ANTIMICROBIAL ACTIVITY OF EQUINE SEMEN EXTENDERS AGAINST *Taylorella equigenitalis*

By

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ANTIMICROBIAL ACTIVITY OF EQUINE SEMEN

EXTENDERS AGAINST *Taylorella equigenitalis*

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CHAPTER I

INTRODUCTION

Taylorella equigenitalis is a microaerophilic gram negative coccobacillus that is the causative agent of equine contagious metritis (CEM). Originally named *Haemophilus equigenitalis*, the contagious equine metritis organism (CEMO) was initially reported in England (Crowhurst 1977), Ireland (Hughes, Bryden et al. 1978), and Australia in 1977 (Hazard, Hughes et al. 1979), as a transmissible venereal disease in equids. Over the next two years, *T. equigenitalis* was isolated in Kentucky, France, Germany, Belgium, and Missouri in various horse breeds (Powell 1981). Since 1978, the United States has encountered additional CEM outbreaks in Missouri (1979), Kentucky (1982), Wisconsin in 2006 and most recently rediscovery of CEM in Kentucky in 2008 (APHIS 2009).

Since the discovery of *T. equigenitalis* in December of 2008, 1,001 horses have been exposed in the United States, including 23 positive stallions and 5 positive mares. Forty eight states have been involved since December 2008, and the origin for this latest incursion of the disease is still under investigation (APHIS 2010). The National Veterinary Services Laboratory performed antimicrobial susceptibility testing and pulsed-field gel electrophoresis (PFGE) on the *T. equigenitalis* isolates from this occurrence in the United States. Of the isolates tested, 26 samples were streptomycin resistant with matching banding patterns between samples, but the PFGE profiles did not match those of strains isolated in 1978 and 2006 (APHIS 2009).

The clinical signs of CEM in mares vary from copious amounts of mucopurulent vaginal discharge, vaginitis, cervicitis, endometritis, and extreme vaginal/cervical hyperemia to mild cervicitis with minimal, sometimes non-existent uterine discharge (Swerczek 1978; Powell 1981; APHIS 2009) . These clinical signs are apparent from 2 days to 2 weeks post-breeding and can persist for days to weeks. Mares commonly return to estrus within 1-2 weeks of infection (Powell 1981), but rarely abortion can occur (APHIS 2009). In mares, the organism can be cultured from the clitoral fossa and sinus, cervix, endometrium, and in some cases the vaginal discharge (APHIS 2009). While

some mares make spontaneous clinical recovery (Swerczek 1978a), many become asymptomatic carriers, harboring the organism in the clitoral fossa and sinuses. Some of these mares can be difficult to detect and often take several treatments to clear the infection. Surgical ablation of the clitoral sinuses may be indicated as a last resort for persistently infected mares (APHIS 2009). In stallions, *T. equigenitalis* is a surface contaminant that does not illicit an inflammatory reaction or identifiable antibody response (Timoney 1996). It has been cultured from the urethral sinus and urethral fossa, terminal urethra, external surface of the penis and sheath, and to a lesser extent, pre-ejaculatory fluid. There is only one report of the bacteria being isolated at necropsy from the testes and various accessory sex glands (Kristula and Smith 2004). Stallions are asymptomatic carriers, and may sometimes be difficult to identify by culture (APHIS 2009).

The United States testing and treatment protocol for exposed and/or infected stallions and mares is available from the United States Department of Agriculture Animal and Plant Health Inspection Services. Currently, Amies transport medium with charcoal is the accepted transport medium for genital swabs, and Eugon agar with 10% chocolated horse blood the preferred culture medium (Timoney, Shin et al. 1982). Swabs should be submitted refrigerated (~4-6°C) within 24-48 hours of collection for culture (Timoney, Harrington et al. 1978; Duquesne, Pronost et al. 2007). Isolates must be submitted to either National Veterinary Services Laboratories (NVSL) or CEM Approved Laboratories depending on the sample being submitted.

The most widely accepted way of diagnosing CEM is bacteriological culture thereby confirming the presence of *T. equigenitalis* in the mare and the stallion (APHIS 2008). Culturing the organism facilitates the characterization of the isolates by their culture requirements, their anti-microbial sensitivity and their pathogenic and antigenic properties. The polymerase chain reaction (PCR) assay of *T. equigenitalis* on swabs has been reported to provide a comparatively rapid diagnosis, with increased sensitivity (Wakeley, Errington et al. 2006; Duquesne, Pronost et al. 2007). Stallions do not mount a detectable humoral antibody response to the bacterium; however, complement fixation (CF) can be used in mares to confirm a recent exposure to *T. equigenitalis*. In mares, antibodies can be found as early as 7 days, reaching peak titers around 21 to 45 days after infection (Timoney 1996). Additional serum agglutination tests and antiglobulin tests have been used, but complement fixation test reactions are detected for longer periods of time post infection compared to the other two tests (Dawson, Benson et al. 1978). In addition, slide agglutination tests and immunofluorescence tests using monoclonal antibodies can help confirm culture results (APHIS 2009). PCR, however, is used as a screening test for mares and stallions in conjunction with cultures, and has been shown to discriminate between *T. equigenitalis* and *T. asinigenitalis*, a genetically closely related species found in the genital tract of donkeys and less frequently horses (Wakeley,

Errington et al. 2006). Unlike PCR, the use of latex agglutination tests will not differentiate these two strains (APHIS 2008).

When repeated negative cultures are obtained from quarantined stallions, test breedings on CEM negative mares are used to rule out the asymptomatic carrier state (APHIS 2009). It is possible, however, for these test bred mares to test positive on test breedings even after the stallion has been cultured negative following treatment.

Taylorella equigenitalis can be transmitted during both natural breeding and artificial insemination, during parturition, as well as indirectly through fomites (Samper 2007). Current investigation as to the source of the 2008 isolation of *T. equigenitalis* in Kentucky has failed to identify whence it originated. Spread of the organism among stallions is believed to have been via contaminated phantoms, artificial vaginas, and various other AI equipment used during semen collection (personal communication). With the increase in shipped semen worldwide, it is critical to develop appropriate semen handling and processing techniques to prevent the dissemination of *T. equigenitalis*.

Equine semen extenders are widely used and are essential for the proper preservation of semen for use in AI. Semen extenders are utilized to maintain plasma membrane integrity while protecting spermatozoa from cold shock, detrimental seminal plasma proteins, and toxic byproducts produced by the sperm cells. In addition, semen extenders maintain a neutral pH and osmotic pressure within 250 mOsm/L – 400 mOsm/L. Additives may include skim milk or non-fat milk solids, egg yolk, glycerol, glucose, Vitamin E, proteinase inhibitors, Methylxanthines, and antibiotics (Katila 1997). Most extenders used today contain antibiotics to decrease bacterial growth in semen; however, additional antimicrobial supplementation of extender is also accepted. Initial antibiotic trials on semen extenders began in 1978, when AI was proposed as a means to control CEM in Thoroughbreds (Timoney, O'Reilly et al. 1978). At that time, the addition of gentamicin to semen extenders proved to be far superior to either ampicillin or penicillin in combination with polymyxin to inhibit the growth of *T. equigenitalis* in "spiked" semen.

The extenders used in this study include INRA 96®, E-Z Mixin BF®, and VMDZ®. INRA 96® is a common internationally used extender that contains defined concentrations of lactose, supplemented with a purified fraction of milk caseins (native phosphocaseinate (NPPC)), glucose, Hank's salts, penicillin, gentamicin and amphotericin B (Fayrer-Hosken, Abreu-Barbosa et al. 2008). INRA 96® is available in solution as provided by the manufacturer. Some extenders, like INRA 96®, can be supplemented with antibiotics to help increase the antibiotic strength of the semen extender. Timentin® has recently become an additive in semen extenders across the country, and antibiotic susceptibility tests for the recent *T. equigenitalis* occurrence have shown susceptibility to ticarcillin (APHIS 2009). E-Z Mixin BF®, also known as "Basic

Formula", is a skim milk based extender with no antibiotics added that requires mixing dry powder with USP purified water. VMDZ® is a newer, internationally used, egg yolk based extender with the antibiotic additives amikacin and potassium penicillin G that requires mixing dry powder with sterile water.

In addition to those mentioned above, there are multiple semen extenders containing various antibiotics available on the market today; however, little is known about their bactericidal capacity against *T. equigenitalis*. Antibiotic susceptibility profiles performed on the 2008 *T. equigenitalis* isolates have shown sensitivity to multiple antimicrobials including penicillin, ticarcillin, amikacin, gentamicin, tetracycline, silver sulfadiazine, nitrofurantoin, and sulfamethoxazole/trimethoprim in various formulations (APHIS 2009). For this reason, we investigated the potential for survival of *T. equigenitalis* in cool-shipped stallion semen inoculated with known concentrations of the organism using specific antibiotic containing extenders.

CHAPTER II

MATERIALS AND METHODS

Sample Collection and Bacteriology

The first part of this study involved 4 replicates with *T. equigenitalis* in semen extender without the addition of semen. The following semen extenders were used and handled as recommended by the manufacturer: INRA 96®, INRA 96® with 0.5 mg/mL Timentin®, INRA 96® with 1.0 mg/mL Timentin®, INRA 96® with 1.5 mg/mL Timentin® , E-Z Mixin BF® "Basic Formula", and VMDZ®. Based on the known geographic distribution of *T. equigenitalis,* INRA 96® was chosen as an internationally used skim milk based semen extender, and VMDZ® was chosen as an internationally used egg-yolk based semen extender.

Two streptomycin resistant strains of *T. equigenitalis*, isolated from England in the late 1970's, were used: Both were "type" strains from the National Veterinary Services Laboratory (NVSL) maintained at -80°C at the Oklahoma Animal Disease Diagnostic Laboratory (OADDL) and University of Missouri Veterinary Diagnostic Laboratory respectively; the strain from Missouri was kindly provided to OADDL by Dr. William Fales for use in this project.

A 3.1g vial of Timentin® powder was mixed with 13 mL sterile water to equal 200 mg/mL ticarcillin (as labeled by the manufacturer). Once thoroughly mixed, three different concentrations (0.5 mg/mL, 1.0 mg/mL, and 1.5 mg/mL) of Timentin® were added to 10 mLs of INRA 96®. Suspensions of *T. equigenitalis* were made to a 0.5 MacFarland standard using the nephalometer of the Sensititre™ autoinoculator (Trek Diagnostics, Cleveland, OH), and 100 µL of suspension was added to 10 mL aliquots of each of the various extenders. This concentration was chosen because it resulted in bacterial growth providing countable colonies routinely in the range of 100-300 colony forming units per mL (CFU/mL). After extender and the suspension of *T. equigenitalis* were mixed, 100 µL of the bacteria/extender preparation was plated onto chocolate Eugon agar plates using a spreader at times 0 hrs, 24 hrs, 48 hrs, and 72 hrs (Timoney, Shin et al. 1982). The plates were incubated at 37° C in 7% CO_2 for approximately 72 to 96 hrs, or until CFUs were visible, up to 7 days. The CFU/mL were calculated by visually counting individual colonies at 24 hour intervals and recorded as negative if no

CFU/mL were observed during the 7 day period. Colonies were confirmed as *T. equigenitalis* based on colonial morphology, gram-negative stain, positive cytochrome oxidase test, and positive catalase reaction. The extender/semen/bacteria replicates were maintained at 4.5 °C between plating to simulate the real life transport conditions of chilled/shipped semen samples utilized by the equine industry.

An additional 10 replicates were prepared as the first 4 replicates, with the addition of semen from two Oklahoma State University-owned quarter horse stallions (5 replicates per stallion). The semen was collected from each stallion using a Missouri model artificial vagina and standard semen collection protocols. Aseptic technique was used with each collection and included pre-warmed non-spermicidal sterile lubricant, a filter to remove the gel fraction of the ejaculate, pre-warmed collection containers, and sterile gloves.

The semen was added to each of the 6 semen extenders listed above at a 1:4 ratio (semen to extender). For replicates 5-10, the 3.1g vial of Timentin® powder was mixed, according to label, using 13 mL sterile water. For the last 4 replicates, the 3.1 g vial of Timentin® was mixed with 13 mL INRA 96® as supplied by the manufacturer instead. The use of INRA 96® was included to ensure that the sterile water was not affecting sperm membrane integrity through changes in extender osmolarity. There was no detectable difference between the two solutions on spermatozoa membrane integrity (unpublished data). Lastly, 100 µL of *T. equigenitalis* suspension was added at the same time the semen was added to the extender. Once each aliquot was prepared, the samples were plated as described above. There were a total of 6 aliquots per stallion per replicate, with 10 replicates total.

Statistics

PC SAS Version 9.1 (SAS Institute, Cary, NC) was used for all statistical analyses. The effects of time and treatment on the number of colony forming units were assessed with analysis of variance procedures using PROC MIXED. A repeated measures model was used with stallion as the subject and time as the repeated factor. The effect of replicate was considered random and all comparisons adjusted for replicate to replicate variation. The simple effects of treatment given time, and time given treatment, were assessed with a SLICE option in an LSMEANS statement. Differences were considered significant if the respective p-value was less than or equal to 0.05. As a secondary analysis, the proportion of samples testing positive, and its relationship with the factors of interest, was further assessed with contingency tables and Fisher Exact Tests.

During the experiment, 14 total replicates were performed; however, replicates 5 and 6 were removed from the statistical analysis due to laboratory error. In addition, the semen sample from replicate 10 was removed from the study due to significant overgrowth of bacterial containments.

For each semen extender, at each particular time period (0, 24, 48, 72 hrs), the results for replicates without semen added (replicates 1-4) were averaged with replicates containing semen from both stallions (replicates 7- 9 and 11-14).

CHAPTER III

RESULTS

The results of bacterial growth over time are presented in Tables 1 and 2 and further illustrated in Figures 1 and 2. Table 1 shows the average number of CFU/mL decreased significantly over 48 hrs in INRA 96® without Timentin® added (from 90-3000 CFU/mL at 24 hrs to 0-40 CFU/mL at 48 hrs). There was no significant decrease in the average CFU/mL over time among the other semen extenders tested (Figure 1) because the CFU/mL was already dramatically reduced within 24h.

Large numbers of *T. equigenitalis* (≥ 3000 CFU/mL) were consistently isolated from replicates of the non-antimicrobial containing extender, E-Z Mixin BF®, at each time point (Figure 1). At all time points evaluated, significantly higher numbers of *T. equigenitalis* were isolated from E-Z Mixin BF® compared to the other semen extenders evaluated (Table 2; Figure 2).

At 24 hrs, significantly more CFU/mL were isolated from INRA 96® than either VMDZ® or INRA 96® with Timentin® added at any of the three concentrations (Table 2; Figure2). There was no significant difference in the number of CFU/mL isolated between VMDZ® and INRA 96® with Timentin® added at any concentration (0.5 mg/mL, 1.0 mg/mL, and 1.5 mg/mL) (Table2; Figure2).

Additionally, the Fisher's Exact Test revealed that the effect of time was significant in the proportion of incidence of *Taylorella equigenitalis*, which was isolated (≥1 CFU) from INRA 96 100% of the time 0 hrs, 64% of the time at 24 hrs, 18% at 48 hrs, and 9% at 72 hrs.

CHAPTER IV

DISCUSSION

Transmission of *T. equigenitalis* remains a concern for many equine practitioners. The primary burden is the major economic loss as a result of decreased fertility and international controls on transport requirements of horses and equine germplasm (Samper 2007). Since the 2008 discovery of *T. equigenitalis* in Kentucky, 5 positive mares have been diagnosed and treated. Of these 5 positive mares, 4 were exposed by AI (APHIS 2010). While *T. equigenitalis* can be transmitted via cooled shipped semen, appropriately selected equine semen extenders can be utilized as a means to help decrease the number of exposed mares bred by AI. However, the question still remains as to how well these extenders can decrease the infectivity of *T. equigenitalis* in semen.

Typically equine semen is cool-shipped overnight, allowing only 24 hrs for antibacterial effectiveness. Because of this, it is important not only for these extenders to decrease and/or inhibit bacterial growth, but to do so in a timely manner (~24 hrs). In addition, it was important in our study to simulate this process by preparing our samples and then refrigerating them until cultures were made at each time point. Our results indicate that INRA 96® without Timentin® added does significantly decrease the bacterial population in semen over 48 hrs, however, it is significantly less effective at 24 hrs than either VMDZ® as supplied by the manufacturer or INRA 96® with Timentin® added at any of the three concentrations used.

The purpose of using Timentin® in this study was to evaluate its antibacterial activity against *T. equigenitalis* compared to current semen extenders, and to determine its mean inhibitory concentration (MIC) against the organism. The ideal concentration of Timentin® to be added to equine semen extenders was not obtained in this study, such that no significant difference in bacterial growth was observed by adding 0.5 mg/mL, 1.0 mg/mL, or 1.5 mg/mL of Timentin® to INRA 96® as supplied by the manufacturer.

Further research to find the MIC of Timentin® in INRA 96® is warranted. In addition, further research is necessary to evaluate how these extenders, with the addition of Timentin®, affect sperm motility and viability (unpublished data). It is important, in the case of AI, to compare semen extenders based on multiple semen parameters to increase longevity of sperm cells, while maximizing fertility and decreasing the spread of pathogens.

In conclusion, this study suggests that using either VMDZ®, as supplied by the manufacturer, or INRA 96® with Timentin® added at one of three concentrations (0.5 mg/mL, 1.0 mg/mL, and 1.5 mg/mL) will reduce the presence of *T. equigenitalis* in semen during equine artificial insemination compared to INRA 96® as supplied by the manufacturer or E-Z Mixin BF®. Furthermore, insemination during the first 24 hours post-collection may allow for infectivity of *T. equigenitalis* if appropriate extenders, such as either INRA 96® with Timentin® added or VMDZ® are not added to the semen.

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APPPENDICES

Table 1. Statistical Comparison (in CFU/mL) of *Taylorella equigenitalis* at 0, 24, 48, and 72 hrs in INRA 96®.

HOUR	TREATMENT	MEAN CFU/mL	STANDARD ERROR
0	INRA 96	2470.91 a	354.925
24	INRA 96	373.64 b	277.444
48	INRA 96	6.36 c	4.322
72	INRA 96	1.82 c	1.818

Table 1. ANOVA results for mean CFU/mL of *Taylorella equigenitalis* isolated in INRA 96®. The data were averaged across all replicates at times 0, 24, 48, and 72 hrs. This table shows level of significance between each time point, using INRA 96®, denoted as a, b, or c.

Table 2. Statistical Comparison (in CFU/mL) of *Taylorella equigenitalis* in 6 Different Equine Semen Extenders at 0, 24, 48, and 72 hrs.

HOUR	TREATMENT	MEAN CFU/mL	STANDARD ERROR
0	E-Z Mixin BF	≥3000 a	0
0	INRA 96	2470.91b	354.925
0	INRA + 0.5mg/ml Timentin	0 c	0
0	INRA + 1.0mg/ml Timentin	0 c	0
0	INRA + 1.5mg/ml Timentin	0 c	0
0	VMDZ	1.82 c	1.818
24	E-Z Mixin BF	≥3000 a	0
24	INRA 96	373.64 b	277.444
24	INRA + 0.5mg/ml Timentin	0 c	0
24	INRA + 1.0mg/ml Timentin	0 c	0
24	INRA + 1.5mg/ml Timentin	0 c	0
24	VMDZ	2.73 c	2.727
48	E-Z Mixin BF	≥3000 a	0
48	INRA 96	6.36 b	4.322
48	INRA + 0.5mg/ml Timentin	18.18 b	18.182
48	INRA + 1.0mg/ml Timentin	0 b	0
48	INRA + 1.5mg/ml Timentin	0 b	0
48	VMDZ	0 b	0
72	E-Z Mixin BF	≥3000 a	0
72	INRA 96	1.82 b	1.818
72	INRA + 0.5mg/ml Timentin	5.45 b	5.455
72	INRA + 1.0mg/ml Timentin	0 b	0
72	INRA + 1.5mg/ml Timentin	0 b	0
72	VMDZ	0.91 b	0.909

Table 2. ANOVA results for mean CFU/mL of *Taylorella equigenitalis* isolated in each extender used. The data were averaged across all replicates at times 0, 24, 48, and 72 hrs. This table compares level of significance between each of the 6 extenders, at each time point, denoted as a, b, or c.

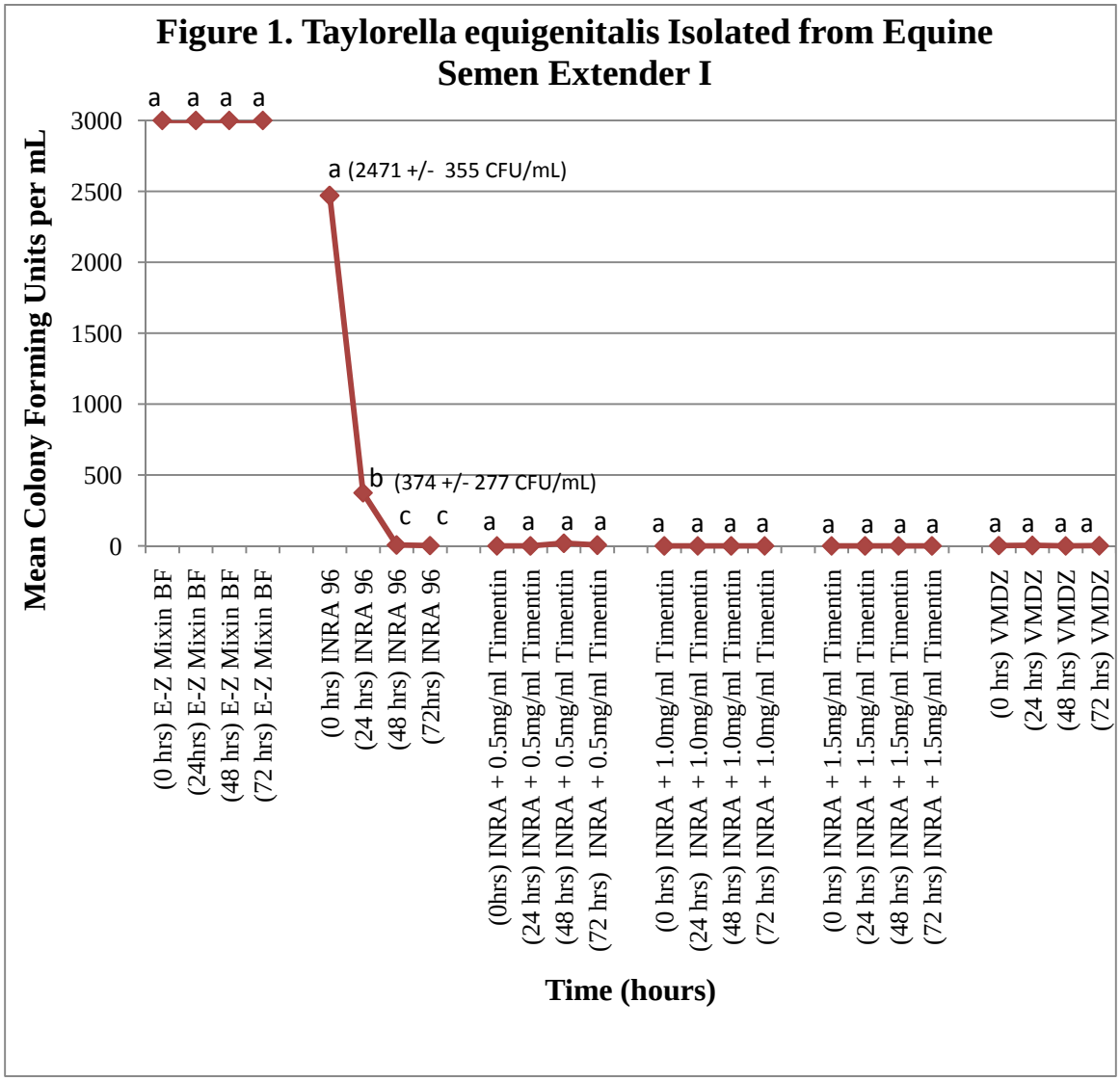

Figure 1. Mean CFU/mL isolated from 6 semen extenders inoculated with *Taylorella equigenitalis* averaged over all replicates. Trends for each semen extender are illustrated over 72 hours. This figure shows level of significance between each time point for each extender used, denoted as a, b, or c.

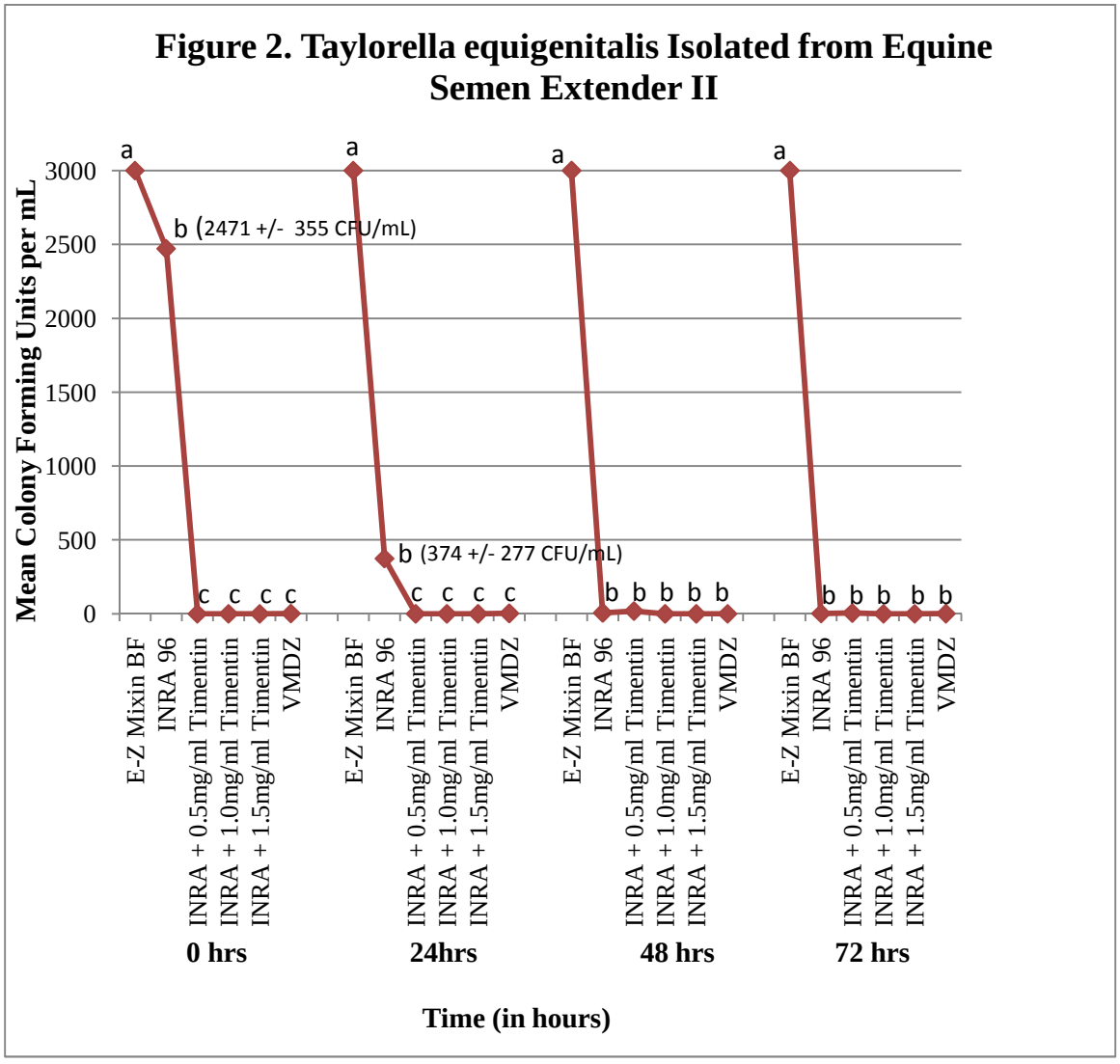

Figure 2. Mean CFU/mL isolated from 6 semen extenders inoculated with *Taylorella equigenitalis* averaged over all replicates at 0, 24, 48, and 72 hours. This figure shows level of significance between each of the 6 extenders, at each time point, denoted as a, b, or c.

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Taylorella equigenitalis is a gram negative coccobacillus and the causative agent of a transmissible venereal disease in horses known as contagious equine metritis (CEM). This disease can be responsible for significant economic loss as a result of decreased fertility and increased rigidity of international controls on shipment of horses and equine germplasm. Outbreaks of this disease have been documented in various countries since 1977, with the most recent discovery of CEM in the United States in December 2008. During disease occurrences / outbreaks, culturing semen samples for *T. equigenitalis* prior to breeding may help to prevent transmission of this disease; however, little is known about the antimicrobial activity of equine semen extenders against the organism. The purpose of this study was to investigate the infectivity levels of *T. equigenitalis* in several equine semen extenders inoculated with known concentrations of the organism. The semen extenders used included INRA 96®, E-Z Mixin BF®, and VMDZ®. In addition, Timentin® was added to INRA 96® at three different concentrations (0.5, 1.0, and 1.5 mg/mL) to further investigate synergistic effects of antibiotic supplementation to extenders.

Findings and Conclusions:

Results were based on visually counting colonies on chocolate Eugon agar plates. Both INRA 96® with added Timentin® and VMDZ® (as supplied by the manufacturer) significantly reduced the numbers of *T. equigenitalis* isolated from semen extenders compared to INRA 96® (as supplied by the manufacturer) or the antibiotic free E-Z Mixin BF®. Our findings indicate that INRA 96® with Timentin® added or VMDZ® may significantly decrease the growth of *T. equigenitalis* in extended semen, however, it is also important to consider possible effects on sperm longevity and fertility while decreasing seminal pathogens.

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