

***In vitro* efficacy of anti-protozoal compounds against *Tritrichomonas foetus***

by

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## Abstract

Bovine trichomoniasis is a sexually transmitted disease caused by *Tritrichomonas foetus*, an obligate parasite of the reproductive tract of cows and the surface of the bull's penis and prepuce. Significant economic losses occur annually in both beef and dairy cattle occur annually due to this disease. In 1991, annual losses approached \$650 million (Speer and White, 1991) in the United States and have continued with some producers reporting income losses of up to 22% (Rae et al., 2004; Villarroel et al., 2004). Infected bulls are asymptomatic carriers of *T. foetus*, but are capable of transmitting the organism to cows during coitus (BonDurant, 1997). Infection in cows results in endometritis, cervicitis, and vaginitis, which may lead to embryonic death, abortion, pyometra, fetal maceration, or infertility (Felleisen, 1999a). The major economic losses associated with *T. foetus* include reduced calf crop due to early embryonic loss or abortion, reduced weaning weights due to delayed conception, and culling and replacement of infected cattle.

Producers invest significant time and expense testing bulls prior to the breeding season. Weekly sampling for six weeks prior to introduction of the bull into a herd is recommended but rarely performed. Vaccination has been considered to be of limited value for protecting the cattle herd (Bondurant, 2005; Herr et al., 1991; Soto and Parma, 1989). Cattle operations currently infected with *T. foetus* struggle with eliminating the pathogen from the herd. With the lack of an effective FDA approved treatment, current recommendations are labor intensive, costly as infected bulls must be slaughtered, and rely heavily upon accurate diagnosis of trichomoniasis.

With the use of simple and accurate testing methods that can reliably screen for infection combined with a safe, effective, and approved treatment for *T. foetus*, all bulls found within an infected herd could be treated. The ability to treat all bulls within an infected herd would eliminate the need to cull valuable animals and/or risk transmission during subsequent breeding seasons via undetected carrier bulls.

Use of nitromidazoles, which have previously proven to be efficacious in clearing infected bulls, has been banned in the United States. Very little research has been conducted with other potential pharmaceuticals for treatment of *T. foetus* infections in bulls.

Benzimidazoles, specifically oxibendazole and oxfendazole, and ponazuril are anti-protozoal drugs that are legal for use in food animals and were found to be the most efficacious of a panel of agents tested against *T. foetus* by this research team in a limited *in vitro* replication study. In this study, ponazuril and oxibendazole combined with a polymer enhancer eliminated 100% of *T. foetus* organisms. Based on these promising results, further investigation of the efficacy and safety of these formulations is warranted and could result in future treatment options for this economically devastating disease of cattle. In this study we formulated an oxibendazole, oxfendazole and ponazuril topical-enhanced were used to establish a dosage for treatment of *T. foetus* infection in bulls and determined the efficacy of the formulations *in vitro*. Assays were evaluated at predetermined time points using Neubauer hemocytometers to determine the number of viable trophozoites and the induction of the pseudocyst form. Cultures were examined for reversibility to the motile trophozoite form or continued replication of organisms following the administration of the drug. In our final study, complete kill of the organism was achieved and all replicates remained negative for reemergence of trophozoites or pseudocysts following 5 days of culture.

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## List of Abbreviations

<i>T. foetus</i>	<i>Tritrichomonas foetus</i>
<i>T. suis</i>	<i>Tritrichomonas suis</i>
PCR	Polymerase chain reaction
FDA	Food and Drug Administration
DM	Diamond's media
PBS	phosphate buffered saline
PLO	pluronic lecithin organogel
OX	oxibendazole
PO	ponazuril
VC	Velvachol
OXF	oxfendazole
EtOH	70% ethanol
DMSO	dimethyl sulfoxide

## **Chapter I: Introduction**

*Tritrichomonas foetus*, an extracellular flagellated protozoa, is the organism responsible for the sexually transmitted disease known commonly as bovine trichomoniasis. This organism is an obligate parasite of the female reproductive tract and surface of the bovine male's prepuce and penis (Roberts, 1956). The bull is an asymptomatic carrier of the *T. foetus* organism and may efficiently transmit the protozoa to the female during coitus. In a study by Parsonson et al., it was found that a single mating with an infected bull resulted in a 95% infection rate among susceptible nulliparous cows (Parsonson et al., 1976). Following infection, the female may experience vaginitis, pyometra, embryonic death, and abortion. Most females clear the infection within three estrous cycles with only 1% of females suspected of maintaining long term carrier status (Skirrow, 1987). In contrast to females, bulls most commonly become chronically infected and maintain a carrier status. Due to the severe economic impact of this disease and the difficulty in eliminating the carrier status, current recommendations are that bulls found to be positive for the disease be culled from the herd as part of disease management (Yule et al., 1989). This recommendation is often codified by state animal health regulations specifying that infected bulls be sent to slaughter.

Culling of *T. foetus* positive bulls presents an economic impact for the producer. Not only must the producer replace the bull but valuable genetics may be lost. Several treatments have reportedly been successful over the years including topical Bovoflavin Salve® and nitromidazoles drugs including ipronidazole, and metronidazole, administered topically and/or systemically (BonDurant, 1985; Gasparini et al., 1963; Hammond et al., 1953; Skirrow and Bondurant, 1988; Skirrow et al., 1985; Williams et al., 1987). Use of

nitromidazoles has become illegal in food animals in the United States and Bovoflavin Salve<sup>®</sup> (active ingredient trypaflavine) is no longer manufactured (Davis et al., 2009). With the increasing value of cattle, the option to treat bulls that have become infected has once again become an attractive option for producers compared to culling and replacing infected bulls.

The objective of this project was to investigate novel chemotherapeutics *in vitro* as potential candidates for the treatment of *Tritrichomonas foetus* infections. Extrapolating from the previous reports of treatment successes with Bovoflavin Salve<sup>®</sup> (Bartlett, 1946; Hammond et al., 1953) applied topically to the prepuce and penis and similar treatments with nitromidazoles, (Gasparini et al., 1963) experiments were designed to test these chemotherapeutics *in vitro* for ability to kill or reduce the number of *T. foetus* organisms within a culture. Various formulations were tested using two types of antiprotozoals, ponazuril and either the benzimidazole drug oxibendazole or oxfendazole. These antiprotozoal drugs were individually combined with pluronic lecithin organogel to form a novel chemotherapeutic that could be applied to the prepuce and penis of infected bulls in similar fashion to the successful Bovoflavin Salve<sup>®</sup>.

## **Chapter II: Literature Review**

## **A. Bovine Trichomoniasis**

### **History of Trichomoniasis**

Originally described in 1888 by Kunstler in France and first diagnosed in the United States in 1932, *Tritrichomonas foetus* has a long standing history of being a cause of disease of the bovine urogenital tract (Morgan, 1944). Emmerson is credited with the initial discovery in the United States diagnosing the condition by microscopic examination of vaginal smears of two infertile cows (Roberts, 1956). Since this initial discovery of *T. foetus*, documented cases of this disease have been made across the United States (BonDurant et al., 1990a; Rae et al., 2004; Rodning et al., 2008; Szonyi et al., 2012) and the world, including Asia, Australia, South America, and South Africa, when natural service by bulls is used as the major means of breeding (Yao, 2013).

### **Taxonomy**

The amitochondriate protozoan flagellate *Tritrichomonas foetus* belongs to the phylum Parabasilia, order Trichomonadida, family Trichomonadidae (Brugerolle G, 2000; Levine, 1973; Schwebke and Burgess, 2004). Three serotypes of *T. foetus* have been described including *var. brisbane*, *var. belfast*, and *var. manley* based on reactions to agglutination, passive hemagglutination, and skin tests (BonDurant and Honigberg, 1994; Dennett et al., 1974). *Tritrichomonas foetus* not only affects the bovine urogenital tracts of both males and females but also is the etiologic agent of a gastrointestinal

infections of domestic cats; however, the relationship between the bovine and feline *T. foetus* strains has been difficult to define (Morin-Adeline et al., 2014). Bovine and feline isolates express near identical functional category distribution of expressed genes with no indication of molecular-level divergence. Therefore, taxonomically, bovine and feline *T. foetus* isolates represent two genotypes displaying intra-specific variation (Morin-Adeline et al., 2014).

*Tritrichomonas suis* is a closely related organism with a worldwide distribution as an inhabitant of the porcine gastrointestinal and nasal mucosa and has historically been differentiated from *T. foetus* (Felleisen, 1999b; Lun and Gajadhar, 1999).

Morphologically *T. foetus* and *T. suis* are nearly identical, although minor differences in size are found. The ultrastructure of *T. foetus* and *T. suis* appears to be identical along with similar host specificity, immunology, biochemistry, and molecular biology.

Comparative analysis of rRNA gene unit sequences and the use of random amplified polymorphic DNA, unambiguously demonstrated that *T. foetus* and *T. suis* are synonymous. They are strains of the same species but are isolated from different hosts and characterized by differences in pathogenicity (Felleisen, 1999a; Lun et al., 2005).

### **Morphology and Life cycle**

Pleomorphic in nature, trichomonads are most commonly described in the trophozoite form consisting of a pyriform shaped protozoa with a rounded anterior and pointed posterior end. *Tritrichomonas foetus* are approximately 10-25  $\mu\text{m}$  x 5-10  $\mu\text{m}$  with a single nucleus and three anterior and one posterior flagella (Rae and Crews, 2006).

Other morphologic characteristics described by electron microscopy include: a pelta supporting the flagellar canal, a single ribbon of microtubules forming the axostyle, hydrogenosomes that line the axostyle, costa, golgi apparatus, rough endoplasmic reticulum, and food vacuoles (Benchimol, 2004; de Andrade Rosa et al., 2013). A recurrent flagellum emerges from the anterior region and runs toward the posterior region forming an undulating membrane with two to five waves that produce the characteristic vibrating or jerky rolling movement of *T. foetus* (Benchimol, 2004; BonDurant and Honigberg, 1994; Rae and Crews, 2006). *Tritrichomonas* and several other trichomonad species found in anaerobic habitats lack mitochondria and utilize hydrogenosomes in cellular metabolism. The hydrogenosomes contain enzymes that participate in the metabolism of pyruvate and are the site of formation of ATP and molecular hydrogen (Benchimol and Engelke, 2003). The oxidative decarboxylation of pyruvate in hydrogenosomes is coupled to ATP synthesis and linked to ferredoxin-mediated electron transport. As we will discuss in forthcoming sections, this pathway is responsible for metabolic activation of 5-nitromidazoles (Kulda, 1999).

The axostyle is a ribbon of longitudinally oriented microtubules running from one end of the cell to the other. The pelta, also formed by microtubules, is a structure present in the anterior region of the cell. The pelta functions as support for the flagellar canal from which the flagella emerge (Benchimol, 2005). *Tritrichomonas foetus* divides by a special type of mitosis called cryptopleuromitosis in which the nuclear envelope persists and the mitotic spindle is extracellular (Pereira-Neves and Benchimol, 2009).



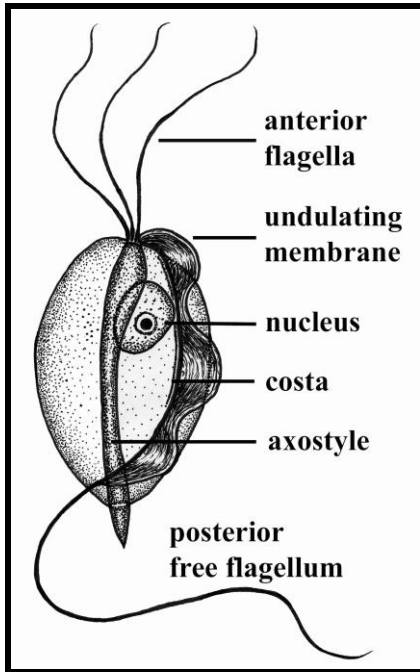


Illustration 1. Anatomy of *Tritrichomonas foetus* (Daly, 2005).

Pleomorphic trichomonads have the ability to enter a pseudocyst stage.

*Tritrichomonas foetus* does not have the ability to enter in to a true cyst form, described as an invagination of external organelles to form a compact, round, non-motile life stage in which the exterior integrity of the cell plasma membrane creates a protective barrier (Pereira-Neves et al., 2003). Instead, *T. foetus* enters into a pseudocyst form. The non-motile pseudocyst stage is characterized by the absence of a cyst wall with internalized flagella and measures approximately 8 $\mu$ m in diameter (Pereira-Neves and Benchimol, 2009). Changes can be noted in the axostyle and costa. The costa may take on different conformations during different stages. It can be a curved shape in multinucleated pseudocysts and the axostyle becomes fragmented during the transformation to the pseudocyst stage (de Andrade Rosa et al., 2015). *Tritrichomonas foetus* may enter into a pseudocyst form in unfavorable conditions such as a decrease in available nutrients, the

presence of drugs, or an abrupt change in temperature (Pereira-Neves et al., 2011). It can re-enter into the trophozoite stage at any time as pseudocysts represent a reversible life form (Pereira-Neves and Benchimol, 2009). The presence of pseudocysts is often associated with unfavorable conditions although it is encountered in fresh preputial smegma from infected bulls and occurs with greater frequency than trophozoites, accounting for 55% of the organisms found upon collection (Pereira-Neves et al., 2011).

Pseudocyst replication is markedly distinct compared to replication of the trophozoite. They undergo asexual reproduction by a process known as budding or a “shizogony like” division (Pereira-Neves et al., 2003). Cytokinesis is arrested during pseudocyst mitosis while the nucleus proceeds with sequential karyokinesis. Binucleated or multinucleated pseudocysts divide by this budding process with trophozoites detaching individually from the pseudocyst. Hence leaving the pseudocyst intact and able to return to the trophozoite form (Pereira-Neves et al., 2003).

The pseudocyst form plays an important role in the life cycle of the parasite. Pseudocysts adhere to vaginal epithelial cells in a higher ratio than their trophozoite counterparts (Mariane et al., 2004). This ability to adhere to epithelium may improve the efficiency of transmission of pseudocysts compared to trophozoites during coitus. Furthermore, the pseudocyst form is more cytotoxic than trophozoites when in contact with vaginal epithelial cells (Mariane et al., 2004; Pereira-Neves et al., 2012). Due to the activity of this cellular form and the fact that it plays an important role in the life cycle of the parasite, it has been recommended that the morphologic form currently termed “pseudocyst” be replaced with the more descriptive “endoflagellar form” (Pereira-Neves et al., 2012).

## **Disease Pathogenesis in the Bull**

An obligate microaerophilic parasite of the bovine reproductive tract, *T. foetus* can be isolated from the nonkeratinized, stratified squamous epithelial surface of the glans penis, prepuce, and distal urethra of bulls (Roberts, 1956). *Tritrichomonas foetus* does not invade the epithelium, but resides only on the epithelial surface where it may rarely cause significant pathologic lesions. A mild purulent discharge within the first two weeks of infection may be recognized as the only clinical sign in bulls (Anderson et al., 1994). Thereafter, the bull is considered an asymptomatic carrier of this true venereal disease (Roberts, 1956). Venereal transmission occurs during coitus when an infected bull breeds a susceptible female or a susceptible bull breeds an infected female. Transmission has also been documented by artificial insemination with infected semen (Bartlett et al., 1953; Bartlett et al., 1947b). Riberio et al. demonstrated that extracellular products secreted by *T. foetus* are cytotoxic to bovine sperm and decrease the progressive motility of sperm (Ribeiro et al., 2010), but no morphologic changes to the sperm have been documented.

The most important feature of the *T. foetus* infection in the bull is the development of the chronic carrier state. It is generally accepted that bulls over three to four years-of-age become chronic carriers of the disease once infected. Chronic infection was once thought to be associated with the age-related changes of the epithelium of the penis and prepuce. As a bull ages, the formation of ever deepening crypts within the preputial or penile epithelium were thought to provide a more favorable microaerophilic environment for survival of the organism (Hammond and Bartlett, 1943). However,

Strickland and colleagues found that neither the area encompassed by the epithelial folds nor the number of epithelial folds differed between age groups of bulls examined in the study (Strickland, 2010). Furthermore, based on examination by scanning electron microscopy there was an absence of structures within the epithelium of the penis and prepuce that can be truly classified as crypts. Due to the absence of crypts or crypt-like structures, the term epithelial folds is suggested to describe the infoldings that are present on the surface of the penis and prepuce (Strickland, 2010).

It has been generally assumed that bulls less than three years of age are usually transient carriers of *T. foetus* organisms and remain infected for only a brief period of time. This is illustrated by the fact that disease transmission by young bulls generally occurs efficiently only if sexual contact with a non-infected cow occurs within minutes to days following breeding of an infected cow (Clark et al., 1977). Breed predilection has been examined as a risk factor for infection in multiple studies, with most concluding that clear breed associations for bull susceptibility to infection with *T. foetus* cannot be made (Abbitt and Meyerholz, 1979; BonDurant et al., 1990a; Rae et al., 2004).

The occurrence of a chronic carrier state in mature bulls indicates that immunity during infection fails to clear the organism from the epithelium. Pathogen-mediated immune evasion may contribute to the persistent infection of *T. foetus* in the lower genital tract of bulls (Cobo et al., 2011). *Tritrichomonas foetus* secretes extracellular cysteine proteinases which digest fibrinogen, fibronectin, albumin, lactoferrin, complement C3, IgG1, and IgG2. IgG1 and IgG2 antibodies bind nonspecifically to the surface of *T. foetus*, masking surface antigens and preventing parasite recognition by the immune system (Corbeil et al., 1991). Since *T. foetus* also sheds surface antigens,

antibodies may be bound away from the parasite surface protecting the organism from antibody-mediated clearance (Singh et al., 1991). Lactoferrin and transferrin from the epithelium may assist in the chronic carrier state by providing sufficient iron to meet the needs of *T. foetus* (Grab et al., 2001; Tachezy et al., 1996).

### **Disease Pathogenesis in the Cow**

Infection with *T. foetus* in the female bovid occurs most commonly following coital exposure from an infected bull or passive transfer from a previously uninfected bull that has had recent coitus with an infected female (Rae and Crews, 2006). Iatrogenic transmission of the disease from one cow to another by contamination of the gloved hand with infected cervical mucous has been reported (Andrews; Andrews and Miller, 1936; Goodger and Skirrow, 1986). A cow may also become infected following artificial insemination with contaminated semen (Roberts, 1956). *Tritrichomonas foetus* can survive procedures that are used to cryopreserve bovine semen. Cows may become infected simply by the use of contaminated insemination equipment (Roberts, 1956).

Infection may be followed by a mild vaginitis that may be undetected. Infection is mitigated by the adherence of the organism to the vaginal epithelial cells and may be mediated by glycoconjugates such as TF 1.17 causing a cytopathic effect on host cells (Corbeil et al., 1989). The organism transverses the cervix and enters the uterine lumen during estrus (BonDurant, 1985). Complete colonization of the entire reproductive tract including the oviduct occurs within 1 to 2 weeks (Bartlett, 1947; Benchimol et al., 2006; Singh et al., 2004). The organism can be cultured from low volume uterine lavages such

as described in a previous study (Gonzalez-Carmona et al., 2012). *Trichomonas foetus* preferentially attaches to keratinized squamous cells (Corbeil et al., 1989; Singh et al., 1999). Ciliated cells present within the bovine oviduct are not a preferential site for trichomonad interaction; instead the secretory cells are the adhesion site of preference for *T. foetus* in the oviduct (Midlej et al., 2009; Vilela and Benchimol, 2011). Repeat breeding or irregular return to estrus due to embryonic or fetal death is often the most recognized clinical sign occurring in infected cows. This common clinical sign may be noticed in tightly managed herds, but can often be missed in large herds and poorly managed herds. This apparent or inapparent infertility is the most economically damaging manifestation of the disease. Currently, the mechanism leading to fetal loss has not been fully elucidated but loss of the conceptus commonly occurs after the maternal recognition of pregnancy at 15-17 days leading to a prolonged interestrus interval in the cow. Pyometra and late term abortion may be associated with *T. foetus* infection but are diagnosed in less than 5% of infected animals (Anderson et al., 1994). By the time pyometra is detected, the uterine endometrium has often suffered significant damage (BonDurant and Honigberg, 1994).

Transmission of *T. foetus* infection at the time of breeding does not prevent conception but replication of the organism within the uterus is followed by death of the embryo or fetus, most commonly between gestation days 15 to 80 (BonDurant, 1985). A few cows abort in the second or third trimester with approximated one-third of the *T. foetus* abortions occurring in the last trimester (BonDurant, 1985). Placental lesions are present due to local or diffuse invasion of the chorionic stroma by *T. foetus*. Fetal bronchopneumonia is also a common finding with identifiable trichomonads in the

airways, and *T. foetus* may be cultured from the abomasum of aborted fetuses (Rhyan et al., 1988; Rhyan et al., 1995; Rhyan et al., 1999)

The occasional presence of a persistent infection in females has been documented previously (Skirrow, 1987). Persistence is defined as any cow that maintains the infection through gestation and into the subsequent breeding season (Skirrow, 1987). Persistently infected cows represent less than 1% of the population. However, they serve as source of infection in subsequent breeding seasons and infect bulls that have been previously free of the disease. (Mancebo et al., 1995; Rae and Crews, 2006; Skirrow, 1987).

Fortunately, persistently infected females are a rarity. Most females mount a humoral immune response throughout the reproductive tract following the initial infection (Parsonson et al., 1976). Time for clearance of the *T. foetus* organism is quite variable with reports as short as six weeks to as long as 22 months (Alexander, 1953; Anderson et al., 1996; BonDurant et al., 1993; Parsonson et al., 1976; Skirrow and BonDurant, 1990). Subsequent infections are cleared in approximately 20 days, indicating an anamnestic response (Peter, 1997). Clark et al. (Clark et al., 1983b) reported that cows exposed to *T. foetus* did not develop long-term immunity to reinfection, estimating that the convalescent period did not persist beyond 15 months. The susceptibility to secondary and tertiary infections was inversely correlated to the elapsed time from the previous infection (Clark et al., 1983b; Parsonson et al., 1974).

## **Diagnostic Technique**

Most states west of the Mississippi River and an increasing number of states east of the Mississippi River have enforced state rules and regulations regarding the testing of bulls entering or moving within the state in order to curtail the disease. Official tests vary from state to state but generally include culture, PCR detected by gel electrophoresis and quantitative real-time PCR (qPCR). The diagnosis of *T. foetus* has historically presented a diagnostic challenge for the cattle industry with the sensitivity and specificity of diagnostic tests for *T. foetus* being compromised by many factors including sample collection and testing.

Clothier et al. demonstrated that large numbers of bacteria which are not inhibited in media interfere with *T. foetus* identification by culture and PCR, and adversely affect diagnostic sensitivity for the fastidious pathogen (Clothier et al., 2015). This is primarily due to the fact the total number of bacterial organisms or specific bacterial species overcome the antimicrobial effects of compounds in the medium leading to depletion of nutrients, a build-up of metabolic end products, and conditions in which the more slowly replicating *T. foetus* organism can no longer survive (Clothier et al., 2015). Temperature can also affect the outcome of culture. Ideally, samples should be collected and placed immediately in media, maintained at 37°C, and protected from extremes in environmental condition during transport to laboratory (Bryan et al., 1999; Davidson et al., 2011; Mukhufhi et al., 2003). Even small factors can make the difference between a positive and negative sample with right-handed people taking samples from the right side of the



bull being four times more likely to obtain a positive sample than if they obtained the sample from the left side of the bull (Parker et al., 2003).

The most common specimens the specimen collected for the diagnosis of *T. foetus* in bovids is preputial smegma in bulls and cervicovaginal mucous in the cow. Diagnostic sampling methodologies vary and include the use of swabs, douches, and pipettes to obtain specimens. Each of these sampling methods have been used with varied success in the bull (Gregory et al., 1990; Irons et al., 2002; Rae and Crews, 2006); (Marquez et al., 1972). While the douche method is the preferred method in obtaining a culture sample in Europe, in the United States the use of the dry pipette scraping method is favored. It should be noted that these two different diagnostic methods have no statistical difference in reliably collecting the organism (Schonmann et al., 1994). Irrespective of the technique used, it is generally recommended that the bulls be sexually rested for one to two weeks before testing for *T. foetus* to avoid false negative results are possible as breeding mechanically reduces the number of organisms on the penis and prepuce of the bull (Bartlett et al., 1947a; Kimsey et al., 1980). The pipette method, commonly referred to as the scraping method, uses a sterile 18 inch plastic mare insemination pipette that is introduced into the preputial opening to just cranial to the preputial fornix and is scraped repeatedly against the epithelium in order to collect smegma within the pipette (Tedesco et al., 1979). The diagnostician should be careful to avoid blood contamination of the sample while ensuring that an adequate sample is achieved. Furthermore, they should be careful not to induce iatrogenic trauma to the prepuce that could induce a retropreputial abscess. The pipette is then removed from the

prepuce, and the sample placed directly into the culture media of choice, typically InPouch™ TF or modified Diamond's media.

Diagnosis was traditionally performed by microscopic examination of preputial smegma or cervicovaginal mucous for the presence of organisms (Parker et al., 2003). Either direct examination of the preputial smegma or by examination of culture following incubation of the diagnostic sample in Diamond's media or modified Diamond's media was the method of choice. Cultivation of live *T. foetus* is still considered the "gold standard" in the diagnosis of bovine trichomoniasis in many countries including the United States. Preputial samples are cultured at 37°C for up to 5-7 days and checked daily by microscopy. The key morphological features of *T. foetus* and the characteristic rolling motion of the live organism have been described. It is also possible to further characterize the organisms by phase contrast microscopy or the use of staining methods to aid in visualization of key diagnostic features of *T. foetus* (Parker et al., 2003).

Several culture media have been found to be suitable for *T. foetus* culture and include Diamond's medium and InPouch™ TF (Biomed Diagnostics; White City, OR). The InPouch™ TF kit contains a proprietary medium and offers a similar sensitivity rate of 81-95% when compared to traditional Diamond's medium. In addition, it also offers a convenient and easy to use packaging system (Appell et al., 1993; BonDurant, 1997; Schonmann et al., 1994). The plastic pouch design is less likely to break or leak compared to tubes containing Diamond's medium and offers an extended shelf life compared to that of traditional Diamond's medium.

With a sensitivity of 80% for a single *T. foetus* culture, false negative results are possible even if a bull has been sexually rested (Kimsey et al., 1980). Only after three

negative cultures at one week intervals is there a 99% sensitivity to detect an infected bull (Kimsey et al., 1980). While culture of the organism offers the advantage of requiring limited equipment, it has several downfalls. Microscopic examination of cultures may be confounded by presence of fecal trichomonads causing the results of the culture to yield false positives. Also the need for live cells for cultivation is a must with the process of culturing taking up to seven days. These downfalls combine to produce a test with low sensitivity. In an effort to reduce the number of times a bull needs to be tested to confirm a positive or negative diagnosis and to mitigate the challenges of microscopic identification, a DNA-based assay was developed to help verify the presence of *T. foetus* (Speer and White, 1991).

The DNA-based test for *T. foetus* was originally designed by workers at Montana State University to extract nucleic acid material, bind the nucleic acid to filters, and then probe for the presence of nucleic acid sequences specific for *T. foetus*. The DNA-probe was initially very promising but due to poor sensitivity when compared to traditional culture, the project was abandoned. Ho et al. (Ho et al., 1994) developed a 0.85kb *T. foetus* probe by identifying conserved sequences in DNA from *T. foetus* isolated from cattle in multiple geographical regions. In order to improve sensitivity, a partial sequence of the probe was used to identify oligonucleotides primers (TF1 and TF2) which could then be used to amplify a 162-bp product for *T. foetus* DNA by PCR. Furthermore, hybridization of a chemiluminescent internal *T. foetus* sequence probe to Southern blots of the amplification product was used. Analysis of this improved PCR showed that 90.4% of samples were correctly identified with no false positives (Ho et al., 1994). The increased sensitivity of the PCR-based amplification system compared to the traditional

culture system led to further development of PCR-based assays for the diagnosis of *T. foetus* (Felleisen et al., 1998; Grahn et al., 2005; McMillen and Lew, 2006).

Polymerase chain reaction assays for the diagnosis of *T. foetus* infections have gained popularity. The higher sensitivity of 95.9% compared to cultures and ability to rule out false-positive cultures in bulls has allowed it to gain popularity as a diagnostic choice (Corbeil et al., 2008; Parker et al., 2003). Polymerase chain reaction assays also offer the advantage of lower detection limits than culture methods for *T. foetus* with only a few organisms needed for a positive result. Another advantage of the use of PCR is the ability to pool multiple preputial smegma samples together without sacrificing sensitivity (Kennedy et al., 2008). This ability to pool samples offers cattle producers a more economical option when testing large numbers of bulls. Garcia Guerra et al. (Garcia Guerra et al., 2014) demonstrated that sensitivities of real time PCR assays for various pooled samples were not significantly different with an overall sensitivity of 94% for the assay. Furthermore, the real-time PCR assay had high specificity and good sensitivity with 94% for the detection of *T. foetus* in pooled cultures of preputial scraping samples from up to 25 animals (Garcia Guerra et al., 2014; Garcia Guerra et al., 2013). Cobo suggests that if sample quality and quantity are adequate and the sample is handled correctly, PCR and culture performed in parallel may offer similar sensitivity results to two cultures or two PCR samples on consecutive weeks (Cobo et al., 2007). These findings provide an attractive option to both veterinarians and cattle producers alike as bulls are handled less often but acceptable test results are achieved.

Test	Sensitivity	Specificity
Parallel Test of PCR & Culture, Single Sample	78.3%	93.3%
2 Cultures, Serial	76.0%	98.5%
2 PCR, Serial	78.0%	96.7%
PCR 3 consecutive weeks	85.0%	95.4%
Parallel of PCR & Culture, 3 consecutive weeks	87.5%	95.6%
6 weekly cultures (Gold Standard)	86.7%	97.5%
Parallel of PCR & Culture, 6 consecutive weeks	93.3%	92.5%

Table 1. Summary of findings from (Cobo et al., 2007)

## **Disease Management**

### **Prevalence**

The prevalence of trichomoniasis has been estimated in different regions of the United States over many years. Johnson, in 1964 reported a 7.5% prevalence in western range bulls (Johnson, 1964). Prevalence studies in Florida (Abbitt and Meyerholz, 1979), Oklahoma (Wilson et al., 1979), California (BonDurant et al., 1990a), Alabama (Rodning et al., 2008) and Texas (Szonyi et al., 2012) found prevalence rates of 7.3%, 7.8%, 4.1%, 0.27%, and 3.7% , respectively.

Rae et al. conducted another epidemiological survey of *T. foetus* in Florida between 1997 and 1999, and determined an overall prevalence of *T. foetus* infected bulls to be 6% (Rae et al., 2004). The overall herd prevalence was 30.4%; and infected bulls were found in 11.1% and 39.5% of herds sampled in North and South Florida,

respectively. Larger herds with 500+, were more likely to be infected (53.9%) than medium-sized herds consisting of 100-499 cows (10%). (Rae et al., 2004)

### **Economic Impact**

Bovine trichomoniasis leads to substantial economic losses for producers with infected herds. This is mainly due to reduced calf crops, culling and replacement of infected bulls, and culling of open cows and heifers (BonDurant et al., 1990a; Rae et al., 1999). The annual calf crop is estimated to decrease as much as 14-50% in herds with 20-40% disease prevalence (Jin et al., 2014; Rae, 1989). The impact of these losses can be quite substantial. Wilson et al. (Wilson et al., 1979) projected an annual calf loss attributable to *T. foetus* in replacement heifers in Oklahoma of \$2.5 million in 1979. Fitzgerald, et al. estimated that each infected bull in a large herd was responsible for a loss of \$800 per year at that time. In 2003, Rae estimated that would have been equivalent to ~\$7.3 million in calf losses and the loss of a bull could be estimated at ~\$3900 (Rae et al., 2004).

Speer and White speculated in 1991 that a 5% calf loss due to trichomoniasis in US beef cattle herds would translate into 1,685,250 calves lost based on the 1989 calf production as estimated by the USDA. This loss was further translated to losses to the industry of \$650 million annually. These figures were based upon a 450 pound calf bringing approximately \$0.85 per pound and totaling \$382 per calf (Speer and White, 1991). Today, the same 450 pound calf would bring approximately \$2.00 per pound bringing the producer \$900. The USDA has estimated that the United States calf crop will be approximately 34.3 million in 2015. If we still assume a 5% loss of calves due to

*T. foetus* infection at current prices, in December of 2015, we could expect a 1.5 billion dollar loss to the industry.

A recent review by Anderson and Hargrove (Rutherford, 2015) estimated that approximately 20% of the roughly 150,000 cattle herds in the state of Texas could potentially be chronically infected with *T. foetus*. They further estimated that approximately 96,000 calves are not born each year that would have been produced in the absence of the disease. This loss, using 2013 price data, added up to \$95 million in revenue to the cow-calf side of the industry in Texas or if extrapolated to the feeder calf industry a loss of \$156 million.

### **Prevention and Control**

Prevention of *T. foetus* in a cattle herd involves the application of basic cattle husbandry practices with the cornerstone being control of animal movement. A recent study from Wyoming looked at risk factors associated with bovine trichomoniasis. The use of public or open allotments by ranchers incurred a 2.9 times higher odds of a having a positive herd than those using private allotments (Jin et al., 2014). This can be compared with a recent study in Argentina showing the odds of a herd becoming infected increased 5.4 times if livestock were housed on common land or pastures at the same time as other herds (Mardones et al., 2008). A closed herd and maintaining good fences will reduce the chances of comingling with infected herds.

The use of younger herd sires may decrease the risk of a herd contracting the disease. The prevalence among herds using bulls younger than three-years-old was 1.8%

as compared to 4.1% among herd using bulls three years-of-age or older (Jin et al., 2014). This has been illustrated multiple times by various authors (Clark et al., 1977; Rae et al., 2004). Therefore, young bulls should be used whenever possible as bulls younger than three years-of-age have been shown to be less susceptible to contracting *T. foetus* and also less likely to become carriers (Rae and Crews, 2006).

The core of all current state regulations on bovine trichomoniasis is to test bulls and cull positive individuals from reproduction. All bulls entering a herd should be tested and declared free of the disease before being pastured with cows. This recommendation applies to all bulls including unexposed or virgin bulls. It has been said that “there is no such thing as a virgin bull” because the libido of young bulls predisposes them to sexual contact even if it is unplanned or unseen (Peter, 1997; Rae and Crews, 2006). To further minimize infection of bulls, open cows and heifers in positive herds should be culled or bred by artificial insemination only. Cows may be tested for *T. foetus*; however, the reduced number of positive animals that are diagnosed makes this less than an attractive option (Goodger and Skirrow, 1986; Skirrow and BonDurant, 1988a). These actions will minimize a bull’s risk of infection, although it does not come without a price to the producer either due to the cost of artificial insemination or due to the cost of replacing the cow or heifer. Ondrak et al. suggest that a combination of culture and the gel PCR assay be performed on 3 sequential preputial scrapings for the best method for identifying bulls that are carriers for *T. foetus* during herd outbreak situations (Ondrak et al., 2010).

Other management options include limiting the length of the breeding season to 60-90 days as long breeding seasons can mask infertility or pregnancy loss. It may be advantageous for owners to retain replacement heifers from the herd in efforts to



maintain a closed herd. If this is not a feasible option or demands for increasing herd size cannot be met, only then should pregnant cows ideally in the second trimester be purchased. Nonetheless, the producer is behooved to maintain strict quarantine of all purchased cows and heifers of unknown status during a breeding season, confirming them pregnant, and then pasture them separately. These management efforts all used in amalgamation will allow the producer to maintain a herd free of *Tritrichomonas foetus*.

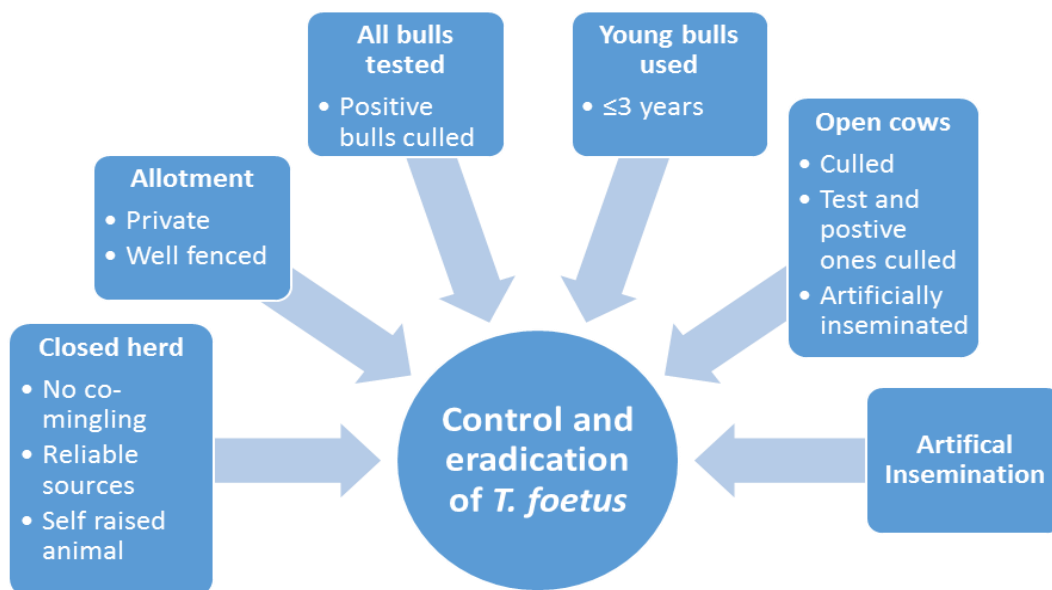


Illustration 2. An integrated approach for control and eradication of *T. foetus* infections.

(Modified from Fig. 2 of Yao, 2013)

### Vaccination

Inoculation and immunization of females against *T. foetus* can be an important management tool in an infected herd or herds at high risk of acquiring *T. foetus*. The vaccine leads to reduced pregnancy wastage that accompanies the disease and quicker

immunity leading to shorter times to rebreeding for infected females. While avoiding the introduction of *T. foetus* into a non-infected herd is superior to vaccination, research trials have confirmed the use of *T. foetus* vaccination in certain situations is beneficial (BonDurant et al., 1993; Cobo et al., 2004; Kvasnicka et al., 1992; Villarroel et al., 2004). Systemic vaccination in females stimulates a mucosal antibody response and mucosal antibodies agglutinate and immobilize *T. foetus* as well as inhibit its adherence to vaginal epithelial cells (BonDurant et al., 1993; Corbeil et al., 1989).

For trichomoniasis, the label for the available *T. foetus* vaccine claims neither prophylactic nor therapeutic efficacy in the bull (Bondurant, 2005). This does not necessarily mean that there is no beneficial effect in the bull, but rather that such efficacy has not been tested by manufacturers (Bondurant, 2005; Clark et al., 1983a; Clark et al., 1984). Cobo et al. (Cobo et al., 2009) demonstrated that bulls systemically immunized with *T. foetus* antigens could resist colonization of the protozoa and had IgG antibodies specific for protective antigens of *T. foetus* in the preputial secretions and serum. They concluded that genital and serum IgG antibodies to *T. foetus* accounts for resistance of vaccinated bulls to *T. foetus* infection. This finding also explains why the absence of IgG response and subsequent lack of antibodies in genital secretions and serum in infected bulls allows for the persistence of infection (Cobo et al., 2011; Cobo et al., 2009).

Despite these findings, a critical review and meta-analysis by Baltzell et al. (Baltzell et al., 2013) found that the evidence bases were designated as moderate to very low quality. As well, they concluded that there was a lack of conclusive evidence to support the use of vaccine in areas where good biosecurity practices are in place. However, this is not to say that in settings where biosecurity is difficult, veterinarians

may elect to use the vaccine. They also concluded that the three studies that have been published assessing the efficacy of *T. foetus* vaccine in bulls are unconvincing.

## **B. Treatment of Bovine Trichomoniasis**

### **Antecedent Treatments of Bovine Trichomoniasis**

Numerous compounds have been used for the treatment of bovine trichomoniasis in bulls. Many are unapproved for the use in food animals by the Food and Drug Administration and others have disadvantages that limit their use. With the ban of nitromidazoles and their derivatives, currently no effective drug is licensed for use in the United States for the treatment of *T. foetus* in cattle. However, many drugs have been used for the treatment of infected bulls since the diagnosis of the disease in the United States in 1932 (Francis and Collins, 1963).

As reported by Hammond et al., Abelein (1938 and 1941) and Swangard (1938, 1939, and 1941) separately reported treatment of *T. foetus* in bulls by application of a tryptaflavine ointment to the penile and preputial membranes and by douching the anterior urethra with tryptaflavine solution (Bartlett, 1946; Hammond et al., 1953). This tryptaflavine salve referred to as Bovoflavin Salve<sup>®</sup> (Farbwerke Hoechst AG, Frankfurt, Germany) contained 0.5% tryptaflavine concentration, and when applied to 57 bulls, 55 of those treated were proven to be cured by examination of cultures two-four weeks following treatment (Hammond et al., 1953). Vandeplassche also obtained successful treatment in three of four bulls with a modification of this method (Hammond et al.,

1953). Bartlett treated eight infected bulls with Bovoflavin Salve<sup>®</sup> and documented that seven were cured by a single series of treatment (Bartlett, 1948). Hammond described the method of treatment for 10 *T. foetus* infected bulls over a four year period with Bovoflavin Salve<sup>®</sup> (Hammond et al., 1953). Each bull was restrained and an epidural using 30-50ml of 2% procaine was administered between the first and second coccygeal vertebrae or first coccygeal and last sacral vertebrae. The penis was then extended, and the penis and prepuce was thoroughly washed with warm water containing a soap or detergent. The area was then dried, and 125mL of Bovoflavin Salve<sup>®</sup> was rubbed into the penis and prepuce for 15-20 minutes. In addition, 20-60 mL of 0.1% trypaflavine solution was introduced into the urethra with a catheter and was allowed to drain out after five minutes. All bulls were tested by preputial scraping, direct examinations, and culture following treatment at intervals of two weeks for varying durations. All bulls were also test-mated to a virgin heifer 45 days after treatment. Of the 10 bulls, nine were cured, three of which required two series of treatments. Only one bull was not cured following two series of treatments (Hammond et al., 1953).

A high percentage of recoveries from *T. foetus* infection had also been reported with hydrogen peroxide applied as a spray and 25% silver nitrate (Fitzgerald, 1963). However, the application of hydrogen peroxide required specialized equipment, and the silver nitrate caused excessive tissue damage (Mariane et al., 2003). Numerous workers reported treatment failures using a variety of different treatments for trichomoniasis in the bull. In 1946, Bartlett discussed the use of multiple formulations that were used as preputial douches or ointments for the treatment of *T. foetus* which included the use of 1% and 5% Lugol's solution, sodium hypochlorite, sodium dioctyl sulfo-succinate and

many others (Bartlett, 1946). Bartlett also discussed the use of potassium iodide orally and potassium sodium intravenously either separately or used as combined treatments. All treatments proved to be unsuccessful. Bartlett further discussed that many workers have studied the action of numerous chemical agents on this pathogen *in vitro*. Their published results demonstrate that while *T. foetus* is rapidly destroyed *in vitro* by these chemical agents, *in vivo* the same agents fail. It was hypothesized this to be primarily due to failure of the various agents to reach all trichomonads (Bartlett, 1946).

Fitzgerald et al. (Fitzgerald, 1963) described the use of acriflavine which consisted of a mixture of 2, 8 diamino-10-methylacridinium chloride and 2, 8-diaminoacridine. In his study, 22 bulls were treated with various acriflavine concentrations prepared in a lanolin base. The penis was washed with an aqueous solution of detergent, dried and the ointment was applied using massage to penis and prepuce. In addition, 60 mL of 0.1% acriflavine solution was infused into the urethra. Success of acriflavine ointment appeared to be dependent upon the strength of the acriflavine ointment with 0.5% and 1% concentrations achieving 100% cure rates. Unfortunately, the 1% concentration instigated severe tissue damage (Fitzgerald, 1963).

In 1963, the use of 1- $\beta$ -hydroxy-ethyl-2-methyl-5-nitro-imidazole, commonly referred to as metronidazole, was first described for the treatment of *T. foetus* in bulls. Gasparini (Gasparini et al., 1963) and colleagues compared metronidazole with the previously described aminitrozole *in vitro* and *in vivo*. *In vitro* studies demonstrated that metronidazole did in fact have trichomonacidal activity and therapeutic trials were initiated (Gasparini et al., 1963). Metronidazole was dosed either by intravenous route at 75 mg/kg every 12 hours for a total of three treatments or bulls were treated topically

with the same compound in the form of a 1% ointment and urethral douche with 30 mL of a 1% solution following a pudendal nerve block. Following treatment, the bulls were cultured six times at an interval of seven days and test-mated to two virgin heifers. All eight bulls were found to be negative following treatment by both preputial culture and culture of heifers following breeding. The authors noted that the drug concentration within blood and urine samples following intravenous administration of metronidazole at 75 mg/kg was considerably higher than those obtained following oral administration of metronidazole at 400 mg/kg. They hypothesized that this was due to the destruction of the compound when given orally by the gastro-intestinal bacterial flora and the subsequent production of non-active metabolites (Gasparini et al., 1963).

In a series of reports, McLoughlin described the use of dimetridazole, an imidazole originally approved for the treatment of histomonads in turkeys. In the first report, (McLoughlin, 1965), dimetridazole was administered either by capsule with the aid of a balling gun or mixed with the daily grain ration at dosages of 25-100 mg/kg with the course of treatment lasting five days. A transitory inappetence was noted at the 100 mg/kg dosage. It was noted that the 25 mg/kg treatment was not only ineffective but resulted in development of resistance to the drug at higher and ordinarily effective levels of the drugs (McLoughlin, 1967). In 1980, Kimsey et al. also described the use of dimetridazole orally by administration through capsules, drench, or stomach tube because of the unpalatability of the drug (Kimsey et al., 1980). Eight bulls were treated orally for five consecutive days with 50 mg/kg dose of dimetridazole; all bulls were found to be culture negative at reexamination. Unfortunately, they were unable to assay dimetridazole in the prepuce. Furthermore, they reported side effects including anorexia, weakness, and

occasional bloating (Kimsey et al., 1980). Jesus et al. compared the use of tryptaflavine, the active ingredient in Bovoflavin<sup>®</sup>, to dimetridazole and found similar results in treatment groups concluding that both drugs were equally efficient at eliminating infection (Jesus et al., 1996)

In a second study performed by McLoughlin in an effort to reduce the cost and frequency of daily oral doses of dimetridazole, intravenous injections of dimetridazole were trialed with 15 experimentally infected bulls (McLoughlin, 1968). A 10-20% solution of sulfuric acid was used to dissolve the drug, and the pH was adjusted to 1.3-1.8. The author noted that reactions of the animals to intravenous injection of the acidic solution was extremely unpredictable and uncontrollable with dyspnea, ataxia, and collapse being noted. Despite these adverse reactions, the author stated that when administered intravenously, dimetridazole was 100% efficacious (Ball et al., 1984; McLoughlin, 1968). McLoughlin (McLoughlin, 1970) continued his research of dimetridazole by treating 11 cows that were experimentally infected with *T. foetus* either by oral or intravenous injection of the drug using dosages comparable to the previous work in bulls. Dimetridazole was shown to clear infections in nine of the 11 cows; however, similar to the bulls the cows receiving intravenous injections demonstrated adverse reactions of ataxia and hyperventilation presumably due to acidity of the injection. While oral administration did not show adverse reactions, the unpalatability of the drug coupled with repeated dosing required makes this drug less than ideal in herd situations (McLoughlin, 1970).

Ipronidazole was used in South Africa to treat trichomoniasis in bulls with a 93% efficacy in clearing trichomonads from more than 100 *T. foetus* infected bulls (Retief,

1978). It was also used by Williams et al. for the treatment of four bulls with 100% efficacy (Williams et al., 1987). Immediately prior to treatment with ipronidazole bulls were treated for two-four days with a broad spectrum antibiotic (Streptopen, Glaxo Aust Pty Ltd, Boronia, Victoria). The purpose of the antibiotic was to reduce the number of preputial bacteria, particularly *Micrococcus* species that have been shown to cleave the imidazole ring *in vitro*. Without the antibiotic pretreatment, ipronidazole only demonstrated a 73% cure rate. Ipronidazole was dissolved in sterile water or saline and administered intramuscularly to bulls at a dose of 30 g of active ingredient in 60-100 mL of sterile water or saline (Williams et al., 1987). Skirrow et al. treated 73 infected bulls with ipronidazole (Skirrow et al., 1985). The approximate cost per bull for treatment with ipronidazole at that time was \$20, approximately 1/5 of the cost of dimetridazole. Forty-two bulls were injected once with 30 g of ipronidazole dissolved in 60 mL of sterile water and injected into three deep intramuscular locations. The remainder of the infected bulls were treated on day one with 30 g of ipronidazole preparation followed on day two and three with an ipronidazole preparation of 15 g mixed in 30 mL of sterile water. The bulls receiving a single treatment demonstrated a 92% clearance rate as compared with bulls that received three treatments demonstrated a 100% clearance rate. The author did note that 12% of treated animals developed injection site abscesses. This was thought to be due to the low pH of 1.1 of the injected compound. An attempt to alkalinize the agent was made but resulted in precipitation of the solution. No other adverse reactions were demonstrated. Due to the fact that ipronidazole was not labeled for use in cattle by the Food and Drug Administration and the drug was used off label, a slaughter withdrawal of 12 weeks was given to the treated animals (Skirrow and Bondurant, 1988). Ipronidazole



had also been advocated for the treatment of infected cows at a dose of 15 g per cow dissolved in 60-100 mL of saline (Ball et al., 1984). By 1987, ipronidazole was withdrawn from the market, primarily due to extra-label use in swine (Skirrow and Bondurant, 1988; Skirrow et al., 1985).

Despite the documented successes of ipronidazole, dimetridazole, and metronidazole in the treatment of bulls infected with *T. foetus*, the drug class of nitromidazoles has been deemed illegal for use in food animals by the Food and Drug Administration. Laboratory studies of members of the nitromidazoles demonstrated mutagenicity and carcinogenicity (Davis et al., 2009). After undergoing reductive activation *in vivo*, metabolites from these compounds attack DNA base pairs resulting in loss of helical structure, strand breakage, and possible inhibition of DNA repair mechanism (Davis et al., 2009; Payne et al., 1999).

Other novel treatments of *T. foetus in vitro* include the use of photodynamic therapy (Machado et al., 2014). Photodynamic therapy using aluminum phthalocyanine tetrasulfonated photosensitizer was efficient in killing *T. foetus* (da Silva et al., 2007). The interaction between the excited photosensitizer and molecular oxygen produces a singlet oxygen as well as other reactive oxygen species. Morphologic changes were investigated by transmission electron microscopy and were found to resemble characteristics found with apoptosis or autophagy of the cell including nuclear fragmentation, chromatic condensation and cytoplasmic vacuolization (Silva et al., 2007). Another novel treatment is the use of tick-borne bacteria from *Rhipicephalus microplus*. Bacteria isolated from the gut, ovary, salivary glands, and gene organ were collected from *R. microplus* females. The bacterium demonstrated efficacy against *T. foetus in*

*vitro*, but did not impair the viability of *T. vaginalis* in the same conditions (Zimmer et al., 2013). Topical therapy with nitrites has also been investigated *in vitro* with hydrogen production causing subsequent cell death (Lloyd et al., 2002).

The effects of microtubule affecting drugs such as taxol, nocodazole, and colchicine have demonstrated the ability to alter the cell cycle and ultrastructure of *T. foetus* during *in vitro* studies (Madeiro da Costa and Benchimol, 2004). Taxol acts as a microtubule stabilizer and promotes the assembly of microtubules in a cell-free system and consequently it is a potent inhibitor of replication of mammalian cells in culture (Madeiro da Costa and Benchimol, 2004). Colchicine is a microtubule depolymerizing agent, and nocodazole is a synthetic microtubule inhibitor. Following administration of taxol to *T. foetus* cultures modifications of the *T. foetus* cells were noticed. Initially, flagella were internalized and pseudocysts were formed with 100% of the cells transformed into the pseudocyst stage. Autophagic vacuoles containing high amounts of microtubular structures were observed in cells submitted to taxol treatment. Autophagy refers to a process by which cells sequester and degrade parts of their own cytoplasm, including organelles (Madeiro da Costa and Benchimol, 2004).

### **Treatment of Trichomoniasis in Other Species**

Treatment of trichomoniasis in other species is similar to that of bovids in that nitromidazoles are a basis of therapy. Perhaps not surprisingly, in research by Michaels and Strube (Michaels and Strube, 1961) the four chemicals that showed the greatest

systemic trichomonocidal activity in hamsters all have a nitro group in the fifth ring position.



Illustration 3: Note nitro group in 5<sup>th</sup> ring position of chemicals shown to have trichomonocidal activity in hamster. (Adapted from Fig. 1 from McLoughlin. Drug Tolerance by *Trichomonas Foetus* 1967)

*Trichomonas vaginalis* (*T. vaginalis*), the most common non-viral sexually transmitted disease in humans, resides in the urogenital tract of both males and females and portrays similar disease characteristics to *T. foetus* in cattle. Similar to *T. foetus*, *T. vaginalis* is also susceptible to 5-nitroimidazole drugs such as metronidazole which have been the mainstay of therapy in human medicine. Metronidazole interferes with electron transfer in the metabolic pathway which leads to the production of molecular hydrogen in the hydrogenosomes and induces the generation of free radicals (Silva-Filho and de-Souza, 1988). Regrettably, metronidazole-resistant *T. vaginalis* has been implicated in an increasing number of refractory cases (Cudmore et al., 2004). This failure of metronidazole brings with it a deep concern as currently it is the only drug approved for the treatment of trichomoniasis in humans in the United States. Refractory cases are often

treated with increased dosages for extended periods of time which does not come without the increased risk of side effects. Metronidazole resistance in humans can either be described as aerobic or anaerobic in nature (Cudmore et al., 2004). In aerobic resistance, oxygen scavenging pathways and possibly ferredoxin are involved. In contrast anaerobic resistance is caused by a reduction or cessation of activity of the enzyme pyruvate: ferredoxin oxidoreductase (PFOR) and hydrogenase. Consequently, decreased hydrogenase activity and its concomitant reduction in hydrogen production lead to impaired oxygen scavenging mechanisms in the hydrogenosomes. Increased levels of cellular oxygen result in impaired reduction and activation of metronidazole (Cudmore et al., 2004).

Tinidazole, a 5-nitromidiazole currently used for treatment of trichomoniasis in countries outside the United States, offers a longer half-life than metronidazole and is eliminated at a significantly lower rate. Furthermore, it shows superior tissue distribution compared to metronidazole as concentrations of drugs in vaginal secretions are similar to that of serum (Cudmore et al., 2004).

Due to the increased incidence of refractory cases and lack of safe, effective and proven alternatives to nitromidazoles, disease prevention through the use of vaccines has been explored. Similar to *T. foetus*, *T. vaginalis* fails to induce long term immunity as 6-12 months after infection neither *T. vaginalis*-specific antibodies nor memory B cells are present in the circulation. Two vaccines have made it to human clinical trials but lack of efficacy caused further investigation to dwindle. Perhaps, the hindrance to the development of solid immunity responses to *T. vaginalis* lies in the fact that infected

women have antibodies against different protein epitopes despite receiving the same trichomonas strain (Chapwanya et al., 2015).

The treatment of *T. foetus* in felines can also be compared to the treatment of the same protozoa in their bovine counterparts. This can be related to the fact the feline and bovine isolates are morphologically indistinguishable and are genetically similar. Therefore the mainstay of therapy in cats consists of 5-nitromidazoles such as metronidazole, tinidazole, and ronidazole. Ronidazole is the treatment of choice in felines as shedding of the trichomonad discontinued within three days of initiating therapy at 10 mg/kg orally every 12 hours and relapses is not reported in cats receiving 50 mg/kg twice daily. Ronidazole is thought to be more efficacious than its nitromidazole counterparts due to improved trapping of the activated compounds within the intestine, and currently is recommended at a dose of 30 mg/kg once daily for 14 days (Davidson, 2006; Gookin et al., 2006; Manning, 2010). Approval of ronidazole for veterinary or human use has not been acquired and consequently the drug should be used with caution as neurologic toxicity has been reported (Manning, 2010). As well, the owner should be aware that relapse is common and cats with resolved clinical signs may continue to carry and shed the organism and thus vigilant and prolonged post-treatment monitoring is indicated (Yao and Koster, 2015).

### **Benzimidazoles**

Following the ban of nitromidazoles in food animals by the Food and Drug Administration, research for alternative chemotherapeutics for the treatment of *T. foetus*

began. Benzimidazoles have been widely used as an anthelmintic in veterinary and human medicine since the 1960s; not only do they offer mild side effects in general but they also offer the attractive option of being approved for use in food animals. Several benzimidazole derivatives have been shown to be active *in vitro* against two protozoan parasites specifically *Trichomonas vaginalis* and *Giardia lamblia* (MacDonald et al., 2004). The microtubule protein  $\beta$ -tubulin subunit has been identified as the benzimidazole target. Benzimidazoles specifically affect the colchicine binding-site of  $\beta$ -tubulin monomers inhibiting microtubule assembly and disassembly (Lacey, 1988). Moreover, benzimidazoles also can act as a lipid-soluble proton ionophore, inducing direct and indirect biochemical changes including inhibition of glucose uptake, glycogen depletion, inhibition of the fumarate reductase system and uncoupling of electron transport-associated phosphorylation (McCracken and Stillwell, 1991).

*In vitro* studies using the benzimidazole derivatives mebendazole and flubendazole were reported to inhibit the growth of the vaginal protozoan *T. vaginalis* (Juliano et al., 1985). *Trichomonas vaginalis* was exposed to three drugs including mebendazole, fenbendazole, and nocodazole for approximately three, eight, and 20 hours to determine the lethal activity against the protozoa. It was observed that lethal activity was strongly correlated with time of exposure and concentration of drug (Katiyar et al., 1994).

Carvalho et al. assessed the effects of thiabendazole, mebendazole, and albendazole on the growth, general morphology and ultrastructure of *T. foetus* using video microscopy and transmission electron microscopy (Carvalho and Gadelha, 2007b). All three benzimidazoles inhibited *in vitro* growth of *T. foetus* to different extents.

Mebendazole was able to inhibit growth at 50% after only four hours of incubation with 14.9  $\mu\text{M}$  and presents a minimum  $\text{IC}_{50\%}$  of 2.3  $\mu\text{M}$  in 24 hours. Albendazole and thiabendazole also presented good trichomonocidal activity with  $\text{IC}_{50\%}$  of 9.4  $\mu\text{M}$  and 142.6  $\mu\text{M}$  respectively at 24 hours of incubation. Treated cultures were incubated in benzimidazole fresh culture medium for 24 hours and most cultures did not restore original growth. Video microscopy demonstrated that thiabendazole and mebendazole treated *T. foetus* presented severe alterations in general morphology with the presence of pseudocysts, as well as vacuolated cells. Transmission electron microscopy also illustrated several changes in the ultrastructure of the protozoa with identification of the pseudocyst stage and altered morphology of the hydrogenosome. Organisms suffering from either or both severe hydrogenosome damage and cytoplasmic vacuolization is postulated to have undergone autophagy and therefore were destroyed. The process of autophagy has been previously described by Benchimol and Mariante when organelles undergo ultrastructure damage (Mariante et al., 2006). In final they reported that benzimidazole derivatives especially mebendazole were successful for the treatment of *T. foetus in vitro*. In conclusion, the ability of benzimidazoles to efficiently alter the microtubules that compose the cytoskeleton of the trichomonad may allow for the degradation and resulting demise of the organism.

### **Toltrazuril sulfone**

Toltrazuril sulfone, commonly known as ponazuril, is a triazine-based anti-protozoal agent with specific actions against apicomplexan group of organisms. Triazine-

based anti-protozoal agents are currently used for a wide variety of diseases caused by Apicomplexans. The exact mechanism of action of the triazine agents is currently unknown. Totrazuril sulfone has been found to have clinical application in the treatment of *Neospora caninum* in cattle and may have clinical application for treatment of other protozoal disease in cattle including *T. foetus*. Pharmacokinetic studies demonstrated that toltrazuril sulfone is relatively well absorbed after oral dosing in cattle and therefore would offer the advantages of easy administration of treatment and legal use in food animals for the treatment of protozoal diseases in cattle (Dirikolu et al., 2009). *In vitro* studies of ponazuril-treated tachyzoites of *Neospora caninum* demonstrated inhibited development after 48 hours exposure to the drug as well as the development of vacuoles and subsequently underwent degeneration. It was also demonstrated that ponazuril was capable of inhibiting cytokinesis and inducing a multinucleate stage in exposed *Toxoplasma gondii*.

### **Pluronic Lecithin Organogel**

Pluronic lecithin organogel (PLO) is a micro-emulsion that has the appearance and feel of gel. A PLO is an opaque, white to yellow preparation that consists of a Pluronic F-127 20-30% and a lipid phase of equal parts lecithin and either isopropyl myristate or isopropyl palmitate. Lecithin acts as a permeating enhancer as it increases the fluidity of the stratum corneum and is used as a dispersing, emulsifying and stabilizing agent. Pluronics, Poloxamers, or Tetronics are all trade names for non-ionic polymers of polyoxyethylene (PEO), polyoxypropylene (PPO), and polyoxyethylene



(PPE) which have many pharmaceutical uses. Pluronic F-127 specifically is a copolymer with a polyoxyethylene to polyoxypropylene or PEO/PPO ratio of 2:1 that is non-toxic. The thermo-responsive nature of Pluronic F-127 allows it to go from a state of low viscosity at 4°C to a semi-solid gel at body temperature 25-29°C. These characteristics demonstrate the ability of PLO to be an ideal candidate for the delivery of drugs at various application sites on the body. With an increase in temperature of PF-127 solution the PPO polymer block tends to dehydrate and form a core with an outer shell of hydrated PEO chains that aggregate into spherical micelles (Almeida et al., 2012b). In aqueous systems, the cores of the micelles consist of hydrophobic blocks, and the shell regions consist of hydrophilic blocks. The core serves as a non-aqueous reservoir for drugs where the drugs are stabilized against chemical modification (Kwon and Kataoka, 1995; Kwon and Okano, 1996). The micellar structure of the copolymer in an aqueous environment can be used for incorporation of hydrophilic and hydrophobic drugs and prolongs drug release (Hosseinzadeh et al., 2012; Leszczynska et al., 2011). These characteristics combined with the low toxicity, biocompatibility with cells and body fluids, and weak immunogenic properties lead to fact that PF-127 is a commonly used polymer in drug delivery systems (Almeida et al., 2012b).

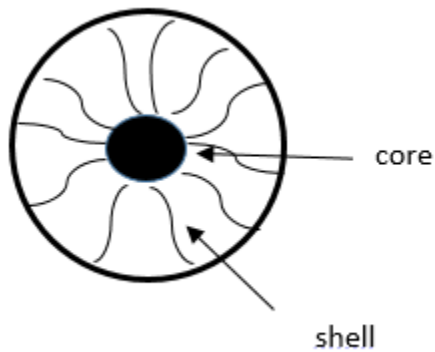


Illustration 4. Spherical micelle demonstrating the shell consisting of hydrophilic blocks and the core consisting of hydrophobic blocks. Adapted from Figure 1 of Kwon and Kataoka, 1995

The Pluronic F-127 and the lipid phase are made separately and can be combined with an active drug and a solvent by shear force from syringe-to-syringe movement of the material. Shear force produces a micelle or bilayer liposome that is small and uniform in size. PLO disrupts the lipid layer of the stratum corneum allowing the medication to slip through the stratum corneum and into the systemic circulation via the dermal-epidermal blood flow (Bramwell and Williams, 2012). PF-127 can be used a carrier for several routes of administration, including oral, subcutaneous, intranasal, vaginal, rectal, ocular, and parenteral (Barichello et al., 1999; Dumortier et al., 2006). To date, multiple drugs or combination of drugs have been incorporated with PLO. Some examples of these drugs include hormones such as estradiol and progesterone, non-steroidal anti-inflammatories such as ketoprofen and piroxicam, selective serotonin reuptake inhibitors, antipsychotic drugs, calcium channel blockers, and insulin (Barichello et al., 1999; Dumortier et al., 2006). PLO gels present unique characteristics that allow these polymers to be excellent

carriers of drug with the ability to efficiently administer concentrations of drugs often avoiding adverse systemic reactions noticed when drugs are given by other routes (Hosseinzadeh et al., 2012; Leszczynska et al., 2011).

### **Chapter III: Journal Article**

*In vitro* efficacy of anti-protozoal compounds as a novel treatment of

*Tritrichomonas foetus*

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## **Abstract**

Bovine trichomoniasis is a sexually transmitted disease caused by *Tritrichomonas foetus*, an obligate parasite of the reproductive tract of cows and folds on the surfaces of the penis and prepuce of bulls. With the ban of efficacious drugs for treatment of *T. foetus* such as the nitromidazoles, veterinarians are currently left without legal means of treatment. Oxfendazole, oxibendazole, and ponazuril are anti-protozoal drugs that can be legally used in food animals. These antiprotozoal drugs were individually combined with pluronic lecithin organogel (PLO) to form a novel chemotherapeutic that could be used for treatment of this protozoal disease. Three *in vitro* experiments were performed using pure cultures of *T. foetus* trophozoites in Diamond's media with the addition of the PLO enhanced drugs and the individual drugs in replicate. Assays were evaluated at predetermined time points using Neubauer hemocytometers to determine the number of viable trophozoites and the induction of the pseudocyst form. Cultures were examined for reversibility to the motile trophozoite form or continued replication of organisms following the administration of the drug. In our final study, complete kill of the organism was achieved and all replicates remained negative for reemergence of trophozoites or pseudocysts following 5 days of culture.

Keywords: *Tritrichomonas foetus*, bull, benzimidazole, ponazuril, treatment

## **Abbreviations**

DM	Diamond's media
PBS	phosphate buffered saline
PLO	pluronic lecithin organogel
OX	oxibendazole
PO	ponazuril
VC	Velvachol
OXF	oxfendazole
EtOH	70% ethanol
DMSO	dimethyl sulfoxide

## 1. Introduction

*Tritrichomonas foetus*, an extracellular flagellated protozoa, is the organism responsible for the sexually transmitted disease commonly known as bovine trichomoniasis. This organism is an obligate parasite of the female reproductive tract and surface of the male's prepuce and penis in cattle (Roberts, 1956). The bull is an asymptomatic carrier of the *T. foetus* organism and transmits the protozoa to the female during coitus (Roberts, 1956). A single mating with an infected bull resulted in infection of 95% of susceptible nulliparous cows (Parsonson et al., 1976). Female cattle may experience vaginitis, pyometra, embryonic death, and abortion. The annual calf crop is estimated to decrease by 14-50% in herds with 20-40% disease prevalence (Jin et al., 2014; Rae, 1989). The impact of these losses can be quite substantial as infection rates amongst herds have been reported from 5-50% with variability based upon geographical location (BonDurant et al., 1990b; Rae et al., 1999; Rae et al., 2004; Rodning et al., 2008). Speer and White speculated that a 5% calf loss due to trichomoniasis in US beef cattle herds would translate into 1,685,250 calves lost based on 1989 calf production as estimated by the USDA (Speer and White, 1991). This equated to a \$650 million annual loss to the industry. These figures were based on the cost of a 450-pound-calf of approximately \$0.85 per pound at market, totaling \$382 per calf (Speer and White, 1991). Today the same calf would cost approximately \$2.00 per pound, grossing \$900. The USDA has estimated the United States calf crop to be approximately 34.3 million in 2015. Assuming a 5% loss of calves due to *T. foetus* infection at current market prices, the loss to the industry would approach 1.5 billion dollars.

Most cows clear the infection within three estrous cycles with only 1% of cows suspected of obtaining carrier status (Skirrow, 1987). In contrast to females, bulls obtain chronic carrier status (Clark et al., 1974). Due to the severe economic impact of this disease, bulls found positive for the disease are culled from the herd as part of disease management, resulting in additional losses to the producer. In addition to the monetary expenditures of replacing the bull, valuable genetics may be lost.

Several treatments for trichomoniasis were used successfully in the past, including: Bovoflavin Salve<sup>®</sup> (active ingredient trypaflavine), ipronidazole, and metronidazole (Hammond et al., 1953) (BonDurant, 1985; Gasparini et al., 1963; Skirrow et al., 1985; Skirrow and BonDurant, 1988b; Williams et al., 1987). Bovoflavin Salve<sup>®</sup> (Farbwerke Hoechst AG, Frankfurt, Germany) contained trypaflavine at a concentration of 0.5% and was applied to the penis and prepuce of infected bulls. When the salve was applied to 57 bulls, 55 bulls proved to be cured by examination two-four weeks following treatment (Hammond et al., 1953). Bartlett (Bartlett, 1948) treated eight infected bulls with Bovoflavin Salve<sup>®</sup> and found that seven were cured by a single series of treatment. Bovoflavin Salve<sup>®</sup> is no longer manufactured and nitromidazoles became illegal for use in food animals (Davis et al., 2009). With the increasing value of cattle, the opportunity to treat bulls infected with *T. foetus* has once again become a feasible option for producers compared to culling and replacing infected bulls.

The objective of this project was to use novel therapeutic drugs with enhancers for the treatment of *T. foetus*. Extrapolating from the previous treatment successes of the use of topical treatments such as Bovoflavin Salve<sup>®</sup> and nitromidazoles, novel therapeutic dosage formulations were tested *in vitro* for ability to kill or reduce the number of *T.*



*foetus* organisms in culture. Various formulations were tested using two types of antiprotozoals, ponazuril and benzimidazoles including oxibendazole and oxfendazole. These antiprotozoal drugs were individually combined with PLO to form a novel chemotherapeutic that could be applied to the prepuce and penis of infected bulls in similar fashion to the successful Bovoflavin Salve<sup>®</sup>.

## **2. Materials and Methods**

### **2.1 Experimental Design**

Antiprotozoal drugs were individually combined with PLO. Three *in vitro* experiments were performed using pure cultures of *T. foetus* trophozoites with the addition of the polymer enhanced drugs or the drugs alone. Samples were evaluated at predetermined time points using Neubauer hemocytometers to determine the number of viable organisms and the induction of the pseudocyst form. Cultures were tested for reversibility to the motile trophozoite form or replication of organisms.

### **2.2 Parasites**

The CDTf3 cloned strain of *T. foetus* was used in this study. The parasites were cultivated in trypticase–yeast extract–maltose (TYM) Diamond’s medium (DM) (Diamond, 1957) and supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologics, Norcross, GA) at 37°C for 24-48 hours until adequate numbers of trichomonads were present to perform the required number of replications. Following

culture, *T. foetus* organisms were washed and re-suspended in 1 mL of sterile phosphate buffered saline (PBS) and then inoculated into 15 mL conical tubes (BD Falcon, Franklin Lakes, NJ) or tissue culture wells (Sarstedt, Inc., Newton, NC) containing DM.

### **2.3 Drug Susceptibility Assay and Reversibility Experiment I**

Trophozoites ( $3.75 \times 10^5 \text{ mL}^{-1}$ ) washed in PBS (VWR, Radnor, PA) were inoculated into 10 mL of fresh DM. At time 0, each of the following treatments were added to culture tubes as follows: 1) control: 0.5 mL DM; 2) 0.5 mL PLO; 3) 75 mg ponazuril (PO) (0.5 mL); 4) 50 mg oxibendazole (OX) (0.5 mL); 5) 37.5 mg PO (0.25 mL) + 0.25 mL PLO; or 6) 25 mg OX (0.25 mL) + 0.25 mL PLO. Pluronic lecithin organogel (Pluronic F127 Letco Medical, Decatur, AL) was combined with OX (Anthelcide EQ<sup>®</sup> Zoetis, Florham Park, NJ) or PO (Marquis<sup>®</sup> Merial, Duluth, GA) paste formulations by shear force, syringe-to-syringe-blending using two luer-lock syringes and a rapid fill connector (Baxter, Inglewood, CA). Each treatment was applied to conical culture tubes in 5 replicates. Following growth at 37°C, samples were taken every 2 hours for a total of 12 hours, the tubes were vortexed prior to removal of a 20 µL sample. From these samples, the surviving organisms were counted utilizing disposable Neubauer hemocytometers (C-Chip<sup>®</sup> NanoEnTek Inc., Pleasanton, CA). At 12 hours post-treatment, each formulation was recultured in fresh DM without drug and evaluated microscopically daily for the presence of *T. foetus* organisms in culture. These assays were performed in order to evaluate if the effects exerted by the antiprotozoal could be reversed.

## **2.4 Drug Susceptibility Assay and Reversibility Experiment II**

Trophozoites ( $9.5 \times 10^5 \text{ mL}^{-1}$ ) were cultured as described above and added to tissue culture wells with 3 mL of DM. At time 0, each of the following treatments were added to tissue culture wells: 1) control 4 mL DM; 2) 4mL PLO; 3) 150 mg oxfendazole (OXF) + 1mL PLO; 4) 450mg PO (3mL) +1 mL PLO. The OXF (U.S. Pharmacopeial Convention Rockville, MD) stock solution was made in EtOH (Humco Texarkana, TX) and mixed with VC (Valeant North America LLC, Bridgewater, NJ) and stored at 150mg  $\text{mL}^{-1}$ . Pluronic lecithin organogel was combined with OXF stock solution or PO by shear force by syringe-to-syringe mixing. Each treatment was applied to tissue culture wells in duplicate. Following growth at 37°C on a rocker plate (Hoefer Inc., Holliston, MA), samples were collected every 4 hours for a total of 24 hours. A 20  $\mu\text{L}$  sample was removed from each well, and the surviving organisms were counted utilizing disposable Neubauer hemocytometers. At 8 hours post-treatment, each formulation was removed from the tissue culture well and centrifuged at 4000g for 10 minutes. The supernatant was removed and the pellet was re-suspended in either PBS + Drug or DM + Drug and re-incubated at 37°C. Assays were microscopically examined every 4 hours for a total of 24 hours for the presence of *T. foetus* organisms in culture by counting organisms as described in Experiment I.

## **2.5 Drug Susceptibility Assay and Reversibility Experiment III**

Trophozoites ( $5 \times 10^4 \text{ mL}^{-1}$ ) were cultured as described above and added to all tissue culture wells along with 3 mL of DM for a total volume of 4mL of DM with 50,000 organisms per well. At time 0, each of the following treatments were added to tissue culture wells as follows: 1) positive control 4 mL DM; 2) negative control 4 mL 70% ethanol (EtOH); 3) 150 mg oxfendazole solution (OXF) (1 mL) + 2 mL Velvachol (VC) + 1 mL PLO; 4) 150 mg OXF (1 mL) + 3 mL VC; 5) 150 mg OXF (1 mL) + 3 mL EtOH; 6) 150mg OXF (1 mL) + 2 mL EtOH + 1 mL PLO; 7) 1 mL EtOH + 2 mL VC + 1 mL PLO; 8) 1 mL EtOH + 3 mL VC; 9) 3 mL EtOH + 1 mL PLO; 10) 4 mL EtOH; 11) 4 mL VC; 12) 150 mg oxfendazole dissolved in 4 mL 99% DMSO; 13) 4 mL 99% DMSO. The OXF (U.S. Pharmacopeial Convention Rockville, MD) stock solution was made in EtOH (Humco Texarkana, TX) and stored at  $150\text{mg mL}^{-1}$  with the exception of formulation 13 which consisted of OXF dissolved in 99% DMSO (VedCo, Saint Joseph, MO). The combinations of PLO (Pluronic F127 Letco Medical, Decatur, AL), VC (Valeant North America LLC, Bridgewater, NJ), EtOH (Humco Texarkana, TX), and OXF stock solution were mixed as described above. Each treatment was applied to tissue culture wells in duplicate. Following culture as described in experiment II, samples were collected every 8 hours for a total of 24 hours and counted as previously described. At 24 hours post-treatment and following counting of organisms, each formulation was removed from the tissue culture well and centrifuged at 4000g for 10 minutes. The supernatant was removed and placed in a labeled vial for evaluation of drug concentration at a later time. The pellet was re-suspended in 4 mL of DM and placed once again in a tissue culture

well before being re-incubated at 37°C. This process was repeated every 24 hours for 5 passages (120 hours) to allow for re-emergence of any organisms. Assays were continued to be microscopically examined during this period for the presence of *T. foetus* organisms in culture by counting of live organisms.

### **3. Results**

All antiprotozoals tested inhibited the *in vitro* growth of *T. foetus* to different extents. In Experiment I, the inhibitory effects of PO and OX significantly inhibited parasite growth at concentrations of 75 mg mL<sup>-1</sup> and 50 mg mL<sup>-1</sup>, respectively. Both PO and OX alone demonstrated a 98% reduction in motile organisms after 2 hours of culture. However, following re-culture at 12 hours and examination at 24 hours, reestablishment of motile organisms was observed. A significant decrease in the number of trichomonads was also noted with PLO combined with OX and PLO combined with PO and their respective cultures were found to be negative for motile trophozoites at 24 hours post treatment. However, motile organisms were once again present at 48 hours with restoration of culture to original growth (Figure 1).

In Experiment II the concentration of benzimidazole was increased with the use of OXF solution at 150 mg mL<sup>-1</sup>. Oxfendazole combined with PLO had substantial inhibitory effects on trichomonad growth, inducing the pseudocyst or endoflagellar state with no motile trichomonads in the culture following an incubation period of 4 hours. The pseudocyst state was maintained for 24 hours and reversibility of the organism was not demonstrated (Table 1).

Experiment III revealed once again that OXF solution when combined with PLO rapidly induced the pseudocyst stage and complete kill of organisms following continued culture of 5 days. Additionally, it was noted that EtOH and 99% DMSO were also capable of inducing death of trophozoites with subsequent negative cultures over a 5 day period (Table 2).

#### **4. Discussion**

In the present study, the *in vitro* susceptibility of bovine *T. foetus* to PO, OX, and OXF was evaluated by susceptibility assays followed by reversibility tests.

Benzimidazoles were chosen for use in this project as a potential treatment for *T. foetus* as they specifically affect the colchine binding-site of  $\beta$ -tubulin monomers inhibiting microtubule assembly and disassembly (Lacey, 1988). Microtubules play a vital role in the cytoskeleton of the trichomonad. The pelta, flagella, spindle and basal bodies as well as the axostyle, the organelle responsible for cell division, are all formed from these structures. Moreover, benzimidazoles also can act as a lipid-soluble proton ionophore, inducing direct and indirect biochemical changes including inhibition of glucose uptake, glycogen depletion, inhibition of the fumarate reductase system and uncoupling of electron transport-associated phosphorylation (McCracken and Stillwell, 1991). The exact mechanism of action the triazine agents such as ponazuril is currently unknown. Totrazuril sulfone, more commonly known as ponazuril, has been found to have clinical application in the treatment of *Neospora caninum* in cattle and may have clinical

application for treatment of other protozoal disease in cattle including *T. foetus* (Dirikolu et al., 2009).

Pluronic lecithin organogels have been previously used in veterinary and human medicine to allow for topical application of drugs (Almeida et al., 2012a; Hosseinzadeh et al., 2012; Kwon and Kataoka, 1995; Kwon and Okano, 1996; Leszczynska et al., 2011). Shear force produces a micelle or bilayer liposome that is small and uniform in size (Bramwell and Williams, 2012). The PLO disrupts the lipid layer of the stratum corneum allowing the medication to slip through the stratum corneum and into the systemic circulation via the dermal-epidermal blood flow (Bramwell and Williams, 2012). These PLO formulations, when combined with a drug forming the unique micelles, not only allow penetration of the drug through the stratum corneum for increased absorption but also allow for time-dependent release of the drug (Bramwell and Williams, 2012). PF-127, a specific PLO, has been used as a carrier for several routes of administration including oral, subcutaneous, intranasal, vaginal, rectal, ocular, and parenteral (Barichello et al., 1999; Dumortier et al., 2006). To date, multiple drugs or combination of drugs have been incorporated with PLO. Examples include: hormones such as estradiol and progesterone, non-steroidal anti-inflammatories such as ketoprofen and piroxicam, selective serotonin reuptake inhibitors, antipsychotic drugs, calcium channel blockers, and insulin (Barichello et al., 1999; Dumortier et al., 2006). The PLO gels present unique characteristics that allow these polymers to be excellent carriers of drugs with the ability to efficiently administer concentrations of drugs often avoiding adverse systemic reactions noticed when drugs are given by other routes (Leszczynska et al., 2011) (Hosseinzadeh et al., 2012).

The efficacy of each anti-protozoal medication was evaluated using a susceptibility technique. In experiment I as hypothesized by the authors, OX and PO in the form of approved drug formulations for horses of Anthelcide EQ<sup>®</sup> (Zoetis, Florham Park, NJ) and Marquis<sup>®</sup> (Merial, Duluth, GA) respectively demonstrated an antiprotozoal effect on *T. foetus*, by severely inhibiting the growth of organisms in the culture. Susceptibility assays determining growth inhibition and reversibility tests demonstrated similar results between OX and PO with OX causing a sharper decline in organisms as compared to PO. However, when the antiprotozoal drugs were combined with PLO the susceptibility periods were significantly longer. Complete inhibition of the trophozoite stage was noted, and the cultures did not demonstrate regrowth until 48 hours after administration of drugs. Perhaps, this could be attributed to the fact that when PLO is combined with a drug a micelle is formed (Leszczynska et al., 2011). The micellar structure prolongs drug release (Hosseinzadeh et al., 2012; Leszczynska et al., 2011). Therefore, the drug was presumably released over a longer period of time exposing the trichomonads to a lower initial concentration of drug as compared to assays that were not combined with the PLO.

In an attempt to induce a permanent reduction in organisms in Experiment II, OXF was substituted for OX in efforts to increase concentration of drug per culture without dilution in the oil-based suspension formulation. When the OXF solution was combined with PLO and added to culture, pseudocyst formation was induced by 4 hours after inoculation. *T. foetus* can enter into this form in unfavorable conditions such as a decrease in available nutrients, the presence of drugs or an abrupt changes in temperature (Pereira-Neves et al., 2011). It can re-enter into the trophozoite stage at any time as



pseudocysts represent a reversible life form (Pereira-Neves and Benchimol, 2009). The non-motile pseudocyst stage is characterized by the lack of a cyst wall with an internalized flagella and measures approximately 8µm in diameter (Pereira-Neves et al., 2003). Changes can be noted in the axostyle and costa which acquire a curved shape. The costa may take on different conformations while in multinucleated pseudocysts. The axostyle is fragmented during the transformation to pseudocyst stage (de Andrade Rosa et al., 2015). Fresh drug was added to the tissue culture wells at 8 hours with the addition of either PBS or DM to satisfy the conclusion that it was indeed the drug causing the reduction in the number of trophozoites. Following continued incubation, the pseudocyst stage persisted for 24 hours without the resumption to the motile trophozoite form.

Experiment III was performed to determine if the higher OXF concentrations in experiment II induced the pseudocyst state or if this observation was due to the additive effects of VC and EtOH to OXF in solution. Oxfendazole in powder form must be dissolved in EtOH and then mixed with VC to allow for a stable formula that can readily be mixed with PLO. All possible combinations of the components of the OXF solution and PLO were tested to determine which component was responsible for inducing death of trophozoites and pseudocyst formation. Multiple formulations rapidly induced the state of non-motile trophozoites and pseudocysts. Non-motile trophozoites were characterized by the presence of the pear-shaped bodies with externalized flagella but lack of motion. These cells were presumed to be non-living cells. Pseudocysts were characterized by the rounding of the cell with internalized flagella as previously described. Following 24 hours of exposure to drug, each culture was re-cultured in drug free DM to test for reversibility of induced states. After 120 hours, no culture demonstrated reversibility of

organisms to the trophozoite state. Interestingly, some cultures were found to have been cleared of cells after 96 hours of incubation. The loss of observable organisms may have been caused by autophagy of cells. Autophagy refers to a process by which cells sequester and degrade parts of their own cytoplasm, including organelles (Madeiro da Costa and Benchimol, 2004). This process of autophagy has been previously described in reference to another microtubule inhibitor, taxol (Madeiro da Costa and Benchimol, 2004).

Ethanol 70% and DMSO 99% both demonstrated efficacy against bovine *T. foetus* causing a rapid and non-reversible death of the organism in *in vitro* cultures. Ethanol 70% is often used to deactivate *T. foetus* cultures in laboratory protocols. Our study demonstrated that EtOH does deactivate cultures of *T. foetus*, and organisms likely undergo autophagy and apoptosis as cells could not be identified following 8 hours of culture. Dimethyl sulfoxide 99% also rapidly induced the death of trophozoites. In previous studies, (Carvalho and Gadelha, 2007a; Kather et al., 2007) DMSO was used to dissolve the benzimidazole and nitromidazoles powder formulations of interest in each study. Several concentrations of DMSO have been used with Carvalho et al. using a 0.1% DMSO solution and Kather et al. used a 99% DMSO (Carvalho and Gadelha, 2007a; Kather et al., 2007). Kather hypothesized that while unlikely, it could not be ruled out that DMSO may enhance drug efficacy even at the small final dilutions (Kather et al., 2007). Our work demonstrated that DMSO may have in fact aided in drug efficacy based on our culture results.

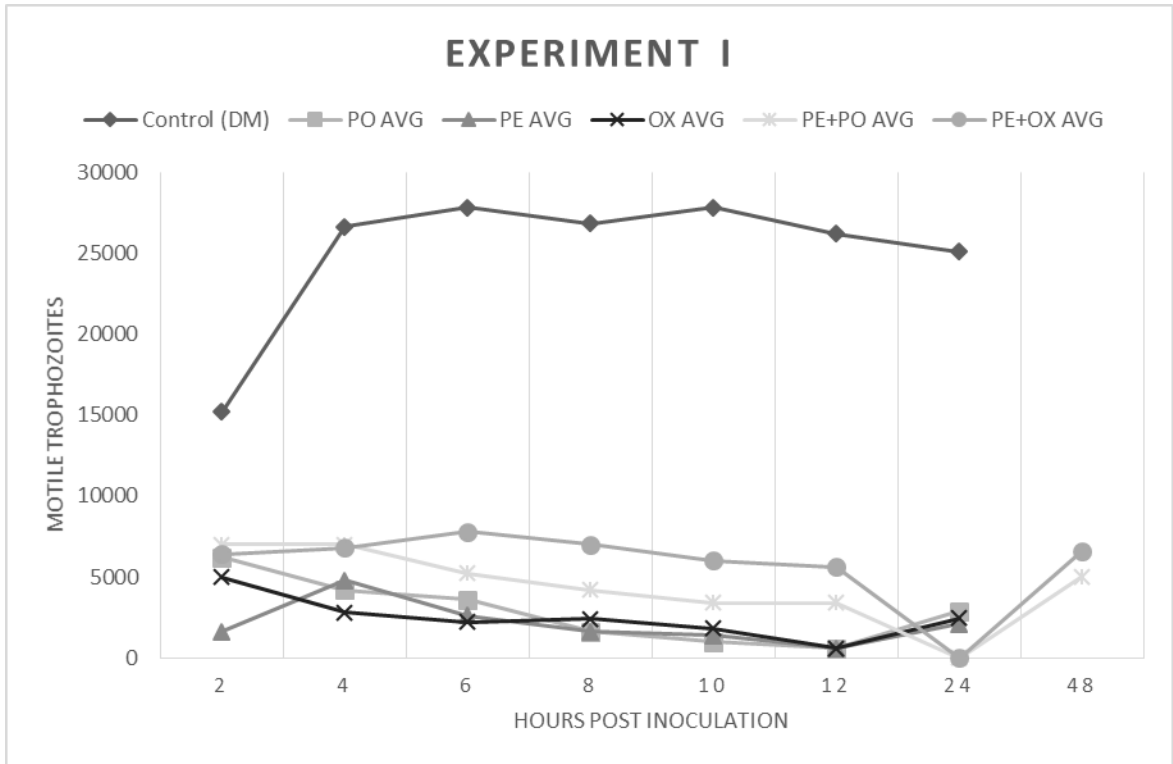
In conclusion, the described experiments demonstrated that OXF solution, when combined with PLO inhibit the growth of the bovine strain CDTf3 of *T. foetus in vitro*.

We have also demonstrated that EtOH can be used to deactivate *T. foetus* cultures as it rapidly induces a non-reversible state. Limitations of this study include not using video microscopy or transmission electron microscopy to further elucidate the effects of our novel chemotherapeutic on the morphology and ultrastructure of the *T. foetus* organism.

## **Acknowledgements**

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**Figure 1**



Averages for each assay was obtained by averaged the 5 replicates at the individual time points. Both PO and OX alone demonstrated a 98% reduction in motile organisms after 2 hours of culture. However, following re-culture at 12 hours and examination at 24 hours, reestablishment of motile organisms was observed. A significant decrease in the number of trichomonads was also noted with PLO combined with OX and PLO combined with PO and their respective cultures were found to be negative for motile trophozoites at 24 hours post treatment. However, motile organisms were once again present at 48 hours with restoration of culture to original growth

**Table 1**

*Experiment II*  
*Hours Post Incubation*

	4hr	8hr	12hr	16h	21h	24h
Control	947,500	952,500	938,500	945,000	927,500	928,000
PO+PLO	no visibility	-	-	-	-	-
PLO	no visibility	-	-	-	-	-
OXF+PLO	pseudocysts	pseudocysts	pseudocysts	pseudocysts	pseudocysts	pseudocysts

OXF solution when combined with PLO and added to culture induced pseudocyst formation 4 hours following inoculation. Cultures remained negative for motile trophozoites for 24 hours with no reversibility noted.

**Table 2**

*Experiment III*  
*Hours Post Incubation*

	8hr	16hr	24h	48h	72h	96h	120h
<u>Control+DM</u>	51,500	49,500	52,000	57,500	55,500	32,000	12,500
<u>Control+EtOH</u>	dead trophs	dead trophs	dead trophs	dead trophs	dead trophs	no cells	no cells
<u>Oxf+EtOH<sup>2</sup>+VC+PLO</u>	pseudocysts	pseudocysts	pseudocysts	pseudocysts	<u>pseudocysts</u>	no cells	no cells
<u>OXF+EtOH+VC</u>	pseudocysts	pseudocysts	pseudocysts	pseudocysts	<u>pseudocysts</u>	no cells	no cells
<u>OXF+EtOH</u>	pseudocysts	pseudocysts	pseudocysts	pseudocysts	pseudocysts	no cells	no cells
<u>OXF+EtOH +PLO</u>	pseudocysts	pseudocysts	pseudocysts	pseudocysts	pseudocysts	no cells	no cells
<u>EtOH+VC+PLO</u>	pseudocysts	pseudocysts	pseudocysts	pseudocysts	<u>pseudocysts</u>	no cells	no cells
<u>EtOH+VC</u>	pseudocysts	pseudocysts	pseudocysts	pseudocysts	pseudocysts	no cells	no cells
<u>EtOH+PLO</u>	pseudocysts	pseudocysts	pseudocysts	pseudocysts	pseudocysts	no cells	no cells
<u>EtOH</u>	dead trophs	dead trophs	dead trophs	dead trophs	dead trophs	no cells	no cells
VC	pseudocysts	pseudocysts	pseudocysts	pseudocysts	pseudocysts	no cells	no cells
OXF+DMSO	no visibility	no visibility	no visibility	pseudocysts	pseudocysts	no cells	no cells
DMSO	dead trophs	dead trophs	dead trophs	dead trophs	dead trophs	no cells	no cells

The formation of pseudocysts was once again rapidly induced by the addition of OXF solution combined with PLO. In addition each component of the OXF solution + PLO formulation was tested in all possible combinations to determine ability to induce pseudocyst formation. Ethanol 70% and DMSO 99% both rapidly induced death of trophozoites. All cultures were negative for reversibility of organisms following 5 days (120hrs) of culture.

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## Appendix I

### Diamond's Media

Stock Solution	(in 100ml dH <sub>2</sub> O)
1.) K <sub>2</sub> HPO <sub>4</sub>	10g
KH <sub>2</sub> PO <sub>4</sub>	6g
NaCl	20g
2.) Bovine Bile	7.5g
3.) Cysteine Hcl	20g
4.) Ascorbic Acid	2g
5.) Fe(NH <sub>4</sub> ) Citrate (keep in dark)	2.2g
6.) 20% Glucose (Autoclave)	20g
7.) 20% Maltose (Autoclave)	20g

\* ALL STOCK SOLUTIONS MUST BE FILTER STERILIZED BEFORE ADDING TO MEDIA EXCEPT (K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>/NaCl) and 20% Sugars

#### Base Media Recipe

To a 500 ml bottle add:

- 1.) 5.0 mLs ((K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>/NaCl)
- 2.) 10 g Trypticase Peptone
- 3.) 5.0 g Yeast extract
- 4.) 2.0 NaOH pellets
- 5.) Adjust pH to 7.0
- 6.) q.s. 415 mLs
- 7.) Autoclave and label base media
- 8.) Can store at room temperature

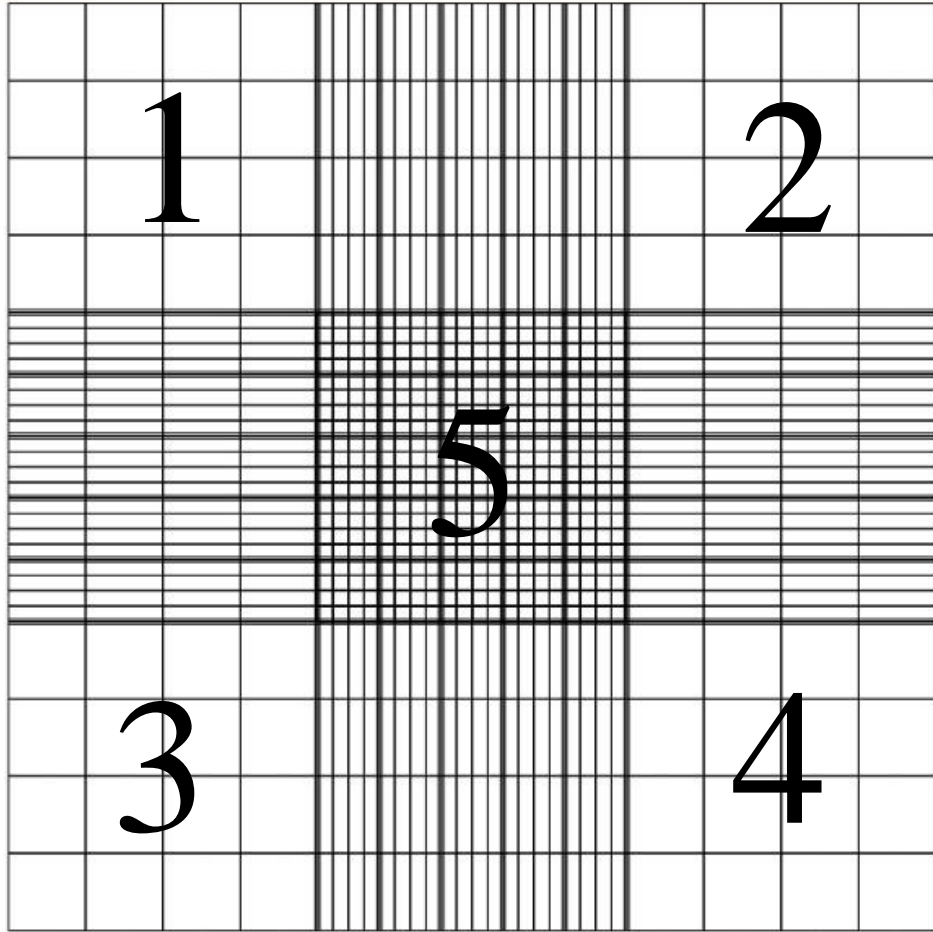
To Base Media Add Accordingly:

#### Diamonds Media

- 1.) 2.5 mL Sterile Cysteine
- 2.) 5.0 mL Sterile Ascorbic Acid
- 3.) 25 mL Sterile 20% Maltose
- 4.) 5.5 mL Sterile dH<sub>2</sub>O
- 5.) 50 mL Fetal bovine Serum
- 6.) 5.0 mL Pen/Strep
- 7.) Store at 4°C

## Appendix II

### Hemocytometer Protocol



- 1.) Count all motile *T. foetus* organisms in squares 1, 2, 3, and 4
- 2.) Multiply number of organisms by 1000
- 3.) This number is equal to the number of *T. foetus* organism per mL

### Appendix III

#### Experiment I

1. 1mL of Ponazuril to 1mL of PLO
2. 1.5mL of Oxibendazole to 1.5mL of PLO
3. 8/28/14: 375,000 organisms was added to conical tube + 10mL of Diamonds media
  - a. At time 0, one of the following treatments will be added to individual culture tubes as follows: 1) 0.5 mL DM (control); 2) 0.5 mL PE; 3) 75 mg PO (0.5 mL); 4) 50 mg OX (0.5 mL); 5) 37.5 mg PO (0.25 mL) + 0.25 mL PE; or 6) 25 mg OX (0.25 mL) + 0.25 mL PE. Each treatment will be applied to individual culture tubes in 5 replicates. The tubes will be vortexed and a 20 µL sample removed every 2 hours for a total of 12 hours. From each sample, the surviving organisms will be counted utilizing disposable Neubauer hemocytometers.
4. At 12 hours post-treatment, each formulation will be recultured in fresh DM, passaged daily for 7 days and evaluated microscopically each day for the presence of *T. foetus* organisms in culture.
5. 8/29/14: Each conical tube was spun for 9min at 4000 rpm the supernatant removed and 10mL of DM added. The tubes were vortexed to resuspend the drug and replaced in incubator
6. 8/30/14: each culture was microscopically examined for *T. foetus*
  - a. All were positive for *T. foetus* except for PE+OX and PE + PO
  - b. Those 2 groups were centrifuged supernatant removed and 10mL of DM added to continue to culture and examine for reemergence
7. 8/31/14: PE+OX and PE+PO were positive

Begin 8/28/14 2PM

8/28/14	4pm	6pm	8pm	10pm	12am	8/29/14 2am	8/30
	T 2hrs	T 4 hrs	T 6 hrs	T 8 hrs	T 10 hrs	T 12 hrs	
DM 1	15000	30000	32000	30000	28000	31000	+
DM 2	15000	18000	24000	20000	22000	21000	+
DM 3	21000	20000	21000	26000	28000	25000	+
DM 4	15000	15000	19000	12000	12000	10000	+
DM 5	10000	50000	43000	46000	49000	44000	+
	T 2hrs	T 4 hrs	T 6 hrs	T 8 hrs	T 10 hrs	T 12 hrs	
PE 1	11000	4000	2000	1000	1000	1000	+
PE 2	27000	10000	4000	4000	3000	2000	+
PE 3	15000	3000	2000	1000	1000	0	+

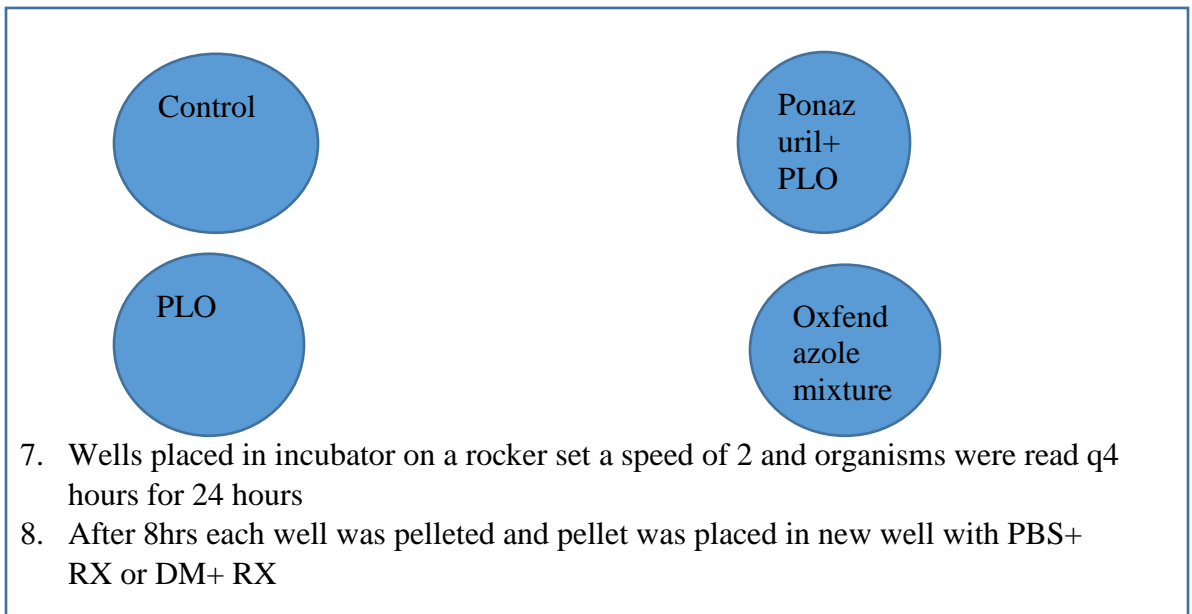
PE 4	12000	4000	1000	1000	1000	0	+	
PE 5	15000	3000	4000	1000	1000	0	+	
	T 2hrs	T 4 hrs	T 6 hrs	T 8 hrs	T 10 hrs	T 12 hrs	8/3 0	
PO 1	8000	7000	6000	2000	2000	1000	+	
PO 2	6000	4000	3000	3000	3000	2000	+	
PO 3	7000	4000	2000	1000	0	0	+	
PO 4	4000	3000	3000	2000	0	0	+	
PO 5	6000	3000	4000	0	0	0	+	
	T 2hrs	T 4 hrs	T 6 hrs	T 8 hrs	T 10 hrs	T 12 hrs		
OX 1	8000	6000	5000	4000	4000	0	+	
OX 2	4000	2000	2000	2000	2000	1000	+	
OX 3	4000	2000	1000	1000	0	0	+	
OX 4	5000	2000	1000	3000	2000	2000	+	
OX 5	4000	2000	2000	2000	1000	0	+	
	T 2hrs	T 4 hrs	T 6 hrs	T 8 hrs	T 10 hrs	T 12 hrs	8/3 0	
							8/3 1	
PE+PO 1	4000	4000	1000	0	0	0	-	+
PE+PO 2	5000	4000	2000	2000	0	0	-	+
PE+PO 3	9000	8000	8000	8000	8000	7000	-	+
PE+PO 4	9000	12000	8000	4000	3000	3000	-	+
PE+PO 5	8000	7000	7000	7000	6000	7000	-	+
	T 2hrs	T 4 hrs	T 6 hrs	T 8 hrs	T 10 hrs	T 12 hrs		
PE+OX 1	7000	8000	6000	4000	3000	4000	-	+
PE+OX 2	6000	8000	8000	8000	7000	5000	-	+
PE+OX 3	7000	7000	8000	6000	6000	5000	-	+
PE+OX 4	8000	6000	6000	7000	5000	4000	-	+
PE+OX 5	4000	5000	11000	10000	9000	10000	-	+



## Appendix IV

### Experiment II

1. 3mL Ponazuril Paste (Marquis) + 1mL of PLO
2. Oxifendazole Powder 600mg
  - a. Dissolved in 4 mL of 70% Ethanol
  - b. Oxifendazole suspension was placed in velvachol water miscible base and qs to 12 mL
  - c. Divided oxfendazole + ethanol+ Velvachol into 3mL aliquots
  - d. Each 3mL aliquot of oxfendazole + ethanol + Velvachol was mixed with 1 mL of PLO
    - i. 150mg of oxfendazole per sample
3. 900,000 of CDTf3 *T. foetus* organism (0.5mL) was added to a tissue culture well
4. 3.5 mL of Diamonds media was added to this well for a final total of 4mLs of Diamonds with 900,000 organisms
5. 4 mLs of one formulation was added to each well already containing organisms and diamonds media
  - a. Ponazuril + PLO gel
  - b. PLO gel
  - c. Oxfendazole + ethanol + Velvachol + PLO
  - d. Control (DM+ organism)
6. 2 plates were made in identical fashion and placed on a rocker in the incubator



## Experiment II Results

Plate 1	4hr	8hr	12hr	16hr
Control	950000	950000	947000	950,000
Ponazuril + PLO	Could Not Read	Could Not Read	Could Not Read	Could Not Read
PLO	Could Not Read	Could Not Read	Could Not Read	Could Not Read
Oxfendazole + PLO	Pseudocysts	Pseudocyst	Pseudocysts	Pseudocysts

Plate 2	4hr	8hr	12hr	16hr
Control	945000	955000	930000	940000
Ponazuril + PLO	Could Not Read	Could Not Read	Could Not Read	Could Not Read
PLO	Could Not Read	Could Not Read	Could Not Read	Could Not Read
Oxfendazole + PLO	Pseudocysts	Pseudocyst	Pseudocysts	Pseudocysts

## **Appendix V**

### Experiment III

1. 50,000 organisms will be placed in each tissue culture well
2. Diamonds media will be added to obtain a total volume of 4mLs of Diamonds media with 50,000 organism/ well
3. Stock oxfendazole solution: dissolving 600mg of oxfendazole in 4mL of 70% ethanol
  - a. Each well will be then treated with one of the following
    - i. Positive Control: Diamonds media + Trichomoniasis = 8mL
    - ii. Negative Control: Trichomoniasis + 70% Ethanol = 8mL
    - iii. Formulation 1: 1mL Stock Oxfendazole solution+2mL VC+ 1mL PLO = 4mL
    - iv. Formulation 2: 1mL Stock Oxfendazole solution + 3mL Velvachol = 4mL
    - v. Formulation 3: 1mL Stock Oxfendazole solution + 3mL 70% Ethanol= 4mL
    - vi. Formulation 4: 1mL Stock Oxfendazole solution + 2mL 70% Ethanol + 1mL PLO= 4mL
    - vii. Formulation 5: 1mL 70% Ethanol + 2mL Velvachol + 1mL PLO = 4mL
    - viii. Formulation 6: 1mL 70% Ethanol + 3mL Velvachol = 4mL
    - ix. Formulation 7: 3mL 70% Ethanol + 1mL PLO = 4mL
    - x. Formulation 8: 4mL 70% Ethanol = 4mL
    - xi. Formulation 9: 4mL Velvachol = 4mL
    - xii. Formulation 10: 150mg Oxfendazole dissolved in DMSO in 4mL 99% DMSO = 4mL
    - xiii. Formulation 11: 4mL 99%DMSO = 4 mL
4. Study will be performed in duplicate
5. Tissue culture plates will be placed in incubator on rocker set a speed of 2
6. Each well be counted at 8hrs and 16hrs post inoculation
7. At 24 hours each well will be read
  - a. Following determination of number of organisms
  - b. Each sample will be spun down
  - c. Supernatant will be removed and placed in labeled vial for evaluation of drug concentration at later time
  - d. And pellet will be re-suspended in 4mL of Diamonds media and re-incubated
8. Process described in number 7 will be continued daily for 5-7 days to allow for re-emergence of any organisms

### Experiment III Results

	8hr	16hr	24h	48h	72h	96h	120h
Control+DM	51,000	49,000	53,000	61,000	59,000	35000	15,000
Control+DM	52,000	50,000	51,000	54,000	52,000	29000	10,000

	8hr	16hr	24h	48h	72h	96h	120h
Control+ EtOH	dead trophs	dead trophs	dead trophs	dead trophs	dead trophs	no cells	no cells
Control+ EtOH	dead trophs	dead trophs	dead trophs	dead trophs	dead trophs	no cells	no cells

Formulation 1	8hr	16hr	24h	48h	72h	96h	120h
Oxf+EtOH <sup>2</sup> +VC+PLO	pseudocysts	pseudocysts	pseudocysts	pseudocysts	pseudocysts	no cells	no cells
Oxf+EtOH <sup>2</sup> +VC+PLO	pseudocysts	pseudocysts	pseudocysts	pseudocysts	pseudocysts	no cells	no cells

Formulation 2	8hr	16hr	24h	48h	72h	96h	120h
Oxf+EtOH <sup>2</sup> +VC	pseudocysts	pseudocysts	pseudocysts	pseudocysts	pseudocysts	no cells	no cells
Oxf+EtOH <sup>2</sup> +VC	pseudocysts	pseudocysts	pseudocysts	pseudocysts	pseudocysts	no cells	no cells

Formulation 3	8hr	16hr	24h	48h	72h	96h	120h
Oxf+EtOH <sup>2</sup>	pseudocysts	pseudocysts	pseudocysts	pseudocysts	pseudocysts	no cells	no cells
Oxf+EtOH <sup>2</sup>	pseudocysts	pseudocysts	pseudocysts	pseudocysts	pseudocysts	no cells	no cells

Formulation 4	8hr	16hr	24h	48h	72h	96h	120h
Oxf+EtOH <sup>2</sup> + PLO	pseudocysts	pseudocysts	pseudocysts	pseudocysts	pseudocysts	no cells	no cells
Oxf+EtOH <sup>2</sup> + PLO	pseudocysts	pseudocysts	pseudocysts	pseudocysts	pseudocysts	no cells	no cells

Formulation 5	8hr	16hr	24h	48h	72h	96h	120h
EtOH <sup>2</sup> +VC+PLO	pseudocysts	pseudocysts	pseudocysts	pseudocysts	pseudocysts	no cells	no cells
EtOH <sup>2</sup> +VC+PLO	pseudocysts	pseudocysts	pseudocysts	pseudocysts	pseudocysts	no cells	no cells

Formulation 6	8hr	16hr	24h	48h	72h	96h	120h
EtOH <sup>2</sup> +VC	pseudocysts	pseudocysts	pseudocysts	pseudocysts	pseudocysts	no cells	no cells
EtOH <sup>2</sup> +VC	pseudocysts	pseudocysts	pseudocysts	pseudocysts	pseudocysts	no cells	no cells
Formulation 7	8hr	16hr	24h	48h	72h	96h	120h
EtOH <sup>2</sup> +PLO	pseudocysts	pseudocysts	pseudocysts	pseudocysts	pseudocysts	no cells	no cells
EtOH <sup>2</sup> +PLO	pseudocysts	pseudocysts	pseudocysts	pseudocysts	pseudocysts	no cells	no cells

Formulation 8	8hr	16hr	24h	48h	72h	96h	120h
EtOH <sup>2</sup>	no organisms	no organisms	no organisms	pseudocysts	pseudocysts	no cells	no cells
EtOH <sup>2</sup>	no organisms	no organisms	no organisms	pseudocysts	pseudocysts	no cells	no cells

Formulation 9	8hr	16hr	24h	48h	72h	96h	120h
VC	pseudocysts	pseudocysts	pseudocysts	pseudocysts	pseudocysts	no cells	no cells
VC	pseudocysts	pseudocysts	pseudocysts	pseudocysts	pseudocysts	no cells	no cells

Formulation 10	8hr	16hr	24h	48h	72h	96h	120h
Oxf+ DMSO	no visibility	no visibility	no visibility	pseudocysts	pseudocysts	no cells	no cells
Oxf+ DMSO	no visibility	no visibility	no visibility	pseudocysts	pseudocysts	no cells	no cells

Formulation 11	8hr	16hr	24h	48h	72h	96h	120h
DMSO	dead trophs	dead trophs	dead trophs	dead trophs	dead trophs	no cells	no cells
DMSO	dead trophs	dead trophs	dead trophs	dead trophs	dead trophs	no cells	no cells