TF in the herd in summer 2008 by the herd veterinarian. TF herd diagnosis occurred after the herd bulls were placed in multiple breeding pastures with the cows. Bulls were removed from the breeding pastures beginning on September 15, 2008 and remained separated from the cows for the duration of tri-sequential bull samplings in order to prevent new bull infections. There was a minimum of one week sexual rest prior to initial preputial sampling. An electronic identification ear tag was placed in each bull to verify bull identity at each sampling event. Bull TF testing began on October 3, 2008 and concluded on December 2, 2008 with an interval of 12 to 27 days between preputial specimen collections. Three bulls were lost to follow-up after the second sampling. At the conclusion of tri-sequential sampling and testing, a subset of herd bulls with unusual test results was sampled and tested a fourth time on January 15, 2009 at owner request.

Preputial specimens were collected with a uterine infusion pipette (0.50 cm outside diameter x 53.34 cm) and a sterile 12 ml syringe. A new pipette and syringe was

used on each bull. The free end of the uterine infusion pipette was introduced into the

prepuce to the level of the fornix with the syringe attached to the opposite end. The free

tip of the pipette was moved back and forth to scrape the surface of the prepuce and penis

while suction was applied with the syringe. After approximately 20 scraping cycles the

suction in the syringe was gently released and the pipette was removed from the prepuce

and examined for specimen adequacy. An adequate specimen was defined as slightly

blood-tinged preputial mucus filling at least 1.3 cm of the lumen of the pipette. If an

inadequate specimen was obtained the same pipette was reintroduced and a second

collection was attempted. The preputial specimen was inoculated into the upper chamber

of an InPouch™TF (Figure 3.2) by tearing off the upper plastic portion of the upper

chamber at the notch and repeatedly drawing media from the upper chamber into the

pipette and flushing it back into the upper chamber until the preputial material was

sufficiently transferred to the pouch. The pouch was then closed according to

manufacturer's directions and placed upright in an insulated container with hot water

bottles which provided an ambient temperature of approximately 20⁰ C until it could be

transferred to an incubator. The environmental temperature across all sampling events

ranged from 0.6⁰ to 27.2⁰ C. Inoculated pouches were placed in an incubator at 37⁰ C

within four hours of field collection.

Laboratory procedures - Inoculated pouches were incubated upright at 37⁰ C for

five days. Daily microscopic examination of pouches started 24 hours after collection

and continued through day five post collection. A single experienced veterinarian blinded

to previous daily examination results and bull identification numbers examined all

pouches. A clip provided by the manufacturer fixed the pouch for examination under a compound light microscope at 100X magnification. The lower chamber of the pouch was systematically scanned along its seam edges starting approximately 1 cm from the bottom on one side, down to and along the bottom, continuing up the other side approximately 1 cm, and then directly across the lower compartment to the original starting point. Approximately 2-3 minutes were required to complete the examination of each negative pouch. Pouches were classified presumptive positive based on visualization of live protozoa with size, morphology and motility patterns consistent with TF on one or more of the five days of examination.

After day five microscopic examinations, the pouch sediment was suspended in the media by gently pulling the pouch up and down across the edge of a counter 3-4 times. Two equal aliquots of approximately 2 ml were then aseptically pipetted into sterile cryogenic vials for submission to two separate American Association of Veterinary Laboratory Diagnosticians (AAVLD) accredited veterinary diagnostic laboratories by overnight delivery. Gel PCR and rt PCR were conducted using standard laboratory protocols at the Colorado State University Veterinary Diagnostic Laboratory at Rocky Ford and the University of Nebraska-Lincoln Veterinary Diagnostic Laboratory, respectively.

Gel-based and rt PCR assays used specific primers targeting the TF 5.8S ribosomal RNA gene and the flanking internal transcribed spacer regions ITS1 and ITS2. the assay was performed using primers TFR3 and TFR4 (Kennedy et al, 2008). Specimens were considered gel PCR positive if a 347 base pair amplicon was visualized

For gel PCR, DNA was extracted by a commercial kit[8] per manufacturer's protocol and following electrophoresis on an ethidium bromide stained agarose gel. Culture-positive,TF gel PCR-negative samples were retested by gel PCR with pan-trichomonal primers TFR1 and TFR2 and were considered gel PCR positive for non-TF trichomonads if a 372 base pair amplicon was visualized following electrophoresis. Specimens that tested pan trichomonad PCR positive but TF PCR negative were considered non-TF trichomonad positive (Campero et al., 2003)

For rt PCR, DNA was extracted using the heat lysis method (McMillen and Lew, 2006) and the assay was performed utilizing commercially available primers TFF2 and TFR2[9] and probe 6FAM[10]. The analysis was carried out in a commercial rt PCR detection and analysis system[11]. Specimens were defined as rt PCR positive when cycle threshold values were less than 38.25.

Pregnancy determination - Breeding cows (n = 2960 crossbred Angus cows) ranging in age from 2½ to 14 years of age were identified and enrolled with owner's consent and included all females that had been exposed to ranch breeding bulls. Replacement breeding females that had not yet given birth to their first calf were managed at an off-ranch site and were not included in the at-risk population. The cows were managed as five distinct groups from the time of breeding beginning July 1, 2008 through the December 2009 pregnancy determination. Pregnancy determination by rectal palpation was performed by the herd veterinarian on all cows beginning on November 7,

[8] DNeasy Blood and Tissue Kit, Qiagen Inc., Valencia, CA.
[9] TaqMan Gene Expression Master Mix, Applied Biosystems, Carlsbad, CA.
[10] TaqMan Probe, Applied Biosystems, Carlsbad, CA.
[11] 7500 Fast PCR System version 2.0.1, Applied Biosystems, Carlsbad, CA.

2008 and concluding on December 9, 2008.

Data analysis - Comparative pouch culture, gel PCR, and rt PCR results were analyzed by a 2x2 contingency table spreadsheet (Mackinnon, 2000) in order to estimate agreement between methods in determining TF status of individual pouches and tri-sequential bull infection status. Cohen's Kappa statistic and McNemar's paired sample Chi square test p values calculated from the 2x2 tables were used to assess agreement and statistical difference between test results or bull tri-sequential infection status results. At a given sampling time, a bull sample was classified as TF positive if the preputial specimen was found to be simultaneously culture and gel-PCR positive (serial test interpretation). Across all sampling events a bull was classified as TF infected if one, two, or three of its preputial specimens were TF positive (parallel test interpretation). This definition of TF infection was used to identify bulls for removal from the herd. Parallel interpretation of tri-sequential rt PCR bull results were compared to culture-gel PCR defined bull TF infection status. Three enrolled bulls were sampled only twice and were excluded from the bull TF infection status analysis.

Simple linear regression using spreadsheet software[12] was used to assess and plot the relationship between non-pregnancy percentages in each of the five cow management groups versus the prevalence of TF positive bulls present in those groups during the 2008 breeding season.

Results

A total of 361 bull preputial mucosal scrapings from 121 herd bulls were

[12] Excel, Microsoft Corp., Redmond, WA.

collected and tested for TF by culture, gel PCR and rt PCR. Sixty-one individual

specimens from 27 different bulls were culture positive. Gel PCR identified 63 positive

specimens from 26 different bulls. Most but not all of the bulls with positive culture or

gel PCR specimens were declared TF positive by our criteria (Tables 4.1 and 4.2).

Table 4.1 - Concatenated Testing Summary. Key: 0 = test negative; 1=test positive.

Status[a]	N [b]	Culture	Gel PCR	rt PCR (n) [c]
A. Bulls sampled three or four time, n=118 bulls.				
Negative	92	0-0-0	0-0-0	0-0-0 (76)
		0-0-0	0-0-0	0-1-0 (1)
		0-0-0-0	0-0-0-0	1-0-0-0 (2) [d]
		0-0-0-0	0-0-0-0	0-1-0-0 (8)
		0-0-0-0	0-0-0-0	0-0-1-0 (5)
	2	0-0-0	0-1-0	0-1-0 (1)
		0-0-0-0	0-1-0-0	0-0-0-0 (1)
	2	0-1-0-0	0-0-0-0	0-0-0-0 (2)
Positive once	3	1-0-0	1-0-0	0-0-0, 0-1-1, 1-1-0
	1	0-0-1	0-1-1	0-0-1
Positive twice	1	1-1-0	1-1-0	1-1-0
	1	1-0-1	1-1-1	0-1-1
	1	0-1-1	0-1-1	0-1-1
Positive thrice	15	1-1-1	1-1-1	1-1-1 (9); 0-1-1 (3); 0-1-0 (1); 1-0-1 (1); 1-1-0 (1)
B. Bulls sampled twice, n=3 bulls.				
Negative	1	1-0	0-0	0-0
Positive once	1	0-1	1-1	1-1
Positive twice	1	1-1	1-1	1-1

[a] Specimens at a given time were classified as *T foetus* positive if both culture and gel PCR were simultaneously test positive (serial test interpretation). Specimens were considered negative if either or both culture and /or gel PCR were test negative. Bull status was defined as *T foetus* negative only if all specimens from a bull were culture and gel PCR negative (parallel interpretation). Positive status bulls had one, two or three dual culture-gel PCR positive test results
[b] Number of bulls with given status
[c] Number of bulls with given status cross-classified by the rt PCR concatenate pattern

Table 4.2 – Fall 2008 Testing Summary.

Status[a]	N[b]	Culture	Gel PCR	rt PCR
A. Bulls sampled three or four time, n=118 bulls.				
Negative	76	0/3[c]	0/3	0/3
	1	0/3	0/3	1/3
	1	0/3	1/3	1/3
	15	0/4	0/4	1/4[d]
	1	0/4	1/4	0/4
	2	1/4	0/4	0/4
Positive once	1	1/3	1/3	0/3
	2	1/3	1/3	2/3
	1	1/3	2/3	1/3
Positive twice	2	2/3	2/3	2/3
	1	2/3	3/3	2/3
Positive thrice	1	3/3	3/3	1/3
	5	3/3	3/3	2/3
	9	3/3	3/3	3/3
B. Bulls sampled twice, n=3 bulls.				
Negative	1	1/2	0/2	0/2
Positive once	1	1/2	2/2	2/2
Positive twice	1	2/2	2/2	2/2

[a] Bull status was defined as T foetus negative only if all preputial specimens from a bull were culture and gel PCR negative. Positive status bulls had one, two or three specimens with simultaneous culture and gel PCR positive test results
[b] Number of bulls with given status
[c] Positive specimens/number of specimens collected
[d] The fourth sampling was a re-test of 17 bulls that were positive by a single test at a single time during the first three samplings with three assays. These represented suspect false positive tests

Cross-classified culture and gel PCR results found 58 specimens positive and 295

specimens negative by both tests. Three specimens were culture positive but gel PCR

negative. Five specimens were culture negative but gel PCR positive. This cross-classification generated a kappa of 0.92 (95% CI 0.87 to 0.97) and Yates corrected McNemar's p = 0.72.

Tritrichomonas foetus infection status determined by combined culture and gel PCR for 118 tri-sequential sampled bulls and for three bisequentially sampled bulls is shown in Table 4.1 and Table 4.2. The three bulls sampled only twice were culled from the herd prior to the third sampling; one was removed due to injury and two were culled for unknown reasons. Among the 118 bulls sampled three times, 92 were culture and gel PCR TF negative on all three samplings. Overall, tri-sequential testing identified the same 22 TF-positive bulls by culture and gel PCR. Only 15 and 16 bulls, respectively, of these 22 TF-infected bulls were positive by culture and gel-PCR on all three samples. The remaining six or seven TF-infected bulls were inconsistently test positive by culture and/or gel PCR at different sampling times (Table 4.1). Combining results of bulls sampled twice and three times with serial interpretation of culture and gel PCR results identified 24 of 121 bulls as TF infected, a prevalence of 19.8%. These 24 TF infected bulls were culled from the herd and sent directly to slaughter in November 2008.

Among 96 tri-sequential and one bisequentially sampled bulls classified as TF negative by culture and gel PCR, three bulls were uniquely one-time culture-positive and two bulls were uniquely one-time gel PCR-positive (Table 4.1). The three culture positive, gel PCR negative samples were pan-trichomonal gel PCR positive, consistent with intestinal trichomonas preputial sample contamination.

Sixty-nine specimens from 38 bulls sampled two or three times were rt PCR positive (Table 4.1). Seventeen specimens were exclusively rt PCR positive i.e. negative by both culture and gel PCR. When combined culture and gel PCR results were compared to rt PCR findings for the 361 individual specimens, 283 specimens were concordant negative and 49 specimens were concordant positive, respectively. Twenty specimens were discordant rt PCR positive but culture/gel PCR negative. Nine specimens were discordant rt PCR negative but culture/gel PCR positive. This cross classified data generated a kappa of 0.72 (0.63-0.82 95% CI) and a Yates corrected McNemar's p value of 0.06.

In terms of classifying 118 tri-sequential sampled bulls as TF infected or not, 79 bulls were culture/gel PCR and rt PCR concordant negative and 21 bulls were concordant positive. Compared to combined culture/gel PCR defined bull status, only one bull was rt PCR false negative but 17 bulls were rt PCR false positive. This data corresponds to a kappa of 0.61 (0.45-0.76 95% CI) and a Yates corrected McNemar's p value of 0.04. The positive predictive value of three rt PCR assays on a bull versus three combined culture/gel PCR tests was 0.55 (0.38 to 0.71 95% CI). The negative predictive value of three rt PCR assays on a bull versus three combined culture/gel PCR tests was 0.99 (0.93 to 1.00 95% CI).

Among the 118 herd bulls sampled three times and tested by the three TF assays (nine total tests), 19 bulls tested TF positive only once by a single test: 2 by culture, 1 by gel PCR and 17 by rt PCR (Tables 4.1 and 4.2). Seventeen of these 19 one time, one test positive bulls were still present on the ranch in January 2009 and were retested for TF at

that point for a fourth time because the owner and herd veterinarian suspected that these were false positive test results. All fourth time specimens were negative by culture, gel PCR, and rt PCR (Table 4.1).

Five cow management groups were present on the ranch. Group size, pregnancy percentages, bull numbers and bull TF infection prevalence are shown in Table 4.3. The

Table 4.3 – Summary of Non-pregnant Cows.

No. of Group	No. (%) non-cows	No. (%) non-pregnant cows	Cow ages (years)	No. of bulls	No. (%) TF infected bulls
1	434	40 (9.2)	2.5	18	0 (0)
2	349	29 (8.3)	5-10	14	0 (0)
3	783	118 (15.1)	4; 10-14	32	6 (18.7)
4	784	114 (14.5)	5-9	32	7 (21.9)
5	610	117 (19.2)	5-9	25	10 (40.0)
All	2960	418 (14.1)	2.5-14	121	24 (21.5)*

* Group membership of one TF-infected bull not determined.

relationship between non-pregnancy percentages in the five cow management groups and TF prevalence in bulls present during the breeding season is shown in Figure 4.1. Bull TF prevalence ranged from 0% to 40% (mean = 21.5%) and non-pregnant cow proportion ranged from 8.3% to 19.2% (mean = 14.1%). Non-pregnant cow proportion correlated positively with herd bull TF prevalence ($r^2 = 0.97$).

Discussion

This study in naturally TF infected beef bulls confirms the findings of Cobo and colleagues (2007) in experimentally TF challenged dairy bulls. Both studies found a

combination of TF tests (culture and PCR) and repeat testing strategies most efficiently identified TF infected carrier bulls.

Tritrichomonas foetus was diagnosed in this well managed beef ranch fortuitously as no signs of reproductive failure were present in the herd at the time of diagnosis in early summer 2008. The herd veterinarian suggested sampling and testing of the herd bulls during scheduled breeding soundness examinations because trichomoniasis was known to be present on nearby ranches. The owner agreed to TF screening of his bulls and was surprised his herd was infected.

Culture and gel PCR classified 361 individual preputial specimens and 118 tri-sequential sampled bulls nearly identically, indicating both techniques are likely functionally equivalent TF detection methods. The data does not support differential weighting of TF results based on whether culture or gel PCR is performed. Based on combined culture and gel PCR from two or three sequential samples, 24 of 121 herd bulls were identified as TF infected and sold for slaughter. In May 2009, the herd veterinarian retested all retained (i.e. TF negative status, Tables 4.1 and 4.2) breeding bulls by culture and pooled PCR as part of the ranch's Spring 2009 pre-breeding herd health program. All bulls were TF negative, suggesting that the tri-sequential sampling and combined culture and gel PCR were successful in eliminating TF carriers from the bull battery. Other studies (Mukjufhi et al., 2003; Cobo et al., 2007) have reported high agreement between results of one-time culture and gel PCR in both naturally-infected and experimentally challenged bulls. We are not aware, however, of any previous study where both tri-

sequential sampling and culture and PCR were compared and applied in a field setting for TF outbreak control.

The study data suggest that false-negative specimens or bull classifications are a significant risk when bulls in TF-infected herds are sampled just one time or by just one test. Some state departments of agriculture including Nebraska and Texas have implemented bull import regulations which require either a single negative PCR or three sequential negative cultures prior to import[13, 14]. Our data does not support the idea or practice of equating results of one PCR assay with three sequential cultures. Sampling sexually rested bulls at least three times is a common recommendation in TF infected herds as a way to increase diagnostic test sensitivity. Using multiple (i.e. culture and PCR) TF tests in series achieved the best positive predictive values for bulls in spite of reports of PCR inhibition due to increasing proteolytic enzyme levels in specimens incubated several days (Mukhufhi et al., 2003). Absence of true culture positive, PCR negative specimens in our study suggests this mode of PCR inhibition was not a significant factor in assay performance under the conditions of the study. However, the benefit of the added positive predictive value must be weighed against the time, cost, and risk to bulls and handlers created by the multiple sampling events and tests. Some producers may be willing to accept the risk of identifying less than 100% of infected bulls in an attempt to avoid the negative aspects of multiple sampling events and tests.

[13] Texas Animal Health Commission news release
http://www.tahc.state.tx.us/news/pr/2009/2009Feb_TrichomoniasisProgramAdopted.pdf. Accessed 09/16/09
[14] Nebraska Department of Agriculture amended trichomoniasis import order
http;//www.agr.state.ne.us/division/bia/trich_order_4.pdf. Accessed 09/16/09

Utilizing 1, 2, or 3 sampling events on the study ranch indentified 21, 23, and 24 positive bulls, respectively. This corresponds to 87%, 95%, and 100% of the positive bulls identified on the ranch. Based on this it is our recommendation to collect and test at least three preputial specimens from each non-virgin bull on infected premises.

The study presented in the previous chapter found similar TF diagnostic agreement between culture and PCR which supports these findings and recommendations. However, the previous study had 62% loss to follow-up primarily due to inadequate, inconsistent bull identification and failure of the herd owners to comply with study protocol which potentially compromises the validity of those findings in some reviewer's opinions. The current study's bull follow-up of 98% was in part due to use of individual electronic identification tags in each bull, meticulous recordkeeping, consistent and methodical specimen collection by the attending veterinarian, and acceptance and nearly complete compliance to the study protocol by the herd owner.

An important clinical question is why truly TF infected bulls do not consistently produce test positive specimens when sampled multiple times. Only 15 of 22 (68 %) of truly infected bulls sampled three times were always culture and gel PCR positive. Our data suggests that laboratory or test factors are not the likely drivers of this phenomenon because culture and gel PCR results were in near uniform agreement at specific sampling event times. Rather, non-test factors may more likely explain sporadic test positivity. These non-test influences could include technical factors such as non-optimized preputial specimen collection devices or protocols, specimen handling, holding or transport issues, or presence of PCR or protozoal growth inhibitors in the collected preputial specimens.

Fluctuations in protozoal population densities or inconsistent TF mucosal spatial distributions in the preputial cavity could also result in inconsistent test positivity in truly infected bulls. These pre-test technical and biological factors merit further investigation as potential approaches for improving TF infection control in cattle.

Nineteen bulls were "one time-one test" positive after tri-sequential sampling: two by culture, one by gel PCR and 16 by rt PCR. Seventeen of these 19 bulls were available to sample a fourth time and all tested negative by all three TF assays (Table 4.). This is strong evidence that sporadic false positive TF results may occur by culture, gel PCR and rt PCR. The ranch owner and herd veterinarian were rightly suspicious that these "one time-one test" positive bulls were false positive animals and thus did not send them to slaughter. The unnecessary sale and slaughter of these 17 bulls would have significantly increased the financial impact of this TF outbreak due to bull replacement costs.

Intestinal trichomonad contamination explained the three false positive culture results. These three bulls were less than two years old and therefore were more likely to have intestinal trichomonad preputial contamination compared to older bulls (BonDurant et al., 1999). We cannot explain the two false positive gel PCR results. However, sporadic false positive gel PCR using the same primers that were employed here has been previously reported (Cobo et al., 2007) More problematic was the occurrence of one-time rt PCR false positive results in 16 of 19 "one time-one test" positive bulls. The rt PCR advantages of higher sample throughput, easier assay performance and faster result reporting must be weighed against the higher likelihood of false positive bull misclassification if this test is utilized as a stand-alone diagnostic test. Real time PCR

has potential diagnostic utility as a herd screening test if combined with culture or gel PCR using serial test interpretation to increase diagnostic specificity.

Rae and colleagues (1999) found no relationship between non-pregnancy percentages and bull TF prevalence between the 11 management units of a large Florida ranch recently diagnosed as TF infected. The authors speculated this was attributable to inaccuracies in pregnancy determination by multiple lay palpators, differences in bull breeds represented in the various management units, and specimen quality concerns. Although only five herds were present on the ranch in our study, the close fit of the data on the linear regression plot of bull TF prevalence versus cow non-pregnancy percentages (Figure 4.1) was striking. Highly similar management group genetics, nutrition, environment, husbandry practices and a single veterinarian palpator on this ranch may have made this intuitive but not previously reported correlation more evident. These findings suggest that pregnancy percentages may be useful as a crude indicator of bull TF prevalence or for prioritizing multiple herd TF test and control strategies on well-managed, properly tested operations based on pretest probabilities (Parker et al., 1999) for bull infection.

In conclusion, we recommend tri-sequential sampling at weekly or greater intervals in sexually rested bulls using combination culture-gel PCR testing to effectively control TF beef cattle outbreaks. Sequential sampling maximizes diagnostic sensitivity while combination testing enhances diagnostic specificity. Findings from just one outbreak are reported here, but we obtained very similar results using tri-sequential sampling and combined culture-gel PCR testing to control the two TF outbreaks
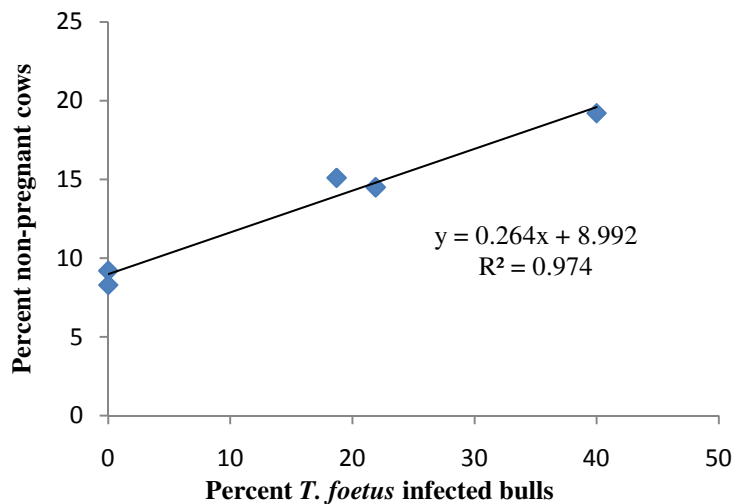
Figure 4.1- Infected Bull Prevalence/Non-Pregnant Cow Linear Regression

discussed in Chapter 3[15]. Our results have implications for states implementing TF

control programs as they attempt to balance the practicality and cost of sequential

sampling and multiple tests with the desire to detect and remove all TF infected bulls.

[15] Ondrak J, Keen J, Rupp G, et al. Serial sampling and comparative testing of bulls for *Tritrichomonas foetus* in two infected Nebraska beef herds by culture, real time PCR and gel PCR (abstr). *Proc 51st Annual Convention of AAVLD*, Greensboro, NC. Oct 23-29, 2008, p 61.

Chapter 5

Conclusions

Outbreak investigations were carried out on three Nebraska ranches to assess the efficiency of currently available diagnostic tests in identifying TF infected bulls in known TF infected herds with the following objectives:

(1) to compare the agreement of the three assays for classifying the status of individual preputial specimens.

(2) to compare the agreement of the three assays in identifying TF infected bulls based on three sequential samples.

(3) to correlate cow herd pregnancy percentages with TF herd bull prevalence. From the data provided through these objectives the overall study goal was to develop tactics for efficiently identifying TF infected bulls in beef cattle herds using one or more of these tests while reducing the number of sampling events per bull.

Two hundred and forty-six extensively managed bulls on three TF infected Nebraska ranches were sampled and tested multiple times and the reproductive performance of 2960 cows from one cooperating ranch were recorded. Comparisons of diagnostic tests were conducted using Cohen's Kappa statistic and McNemar's paired sample Chi square test p values. Simple linear regression was used to assess the relationship between non-pregnancy percentages and prevalence of TF positive bulls.

No significant differences between culture and gel PCR for individual specimen and bull TF classification were found. The thorough microscopic examination technique used in the study may have accounted for the high level of diagnostic agreement between

culture and gel PCR. Real time PCR had a high rate of apparent false positives relative to culture and gel PCR for individual specimen and bull TF classification which may have been the result of newly implemented protocols at the rtPCR diagnostic laboratory. However, all assays required multiple, sequential specimens to adequately identify all TF infected bulls in the study herds due to inconsistent positivity of infected bulls indicating tri-sequential sampling at weekly or greater intervals in sexually rested bulls using combination culture-gel PCR testing may be necessary to effectively control TF beef cattle outbreaks in an efficient manner. Cow non-pregnancy rates correlated linearly with TF positive bull prevalence and may be a tool to develop pre-test probabilities for TF testing programs.

Mukhufi et al. (2003) stated, "While much emphasis has fallen on primer selection and the optimization of laboratory protocols to render satisfactory results, little attention has been given to sample collection and handling procedures." Our findings of similar diagnostic assay performance for culture, gel PCR, and real time PCR suggests opportunities for improved TF control tactics may be found by focusing on pre-analytical aspects of diagnostic testing such as consistent bull identification, optimization of specimen collection techniques, and pre-incubation specimen handling conditions. Unfortunately, most recent interest in TF diagnosis has been in the analytical area with an emphasis on developing and improving rt PCR which proved to be the least repeatable test in our study.

Bibliography

1. Abbitt B, Meyerholz GW. 1979. *Trichomonas foetus* infection of range bulls in South Florida. *Vet Med Sm Anim Clin.*74:1339-1342. (154)

2. Alexander GI. 1953. An outbreak of bovine trichomoniasis in Queensland and its control. *Aust Vet J.*29:61-66. (1)

3. Alstad AD, Krogh D, Fischer K, et al. 1984. Trichomoniasis in a beef herd. *Vet Med.* 79:708-709. (2)

4. Appell LH, Mickelsen WD, Thomas MW, et al. 1993. A comparison of techniques used for the diagnosis of *Tritrichomonas foetus* infections in beef bulls. *Agri-Pract.* 14:30-34. (52)

5. Aydintug MK, Leid RW, Widders PR. 1990. Antibody enhances killing of *Tritrichomonas foetus* by the alternative bovine complement pathway. *Infect and Immun*. 58:944-948. (194)

6. Aydintug MK, Widders PR, Leid RW. 1993. Bovine polymorphonuclear leukocyte killing of *Tritrichomonas foetus*. *Infect and Immun.* 61:2995-3002. (197)

7. Ball L, Mortimer RG, Cheney JM. 1984. Trichomoniasis: Diagnosis, pathogenesis, treatment and control. *Proc Am Assoc Bov Pract.* 16:163-165. (3)

8. Ball L, Dargatz DA, Cheney JM, et al. 1987. Control of venereal disease in infected herds. *Vet Clin North Am, Food Anim Pract*. 3:561-574. (5)

9. Barling KS, Field RW, Snowden KF, et al. 2005. Acute trichomoniasis and sub-optimal fertility in a cow/calf herd: an investigation and case management. *Bov Pract.* 39:1-5. (132)

10. Bartlett DE. 1947. *Trichomonas foetus* infection and bovine reproduction. *J Am Vet Med Assoc.* 8: 343-352. (168)

11. Bartlett DE, Hasson EV, Teeter KG. 1947. Occurrence of *Trichomonas foetus* in preputial samples from infected bulls. *J Am Vet Med Assoc.* 110:114-120. (53)

12. Bartlett DE, Dikmans G. 1949. Field studies on bovine venereal trichomoniasis effects on herds and efficacy of certain practices in control. *Am J Vet Res*. 10:30-39. (169)

13. Bier PJ, Hall CE, Duncan JR, et al. 1977. Measurement of immunoglobulins in reproductive tract fluids of bulls. *Vet Micro.* 2:1-11. (211)

14. Blackshaw AW, Beattie HER. 1955. The preservation of *Trichomonas foetus* at -79°C. *Aust Vet J.* 31:214-216. (224)

15. BonDurant RH. 1985. Diagnosis, treatment and control of bovine trichomoniasis. *Compend Contin Educ Pract Vet (Supplement).* 7:S179-S186. (7)

16. BonDurant RH, Anderson ML, Blanchard P, et al. 1990. Prevalence of trichomoniasis among California beef herds. *J Am Vet Med Assoc.* 196:1590-1593. (8)

17. BonDurant RH, Honiberg BM. 1994.Trichomonads of veterinary importance. In: Kreier, JP, editor. *Parasitic Protozoa*. Academia Press, Inc. San Diego, CA: 111-188. (219)

18. BonDurant RH, Gajadhar A, Campero CM, et al. 1999. Preliminary characterization of a *Tritrichomonas foetus*-like protozoan isolated from preputial smegma of virgin bulls. *Bov Pract.* 33: 124-127. (133)

19. BonDurant RH, Campero CM, Anderson ML, et al. 2003. Detection of *Tritrichomonas foetus* by polymerase chain reaction in cultured isolates, cervicovaginal mucus, and formalin-fixed tissues from infected heifers and fetuses. *J Vet Diagn Invest.* 15:579-584. (54)

20. Borchardt KA, Norman BB, Thomas MW, et al. 1992. Evaluation of a new culture method for diagnosing *Tritrichomonas foetus* infection. *Vet Med.* 87:104-112. (134)

21. Bryan LA, Campbell JR, Gajadhar AA. 1999. Effects of temperature on the survival of *Tritrichomonas foetus* in transport, Diamond's and InPouch TF media. *Vet Rec.* 144:227-232. (57)

22. Burgess DE, Knoblock KF, Daugherty T, et al. 1990. Cytotoxic and hemolytic effects of *Tritrichomonas foetus* on mammalian cells. *Infect and Immun.* 58:3627-3662. (196)

23. Burgess DE, McDonald CM. 1992. Analysis of adhesion and cytotoxicity of *Tritrichomonas foetus* to mammalian cells by use of monoclonal antibodies. *Infect and Immun.* 60:4253-4259. (195)

24. Campero CM, Dubra CR, Bolondi A, et al. 2003. Two-step (culture and PCR) diagnostic approach for differentiation of non-TF trichomonads from genitalia of virgin beef bulls in Argentina. *Vet Parasitol.* 112:167-175. (9)

25. Chakrabarti D, Dame JB, Gutell RR, et al. 1992. Characterization of the rDNA unit and sequence analysis of the small subunit rRNA and 5.8S rRNA genes from *Tritrichomonas foetus*. *Mol Biochem Parasit.* 52:75-84. (61)

26. Christensen HR, Clark BL, Parsonson IM. 1977. Incidence of *Tritrichomonas foetus* in young replacement bulls following introduction into an infected herd. *Aust Vet J.* 53:132-134. (10)

27. Christensen HR, Clark BL. 1979. Spread of *Tritrichomonas foetus* in beef bulls in an infected herd. *Aust Vet J.* 55:205. (11)

28. Cima, G. 2009. Texas joins states with bovine trichomoniasis regulations: disease impairs fertility, reduces producer income. *J Am Vet Med Assoc.* 234:862-863. (12)

29. Clark BL, White MB, Banfield JC. 1971. Diagnosis of *Trichomonas foetus* infection in bulls. *Aust Vet J.* 47:181-183. (183)

30. Clark BL, Parsonson IM, Dufty JH. 1974a. Infection of bulls with *Tritrichomonas foetus* through mating with infected heifers. *Aust Vet J.* 50:180. (13)

31. Clark BL, Parsonson IM, Dufty JH. 1974b. Experimental infection of bulls with *Tritrichomonas foetus*. *Aust Vet J.* 50:189-191. (14)

32. Clark BL, Parsonson IM, White MB, et al. 1974c. Control of trichomoniasis in a large herd of beef cattle. *Aust Vet J.* 50:424-426. (15)

33. Clark BL, Dufty JH, Parsonson IM. 1977. Studies on the transmission of *Tritrichomonas foetus*. *Aust Vet J.* 53:170-172. (16)

34. Clark BL, Dufty JH, Parsonson IM. 1983. The effect of *Tritrichomonas foetus* infection on calving rates in beef cattle. *Aust Vet J.* 60:71-74. (17)

35. Clark BL, Dufty JH, Parsonson IM. 1986. The frequency of infertility and abortion in cows infected with *Tritrichomonas foetus* var. brisbane. *Aust Vet J.* 63:31-32. (181)

36. Cobo ER, Campero CM, Mariante RM, et al. 2003. Ultrastructural study of a tetratrichomonad species isolated from preputial smegma of virgin bulls. *Vet Parasitol.* 117:195-211. (159)

37. Cobo ER, Canton G, Morrell E, et al. 2004. Failure to establish infection with *Tetratrichomonas* sp. in the reproductive tracts of heifers and bulls. *Vet Parasitol.* 120:145-150. (160)

38. Cobo ER, Favetto PH, Lane VM, et al. 2007. Sensitivity and specificity of culture and PCR of smegma samples of bulls experimentally infected with *Tritrichomonas foetus*. *Therio.* 68:853-860. (63)

39. Corbeil LB, Campero CM, Van Hoosear K, et al. 2008. Detection of trichomonad species in the reproductive tracts of breeding and virgin bulls. *Vet Parasitol.* 154:226-232. (65)

40. Diamond LS. 1957. The establishment of various trichomonads of animals and man in axenic cultures. *J Parasitol.* 43:488-490. (212)

41. Dikmans G, Poelma LJ. 1938. Bovine genital trichomoniasis with special reference to diagnosis. *J Am Vet Med Assoc.* 93:362-366. (66)

42. Dufernez F, Walker RL, Noel C, et al. 2007. Morphological and molecular identification of non-*Tritrichomonas foetus* trichomonad protozoa from the bovine preputial cavity. *J Eukaryot Microbiol.* 54:161-168. (67)

43. Emmerson MA. 1932. Trichomoniasis in cattle. *J Am Vet Med Assoc.* 81:636-640. (19)

44. Felleisen RSJ. 1997. Comparative sequence analysis of 5.8S rRNA genes and internal transcribed spacer (ITS) regions of trichomonadid protozoa. *Parsitology.* 115:111-119. (71)

45. Felleisen RSJ, Schimid-Lambelet N, Walubengo J. 1997. Comparative evaluation of methods for the diagnosis of bovine *Tritrichomonas foetus* infection. *J Protozool Res.* 7:90-101. (69)

46. Felleisen RSJ, Lambelet N, Bachmann P, et al. 1998. Detection of *Tritrichomonas foetus* by PCR and DNA enzyme immunoassay based on rRNA gene unit sequences. *J Clin Micro.* 36:513-519. (68)

47. Fitzgerald PR, Hammond DM, Miner ML, et al. 1952. Relative efficacy of various methods of obtaining preputial samples for diagnosis of trichomoniasis in bulls. *Am J Vet Res.* 13:452-457. (72)

48. Fitzgerald PR, Hammond DM, LeGrande Shupe J. 1954. The role of cultures in immediate and delayed examinations of preputial samples for *Trichomonas foetus*. *Vet Med.* 49:409-412. (214)

49. Fitzgerald PR, Johnson AE, Thorne J, et al. 1958. Trichomoniasis in range cattle. *Vet Med.* 53:249-252. (20)

50. Gay JM, Ebel ED, Kearley WP. 1996. Commingled grazing as a risk factor for trichomonosis in beef herds. *J Am Vet Med Assoc.* 209:643-646. (21)

51. Goodger WJ, Skirrow SZ. 1986. Epidemiologic and economic analyses of an unusually long epizootic of trichomoniasis in a large California dairy herd. *J Am Vet Med Assoc.* 189:772-776. (22)

52. Grahn RA, BonDurant RH, van Hoosear KA, et al. 2005. An improved molecular assay for *Tritrichomonas foetus*. *Vet Parasitol.* 127:33-41. (73)

53. Greenland S. 1977. Response and follow-up bias in cohort studies. *Am J Epi.*106:184-187. (164)

54. Gregory MW, Ellis B, Redwood DW. 1990. Comparison of sampling methods for the detection of *Tritrichomonas foetus* infection in bulls. *Vet Rec.* 7:16. (166)

55. Grotelueschen DM, Cheney J, Hudson DB, et al. 1994. Bovine trichomoniasis: results of a slaughter survey in Colorado and Nebraska. *Therio.* 42:165-171. (23)

56. Hall MR, Kvasnicka WG, Hanks D, et al. 1993. Improved control of trichomoniasis with *Trichomonas foetus* vaccine. *Agri-Pract*; 14:29-34. (118)

57. Hammond DM, Bartlett DE. 1943a. Establishment of infection with *Trichomonas foetus* in bulls by experimental exposure. *Am J Vet Res.* 4:61-65. (25)

58. Hammond DM, Bartlett DE. 1943b. The distribution of *Trichomonas foetus* in the preputial cavity of infected bulls. *Am J Vet Res.* 4:143-149. (24)

59. Hammond DM, Bartlett DE. 1945. Pattern of fluctuations in numbers of *Trichomonas foetus* occurring in the bovine vagina during initial infections. I. Correlation with time of exposure and with subsequent estrual cycles. *Am J Vet Res.* 6:84-90. (179)

60. Hammond DM, Bishop VR, Jeffs G, et al. 1950. A quantitative study of *Trichomonas foetus* in preputial samples from infected bulls. *Am J Vet Res.* 11:308-314. (225)

61. Harding HP. 1950. Infertility in cattle with special reference to trichomoniasis: I. The clinical aspect and diagnosis of trichomoniasis, with particular reference to the bull. *Vet Rec.* 62:539-540. (26)

62. Ho MSY, Conrad PA, Conrad PH, et al. 1994. Detection of bovine trichomoniasis with a specific DNA probe and PCR amplification system. *J Clin Micro.* 32:98-104. (76)

63. Hoevers J, Snowden K, Graham S, et al. 2003. A comparison of PCR and in vitro culture for detection of *Tritrichomonas foetus* in bovine preputial scrapings. *J Eukaryot Microbiol (Supplement)*. 50:S699-S700. (77)

64. Huang JC, Hanks D, Kvasnicka W, et al. 1989. Antigenic relationships among field isolates of *Tritrichomonas foetus* from cattle. *Am J Vet Res*. 50:1064-1068. (175)

65. Huby-Chilton F, Scandrett BW, Chilton NB, et al. 2009. Detection and identification of *Tetratrichomonas* in a preputial wash from a bull by PCR and SSCP. *Vet Parasitol.* 166:199-204. (233)

66. Jeffries L, Harris M. 1967. Observations on the maintenance of *Trichomonas vaginalis* and *Trichomonas foetus*; the effects of cortisone and agar on enhancement of severity of subcutaneous lesions in mice. *Parasitology*. 57:321-334. (209)

67. Johnson EA. 1964. Incidence and diagnosis of Trichomoniasis in western beef bulls. *J Am Vet Med Assoc.* 14:1007-1010. (144)

68. Kennedy JA, Pearl D, Tomky L, Carman J. 2008. Pooled polymerase chain reaction to detect *Tritrichomonas foetus* in beef bulls. *J Vet Diagn Invest*. 20:97-99. (79)

69. Kimsey PB, Darien BJ, Kendrick JW, et al. 1980. Bovine trichomoniasis: diagnosis and control. *J Am Vet Med Assoc*. 177:616-619. (80)

70. Kimsey PB. 1986. Bovine trichomoniasis. In: Morrow, DA, editor. *Current Therapy in Theriogenology,* 2nd edition. WB Saunders, Inc. Philadelphia, PA: 275-279. (27)

71. Kvasnicka WG, Taylor REL, Huang JC, et al. 1989. Investigations of the incidence of bovine trichomoniasis in Nevada and of the efficacy of immunizing cattle with vaccines containing *Tritrichomonas foetus*. *Therio*. 31:963-971. (120)

72. Kvasnicka WG, Hall MR, Hanks DR. 1999. Bovine Trichomoniasis In: Howard and Smith, editors. *Current Veterinary Therapy Food Animal Practice 4*. W.B. Saunders, Co. Philadelphia, PA: 420-425. (00)

73. Ladds PW, Dennett DP, Glazebrook JS. 1973. A survey of the genitalia of bulls in northern Australia. *Aust Vet J*. 49: 335-340. (29)

74. Levine ND, Marquardt WC. 1955. The effect of glycerol and related compounds on survival of *Tritrichomonas foetus* at freezing temperatures. *J Protozool.* 2:100-107. (210)

75. Levine ND. 1973. *Protozoan Parasites of Domestic Animals and of Man,* 2nd edition. Burgess Publishing Company. Minneapolis, MN: 88-110. (223)

76. Levine ND. 1985. *Veterinary Parasitology,* 1st edition. Iowa State University Press. Ames, IA: 61-64. (30)

77. Lun ZR, Parker S, Gajadhar AA. 2000. Comparison of growth rates of *Tritrichomonas foetus* isolates from various geographic regions using three different culture media. *Vet Parasitol*. 89:199-208. (83)

78. Mackinnon A. 2000. A spreadsheet for the calculation of comprehensive statistics for the assessment of diagnostic tests and inter-rater agreement. *Computers in Biology and Medicine*; 30(3):127-134. [DAG_Stat, www.mhri.ecu.au/biostats/DAG_Stat]. Accessed June 16, 2009.

79. Mancebo OA, Russo AM, Carabajal LL, et al. 1995. Persistence of *Tritrichomonas foetus* in naturally infected cows and heifers in Argentina. *Vet Parasitol.* 59:7-11. (171)

80. McMillen L, Lew AE. 2006. Improved detection of *Tritrichomonas foetus* in bovine diagnostic specimens using a novel probe-based real time PCR assay. *Vet Parasitol.* 141:204-215. (84)

81. Mickelsen WD. 1983. Diagnosis of trichomoniasis: herd history and culture techniques. *Proc Am Assoc Bov Pract.* 15:134-135. (189)

82. Mickelsen WD. 1984. The incidence of post-service pyometra in a herd of 597 beef cows infected with trichomoniasis. *Proc Soc Therio.* :245-248. (172)

83. Mickelsen WD, Paisley LG, Anderson PB. 1986. Survey of the prevalence and types of infertility in beef cows and heifers. *J Am Vet Med Assoc.* 189:51-54. (31)

84. Morgan UM, Thompson RCA, Smith HV, et al. 1998. Molecular detection of parasitic protozoa. *Parasitology* (Supplement). 117:S73-S85. (85)

85. Mukjufhi N, Irons PC, Michel A, Peta F. 2003. Evaluation of a PCR test for the diagnosis of *Tritrichomonas foetus* infection in bulls: effects of sample collection method, storage and transport medium on the test. *Therio.* 60:1269-1278. (86)

86. Murname D. 1959. Field and laboratory observations on trichomoniasis of dairy cattle in Victoria. *Aust Vet J.* 35:80-83. (174)

87. Mutto AA, Giambiaggi S, Angel SO. 2006. PCR detection of *Tritrichomonas foetus* in preputial bull fluid without prior DNA isolation. *Vet Parasitol.* 136:357-361. (198)

88. Nickel DD, Olson ME, Schultz GA. 2002. An improved polymerase chain reaction assay for the detection of *Tritrichomonas foetus* in cattle. *Can Vet J.* 43:213-216. (87)

89. Parker S, Campbell J, Ribble C, Gajadhar A. 1999. Comparison of two sampling tools for diagnosis of *Tritrichomonas foetus* in bulls and clinical interpretation of culture results. *J Am Vet Med Assoc.* 215:231-235. (92)

90. Parker S, Lun Z, Gajadhar A. 2001. Application of a PCR assay to enhance the detection and identification of *Tritrichomonas foetus* in cultured preputial samples. *J Vet Diagn Invest.* 13:508-513. (90)

91. Parker S, Campbell J, Gajadhar A. 2003. Comparison of the diagnostic sensitivity of a commercially available culture kit and a diagnostic culture test using Diamond's media for diagnosing *Tritrichomonas foetus* in bulls. *J Vet Diagn Invest.* 15:460-465. (91)

92. Parsonson IM, Clark BL, Dufty J. 1974. The pathogenesis of *Tritrichomonas foetus* infection in the bull. *Aust Vet J.* 50:421-423. (33)

93. Parsonson IM, Clark BL, Dufty JH. 1976. Early pathogenesis and pathology of *Tritrichomonas foetus* infection in virgin heifers. *J Comp Path.* 86:59-66. (34)

94. Perez A, Cobo E, Marinez A, et al. 2006. Bayesian estimation of *Tritrichomonas foetus* diagnostic test sensitivity and specificity in range beef bulls. *Vet Parasitol.* 142:159-162. (93)

95. Peter DA, Fales WH, Miller RB. 1995. *Tritrichomonas foetus* infection in a herd of Missouri cattle. *J Vet Diagn Invest.* 7:278-280. (94)

96. Peter D. 1997. Bovine Venereal Disease. In: Youngquist RS, editor. *Current Therapy in Large Animal Theriogenology,* 1st edition. W.B. Saunders Co, Philadelphia, PA: 355-363. (18)

97. Rae DO. 1989. Impact of trichomoniasis on the cow-calf producer's profitability. *J Am Vet Med Assoc.* 194:771-775. (37)

98. Rae DO, Chenoweth PJ, Genho PC, et al. 1999. Prevalence of *Tritrichomonas fetus* in a bull population and effect on production in a large cow-calf enterprise. *J Am Vet Med Assoc.* 214:1051-1055. (38)

99. Rae DO, Crews JE, Greiner EC, Donovan GA. 2004. Epidemiology of *Tritrichomonas foetus* in beef bull populations in Florida. *Therio.* 61:605-618. (36)

100. Rae DO, Crews JE. 2006. *Tritrichomonas foetus*. *Vet Clin North Am, Food Anim Pract.* 22: 595-611. (39)

101. Reece RL, Dennett DP, Johnson RH. 1983. Some observations on cultural and transport conditions for *Tritrichomonas foetus* var. *brisbane*. *Aust Vet J.* 60:62-63. (95)

102. Rhyan JC, Stackhouse LL, Quinn WJ. 1988. Fetal and placental lesions in bovine abortion due to *Tritrichomonas foetus*. *Vet Pathol.* 25:350-355. (184)

103. Rhyan JC, Wilson KL, Burgess DE, et al. 1995a. Immunohistochemical detection of *Tritrichomonas foetus* in formalin-fixed, paraffin-embedded sections of bovine placenta and fetal lung. *J Vet Diagn Invest.* 7:98-101. (96)

104. Rhyan JC, Blanchard PC, Kvasnicka WG, et al. 1995b. Tissue-invasive *Tritrichomonas foetus* in four aborted bovine fetuses. *J Vet Diagn Invest.* 7:409-412. (187)

105. Rhyan JC, Wilson KL, Wagner B, et al. 1999. Demonstration of *Tritrichomonas foetus* in the external genitalia and of specific antibodies in preputial secretions of naturally infected bulls. *Vet Pathol.* 36:406-411. (97)

106. Ribeiro LMM. 1990. An efficient medium for the isolation of *Trichomonas foetus*. *Onderstepoort J Vet Res.* 57:209-210. (98)

107. Riley DE, Wagner B, Polley L, et al. 1995. PCR-based study of conserved and variable DNA sequences of *Tritrichomonas foetus* isolates from Saskatchewan, Canada. *J Clin Micro.* 33:1308-1313. (99)

108. Rothman KJ, Greenland S, Lash TL. 2008. *Modern Epidemiology*. Lippincott, Williams, and Wilkins. Philadelphia, PA: 109. (165)

109. Rodning SP. 2007. Trichomoniasis in beef herds. *Proc Am Assoc Bov Pract*. 40:71-76. (40)

110. Saah AJ, Hoover DR. 1997. "Sensitivity" and "specificity" reconsidered: the meaning of these terms in analytical and diagnostic settings. *Annals Internal Med.* 126:91-94. (143)

111. Samuelson JD, Winter AJ. 1966. Bovine vibriosis: The nature of the carrier state in the bull. *J Infect Dis.* 116:581-592. (41)

112. Schmidt H. 1937. Trichomoniasis or trichomonad abortion in cattle. *J Am Vet Med Assoc.* 90: 608-618. (42)

113. Schonmann MJ, BonDurant RH, Gardner IA, et al. 1994. Comparison of sampling and culture methods for the diagnosis of *Tritrichomonas foetus* infection in bulls. *Vet Rec.* 134:620-622. (100)

114. Skirrow SZ, BonDurant RH, Farley J, et al. 1985. Efficacy of ipronidazole against trichomoniasis in beef bulls. *J Am Vet Med Assoc.* 187: 405-407. (47)

115. Skirrow SZ. 1987. Identification of trichomonad-carrier cows. *J Am Vet Med Assoc.* 191:553-554. (45)

116. Skirrow SZ, BonDurant RH. 1988. Bovine Trichomoniasis. *Vet Bull.* 58:591-603. (147)

117. Skirrow SZ, BonDurant RH. 1990. Induced *Tritrichomonas foetus* infection in beef heifers. *J Am Vet Med Assoc.* 196:885-889. (46)

118. Soto P, Parma AE. 1989. The immune response in cattle infected with *Tritrichomonas foetus*. *Vet Parasitol*. 33:343-348. (124)

119. Sutka P, Katai PL. 1969. Rapid demonstration of bull trichomonadosis in unstained smear preparations from preputial scrapings. *Acta Vet Hung.* 19:385-389. (221)

120. Tedesco LF, Errico F, Del Baglivi LP. 1979. Diagnosis of *Tritrichomonas foetus* infection in bulls using two sampling methods and a transport medium. *Aust Vet J.* 55:322-324. (105)

121. Todorovic R, McNutt SH. 1967. Diagnosis of *Trichomonas foetus* infection in bulls. *Am J Vet Res.* 28:1581-1590. (108)

122. Thomas MW, Harmon WM, White C. 1990. An improved method for the detection of *Tritrichomonas foetus* infection by culture in bulls. *Agri-Pract.* 11:13-17. (106)

123. Van Eenennaam AL, Weaber RL, Drake DJ, et al. 2007. DNA-based paternity analysis and genetic evaluation in a large, commercial cattle ranch setting. *J Anim Sci.* 85:3159-3169. (227)

124. Villarroel A, Carpenter TE, BonDurant RH. 2004. Development of a simulation model to evaluate the effect of vaccination against *Tritrichomonas foetus* on reproductive efficiency in beef herds. *J Am Vet Med Assoc.* 65:770-775. (51)

125. Walker RL, Hayes DC, Sawyer SJ, et al. 2003. Comparison of the 5.8S rRNA gene and internal transcribed spacer regions of trichomonadid protozoa recovered from the bovine preputial cavity. *J Vet Diagn Invest.* 15:14-20. (110)

126. Williamson SH. 2009. Six Ways to Compute the Relative Value of a U.S. Dollar Amount, 1790 to Present. *Measuring Worth.* URL http://www.measuringworth.com/uscompare/. Accessed December 6, 2009.

127. Wilson SK, Koccan AA, Gaudy ET, et al. 1979. The prevalence of trichomoniasis in Oklahoma beef bulls. *Bov Pract.* 14:109-110. (149)

Appendix A

InPouch™ TF
Tritrichomonas foetus Test

INTRODUCTION

Bovine trichomoniasis is a venereally transmitted infection by the protozoan, *Tritrichomonas foetus*. The major pathological manifestation of this infection is early embryonic death or abortion in an impregnated cow. The cow shows few other signs of infection. An infected bull is asymptomatic. The organism can only infrequently be found in direct microscopic examination of clinical specimens and serological methods of diagnosis are not reliable.

PRINCIPLES OF THE PROCEDURE

The InPouch™ TF is a self-contained system for the detection of *T. foetus* from bovine preputial or vaginal samples. The proprietary medium is selective for the transport and growth of the trichomonad, while inhibiting the growth of yeast, mold and bacteria which might interfere with a reliable diagnosis.

The InPouch consists of a high barrier, oxygen resistant, plastic pouch with two V-shaped chambers connected by a narrow passage. This two-compartment system allows direct observation (**wet mount**) of a newly collected specimen in the upper chamber before expressing the contents into the lower chamber for culture.

The InPouch™ is sensitive enough that **an inoculum containing as little as 1 organism is sufficient to potentially result in a presumptive positive test.**

Presumptive positive pouches for *T. foetus* can be tested via a PCR procedure to verify the result. Transport and off-site testing can be performed easily due to the flexible packaging and integral design of the pouch.

REAGENTS

The InPouch medium contains the following: trypticase, protease, peptone, yeast extract, maltose and other nutrients, amino acids, salts, antifungal and antimicrobial agents in a normal saline phosphate buffer.

PRECAUTIONS

For *in vitro diagnostic use* only. **The pouch is labeled for veterinary microbial identification and test results only.**
NOTE: State Departments of Agriculture may require that only state certified veterinarians collect and read bovine cultures. Only state certified laboratories may read bovine cultures and perform PCR confirmation testing. All specimens should be handled according to CDC-NIH recommendations for potentially infectious organisms at **BIOHAZARD LEVEL 2.**

STORAGE AND SHELF LIFE

Never refrigerate or freeze the InPouches. Store uninoculated InPouches at room temperature (18°C to 25°C) horizontally, away from direct sunlight. Product shelf life is 12 months from the date of manufacture. Do not use an InPouch if it appears to be cloudy, leaky, dark brown, dry, or if the medium has thick syrup-like consistency.

SPECIMEN COLLECTION

MATERIALS NEEDED
- InPouch™ TF pouches
- Package Insert (this document)
- Microscope clips (optional and sold separately)
- Disposable gloves
- Infusion pipette
- 20 ml syringes or
- Wooden applicator sticks/sterile cotton-tipped swabs
- Microscope

The specimen collection technique is important regardless of the nutrient medium used for culturing.

Step 1..........Use a clean, dry infusion pipette with a 20mL syringe on each bull or cow, **OR**, a satisfactory sample can also be obtained using a long, sterile cotton swab for collection.

Step 2..........Clip the hair around the prepucial orifice in bulls.

Step 3..........If necessary, rinse out the prepucial cavity with sterile saline (not water) to clean out mud and manure. This will help prevent contamination from non-pathogenic intestinal trichomonads and coliform bacteria.

Step 4........... Direct the pipette to the distal penis in the sheath. Obtain the specimen by scraping the mucosa of the distal penis and the fornix area while applying suction with the syringe.

Step 5..........For cows, advance the pipette gently to the floor of the vaginal fornix, and patiently aspirate mucus. This may take 20-60 seconds, due to the viscosity of mucus. Some persistence may be required to get a thick, slightly yellow material that tends to stick to the pipette.

The specimen should NOT be refrigerated or frozen.

INOCULATION

Step 1..........Remove the pouch from the bag, and manually express the liquid so that there is approximately 1 ml in the upper chamber. **Be sure that the liquid in the upper chamber is below the closure tape** to prevent fluid from leaking upon opening.

Step 2..........Tear the pouch open at the notch just above the closure. Open the pouch by pulling the closure tape's middle tabs apart.

Step 3..........**Bovine infusion pipette (for swab, see below)** - insert the specimen pipette tip into the liquid of the pouch's upper chamber and expel 0.5 -1.0 cc of the sample into the pouch. If the collected material adheres to the wall of the pipette, rinse the pipette by flushing a small amount of the liquid medium back and forth into the pouch. Minimize the production of bubbles. Dispose of both pipette and syringe.

Step 4..........**Bovine swab** - insert the swab into the liquid of the pouch's upper chamber. The swab can be "milked" out by pressing the tip of the swab between the fingers through the flexible walls of the pouch. Dispose of the swab.

Step 5..........For wet mount examination, squeeze the top to close, roll the top edge down twice and fold over the end tabs. For culture or transport, express the media into the lower chamber and roll the pouch down until the tape is at the top of the label. Fold the wire tape's end tabs to lock the roll.

Use the BioMed label to write animal identification information.

Transport

InPouch TF is an excellent device for the transport of samples. The plastic pouch resists damage in transport and maintains viability. Inoculated InPouches should be maintained between 15°C - 37°C.

Laboratory Procedures

Wet Mount - Immediate microscopic examination. Place the viewing clip horizontally over the upper chamber and close the clip. (Using the clip is optional). Observe with a microscope under low power 10x, using 20x or 40x if necessary for confirmation.

Immediate Specimen Concentration - It is possible to concentrate the cellular material by standing the pouch vertically or by placing it in a shirt pocket for at least 15 minutes prior to microscopic evaluation. The trichomonads will concentrate at the bottom of the chamber.

Incubation and Examination

Incubate the pouch vertically at 35°C - 37°C for 18-24 hours.

Mixing - before reading the pouch, pull the pouch up and down across the edge of a table approximately 3-4 times.

Microscopic Evaluation - Place the viewing clip horizontally over the lower chamber and close the clip. (Using the clip is optional). Observe with a microscope under low power (10x). Use a higher power (20x or 40x) if necessary for confirmation.
READING TIP: Trichomonads gravitate to the edges of the InPouch. If none are seen along the edges, briefly scan the liquid. Make sure to focus on the liquid and not the textured plastic of the pouch. Do not mistake Brownian motion of small debris particles for evidence of trichomonad activity.

1 to 10 trichomonads observed are enough for a presumptive positive.

Repeat the evaluations daily for the presence of motile trichomonads for 6 days (after inoculation).
Field studies indicate that if
T. foetus is present, 98% of InPouches will be positive at 5 days, 2% of InPouches will be positive at 6 days.

DISPOSAL

All InPouches should be autoclaved or disposed of by equivalent means at **BIOSAFETY LEVEL 2.**

QUALITY CONTROL

The InPouch diagnostic medium is manufactured in small lots. Each lot is released only after a QC test of viability, doubling time, and sterility. QC testing is repeated through the end of shelf life by BioMed. Refer to BioMed Diagnostics Quality Control Procedure (Doc.# 100- 135).

Tritrichomonas foetus LIVE CULTURE

BioMed Diagnostics maintains a live culture of *T. foetus* (clinical isolate) for QC purposes. This live culture (positive control) can be purchased from BioMed Diagnostics (**Catalog #11-1015**).

To maintain an active culture, inoculate a new pouch with one drop (approximately 40µl) of the actively growing culture and incubate at 37°C for 24 hours. They can then be moved to a 32°C incubator or to room temperature. Subculture every 3-4 days when the organisms reach a concentration of 1×10^5/ml.

Specificity
InPouch TF medium is known to be effective in culturing *T. foetus*, *T. suis*, *T. gallinae* and *P. hominis*.

Limitations to the Procedure
In bovine samples *P. hominis*, is a contaminant. While differential staining can sometimes distinguish trichomonad species based on the number of flagellae, PCR testing is the best way to distinguish among trichomonads.

The InPouch medium suppresses but does not eliminate yeast and bacterial growth. A build-up of gas from bacterial growth can be vented by opening the pouches inside a **BIOHAZARD LEVEL 2 HOOD.**

Too much fecal material can make the medium too cloudy to examine. Subculture, if necessary, into another InPouch.

WARNING: This product contains a chemical known to the State of California to cause cancer, birth defects and other reproductive harm.

References
1. Thomas, et al., <u>Agri-Practice Journal</u>, 11:13-17,1990.
2. Borchardt, Norman, Thomas, Harmon, <u>Veterinary Medicine</u>, Feb: 104-112, 1992.

InPouch™ TF

Tritrichomonas foetus Test

Catalog No. 11-1001   20 Test Kit

Catalog No. 11-1003   100 Test Kit

A SELECTIVE CULTURE SYSTEM FOR
THE DIAGNOSIS OF
Tritrichomonas foetus

For Veterinary Use Only
For *In Vitro Diagnostic* Use Only

Manufactured by

BIOMED
DIAGNOSTICS

1388 Antelope Road
White City, OR 97503
1-800-964-6466
(541) 830-3000
fax: (541) 830-3001
www.biomeddiagnostics.com
email: info@biomeddiagnostics.com

Appendix B

CSU Veterinary Diagnostic Lab
Standard Operating Procedure

Tritrichomonas foetus Polymerase Chain Reaction Testing

Next Review Date: 09/12/2008

Brief purpose and application: To demonstrate the presence of *T. foetus* in preputial scrapings and/or cervical mucus using Polymerase Chain Reaction methods.

Justification: Modification to worksheet to print on one page.

Groups using this SOP: RF Laboratory

Required precursor SOPs: None

Procedure:
SAMPLE PREPARATION:
1) Wipe down work area with ELIMINase® prior to starting.

2) Obtain three 250 ml plastic beakers. One will contain ELIMINase® with sufficient volume to cover the blades of the scissors, and two will contain distilled water with sufficient volume to rinse off all residual ELIMINase®.

3) Upon arrival, samples are placed in 37°C incubator to allow organisms to settle to the bottom of the InPouch^{TM} TF test pouch. Allow them to remain there for 2-3 hours. If the sample is culture positive, no further incubation is required, but there should be time allowed for organisms to settle to the bottom of the pouch. Samples received in lactated ringers solution are inoculated into Diamond's Media or InPouch (see Trichomoniasis Culture SOP) and allowed to incubate at 37°C for 24 hours.

4) Label enough NALGENE® Cryogenic Vials to accommodate individual specimens and pools when requested. Also prepare a worksheet numbered with accession numbers and label 2.0 ml Eppendorf Tubes to coordinate with the worksheet.

5) Use clean, sterile scissors to cut the top off of the InPouch^{TM} TF. After cutting the top off of the first pouch, place the scissors in the beaker containing ELIMINase®. Before

using the scissors for the next pouch, rinse them in two changes of distilled water. This must be done between each specimen to avoid cross contamination.

6) Using a sterile disposable transfer pipette, remove 1 ml of sediment from the bottom of the pouch. Transfer sediment to the appropriately labeled Cryogenic Vial. Mix well and remove 200 µl and transfer to the coordinating Eppendorf Tube.

7) When pooling specimens, remove 1 ml from individual pouches as previously described, but transfer 250 µl from the Cryogenic Vial to the vial labeled for the pool. Pool size should be no greater than 5 samples. Once all individual samples have been added, mix pool well by pipetting gently with a transfer pipette, then remove 200 µl from the pool and transfer to appropriate Eppendorf Tube.

8) Once all specimens have been pulled from pouches and/or tubes, Cryogenic Vials are frozen at -70°C in labeled boxes. A log is kept on computer according to box location and accession number. Samples are kept for a minimum of one month.

9) Samples in Eppendorf Tubes are ready for extraction. If extracting will not be performed the same day, samples may be frozen at -70°C overnight.

T. foetus PCR EXTRACTION:
1) Wipe down work area with ELIMINase® prior to starting. Preheat water bath to 56°C.

2) Use the QIAGEN DNeasy® Blood & Tissue Kit. Kit is stored at room temp. Use pipettors and sterile aerosol resistant tips reserved for extractions.

3) If opening a new kit, AW1 and AW2 are supplied as concentrates and must be diluted by adding ethanol (96-100%) as indicated on the bottle.

4) Add 20 µl proteinase K solution to each sample, followed by 200 µl Buffer AL®, and mix thoroughly by pipetting up and down. Incubate at 56°C in water bath for 10 min. Gently mix by inversion throughout the incubation period. While samples incubate, remove DNeasy Mini spin columns (blister packs) from kit and label lids with appropriate numbers according to worksheet.

5) Remove from water bath and add 200 µl ethanol (96-100%) to each sample and mix thoroughly, but gently.

6) Pipette ethanol/template mixture into DNeasy Mini spin columns. Centrifuge at 8000 rpm for 1 min. Discard flow-through and collection tube.

7) Place DNeasy Mini spin column in a new 2 ml collection tube, add 500 µl Buffer AW1, and centrifuge for 1 min at 8000 rpm. Discard flow-through and collection tube.

8) Place DNeasy Mini spin column in a new 2 ml collection tube, add 500 μl Buffer AW2, and centrifuge for 3 min at 14,000 rpm to dry the membrane.

9) Label a 1.5 or 2 ml PCR grade collection microfuge tube with lid (not included in kits) for each sample with accession numbers and animal/sample ID, where appropriate.

10) Remove each tube from centrifuge carefully and check nipple for carryover fluid. If present, transfer to a new 2 ml collection tube and centrifuge again. Be sure to balance the centrifuge before operating.

11) Transfer each spin column to the appropriately labeled 1.5 or 2 ml collection tube. Pipette 100-200 μl Buffer AE® directly onto the DNeasy membrane. Incubate at room temp for 1 min. Centrifuge for 1 min at 8000 rpm to elute.

12) Carefully remove spin columns and discard, saving tubes and their contents. Refrigerate tubes if running PCR within 24 hours or freeze at -70°C (avoid repeated freezing and thawing).

13) Wipe down work surfaces and pipettors with ELIMINase®.

T. foetus **PCR PROCEDURE:**
Hood Protocol:
a. Wipe down hood with a Kimwipe® moistened with ELIMINase® before and after each use while wearing gloves. Gloves should be worn at all times while working in the hood. Change gloves each time the hood is to be re-entered.

b. The pipettors in the PCR hood are for PCR use only. Do not remove from hood.

c. UV light will not come on with hood door open. To turn on UV, close hood door and turn timer knob on top of hood clockwise all the way.

Reagents Needed:
All PCR reagents that are stored at -20°C should be left at -20°C until ready for use or stored in 0°C ice block. Immediately return to -20°C storage after use.

Promega dNTPs®: 10mM Each:
  dATP
  dCTP
  dGTP
  dTTP

BIOLASE^{TM} DNA Polymerase
  Bioline Taq Polymerase
  50 mM MgCl$_2$ Solution
  10X NH$_4$ Reaction Buffer

DEPC-treated Water

IDT® TFR3 Primer (50mM)

IDT® TFR4 Primer (50mM)

Filtered Mineral Oil

Procedure:
1) Put on clean designated PCR lab coat.
2) Wipe down hood with ELIMINase®, remove foil from beakers of tubes, remove lids from pipette tip boxes in hood, place ice blocks from PCR freezer into the hood, and close the hood door. Turn on UV light.

3) While hood is under UV (at least 10 min), fill out *Tritrichomonas foetus* PCR Worksheet (sample attached):

> **List all samples being run.** List DEPC-treated water (aliquots in small box in hood) as sample #1. List sample extractions next. If running more than 10 samples, list another water between them (about every 10 samples). Follow the last sample extraction with water. Then list positive controls. The water samples serve as negative environmental controls.

> **Calculate the amount of each reagent needed for each reaction mixture** (Master Mix and Taq Mix). Prepare enough of each mixture for the number of samples being run, plus enough extra for 1 additional sample for every 5 being run. Example: If running 10 samples (including controls), prepare enough of each reagent for 12 reactions. Write the amounts needed for each mixture on the worksheet under "Amount of Reagent." To check the math, add up the amounts of all the individual additives and divide by the number of reactions. This should equal the total amount shown on the worksheet for each reaction mixture (e.g., 40 µl for Master Mix).

4) When the hood UV light shuts off, prepare reaction tubes for testing by labeling lids according to the worksheet. Also label a tube for Master Mix (MM) and one for Taq Mix (TM). Remove the TF PCR box from the PCR freezer and place in the hood. Prepare mixtures in order, adding reagents in order as listed on the worksheet (exception: add water to Master Mix last) using the amounts calculated on the worksheet and working in the ice block (always keep enzymes in the ice block while working with them). Return the TF PCR box to the freezer as soon as each mix is complete.
> a. **Master Mix (MM)**: Use the same aliquot of DEPC-treated water as will be used for negative environmental controls. Aliquot tubes in the TF PCR box are labeled as follows: "B" (10X Buffer), "M" ($MgCl_2$), "N" (dNTPs), "TFR3" & "TFR4" (Primers). Taq ("T") should be centrifuged and kept in the ice block.

b. **Taq Mix (TM)**: Combine DEPC-treated water, 10X Buffer, and Taq Polymerase in the amounts indicated on the worksheet. Place in refrigerator for later use.

5) Add 40 μl of MM to each reaction tube.

6) Add 5 μl of template to the appropriately labeled reaction tube according to the worksheet.

7) Overlay each reaction mixture with 60 μl of Mineral Oil.

8) Denature in the thermocycler at 94°C for 4 min. Add 5 μl TM to each tube at 90°C hold using pipettor located by the thermocyclers.
9) Run thermocycler program TF. After cycling is complete, thermocycler will hold at 10°C, so this can be set up to run overnight. When removed from thermocycler, store in refrigerator until ready to run gel.

10) Wipe down hood with ELIMINase® and set UV light for 20 min (full turn).

11) Wipe off ice blocks with paper towel to remove excess moisture and put back in PCR freezer.

T. foetus PCR AGAR GEL:
Reagents needed:
5X TBE Buffer (Dilute 1:10 for use)
      1 Liter DEPC-treated H2O
      SIGMA Tris-Borate-EDTA Buffer® Powdered Blend

GenePure LE Agarose®

Ethidium Bromide (10mg/ml solution)

Promega Bromophenol Blue Loading Solution®

New England BioLabs 100 bp DNA Ladder®

Prepare gel:
For large casting tray (halve quantities if using the small casting tray):

Combine in 500 ml flask with screw cap:
      140 ml 0.5X TBE Buffer (5X TBE Buffer concentrate dissolved in 1 liter DEPC water)
      5 gm GenePure LE Agarose® *

* Note: gel may be from 1.5% to 4% agarose

Heat to boiling, using stir bar. Let it boil continuously for about 1 min, until clear.

Cool with cap off, swirling frequently to keep from setting up, until no steam is given off.

Prepare tray by placing autoclave tape across each end to make a barrier for the gel and placing one comb in the slot nearest the end, and the other comb in the slot half way along the tray. Set tray on level counter.

Add 15 µl Ethidium Bromide (HIGHLY TOXIC, keep in plastic Ziploc bag) to liquid agar gel once it has cooled enough so that steam is not visible, being careful not to contaminate pipetor. Swirl gently to mix.

Slowly pour gel into prepared tray and use a pipette tip to move any bubbles away from the teeth of the combs to the edge of tray.

Let gel sit for about 1 hour.

Loading Gel:
When gel is set (bottom feels cool to the touch), gently push each comb in slightly from each end to loosen and then pull up to remove the comb from gel. Remove tape from ends of tray. Reverse orientation of gel tray to load it in the buffer solution so that wells are closest to you. Make sure 0.5X TBE Buffer covers wells, adding more if necessary (fill to the fill line on the electrophoresis tray).

Get small plastic beaker containing Ladder and Loading Solution located in the gel room next to the power supply on the counter.

Load gel:
Pipette 1 µl of the Loading Dye Solution into 1 well of a microtiter plate on the bench for each product being run.

Working on the half of the gel closest to you and working from right to left, load 5 µl of ladder into the first well (lower right corner). Pipette 10 µl of the first sample and mix with the Loading Solution in first well of the microtiter plate by pipeting up and down until homogenous. Then load 10 µl of the reaction mixture into the next well of the gel. To load, insert pipette tip through the buffer and partly into the well (about 3 mm) and expel slowly. If only using half of the gel, use the row of wells furthest from you first.

Continue from right to left until all products have been loaded. Make sure positive control is loaded last in each row of wells (i.e. load positive control for each top and bottom half of the gel). Load 5 µl of Ladder in well after positive control. Once all products are loaded, let them settle for a few minutes.

Put lid on electrophoresis unit (red furthest from you, black closest). Make sure it locks in snugly on the left side. Turn on power unit with toggle switch (back left). Push "up" arrow until display shows "100," then push "Start" (right front of unit). Once the display shows 100, count for 5 sec to allow products to "stack," then push the "down" arrow until display reads 80 ($\pm$5). Look for bubbles forming along bottom at front of unit to make sure it is working. Allow to run for 1 hour or until dye has reached a point between 2nd and 3rd comb slots on the tray (from the front).

Turn power unit off by pushing the start button again, and then shutting off the toggle switch on the back.

Remove cover. Lift out gel tray. Tilt slightly to pour off buffer. Be careful, as gel can slide out of tray. Blot excess buffer off bottom of gel tray with a paper towel. Reverse tray orientation (filled wells at top) and slide gel onto transilluminator.

Turn on the transilluminator, turn off lights, and observe bands wearing protective eyewear.

Interpretation:
Use the Ladder and the *T. foetus* control band to classify test bands as "*T. foetus* Not Detected" or as Positive (*T. foetus* product will be 347 bp). A *T. foetus* positive band should be present for each control sample.

Quality control methods: A *T. foetus* positive extraction control is included in a run with each new kit that is opened. A positive control is included at the end of each run. DEPC-treated water samples are run as negative environmental controls.

References, ancillary materials:
BonDurant, Robert H., Carlos M. Campero, Mark L. Anderson, Karen A. Van Hoosear. Detection of *Tritrichomonas foetus* by polymerase chain reaction in cultured isolates, cervicovaginal mucus, and formalin-fixed tissues from infected heifers and fetuses. *J Vet Diagn Invest* 2003; 15:579-584.

Felleisen, Richard S. J., Natacha Lambelet, Philipp Bachmann, Jacques Nicolet, Norbert Müller, Bruno Gottstein. Detection of *Tritrichomonas foetus* by PCR and DNA Enzyme Immunoassay Based on rRNA Gene Unit Sequences. *Journal of Clinical Microbiology* Vol. 36, No. 2; February 1998, p. 513-519.

QIAGEN® DNeasy® Blood & Tissue Handbook. July 2006, p. 25-27.

Associated SOPs: Trichomoniasis Culture, Sample Storage, Sample Disposal, Hazardous Waste Disposal

Key Words: *Tritrichomonas foetus,* Polymerase Chain Reaction

Generator: Dayla Pearl   Date: 09/06/2007

Reviewed by: James A. Kennedy   Date: 09/06/2007

Approved by: Dwayne Hamar   Date: 09/12/2007

Appendix C

Nebraska	SOP NUMBER MD-518-00	PAGE Page 1 of 3
Lincoln VETERINARY DIAGNOSTIC CENTER	AUTHOR Debra K Royal	AREA Bacteriology
STANDARD OPERATING PROCEDURE	EFFECTIVE DATE 21Jul08 / REVIEW DATE Jul2011	SUPERCEDES New
TITLE:	PCR: Tritrichomonas foetus RT DNA Extraction	

1. **Purpose**

   To describe the procedure for DNA isolation from InPouches for Tritrichomonas foetus real-time PCR.

2. **Associated documents**:

   2.1. MD-519, PCR: Tritrichomonas foetus RT

   2.2. BP-013, Culture: Tritrichomonas foetus

   2.3. MD-012, PCR: Extraction

3. **Definitions**:

   3.1. None

4. **Responsibilities**

   4.1. Lab Manager: It is the responsibility of the lab manager to ensure proper training of laboratory staff.

   4.2. Laboratory Staff: It is the responsibility of the laboratory staff to follow this procedure.

5. **Materials**

   5.1. InPouches to be tested

   5.2. 1.5ml & 2ml microcentrifuge tube

   5.3. Molecular grade sterile distilled H_2O

   5.4. Micro-centrifuge (Eppendorf 5415D or Eppendorf 5415C)

   5.5. Vortex

   5.6. Pipettes

   5.7. Filtered pipette tips

   5.8. 96-well plate

6. Procedure

⬚ Method

Extraction processes are performed in the extraction lab (the extraction process must be performed in a separate location from the master mix and gel electrophoresis procedures). Always wear gloves when handling DNA and reagents. Filtered pipet tips only.

6.1. Extraction:

6.1.1. Collect appropriate amount of all of the above reagents prior to touching any samples.

6.1.2. Preputial wash samples must be inoculated to an Inpouch and incubated from 1-5 days before testing. See SOP # BP-013, Tritrichomonas foetus culture, for inoculation instructions.

   6.1.2.1. To prepare a swab swirl it in 1ml of molecular grade sterile distilled H_2O and skip to step 6.1.4.

   6.1.2.2. For all other samples such at tissue or secretions follow the general extraction procedures in SOP # MD-512 for that type of sample.

6.1.3. Transfer 1ml Inpouch media (inoculated with sample) to a 2ml tube.

6.1.4. Centrifuge at 12,000 x g (11,400 rpms-Eppendorf 5415D, 12,000 rpms-Eppendorf 5415C) for 5 minutes.

6.1.5. Remove the supernatant using a pipet or decanting making sure not to disturb the pellet and discard.

6.1.6. Resuspend the pellet with 500μl molecular grade sterile distilled H_2O. The pellet will be firm so you may use the pipet tip to scrape the pellet lose if necessary

6.1.7. Vortex to mix.

6.1.8. Boil in 100°C heat-block for 10 minutes.

6.1.9. Centrifuge at max speed for 2 minutes.

6.1.10. Remove the supernatant and pipet in a clean 1.5ml microcentrifuge tube. Discard the pellet.

   6.1.10.1. The supernatant can be pipetted into a 96-well plate instead of a 1.5 ml microcentrifuge tube to make transfer to the 96-well PCR reaction plate easier.

6.1.11. The sample is ready to PCR.

⊞ Quality Control

6.1.12. None

ⓘ Additional Notes

- none

Appendix D

SOP NUMBER		PAGE	
MD-519-01		Page 1 of 1	
AUTHOR Debra K Royal		AREA Bacteriology	
EFFECTIVE DATE 15Dec08	REVIEW DATE 21Mar11	SUPERCEDES MD-519-00	

Nebraska UNIVERSITY OF
Lincoln
VETERINARY DIAGNOSTIC CENTER
STANDARD OPERATING PROCEDURE

TITLE:	PCR: Tritrichomonas foetus Real-time

1. Purpose

To describe the procedure for real-time PCR for Tritrichomonas foetus

2. Associated documents

Forms

2.1. *Molecular Diagnostic Lab – PCR Test Request* (CA)

2.2. *Real-time Trich PCR Worksheet* (CA)

SOPs

2.3. MD-518, *PCR: Tritrichomonas foetus RT DNA Extraction*

3. Definitions

3.1. PCR: Polymerase Chain Reaction

4. Responsibilities

4.1. Lab Manager: It is the responsibility of the lab manager to ensure proper training of laboratory staff.

4.2. Laboratory Staff: It is the responsibility of the laboratory staff to follow this procedure.

5. Materials

5.1. Stock solutions (Applied Biosystems)

Probe: 100μM (4316032)

Primers: ...100pmol/μl (4304971)

Xeno Internal Control............150.0ul (1026G)

Gene Expression Master Mix: 5ml (4369016)

5.2. Master Mix Solution (1 reaction, Total reaction volume: 21.13 ul):

Gene Expression Master Mix 10.0ul   Probe..0.05ul

Nuclease free H_2O.............. 7.35ul   Xeno Internal Control.....................3.0ul

TFF2...............................0.365ul   Template..............................2.0ul

TFR2 .... 0.365ul   Total...................................21.13ul

5.3. Primers available:

TFF2, GCG GCT GGA TTA GCT TTC TTT

TFR2, GGC GCG CAA TGT GCA T

5.4. Probes available:

6FAM: ACA AGT TCG ATC TTT G

5.5. Set up materials:

5.5.1. 0.5-10, 2-20, 10-100, 50-200, 100-1000ul pipettors

5.5.2. multi-channel pipettor

5.5.3. Sterile reagent reservoir

5.5.4. Alcohol spray

5.5.5. Biohazard waste bag

5.5.6. Clean lab coat

5.5.7. Gloves

5.5.8. MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 ml (4346906)

5.5.9. MicroAmp™ Optical Adhesive Film (4360954)

5.5.10. Isofreeze 96-well Cool Block

5.5.11. Tips (sterile) 1-10ul, 1-50ul, 20-200ul, 1000ul

5.5.12. Applied Biosystems 7500 Fast PCR System

5.5.13. TaqMan Probe (4316034) Applied Biosystems

5.5.14. Custom Oligonucleotides Applied Biosystems

5.5.15. TaqMan Gene Expression Master Mix Applied Biosystems

6. Procedure

Method

6.1. **Master Mix** (this should be performed in a DNA free environment)

6.1.1. Extract the DNA according to SOP-MD-518 for Tritrichomonas foetus extraction.

6.1.2. Create a worksheet by entering the ID number for each sample underneath 'lane designation'. Enter the number of samples to be done +2. Always add at least 2 extra reactions to your calculations to allow for pipette error. Print out sheet.

    6.1.2.1. If using a multi-channel pipet add 5-10 extra reactions and pour master mix into a sterile reagent reservoir.

6.1.3. PCR is a very sensitive procedure and DNA and RNA are easily degraded by nucleases that are all around our environment. Therefore, it is important to wear gloves and a clean lab coat while making the PCR master mix in the "clean box".

6.1.4. Use only the pipettors and blocked pipette tips provided in the "clean box" when making the PCR master mix. NEVER REMOVE PIPETTES FROM THE CLEAN BOX OR BRING OTHER PIPETTES INTO THE CLEAN BOX.

6.1.5. Remove reagents from the freezer and allow them to thaw.

6.1.6. Fill an ice box with ice.

6.1.7. Place Gene Expression Master Mix and thawed reagents into the ice. **It is important that these reagents never get warm and that the Gene Expression Master Mix is not frozen.**

6.1.8. Place 96-well plate into cool block.

6.1.9. Open a 1.5 or 2ml microcentrifuge tube and place in the ice.

6.1.10. Add the appropriate amount of master mix/water/primers/probe/Xeno control to the tube. These amounts are calculated on the *Real-time Trich PCR Worksheet.*

6.1.11. Using either a multi-channel or standard pipette; pipette 18µl of the mixture into each well.

6.1.12. Then add 2ul of template to the appropriate wells and seal with adhesive film .

6.2. **DNA Amplification**

6.3.

Stage 1	Stage 3
95°C for 10 min.	95°C for 15 sec
1 repeat	60°C for 1 min.
	40 repeats
Dye of interest is FAM	

6.3.1. If ABI computer is not turned on do so and turn on the 7500 Software v 2.0.1.

Nebraska Lincoln VETERINARY DIAGNOSTIC CENTER STANDARD OPERATING PROCEDURE	SOP NUMBER MD-519-01		PAGE Page 4 of 8	
	AUTHOR Debra K Royal		AREA Bacteriology	
	EFFECTIVE DATE 15Dec08	REVIEW DATE 21Mar11	SUPERCEDES MD-519-00	
TITLE:	PCR: Tritrichomonas foetus Real-time			

6.3.2. Log in as Guest.

6.3.3. Software will ask to open program using the Instrument Maintenance Manager or to ignore and continue startup, choose the latter.

6.3.3. Software will ask to open program using the Instrument Maintenance Manager or to ignore and continue startup, choose the latter.

6.3.4. Under the "Set up" column on the following screen, select the "Template" option.

6.3.5. When running a full plate of samples select the "Agpath Trich with wells" template. This template has 96 wells already selected and assigned.

6.3.6. When running less than 96 samples select the "Agpath Trich" template. When using this template the number of samples must be added and assigned to each well on the plate.

6.3.7. To add samples select "Add New Sample" under the "Define Samples" tab. Once the correct number of samples is added, enter in sample information for each.

6.3.8. To assign samples to wells, click on the tab marked "Assign Targets and Samples". Select wells one by one and click on box marked "Assign" next to the corresponding sample.

6.3.9. Once sample information is entered save the document using the title "Trich" followed by today's date in the title.

6.3.10. Remove the plate from the cool block and quickly spin the plate in the salad spinner. Make sure you use a balance.

6.3.11. Place the 96-well plate in the drawer of the 7500 Fast and close the drawer.

6.3.12. Select the "Start Run" button which is located in the upper right hand corner of the screen. The assay will begin and will run for approximately 1.5 hours.

6.3.13. When the run is complete select "Analysis".

6.3.14. Under the tab "Options" on the bottom of the screen select "T. foetus Ag" under the

6.3.14. Under the tab "Options" on the bottom of the screen select "T. foetus Ag" under the "Target Field" option and then deselect the threshold auto option.

6.3.15. Enter in a threshold amount which is above the background on the graph.

6.3.16. After selecting a new threshold, highlight the wells that were tested and select "Print Report" on the top of the screen. Deselect all options excluding "Results Table (By Well)" and then select "Print Preview".

6.3.17. If values and graphs for your controls look accurate select print.

Interpretation

6.4. A Ct value of <38.25 is considered positive, 38.25-39.20 in inconclusive, and >39.20 is negative. All results of 38.25-39.20 will be automatically retested one time and reported at the new value.

6:5. Ct < 38 corresponds to 96% confidence in positive test

6.6. Ct >38 corresponds to >90% confidence once confirmed as positive

6.7. A real-time PCR sample is positive when it passes the cycle threshold of the run. The Ct value is visible as a horizontal red line across the graph page. However, some samples may flag as false positives.

Quality Control

6.8. Positive and negative controls are included in each run.

Reporting

6.9. The following comment will print on each report:

6.9.1. As more data has become available we have been able to eliminate the suspect range. Due to these changes a small number of samples may test as inconclusive. If this happens they will be automatically retested for status confirmation at no charge. The confidence level of both positive and negative results is 96%. As with any Tritrichomonas foetus testing procedure proper collection and transport are vital to this confidence level.

6.10. Enter results into LIMS and give report to the case coordinator.

6.11. Sign and date the *Real-time Trich PCR Worksheet* and attach the 7500 fast report.

Documentation

6.12. Place the *Real-time Trich PCR Worksheet* into the proper section of the "Diagnostic PCR Report Book".

6.13. File the *Molecular Diagnostic Lab – PCR Test Request* form in top drawer of filing cabinet in Rm VDC 119.

Additional Notes

- Stock primer solutions are located in the small freezer.