Efficacy of disinfection of endoscopes contaminated by *Streptococcus e*qui subspecies *equi.*

by

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Abstract

Prevention of spread of *Streptococcus equi* subspecies *equi* (*S. equi*) following an outbreak is best accomplished by confirming negative bacterial status through endoscopic lavage of the guttural pouch, with samples tested by culture or/and real time polymerase chain reaction (qPCR). It is critical that high-level disinfection of endoscopes eliminates live bacteria and DNA to avoid the false diagnosis of carrier horses of *S. equi*. The objective of this study was to report the failure rate (by culture and qPCR) of high-level disinfection of endoscopes contaminated with *S. equi* with two different disinfectants, accelerated hydrogen peroxide (AHP) and ortho-pthaldehyde (OPA). The second objective of this study was to determine if AHP and OPA disinfectants were equivalent for disinfection of endoscopes.

Two endoscopes were contaminated with a standard S. equi broth suspension. The endoscopes were disinfected with AHP, OPA or control (water). In a randomized block design, endoscopes were contaminated and disinfected 30 times for each disinfectant (15 disinfections performed per day for 6 days). Samples were collected before and after disinfection and submitted for detection of *S. equi* by culture and qPCR assay. Using multivariable logistic regression model adjusted probability with endoscope and day as controlled variables the probability of an endoscope being qPCR was determined.

Following contamination all endoscopes were positive for *S. equi* culture and qPCR. After disinfection, all endoscopes were culture negative. However, qPCR testing was positive for *S. equi* for endoscopes treated with AHP, 10/30, with OPA, 22/30, and with water, 21/30 following disinfection. Use of the AHP disinfectant resulted in a significantly lower probability

of being PCR-positive after disinfection (31%) compared to disinfection with OPA (81%) and control (72%).

High-level disinfection of endoscopes contaminated with *S. equi* resulted in high failure rate in successfully removing DNA. The AHP disinfectant was more effective than the OPA product, however, it was less effective in removing DNA. Therefore, horses could be falsely diagnosed as carriers of *S. equi*, if the testing is based on qPCR assay only. Further studies are needed to better understand the carrier *S. equi* horses.

Table of Contents

List of Figures

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"When you do things from the soul, you feel a river moving in you, a joy." Rumi

Chapter 1 - Introduction

Streptococcus equi subspecies *equi* (*S. equi*) is a worldwide infectious disease in horses that causes strangles, resulting in significant morbidity. Carrier horses, which can be clinically normal, harbor the organism in their guttural pouch, resulting in further outbreaks (Boyle et al. 2018; Newton et al. 1997). Prevention of spread of *S. equi* following an outbreak is best accomplished by confirming negative bacterial status through endoscopic lavage of the guttural pouch with samples tested by culture and real time polymerase chain reaction (qPCR) (Boyle et al. 2016; Boyle et al. 2018).

Devices that come into contact with mucous membranes, such as endoscopes, are considered semi-critical devices, which must undergo high level disinfection between uses, as contaminated endoscopes are linked to outbreaks of nosocomial infections in human medicine (Keswani et la. 2015; Kovaleva et al. 2013; Olfstead et al. 2020; Olfstead et al. 2018). There is only one published study evaluating efficacy of disinfection with contaminated endoscopes in veterinary medicine (Svonni et al. 2020). Most endoscopes were culture negative, following several different methods of disinfection after experimental contamination with *S. equi* (Svonni et al. 2020). However, qPCR results were positive for 6/6 of endoscopes disinfected with alcohol, 5/6 disinfected with quaternary ammonium, 2/6 cleaned with an enzymatic cleaner followed by disinfection with an *ortho*-phthalaldehyde (OPA), and 1/6 disinfected with an automated endoscope cleaner (Svonni et al. 2020). Researchers concluded that horses could be falsely diagnosed as strangles carriers based on these results (Svonni et al. 2020).

It is common for veterinarians to perform multiple endoscopic exams on several horses in a field setting following an outbreak, making manual high-level disinfection necessary.

Manufacturer's guidelines for manual high-level disinfection include cleaning with an enzymatic cleaner followed by use of a disinfectant. The disinfectants that are most currently used are the OPA products, which require 12 minutes of contact time with the endoscope. Commercial hydrogen peroxide is another broad-spectrum antimicrobial; however, these products are highly corrosive to endoscopes. A newly developed product is accelerated hydrogen peroxide (AHP) which is a blend of 2% hydrogen peroxide, surfactants, and stabilizers (Omidbakhsh 2006). This AHP product is compatible with flexible endoscopes, and only requires an 8-minute contact time (Omidbakhsh 2006). Using a disinfectant with a shorter contact time would be advantageous to clinicians when performing endoscopic exams of several horses in a single day.

The qPCR is currently considered the gold standard for detection of *S. equi*, even if culture negative (Boyle et al. 2018). A commonly asked question when horses are positive by qPCR test alone is, "What are the chances that this is real or could this be due to contamination?" It does appear from previous work that iatrogenic spread of *S. equi* with disinfected endoscopes is unlikely; however, disinfection does not always remove all DNA (Svonni et al. 2020).

Objectives:

The first objective of this study was to report the failure rate (by culture and qPCR) of high-level disinfection of endoscopes contaminated with S. equi with two different disinfectants, accelerated hydrogen peroxide (AHP) and ortho-pthaldehyde (OPA). The second objective was to determine if efficacies of AHP and OPA disinfectants were similar. If these products are equivalent, then the clinician could pick a particular product based on their needs.

Chapter 2 - Literature Review

Streptococcus equi subspecies equi and Strangles

Etiology

Streptococcus equi subsp. equi is a gram-positive, chain-forming cocci that belongs to the Lancefield group C Streptococcus. The ß-hemolytic pathogen is highly infectious and is responsible for upper respiratory disease in equids called "strangles." Unlike its ancestor, Streptococcus equi subsp. zooepidemicus (S. zooepidemicus), S. equi is not a commensal organism. Strangles was first described in 1251 by Jordanus Rufus as a disease seen as inevitable (Waller 2014, Boyle et al. 2018). In 1811, a letter written by Napoleon, the Emperor of France, requested that 543 horses being transferred to his army should be "at least 60 months of age and should already have recovered from strangles" so that they would be less likely to fall ill from this disease on the battlefront (Waller 2018). Strangles affects equids of all age groups but is usually more severe in young animals, with a morbidity rate of up to 100%. Fatality rates are estimated as 1% but can be as high as 10% (Boyle et al. 2018). Clinical signs include fever, lethargy that typically occur 3-14 days after exposure, followed by pharyngitis and lymphadenopathy (Boyle et al. 2018). The lymph nodes will abscess and typically rupture between 7 days and 28 days after the infection.

Zoonotic potential

Although rare, 3 subspecies of *Streptococcus equi* are reported to cause zoonotic disease in humans: *S. equi*, *S. zooepidemicus*, and *Streptococcus equi* subsp. *ruminatorum* (Pelkonen et al. 2013). Zoonotic infections with these subspecies of *Streptococcus equi* can cause severe and invasive infection in immunocompromised humans, following close contact with horses,

contaminated environments, or consumption of unpasteurized milk products (Kim et al. 2021; Pelkonen et al. 2013). The most frequent case reports include septicemia, septic arthritis, and meningitis due to *S. zooepidemicus* (Kim et al. 2021). Less frequently, there are case reports of *S. equi* causing bacteremia, sepsis, and meningitis associated with significant mortality and morbidity, including deafness, blindness, amnesia, and ataxia (Torpiano et al. 2020). Complications such as brain abscesses and endophthalmitis are common following *S. equi* meningitis (Torpiano et al. 2020) In addition to zoonotic infections, *S. zooepidemicus* has been increasingly recognized causing opportunist infection in in several domestic animals (Corpa et al. 2018; de Oliveira Costa et al. 2022; Verlnen et al. 2014).

Epidemiology

With a worldwide distribution, strangles is one of the most frequently identified infectious diseases and responsible for considerable economic and welfare cost. *Streptococcus equi* subsp. *equi* has limited capacity for survival outside the horse and may persist in water for 4-6 weeks but not in feces or soil (Boyle et al. 2018). Transmission of *S. equi* can occur by direct horse-to-horse contact or indirectly by fomites contaminated with purulent discharges. In most horses, nasal shedding persists for 2 to 3 weeks, but in some cases intermittent shedding may persist longer. In an outbreak of strangles, Newton et al. detected persistence of *S. equi* in a few horses for up to 39 months (Newton et al. 1997). These persistent shedders or carriers appear clinically normal (silent carriers) by harboring the organism in their guttural pouch. Approximately 10% of recovered horses will fail to clear all the purulent material from the guttural pouches resulting in empyema or chondroids (Boyle et al. 2018; Newton et al. 2000; Waller 2014). Although this asymptomatic carrier state for strangles was acknowledged as early

as 1910 (Todd 1910; Newton et al. 1997), the role of the carrier state is recognized to be of paramount importance in outbreaks and disease control. Approximately 75% of strangles outbreaks result in the development of new carrier horses which then results in further outbreaks (Rendel et al. 2022).

Pathogenesis

After entering the mouth or nose, the organism gains access to the pharynx where it attaches and colonizes the crypt cells of the tonsils (Timoney et al. 2008). Within a few hours, the bacteria can be found within the epithelial cells and subepithelial tonsillar follicle but cannot be detected on the mucosal surface (Boyle et al. 2018; Rendle et al. 2021; Timoney 1993). Translocation to the mandibular and retropharyngeal lymph nodes will occur causing influx of neutrophils (Mallicote 2015; Timoney et al. 2008). Because of the ability of *S. equi's* to resist phagocytosis due a combination of virulence factors (SeM protein, hyaluronic acid capsule, H factor binding Se 18.9, Mac protein and other antiphagocytic factors), the organism continues to multiply and colonize the lymph nodes (Boyle et al. 2018; Timoney et al. 2014). Accumulated extracellular *S. equi*, and its virulence factors such streptokinase and streptolysin S, combined with large numbers of neutrophils will contribute to abscess development (Boyle et al. 2018; Mallicote 2015 Rendle et al. 2021; Timoney 2004) Abscesses in the retropharyngeal lymph node usually rupture into the guttural pouches (Boyle et la. 2018; Waller 2014). Rarely, the spread of the organism to other sites through different routes (lymphatic migration, hematogenous, or close association with the septic foci) may occur being referred as bastard strangles (Boyle et al. 20121; Mallicote 2015; Rendle et al. 2021; Timoney 2004). About 75% of the horses will develop immunity and the remaining 25% of the convalescent horses will be at risk for infection within several months (Boyle et al. 2018; Rendle et al. 2021; Timoney 1993).

Diagnosis of strangles: active disease versus carrier horses

Testing for strangles requires detection of *S. equi* by bacterial culture, PCR, or both. The sampling technique depends on the stage of infection, the anatomical location from which the sample is taken, and the test used. Various sampling methods include guttural pouch lavage or nasopharyngeal wash, nasopharyngeal swab, needle aspirate from an abscessed lymph node or nasal swab (Boyle et al. 2018). The ideal sampling method depends on if the clinician is trying to diagnose an active infection or carrier horses. Diagnosis of an active infection is most commonly achieved by needle aspirate of abscessed lymph nodes. In early cases, sampling of the upper respiratory tract (either via nasopharyngeal wash and/or guttural pouch lavage) may fail to identify *S. equi* as lymph nodes have not yet ruptured into the guttural pouch. There are atypical cases of strangles in horses with partial immunity that do not develop lymph node abscessation but will have purulent exudate in the upper respiratory tract and do shed organism (Boyle et al. 2018). There are a number of publications documenting the ideal methods for identification of silent carrier horses following an outbreak (Boyle et al. 2016; Boyle et al. 2017; Boyle et al. 2018; Lindahl et al. 2013; Newton et al. 2000; Pringle et al. 2019; Pringle et al. 2021; Waller 2014). The sensitivity and specificity of identifying carrier horses depend on which site is sampled, method of detection, if endoscopy is used, and in some cases frequency of sampling. It is recommended to wait for at least 3 weeks following an outbreak (with no new cases of strangles being detected) to test horses for carrier status of *S. equi*.

Carrier Horses: Site of Sampling

The sampling method and site for detection of *S. equi* to detect carrier horses, have been evaluated in a number of studies (Boyle et al. 2017; Lindahl et al. 2013; Pringle et al. 2021). Nasopharyngeal swabs are more sensitive than nasal swabs (Boyle et al. 2017; Boyle et al. 2018; Lindahl et al. 2013). Nasopharyngeal washes are even more sensitive than nasopharyngeal swabs, as they sample a larger area of the pharynx (Lindhal et al. 2013; Boyle et al. 2017; Boyle et al. 2018). However, nasopharyngeal washes can be challenging during collection due to potential coughing and sneezing of horses resulting in loss of the contaminated sample into the environment and onto the sampler (Boyle et al. 2018). Horses may intermittently shed bacteria from the guttural pouch which may yield false negatives from samples taken from the nasopharynx. Guttural pouch lavage, via endoscopy, is currently considered the most sensitive method to identify carrier horses (Boyle et al. 2016; Boyle et al. 2017; Boyle et al. 2018; Newton et al. 2000; Pringle et al. 2019). Detection of *S. equi* DNA was 51 times more likely from samples taken from guttural lavage compared to nasopharyngeal wash samples (Boyle et al. 2017). The most recent American College of Veterinary Internal Medicine (ACVIM) consensus statement (Boyle et al. 2018) recommends endoscopy of the guttural pouches and lavage to obtain samples for culture and qPCR to detect carrier horses (Boyle et al. 2018). Endoscopy is also useful for identifying gross pathology (empyema or chondroids) which often requires additional medical treatment to resolve. However, even if the guttural pouch is grossly normal, lavage of the guttural pouch (with testing) is still recommended, as *S. equi* can be present in a biofilm and result in disease transmission (Boyle et al. 2018; Waller 2014).

Culture vs PCR

Culture has been a traditional method to confirm the presence of *S. equi*. It is widely available at low cost; however, confirmatory results may take a minimum of 1-2 days. Other ß -hemolytic bacteria, such as *S. zooepidemicus* and *Streptococcus equisimilis* may complicate interpretation of cultures (Boyle et al. 2018; Rendle et al. 2021; Waller 2014). Other disadvantages include false negative results due to early clinical phases of the disease, non-viable organisms, or low bacterial count. Cultures are much less sensitive to detect *S. equi* when compared to qPCR (Boyle et al. 2016; Boyle et al. 2018; Lindahl et al. 2013; Newton et al. 2000; Pringle et al. 2019). Boyle et al. 2016 reported the sensitivity and specificity of culture (when compared to PCR) to be 40% and 99% respectively. In previous studies, culture method failed to identify 39.7% of qPCR-positive samples (Waller 2014). There is concern that culture-negative, qPCR-positive samples can be due to dead or nonviable bacteria. However, there are reports of horses being initially qPCR positive but culture negative; but when retesting at a later date, both the culture and qPCR are positive, supporting the idea that solely qPCR positive horses should be considered carriers (Pringle et al. 2019). Boyle et al. (2018) reports clinical evidence of transmission of strangles from horses with qPCR-positive, culture-negative guttural pouch lavage samples to naïve horses. Despite disadvantages, culture will still occasionally pick up aberrant genetic strains that are not detected by PCR.

Types of Polymerase Chain Reaction (PCR) assays

Polymerase chain reaction is a molecular diagnostic test that detects bacterial DNA or RNA. The assay is three times more sensitive than culture and can be completed in less than 2 hours with results expected perhaps at the same day (Boyle et al. 2018). Several studies that have evaluate PCR assay for detection of *S. equi* have shown PCR to be more sensitive than bacterial culture (Newton et al. 2000; Boyle et al. 2016; Boyle et al. 2018; Lindall et al. 2013; Pringle et al. 2019). The first PCR test developed for *S. equi* targeted a highly variable region of the *SeM* gene that some strains isolated from persistent carriers lacked (Chanter et al. 2000; Waller 2014). The next challenge consisted in differentiating closely related streptococcal species. A multiplex PCR was later developed by targeting genes *seeH* and *seeI*, which encode exotoxins for identification and differentiation of *S. equi* and *S. zooepidemicus* (Albert et al. 2004). Advances in technology led to the development of a qPCR that involved the detection of the organism targeting different superantigen-encoding genes. Quantitative PCR has increased sensitivity and rapid turn-around time (Boyle et al. 2018). Recently, Webb and colleagues developed a triplex qPCR targeting *eqbE* and *SEQ2190* genes, and a synthetic DNA sequence SZIC (control) with overall sensitivity of 93.9% and specificity of 96.6%. (Webb et al. 2013) Moreover, a nested PCR (nPCR) targeting *SeM* and an 18S rRNA internal control gene was developed with increased sensitivity when compared to qPCR providing superior detection of *S. equi* during low bacteria shedding periods (Noll et al. 2020). Attempts in developing novel diagnostic methods, such as a loop-mediated isothermal amplification (LAMP) assay that targets the *S. equi*-specific *eqbE* gene, demonstrated reliable results in comparison to qPCR assay that targets the *S. equi*-specific *seeI* gene (Boyle et al. 2017). The PCR test does not distinguish between live and dead organisms and false positive diagnosis can result from the organism's degrading DNA or

carryover of non-viable organisms (Boyle et al. 2018), Very rarely, cultures may identify the presence of *S. equi* when PCR has failed (false negative results) due to samples containing polymerase inhibitors or abundant *S. equi*. (Boyle et al. 2018).

Summary of detection of carriers

In summary, the ACVIM 2018 consensus statement recommends that detection of carrier horses is best accomplished by a single guttural pouch lavage via endoscopy or via repeated nasopharyngeal washes (Boyle et al. 2018) and samples being tested by both culture and qPCR. The advantages of endoscopy include only having to perform testing once. The nasopharyngeal wash should be repeated three times over a 3-week period, and all 3 samples should be negative in order to correctly identify non-carrier horses (Boyle et al. 2017; Boyle et al. 2018; Pringle et al. 2019). Some disadvantages of endoscopy include the need of special equipment and technical experience (guidance of the scope into the guttural pouches) and time consumption. In addition, endoscopes are considered fomites and may remain contaminated with live *S. equi* or DNA with the potential for spreading disease between horses or resulting in false positives (Svonni et al. 2020). Finally, there are reported failures of guttural pouch lavage to identify all carrier horses with strangles (Pringle et al. 2019; Pringle et al. 2021). A recent study reports that solely relying on a single guttural pouch lavage sample would have misdiagnosed 4/14 horses which were solely positive on nasopharyngeal wash samples (Pringle et al. 2021). The researchers suggested that *S. equi* may be present in non-guttural pouch sites such as nasal sinuses (Pringle et al. 2021).

Endoscopy linked to infection

In 1805, Philip Bozzini made the first attempt to observe the living human body directly through a tube he created known as a "Lichtleiter" (light conductor). But it was not until 1853 when Antoine Jean Desormeaux of France developed a special instrument named "endoscope", using this term for the first time in history (Olympus website). Flexible endoscopes are diagnostic and therapeutic (Benowitz et al. 2020) and as previously mentioned, are semi-critical devices (Spalding classification) having a moderate degree of risk of transmission of bacteria if contaminated at the time of use (Gonzales et al. 2019). Since endoscopes contain heat-sensitive components, they must undergo high-level disinfection for the elimination of all microorganisms (Benowitz et al. 2020). In human literature, the consequences of contaminated endoscopes are a recurrent topic and raise concerns in recent years of increased multi-drug resistant bacterial outbreaks (Keswani et al. 2015; Kovaleva et al 2013; Olfstead et al. 2020; Olfstead 2018). Endoscope reprocessing failure is more frequently reported over the last 10 years (Olfstead et al. 2020; Olfstead 2018). As mentioned in the introduction, there is only one published study evaluating efficacy of disinfection with contaminated scopes in veterinary medicine (Svonni et al. 2020). In that report, disinfection was likely successful in eliminating live bacteria (detected by culture). However, disinfection was unsuccessful in completely eliminating bacterial DNA, which could result in the false diagnosis of carrier horses for *S. equi*.

Chapter 3 - Materials and Methods

Two different disinfectant products were tested to determine efficacy of manual high-level disinfectants of endoscopes and included: 1) AHP (Prevention HLD Virox Technologies Ontario Canada) 2) OPA (Metricide OPA Plus, Metrex, Orange, CA) and 3) control (water). Endoscopes were contaminated and disinfected 30 times for each disinfectant with a randomized block design (15 disinfections performed per day for 6 days). Two different endoscopes were used, which were leak tested at the start of each testing day.

Contamination and Sample Collection: A broth culture of S. equi was prepared by adding one colony of S. equi (obtained from the Kansas State University Veterinary Diagnostic Laboratory (KSU-VDL)) into 15 ml of a brain heart infusion (BHI) broth and incubated at 37 ºC for 15-18 hrs. Prior to starting the experiment, the S. equi was confirmed by testing one colony on a plated and incubated sample, by Matrix-assisted laser desorption of ionization-time of flight mass spectrometry for microbiological identification and qPCR. For each testing day, 5- 15 ml BHI S. equi broths were prepared. The bacterial growth was greater than 100,000 colony forming units/ml. The distal end of the endoscope was placed in a 50 ml conical tube. Five ml of S. equi broth was pipetted over the distal end of the endoscope which accumulated in the bottom of the conical tube. A sterile endoscopic catheter was passed through the biopsy channel until it emerged from the distal end of the endoscope. Saline (30 ml) was flushed through the catheter and the fluid was collected into the conical tube, and the distal 5 cm of the endoscope remained immersed in the contaminated saline in the conical tube. The saline was aspirated back into the syringe and then flushed back through the catheter and collected back into the conical tube (with the tip of the endoscope remaining saline filled conical tube for 5 minutes). The 50 ml conical tube with the contaminated saline was submitted for culture and qPCR testing.

Disinfection: There were three separate stations set up for each disinfectant product. Each station had 4 different containers with 1) enzymatic cleaner (Endozime AW Triple Plus with APA, Mineloa NY), 2) water, 3) disinfectant, and 4) water. The endoscopes were first immersed in the enzymatic cleaner, then water, then disinfectant, and finally water. The exterior of the endoscopes was manually cleaned with a 4x4 gauze sponge in each tub. The interior channels of the endoscopes were cleaned with endoscopic brushes twice and flushed twice with a 60 ml syringe in each tub. The endoscopes were immersed into each disinfectant for 12 minutes for the OPA and control and 8 minutes for the AHP. Test strips were used to test the OPA and AHP each time the endoscope was disinfected. The endoscopes were than rinsed (exterior and interior) with 70% isopropyl alcohol. The endoscopes were dried with a 4x4 gauze sponge and air was injected through the interior channels for drying. The enzymatic cleaning solution and water in each tub was discarded and replaced each time between disinfecting each endoscope. The disinfectants (AHP and OPA) were used for only one testing day and replaced with a new gallon at the start of each testing day. Sample collection after endoscope disinfection was identical to sample collection after contamination, (using a conical tube, endoscopic catheter, and 30 ml of saline).

It was not possible to blind individuals performing the contamination, disinfection, and sample collection due to the differences in time and smell for each disinfectant. To try to prevent contamination of disinfected endoscopes, only one-person contaminated endoscopes and collected samples after contamination for all six testing days. The same two people disinfected the endoscopes, and only one person collected the sample after disinfection for all six testing days. All personnel wore disposable protective gowns, gloves (double gloved), and plastic boots. The personnel disinfecting the endoscopes and collecting samples after disinfection

changed protective clothing every time an endoscope was disinfected. The endoscopes were disinfected twice at the end of each testing day. At the end of each testing day, all equipment (tubs, tables, floor, and walls) were rinsed with water, disinfected with a different AHP product (Intervention Farm Animal care, Virox Technologies, Ontario Canada) used by the hospital, and allowed to air dry for at least 7 days.

Endoscope testing included aerobic culture and qPCR for S. equi and was performed after contamination and following disinfection. The conical tubes were centrifuged at 3.320 g for 15 minutes. Following centrifugation, approximately 25 ml of supernatant was removed. Culture was performed by plating one µl of the pellet directly onto blood agar plates (tryptic soy agar with 5% defibrinated sheep blood; Remel Inc., San Diego, CA), and incubated in a 5% CO2 atmosphere at 37 ºC for 24 hrs. The remaining saline fluid was frozen at -70 ºC until qPCR could be performed. The qPCR was designed from a conserved region in the M protein (SeM) gene of S. equi. It specifically detects the pathogen and does not cross-react with closely related pathogens including S. zooepidemicus. The assay has been fully validated analytically, and diagnostically validated with 42 positive and 280 negative clinical samples. All 42 positive samples were confirmed by DNA sequencing. The assay is currently an official diagnostic test at KSVDL. Personnel performing culture and qPCR were blinded to which disinfectant was used.

Statistical analysis

In order to provide evidence for substantial equivalence for treatments that result in rare binomial "events" (i.e. failure to sterilize; probability (P) of failure = 0.001), the sample size was computed using the equation $N = (Z_\alpha + Z_ß)^2 [P_S (1 - P_S) + P_T(1 - P_T)]$ where N = the sample size for each treatment group, Z_α = the standard normal variate corresponding to the α significance

level (0.05), $Z_{ß}$ = the standard normal variate corresponding to the tail probability of size ß (0.10), and d = that difference between the standard and new treatment effects that is considered to be clinically meaningful (0.025). The sample size calculated when using these assumptions was 30 replications per treatment. Using multivariable logistic regression model adjusted probability with endoscope and day as controlled variables the probability of an endoscope being qPCR was determined

Chapter 4 - Results

All samples were culture and qPCR positive following contamination. All samples were culture negative after disinfection. Following disinfection 10/30 AHP, 22/30 OPA and 21/30 control samples were qPCR positive. The disinfectants were not equivalent regarding qPCR positive test results following disinfection. Disinfection with the AHP resulted in a significantly lower probability of being PCR-positive after disinfection (31%) compared to disinfection with OPA (81%) and control (72%) (Figure 4.1). The mean cycle threshold (CT) values +/- standard deviation for the qPCR after contamination were 20.8 +/- 2.5 for AHP, 22.3 +/- 1.8 for OPA, and 21.8 +/- 2.2 for control samples. The mean CT values for the qPCR after disinfection were 37.0 +/- 0.8 for AHP, 35.8 +/- 1.5 for OPA, and 35.6 +/- 1.3 for control samples.

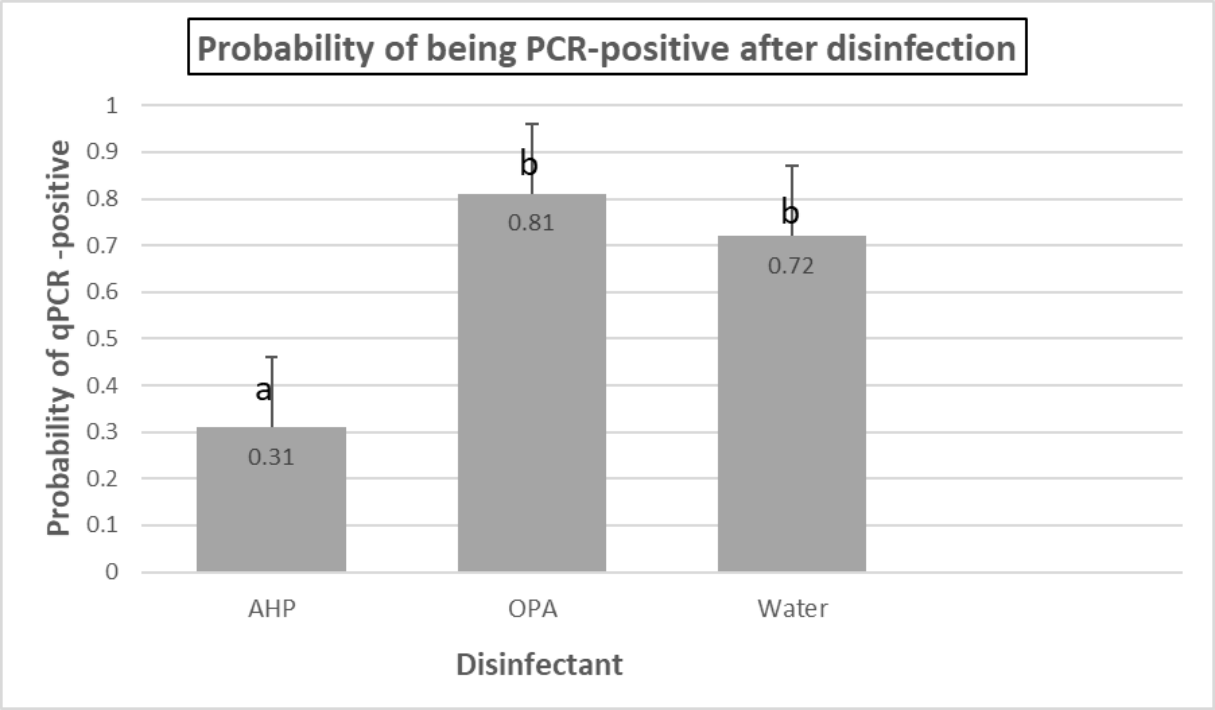

Figure 4.1: Probability of being PCR positive after disinfection with accelerated hydrogen peroxide (AHP), ortho-pthaldehyde (OPA) and water

Chapter 5 - Discussion

This study documented that high level manual disinfection with either AHP, OPA or even water was successful at eliminating live S. equi bacteria, at least by culture from endoscopes, which is similar to what has been previously reported (Svonii et al. 2020). However, none of the disinfectants used completely eliminated DNA following disinfection, with similar results reported by Svonni et al. 2020. Surprisingly, disinfection with the AHP product resulted in significantly lower probability of the endoscope being qPCR positive compared to the OPA product and water. However, even with use of the AHP product, the disinfected endoscopes were still qPCR positive 33% of the time.

The current study is similar to a previous report that documents residual DNA contamination of endoscopes after disinfection (Svonni et al. 2020). The current study resulted in qPCR positive following disinfection with OPA 81% of the time, which was much higher than the previous report having qPCR positive 36% of the time using the same disinfectant (Svonni et al. 2020). One likely reason for the difference is the current study performed more replicates of endoscope testing (30) compared to the previous study (6) (Svonni et al. 2020). In addition, the endoscope testing was spread out over six different testing days. There was significant variability in the results from day to day for both disinfectants (figure 5.2). The OPA product resulted in a qPCR positive rate from 40% on day one to a high of 100% on days 5 and 6. The AHP product had a qPCR positive rate from 20% on days 2, 3, and 4, to a high of 80% on day 5. False positives maybe less likely in a field setting as only 1 of 14 disinfected endoscopes tested positive by qPCR from horses that were documented carriers (Svonni et al. 2020). One thing to note in that field setting, the endoscopes were in contact with the OPA for a longer period of time (20 minutes) versus the experimental contamination part of that study (15 minutes contact

time) (Svonni et al. 2020). Based on the current and the previous study, it is likely that false diagnosis of S. equi carrier can occur if endoscopes are not known to be qPCR negative prior to testing horses. Therefore we recommend, when possible, performing qPCR testing of endoscopes after disinfection in horses suspected of having strangles, in order to prevent false diagnosis of carrier horses.

All of the culture results were negative after disinfection with the AHP and OPA. Additionally and even when water was used as a disinfectant. Based on the current and previous studies, it is extremely unlikely that transmission of S. equi occurs after high level disinfection with endoscopes. The CT values for the positive qPCR samples post disinfection were very high (all greater than 35). The numbers of bacteria recovered on culture was proportional to the strength of the qPCR, such that samples positive on qPCR with a CT above 34 were culture negative (Pringle et al.2019). According to the current ACVIM consensus statement, the recommendations are to consider horses that are qPCR positive (but culture negative) to be considered carriers of S. equi regardless of how high the CT values are (Boyle et al. 2018). Therefore, even with the culture negative results and high CT values post-disinfection, results of our study indicated that we cannot confirm with 100% certainty that all live bacteria were removed.

The reason that the AHP disinfectant significantly resulted in a lower probability of having the endoscope test positive on qPCR compared to the OPA product is likely related the differences of mechanism of action of the disinfectants. The OPA product is an aromatic aldehyde that results in amino-acid interactions, cross-linking and increases membrane permeability by cytoplasmic membrane damage (Simoes et al. 2007). Interferences at the DNA level were restricted to extremely high OPA concentrations (Simoes et al. 2007). Hydrogen

peroxide is an oxidizing agent and works by producing free hydroxyl radicals, a powerful oxidant, which can initiate oxidation and damage to nucleic acids, proteins, and lipids (Juven et al. 1996).

Traditional hydrogen peroxide disinfectants have a higher concentration (7%) than used in the AHP product (2%). These traditional hydrogen peroxide products, having a high concentration, are corrosive to many medical instruments including flexible endoscopes. The AHP product is reported to be much less corrosive to medical equipment such as endoscopes (Omidbakhsh 2006). The AHP product was tested with an endoscope for its compatibility by soaking the endoscope 1000 cycles for 5 minutes of contact time, with the endoscope being evaluated every 24 hours (Omidbakhsh 2006). No functional or material cosmetic damage was reported on the endoscope at any time (Omidbakhsh 2006). The particular product used (Prevention HLD) is no longer on the market. However, a similar product (Revital-OxTM Resert High Level Disinfectant, Steris, Mentor, OH) is currently available and is designed for use on flexible endoscopes. According to manufacturer's data, material compatibility was tested through hundreds of cycles of exposure to this product with flexible endoscopes (Revital-OX RESERT). The endoscopes were evaluated during testing by an independent third party or the manufacturer and were found to have to no functional or material cosmetic damage. Despite this published information regarding compatibility of AHP products with endoscopes, not all endoscope manufacturers recognize these products as being compatible with their endoscopes. According to materials compatibility list from Karl Storz Endoscopes, this product is compatible with their flexible video endoscopes. However, a customer letter from Olympus dated in 2014, states that "Olympus has not tested the compatibility of Revital-OxTM Resert High Level Disinfectant STERIS with Olympus endoscopy equipment (Compatibility of Revital-OX). As a

result, Olympus does not list this particular product as compatible or incompatible product for reprocessing of Olympus endoscopy equipment" (Compatibility of Revital-OX). Veterinarians are ncourageed to check with their endoscopic manufacturer prior to the use of these products.

Finally, there are some additional differences between the OPA and AHP disinfectants. The OPA products are considered much less toxic than the previously used glutaraldehyde products. However, exposure to OPA products has resulted in asthma in humans (Robitaille et al. 2015). Anaphylaxis has also been reported in patients following endoscopic examinations with endoscopes disinfected with OPA products (Atiyeh et al. 2015; Suzukawa et al. 2007). The AHP product is considered much less toxic and with minimal eye or skin irritation reported in a rabbit model (Omidbakhsh 2006). However, the AHP product was more expensive than the OPA product at the time of this study with the AHP product costing approximately twice as much as the OPA product. The AHP product has the longest duration of efficacy (21 days) when compared to the OPA product (14 days).

The main limitation of the current study is due to the experimental nature using a BHI broth of a S. equi solution. Certainly, the BHI broth is thin, and probably easier to remove than the purulent exudate from horses with S. equi. This study did serially contaminate endoscopes many times (a total of 30 times for each three disinfectants with a live bacterial broth). When performing endoscopies in a field setting following an outbreak, it would be unusual to have this many horses be truly positive. It would be likely that the endoscopes could be disinfected several times before being truly contaminated again. The previous publication which did include some field trial data found that only one out of 14 endoscopes used on carrier horses were positive on qPCR after disinfection (Svonni et al. 2020). The authors pointed out that only 5/14

horses were culture and qPCR positive, with the other nine horses being only qPCR positive, but culture negative (Svonni et al. 2020).

In conclusion, based on this study and previous work, there is real concern that DNA may still be present on endoscopes after high level disinfection, which could result in the false diagnosis of a healthy horse as a carrier of S. equi. If horses are being tested for S. equi (by qPCR), it is important to ensure that endoscopes are known to be qPCR negative prior to testing. Veterinarians should keep this in mind when performing multiple endoscopies following an S. equi outbreak in a field setting, when it is not possible to test the endoscopes between horses and have the test results back. Veterinarians should consider the use of AHP products in this situation, as there was a significantly reduced probability of the endoscope testing positive following disinfection. We suggest that veterinarians should contact their endoscope manufacturer prior to the use of this disinfectant, as warranties may not be covered if there is any evidence of corrosion of the endoscope when this product is used. Future studies on how to best eliminate DNA from endoscopes after disinfection would be ideal to address this issue.

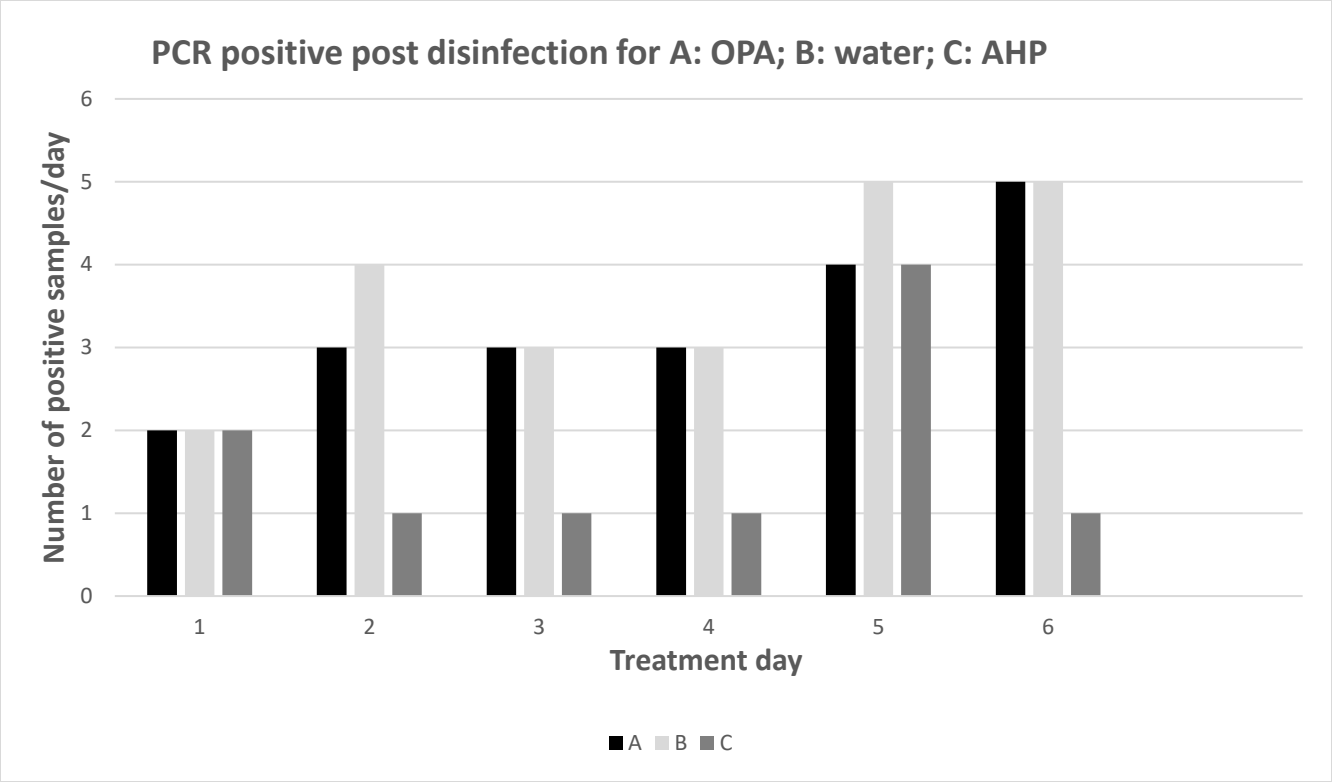

Figure 5.2: PCR positive samples post disinfection with ortho-pthaldehyde (OPA), water, and accelerated hydrogen peroxide (AHP) by day

Chapter 6 - Future directions

Strangles caused by *Streptococcus equi* is the most common disease in horses 6 to 10 years of age in the USA (Boyle et al 2018). Interestingly strangles is not recognized by the OIE (Office International des Epizooties) although the disease is problematic worldwide. While molecular advances allow rapid test results with greater sensitivity and quantification of initial concentration of target DNA, it cannot discriminate dead from live bacteria and consequently bacterial viability is questionable. While different methods of analyzing viability of bacterial cells of PCR test are being studied, PCR positive and culture negative sample results for *S. equi* should be interpreted as a positive diagnosis and biosecurity measures should be adopted (Boyle et al. 2018, Pusterla et al. 2018, Riihimäki et al. 2018). When analyzing PCR results, interpretation of bacterial viability, in other words: whether the bacteria is able to form progeny, is not only infectious disease clinicians' concern, but also food or water safety analysts and microbiologists. (Cangelosi et al. 2014) Strategies have been proposed to overcome this challenge. Pusterla et al. (2018) investigated the molecular viability of *S. equi* by detecting mRNA for specific target genes. Unlike DNA-based detection, mRNA would only be limited to the viable and active cells within the population since its half-life is less than 60 minutes for most bacteria (Cangelosi et al. 2014, Pusterla et al. 2018). Interestingly the research showed qPCR-positive and culture-negative samples were significantly associated with the presence of molecular viability (Pusterla et al. 2018). Although the study's lack of sample standardization and delayed sample collection affected cultivability of S. equi and mRNA quality, molecular viability could be investigated (Pusterla et al. 2018). Recently, detection of mRNA transcripts for the *SeM* gene of *S. equi* in pre- and post-cultured samples by qPCR was investigated to determine microbial viability (Pusterla et al. 2020). However, the overall agreement between

culture alone and the three of the criteria used to determine viability was only 59% (Pusterla et al. 2020).

Detection of bacterial viability has been described using a membrane-impermeative reagent such as propidium monoazide (PMA), a derivative of propidium iodide (PI) that penetrate membranes that have lost their integrity (Alvarez et al. 2013). However, limitations of PMA use are due to its incomplete penetration of the dead cell membrane that can result in a false positive (Zhao et al. 2019).

Currently, discernment between presence of DNA versus viable bacteria after positive guttural pouch lavage PCR samples, yet culture negative, is not possible. Consequently, false positive diagnosis of a healthy horse as a carrier is a possibility.

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