

Presumptive identification of smooth *Brucella* strain antibodies in canines

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ACADEMIC ABSTRACT

Brucellosis is a zoonotic disease caused by a Gram-negative coccobacillus. There are four *Brucella* strains of zoonotic importance in our domestic species, subdivided by their culture phenotypes: *Brucella abortus* (*B. abortus*, *B. melitensis*, *B. suis* (smooth strains) and *B. canis* (rough strain). Dogs can serve as hosts for all four of the zoonotic strains; however, routine serologic testing in dogs has been limited to the identification of antibodies to *B. canis*. The aim of our study was to identify a serologic test that can be utilized to identify smooth *Brucella* strain antibodies in canines. We hypothesize that the *Brucella abortus* Fluorescence Polarization Assay would be successful in identifying antibodies to smooth *Brucella* strain in canines. Ninety-five dogs, including forty-five hog hunting dogs were screened for circulating antibodies to any of the four zoonotic strains of the bacteria utilizing a combination of Canine *Brucella* Slide Agglutination Test (CBSA), *Brucella canis* Agar Gel Immunodiffusion II test (AGID), *Brucella abortus* Card Agglutination Test (BCA), and the *Brucella abortus* Fluorescence Polarization Assay (FPA). Test interpretation results yielded a 0% (0/95) smooth *Brucella* strain seropositivity rate, with 2% (2/95) of dogs yielding inconclusive rough *Brucella* strain serology results (0-2% rough strain seropositivity rate). Additionally, a retrospective portion of the study was performed to identify sera containing circulating antibodies to any of the smooth strains of *Brucella* by testing previously banked canine serum samples stored at Cornell's Veterinary Diagnostic Laboratory from 2018-2019 via *Brucella abortus* FPA. Of the 769 serum samples tested, 13/769 (1.7%) yielded an inconclusive result, 725/769 (94.2%) were negative, 30/769 (4%) yielded a positive FPA test result, and 1/769 (0.1%) had to be excluded due to insufficient

sample remaining to perform the diagnostic test. Of the 30 FPA positive canine serum samples, 97% (29/30) also tested positive on the CBSA test. Additionally, there was a statistically significant ($p < 0.0001$) likelihood of altered (spayed/neutered) and mixed breed dogs to be FPA positive when compared to intact, purebred dogs.

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GENERAL AUDIENCE ABSTRACT

Brucellosis is a bacterial disease that can cause severe reproductive, orthopedic and general illness in both dogs and humans. There are four different species of *Brucella* that can be transmitted between animals and people: *Brucella canis*, *abortus*, *suis*, and *melitensis*. Although *Brucella canis* is the species that is widely recognized and breeding dogs are routinely tested for this strain, we have vastly under recognized the ability for dogs to contract and transmit the other three (smooth) *Brucella* species. Of added concern is the fact that the test currently used to screen dogs for brucellosis only identifies *Brucella canis* infection. Thus, veterinarians may be missing cases where dogs are infected with other *Brucella* species. This study revealed promising evidence in identification of smooth *Brucella* strain antibodies in canines, particularly altered and mixed breed canines, via the *Brucella abortus* Fluorescent Polarization Assay. The contributions of this study are threefold. First, to heighten awareness that both smooth and rough strains of brucellosis infection in dogs are infectious diseases of zoonotic concern. Second, it demonstrates that smooth *Brucella* strain infection along with the traditional strategy of selectively screening dogs breeding dogs may be underestimating the prevalence of brucellosis among the canine population in the United States thus, supporting the need for broadened screening recommendations. Third, it reveals the need for a commercially-available, validated test for the smooth strains of brucellosis in dogs and offers direction for future research efforts to likely focus on the validation of the *Brucella abortus* Fluorescent Polarization Assay.

Attributions

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Dr. Cecere is a Professor of Theriogenology in the Small Animal Clinical Sciences department at the Virginia-Maryland College of Veterinary Medicine. She is the chair of this committee and was highly active in obtaining funding and assisting with the design and implementation of the project. She also contributed in the writing and revisions of this thesis and the manuscript for publication.

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Chapter 1: Thesis Organization

This thesis is written to confer a succinct overview of brucellosis in canines, with a specific focus on the less widely considered potential for smooth *Brucella* strain infection in canines. A comparison of *Brucella* strain differences is discussed, followed by the challenges of diagnosing the disease, and lastly an explanation for the need of a commercially available, validated serologic test for the diagnosis of smooth *Brucella* strain infection in canines. This thesis contains a manuscript that has been accepted for publication/published in *Frontiers in Veterinary Science* as the fundamental portion of the document. The manuscript is entitled “Presumptive identification of smooth *Brucella* strain antibodies in canines”.

Chapter 2: Literature Review

Brucella: Background and Pathophysiology

Brucella bacteria are small, intracellular, gram-negative, facultative aerobic coccobacilli, belonging to the family *Brucellaceae* (GŁOWACKA, ŻAKOWSKA, Naylor, Niemcewicz, & Bielawska-Drozd, 2018). *Brucella* is the causative agent for a disease known widely as brucellosis. Brucellosis is an incurable, zoonotic, febrile disease that is most notably known for causing reproductive loss in a myriad of animal hosts (GŁOWACKA et al., 2018). Brucellosis is a worldwide problem with the highest incidence of human disease reported in developing countries such as South America, central Asia, the Mediterranean and the Middle East (Organization, 2021). It is believed that the disease is more prevalent in these regions due in large part to the mass consumption of raw dairy products coupled with a lack of organized animal health regulations (GŁOWACKA et al., 2018; Rubach, Halliday, Cleaveland, & Crump, 2013). Specific clinical diagnoses that are indicated with brucellosis infection in animals include: late-term abortion, epididymitis, orchitis, infertility, diskospondilitis, encephalitis and undulating fever among others (Greene, 2006). Recently, twelve species of *Brucella* have been recognized and grouped according to primary host preferences, including, *B. abortus*, cattle; *B. suis*, swine; *B. canis*, canines; *B. ovis*, sheep; *B. melitensis*, sheep and goats; *B. neotomae*, dessert rats; *B. inopinata*, humans; *B. microti*, voles; *B. pinnipedialis* and *B. ceti*, aquatic mammals; *B. vulpis*, red fox; *B. papionis*, non-human primates (de Figueiredo, Ficht, Rice-Ficht, Rossetti, & Adams, 2015; Hull & Schumaker, 2018). The traditional understanding that *Brucella* has a basis for host preference is recently debated and questioned due to the bacteria's host adaptation capabilities,

as well as the wide spectrum of case reports indicating much variability in host infection with different strains of the bacteria.

Brucella is shed from mammal to mammal via mucous membrane contact with infected bodily secretions and excretions. The bacteria is transmitted in highest numbers within infected milk, reproductive fluids and aborted material, but may also be transmitted through infected urine, saliva, and respiratory secretions to a lesser degree (Carmichael & Joubert, 1988; Hensel, Negron, & Arenas-Gamboa, 2018; Moore & Gupta, 1970). Therefore, in animal hosts, *Brucella* can be contracted through ingestion, venereal contact, inhalation, or via conjunctiva and skin abrasions (María Pía, Maximilian, Gilman, & Smits, 2007). Once infection is established, the bacteria become sequestered within the cells of the reticuloendothelial system evading host's immune response (María Pía et al., 2007). *Brucellae* do not contain classic virulence factors known to other common bacteria; instead, the bacteria have evolved many mechanisms to avoid the host's innate and adaptive immune systems.

After mucosal invasion, *Brucellae* are ingested by phagocytes, relying specifically on macrophages during the early establishment of disease. After gaining entrance into the phagocytic cell, the bacteria sequester into early phagosomes and suppress intracellular trafficking pathways (Gomez, Adams, Rice-Ficht, & Ficht, 2013). This suppression and sequestration allows the bacteria to avoid fusion and degradation by cellular lysosomes (Gorvel & Moreno, 2002). Within these phagocytic cells, the bacteria evade killing in phagolysosomes and replicate within the endoplasmic reticulum (Byndloss & Tsolis, 2016; Gorvel & Moreno, 2002; Starr, Ng, Wehrly, Knodler, & Celli, 2008). *Brucellae* are proficient in their proliferation within the endoplasmic reticulum, as replication occurs without causing noticeable cell damage or restricting cellular function (Gorvel & Moreno, 2002). The bacteria also possess strong tissue

trophism, depending on macrophages, dendritic cells and placental trophoblasts for survival and replication (Copin et al., 2012). *Brucellae*'s tissue tropism, particularly for tissues comprising the genital system, are due to the production of high amounts of fructose and erythritol within these tissues which favor growth and proliferation of the bacteria (Letesson et al., 2017).

Approximately one week after a human host is infected with *Brucella*, the IgM antibody response will begin to rise sharply, reaching its peak approximately one to three months postinfection (Avijgan, Rostamnezhad, & Jahanbani-Ardakani, 2019). IgM subsequently begins to decline after reaching its peak, and disappears approximately one to two years after the onset of disease; although a recent paper suggests that IgM could be present up to 20 years post-infection (Al Dahouk & Nöckler, 2011; Avijgan et al., 2019; Mantur et al., 2006; Solís García del Pozo, Lorente Ortuño, Navarro, & Solera, 2014). In regards to the IgG antibody response, there is a sharp rise in IgG production starting approximately two weeks after the onset of disease, with a continual rise until six to eight weeks post infection, after which it begins to decline around 6 months and becomes undetectable after 1 year in treated patients (Avijgan et al., 2019; Solís García del Pozo et al., 2014). However, if treatment is inappropriate, IgG antibodies will remain in blood serum and maintain constant levels in patients with chronic brucellosis (Avijgan et al., 2019).

Brucellae contain a smooth, non-endotoxic lipopolysaccharide coating that aids in blocking the innate immune response during the acute stage of infection (María Pía et al., 2007). It is believed that the smooth lipopolysaccharide [LPS] (those containing an O-side chain on the LPS) alters the infected cell's MHC class II antigen presenting system and causes it to present foreign antigens to cytotoxic T-cells, thus preventing the death of the infected cell (Lapaque,

Moriyon, Moreno, & Gorvel, 2005; María Pía et al., 2007). However, not all strains of *Brucella* contain these evasive LPS qualities. Rough strains of the bacteria (those lacking an O-side chain on the LPS) are historically considered less virulent because they do not possess the ability to overcome the host's innate immune defense system in this way (Porte, Naroeni, OuahraniBettache, & Liautard, 2003). Nonetheless, both strains have been known to cause significant disease in animals and humans.

Comparison of Strain Differences

There are several criteria that are used to divide the *Brucella* genus, including: structural cell envelope features, preferred host, physiological differences and phage susceptibility (Mancilla, 2015). The broadest category of differentiation of *Brucella* is into that of a strain type, consisting of either smooth or rough. Strain type is determined based on the cell surface lipopolysaccharide (LPS) structure with smooth strains expressing a full LPS molecule linked to an O-antigen, and rough strains containing an LPS molecule that lacks an O-antigen side chain (Foster, Osterman, Godfroid, Jacques, & Cloeckert, 2007; Mancilla, 2015). LPS structure has classically been known to be linked to virulence, with smooth strains of brucellosis being considered more virulent than rough strains. The reason behind the differences in virulence is tied to the role that the LPS plays in host interactions, in that the O-antigen (found on the LPS of smooth strains) is a key molecule for invasion, impairing cytokine production and protecting the bacteria from apoptosis, complement-mediated lysis, peptide metabolism and oxygen metabolites (Jiménez de Bagüés, Terraza, Gross, & Dornand, 2004). Of the *Brucella* species

that affect our domestic animal populations, four are of zoonotic importance (*B. abortus*, *B. suis*, *B. melitensis*, and *B. canis*), and all but *B. canis* are classified as smooth strains.

Brucella abortus is considered to be one of the most virulent strains of the bacteria with its primary host being cattle. *B. abortus* infection is well known to cause reproductive disease in cattle including orchitis and epididymitis in bulls, and late term abortion, stillbirth, metritis, mastitis, and subsequent decline in milk production in cows and heifers (Meador & Deyoe, 1989; Xavier, Paixão, Poester, Lage, & Santos, 2009). The bacteria are shed in highest numbers through infected milk and reproductive tissues, secretions and excretions. Thus, infection causes major production losses on the agriculture industry and poses a huge human health risk for those consuming unpasteurized dairy products and those that work in the livestock and dairy industry. Because of the zoonotic risk, much effort has been expended into the control of brucellosis in cattle, with one of the most influential control efforts being the development of *B. abortus* vaccines. Although multiple *B. abortus* vaccines now exist, including SR19, RB51, 45/20 and SR82; the RB51 vaccine strain is the most widely used at this time (Dorneles, Sriranganathan, & Lage, 2015). *B. abortus* strain RB51 is a rough, natural mutant strain derived from a virulent, smooth *B. abortus* biovar 1 strain 2308 (Dorneles et al., 2015; Schurig et al., 1991). The vaccine has sufficiently demonstrated protection against abortion and infection, and does not revert to the virulent phenotype (Cheville et al., 1996; Cheville, Stevens, Jensen, Tatum, & Halling, 1993; Olsen, 2000; Olsen, Bricker, Palmer, Jensen, & Cheville, 1999; Poester et al., 2006; Schurig et al., 1991). Another benefit to the rough phenotype of RB51 is that it does not induce production of anti-O antibodies after immunization (Olsen, 2000; Olsen et al., 1999; Schurig et al., 1991). Thus, vaccinated cattle can be distinguished from naturally infected cattle, because the

antibodies generated from vaccination with RB51 do not cross-react with serologic brucellosis diagnostic tests (Olsen, 2000; Olsen et al., 1999; Schurig et al., 1991).

Sheep and goats are the nature host of *Brucella melitensis*, although other species such as cattle and other ruminants can become infected (Byndloss & Tsolis, 2016; Xavier et al., 2009). Similar to *B. abortus* in cattle, *B. melitensis* causes significant reproductive disease (orchitis, abortion, epididymitis, metritis). An interesting characteristic of *B. melitensis* is that its primary target tissue during the acute phase of infection is the mammary gland, which leads to the excretion of high numbers of the bacteria during lactation (Byndloss & Tsolis, 2016). *B. melitensis* is considered to be the most common strain of acquired zoonotic infection in humans, accounting for 70% of human brucellosis infections (Health, 2018a). This is in large part due to the widespread consumption of unpasteurized goat's milk within developing countries.

Brucella suis prefers both domestic and wild swine as its host and is a pathogen of growing concern within the United States. Similar to all *Brucellae*, the primary clinical signs seen with *B. suis* are reproductive in nature such as: abortion, infertility, orchitis and epididymitis. Historically, *B. suis* was responsible for large numbers of human cases of brucellosis around the twentieth century (Franco-Paredes, Chastain, Taylor, Stocking, & Sellers, 2017). However, after the implementation of rigorous control strategies and government regulated swine farming practices, *B. suis* is now considered eradicated from the domestic swine population in the United States (Franco-Paredes et al., 2017). However, the disease still exists within the domestic swine populations in developing countries. Today, an important reservoir for the disease is found in the ever-growing feral swine population. Current estimated seroprevalence rates for *B. suis* in the feral swine population in the United States are not fully

known. Recent studies have shown suspected seroprevalence rates to be highly variable, with study populations indicating estimated seroprevalence rates between 3.5- 28% (Health, 2018b; Pedersen et al., 2012). Positive samples are typically found in clusters (V. R. Brown, Bowen, & Bosco-Lauth, 2018), thus eluding to the establishment and spread of the disease through specific sounders of swine.

Brucella canis is the only rough strain of brucellosis of zoonotic importance that is known to affect domestic animals. The bacterium causes abortion, infertility, epididymitis/orchitis, and diskospondylitis in its preferred host, the canine. *B. canis* is primarily found in commercial breeding facilities with seroprevalence rates estimated to be as high as 83%, but as low as 0.4% in noncommercial breeding facilities (Johnson et al., 2018). The disease is not considered a treatable condition with chronic, recurrent infection and intermittent shedding of the bacteria posing a real risk to animal and human health. Thus, the recommended treatment of confirmed positive animals is euthanasia.

Diagnosis of Brucellosis

The definitive diagnosis of brucellosis in any species is considered difficult due to the bacteria's intracellular qualities, affinity for tissue trophism, and slow-growing nature. A battery of tests are typically involved in the diagnostic strategy due to the fact that a single perfect diagnostic test does not exist. The options for laboratory testing that may be utilized in the diagnosis of brucellosis includes the detection of brucella antibodies, molecular diagnosis by polymerase chain reaction (PCR), and isolation of the bacteria via culture.

Serologic tests, which utilize the detection of circulating antibodies to *Brucella*, are widely used, especially as first line screening tests in the diagnosis of brucellosis. A large number of serologic assays exist. The majority of these tests evaluate the host's antibody response against *Brucella* cell wall surface antigens, with two cell wall morphologies existing: smooth (O-antigen subunit) and rough (lacking O-antigen subunit) (Hensel et al., 2018). It is crucial to recognize the differences between these two morphologies, because serologic tests designed to detect smooth *Brucella* species (such as *B. abortus*, *B. suis*, and *B. melitensis*) do not cross react with the rough strains of *Brucella* (*B. canis*, *B. ovis*) (Hensel et al., 2018). There are serologic tests that detect the antibody response against *Brucella* internal cytoplasmic antigen, but these tests are developed to be species specific tests (Bramlage et al., 2015; Wanke, Delpino, & Baldi, 2002). Because most serologic tests are readily available, simple to run, yield rapid results, and are highly sensitive, serology remains the mainstay for initial brucellosis screening. However, it is important to recount that although serologic testing is a vital tool in the initial diagnostic screening for brucellosis, testing for the presence of antibodies does not always indicate an active case of brucellosis. As such, many other organisms can cause cross-reactivity on serologic testing leading to false positive results. Thus, positive serologic test results should be confirmed with either other testing modalities, or with repeated serologic testing.

Polymerase chain reaction assays (PCR) also exist as a diagnostic tool for brucellosis infection. The basis of the PCR test is the detection of *Brucella* DNA via DNA extraction and amplification (Navarro, Casao, & Solera, 2004). *Brucella* DNA is most commonly detected in whole blood, however, other fluids and tissues can be utilized as well (Navarro et al., 2004). Although PCR is overall considered a fairly sensitive test (Hensel et al., 2018), individual test sensitivities and specificities vary depending on the laboratory that is performing the test

(Navarro et al., 2004). Due to the possibility of false negative results with PCR, the test is not recommended nor routinely used as a screening test in the initial diagnosis of brucellosis (Hensel et al., 2018). Instead, it is most often utilized to aid in the confirmation of disease.

The isolation of *Brucella* bacteria via culture is considered the gold standard for the diagnosis of brucellosis, as it provides unequivocal proof of an active brucellosis infection. However, conventional culture techniques used for the isolation of *Brucella* present several problems including failure to detect the pathogen, and long incubation periods (Mantur, Mulimani, Bidari, Akki, & Tikare, 2008). Because the nature of the disease being an intracellular pathogen that prefers to sequester in the cells of the reticuloendothelial system, and its fastidious nature, the organism can be difficult to isolate. The disease also exhibits a pattern of intermittent bacteremia, which makes the yield of a positive culture result more likely during the acute stage of infection rather than the subacute and chronic stages of infection (Mantur et al., 2008). Because of these factors, culture is overall considered to be poorly sensitivity. With an estimated average sensitivity rate of approximately 70.1% (Avijgan et al., 2019; Morata et al., 2003), and a reported range of sensitivities from 10-90% (Avijgan et al., 2019; Mantur & Mangalgi, 2004; P Yagupsky, 1999; Pablo Yagupsky, 2015). Although a positive culture result is considered the gold standard definitive diagnosis for brucellosis, false negative results are very possible (Hensel et al., 2018). Therefore, culture is considered a poor screening test for brucellosis, and should instead be utilized to aid in the confirmation of disease diagnosis.

Zoonotic Concerns with Brucellosis

Although the true incidence of human brucellosis is unknown (M. J. Corbel, 1997), according to the World Health Organization (WHO), brucellosis is the world's most widespread

zoonosis and ranks as the seventh most neglected disease with 500,000 human cases reported annually (Hull & Schumaker, 2018). However, it is estimated that a vast number of infections are under-reported, and that the true estimated incidence of the disease is between 5,000,000 to 12,500,000 cases annually (Berger, 2016; Michael J Corbel, 2006; Godfroid et al., 2013; Mustafa & Nicoletti, 1995). *B. abortus*, *B. melitensis* and *B. suis* are considered the most common strains of the disease reported with domestic animal and human brucellosis infections; however brucellosis is also considered the most common laboratory-acquired infection in people (Hull & Schumaker, 2018; Weinstein & Singh, 2009). This is likely due to the bacteria having a very low infectious dose (as low as 10 bacterial cells) as well as its ability to gain access to the host via inhalation and mucous membrane contact (Mense, Borschel, Wilhelmsen, Pitt, & Hoover, 2004). In addition to laboratory exposure, rural workers, veterinarians, slaughterhouse workers, and hunters are all considered groups at higher risk of exposure, with a recent meta-analysis revealing that laboratory workers, animal breeders and abattoir workers are 3.47 times more likely to acquire *Brucella* infection (Pereira et al., 2020).

Although brucellosis has been well documented as a worldwide zoonotic concern, it continues to be neglected by both physicians and veterinarians as a possible cause of chronic, debilitating disease. In human medicine, it is suspected that brucellosis often goes underdiagnosed or misdiagnosed due to its non-descript clinical presentation with the main clinical symptoms in humans being fever, sweating, arthralgia (joint pain), and back pain (Aygen, Doğanay, Sümerkan, Yildiz, & Kayabaş, 2002; Roushan, Ebrahimpour, & Moulana, 2016). Acute, sub-acute, chronic, relapsing, and asymptomatic infection have all been described, with asymptomatic infection existing in individuals with a high risk of exposure: people living in

brucella endemic countries with animal/animal product contact (Roushan et al., 2016; Zhen et al., 2013). Brucellosis is reported to primarily affect individuals in the 15-40 year old age group, likely due to the fact that the working-aged group of people are more likely to have livestock and animal exposure (Buzgan et al., 2010; Roushan et al., 2016). However, the disease is also well known to affect children and elderly adults.

First line treatment of brucellosis in people includes combination bactericidal antimicrobial therapy along with supportive care such as anti-inflammatories and fluid therapy. The combination of doxycycline with an aminoglycoside is currently considered first line therapy for uncomplicated brucellosis in adult patients (Buzgan et al., 2010). However, other combinations including doxycycline paired with rifampin or clotrimazole have also been used alternatively (Buzgan et al., 2010). Nonetheless, brucellosis is considered to have a high rate of relapse within the year following diagnosis, with an estimated 5-40% of patients experiencing a relapse in infection (Alavi & Alavi, 2013). If the disease is not recognized and treated early and effectively, chronic carrier status and even death can ensue. Longterm consequences of brucellosis can include arthritis, spondylitis, bursitis, arthralgia, and occasionally respiratory, central nervous system and/or gastrointestinal involvement (Roushan et al., 2016). Brucellosis infection in people is a nationally notifiable condition, as treatment is very difficult and sometimes not achievable resulting in recurrent infection (WHO., 2006). Thus, preventing the spread of disease is paramount for both human and animal health.

Brucellosis in Dogs

Brucellosis infection in canines has historically been known to be caused by the rough strain of the bacteria, *B. canis*. Because brucellosis is well-known to be transmitted venereally

and cause reproductive pathology, regular testing is recommended (though not mandated) for all breeding dogs. A recent prevalence study in breeding dogs revealed that the incidence of *B. canis* is quite low in noncommercial breeding facilities (0.4%), but endemic among commercial dog breeding facilities with a reported prevalence ranging between 9% - 83% (Johnson et al., 2018). This raises cause for alarm, as many puppies sold from commercial breeding facilities are subject to inter-state commercial pet trade and destined to become household pets. Thus, given the nature of the disease, the fact that asymptomatic carrier status exists, and the potential for transmission to occur via close contact with infected individuals, the prevalence of brucellosis in the canine population could be more widespread than currently realized. Although the unrestricted movement of sexually intact dogs and puppies has been identified as an inherent risk factor for the spread of brucellosis to dogs and people alike, there is currently no mandated testing for the movement of dogs from *Brucella* endemic kennels, nor for dogs imported from *Brucella* endemic countries (Brower et al., 2007; Hensel et al., 2018).

Additionally, case reports of dogs infected with the smooth forms of *Brucella* (*B. suis*, *B. melitensis*, and *B. abortus*) have been reported worldwide (Barr, Eilts, Roy, & Miller, 1986; Hinic, Brodard, Petridou, Filioussis, & Contos, 2010; Mor et al., 2016; Ramamoorthy et al., 2011; Taylor, Renton, & McGregor, 1975; Wareth et al., 2018). *Brucella suis* is considered endemic within the ever growing feral swine population within the US (Pedersen et al., 2014). With the feral swine population on the rise and the growing number of pet pigs and hog hunting dogs developing into a sport, our canine population is likely at a much higher risk of exposure to this pathogen than previously considered. Currently, no validated serologic test exists for the identification of the smooth *Brucella* strains in canines, and there have been no studies on the prevalence of the smooth forms of brucellosis in dogs in the US to date (Johnson et al., 2018).

Furthermore, the first-line serologic screening test most commonly used in the diagnosis of brucellosis in dogs, the Rapid Slide Agglutination Test (RSAT) and the 2-ME RSAT, will only identify *B. canis* infection and do not cross react with the smooth species of *Brucella* due to differences in the cell wall morphology (L. Keid et al., 2007; L. B. Keid et al., 2009). Therefore, canine infection with the smooth forms of *Brucella* is likely being missed by veterinarians due to a low index of suspicion, and the lack of a readily available validated test for canines.

Previous literature has identified that the stray dog population has a higher reported seropositivity level for *B. canis* than privately owned dogs (J. Brown, Blue, Wooley, & Dreesen, 1976). Since many of these stray dogs are roaming and subject to being placed into shelters or adopted out into homes without being tested for brucellosis, the potential for brucellosis infection to spill over into the human population from contact with domestic dogs is evident (Hensel et al., 2018). Currently, it is unknown how common infection with the smooth form of brucellosis is within the domestic dog population in the United States.

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Chapter 3: Manuscript

Presumptive identification of smooth *Brucella* strain antibodies in canines

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Abstract

Brucellosis is a zoonotic disease caused by a Gram-negative coccobacillus. There are four *Brucella* strains of zoonotic importance in our domestic species, subdivided by their culture phenotypes: *Brucella abortus* (*B. abortus*), *B. melitensis*, *B. suis* (smooth strains) and *B. canis* (rough strain). Dogs can serve as hosts for all four of the zoonotic strains; however, routine serologic testing in dogs has been limited to the identification of *B. canis* antibodies. The aim of our study was to identify smooth *Brucella* strain antibodies in canines. We hypothesize that the *Brucella abortus* Fluorescence Polarization Assay would be successful in identifying smooth *Brucella* strain antibodies in canines. Ninety-five dogs, including forty-five hog hunting dogs were screened for circulating antibodies to any of the four zoonotic strains of the bacteria utilizing a combination of Canine *Brucella* Slide Agglutination Test (CBSA), *Brucella canis* Agar Gel Immunodiffusion II test (AGID), *Brucella abortus* Card Agglutination Test (BCA), and the *Brucella abortus* Fluorescence Polarization Assay (FPA). Test interpretation results yielded a 0% (0/95) smooth *Brucella* strain seropositivity rate, with 2% (2/95) of dogs yielding inconclusive rough *Brucella* strain serology results (0-2% rough strain seropositivity rate). Additionally, a retrospective portion of the study was performed to identify sera containing circulating antibodies to any of the smooth strains of *Brucella* by testing previously banked canine serum samples stored at Cornell's Veterinary Diagnostic Laboratory from 2018-2019 via *Brucella abortus* FPA. Of the 769 serum samples tested, 13/769 (1.7%) yielded an inconclusive result, 725/769 (94.2%) were negative, 30/769 (4%) yielded a positive FPA test result, and 1/769 (0.1%) had to be excluded due to insufficient sample remaining to perform the diagnostic test. Of the 30 FPA positive canine serum samples, 97% (29/30) also tested positive on the CBSA test. Additionally, there was a statistically significant (p-value: <0.0001) likelihood of altered (spayed/neutered) and mixed breed dogs to be FPA positive when compared to intact, purebred dogs respectively.

Introduction

Brucella is a small gram-negative, intracellular, facultative aerobic coccobacillus that is the causative agent of the disease known as brucellosis (Głowacka et al., 2018). Brucellosis is a zoonotic disease most well-known for causing reproductive loss in many mammalian species across the globe (Głowacka et al., 2018). The highest incidence of disease in humans has been reported in developing countries due to the consumption of raw and unpasteurized animal products (Rubach et al., 2013; GŁOWACKA et al., 2018). In animal hosts, the bacteria is most well-known to be transmitted venereally due to tropism for reproductive tissues. However, *Brucella* has also been documented to be transmitted through mucous membrane contact with bodily secretions such as reproductive fluids, aborted materials and milk predominately, but also urine, respiratory secretions and saliva to a lesser degree (Moore and Gupta, 1970; Carmichael and Joubert, 1988; Hensel et al., 2018). Following host infection, *Brucella* bacteria become sequestered within phagosomes of the reticuloendothelial system, by evading or suppressing host bactericidal mechanisms and beginning replication within these cells (Gorvel and Moreno, 2002; Starr et al., 2008; Byndloss and Tsolis, 2016). *Brucella* especially prefers the cells contained within the reproductive tract, due to the presence of erythritol and fructose which promote bacterial growth (Letesson et al., 2017). In general, clinical signs of infection may include: infertility, abortion, epididymitis, orchitis, diskospondylitis, and undulating fever (Aygen et al., 2002; Johnson et al., 2018). Additionally, asymptomatic carrier status is also described (Zhen et al., 2013), and infection is often considered to be life-long due to the inability of current antimicrobial strategies to fully clear the organism from the body (Roushan et al., 2016).

There are currently twelve recognized species of *Brucella* that have been identified and named including *Brucella abortus*, *Brucella suis*, *Brucella canis*, *Brucella ovis*, *Brucella*

melitensis, *Brucella neotomae*, *Brucella inopinata*; *Brucella microti*, *Brucella pinnipedialis*, *Brucella ceti*, *Brucella vulpis*, and *Brucella papioni* (Hull and Schumaker, 2018). However, the five *Brucella* species that are considered to be of utmost importance in our domestic animal species along with their preferred host include *Brucella abortus* in cattle; *Brucella suis* in pigs and feral swine; *Brucella melitensis* in goats and sheep; *Brucella ovis* in sheep, and *Brucella canis* in dogs (de Figueiredo et al., 2015). Historically, *Brucella* species have been identified to have a distinct host preference, which allowed scientists to name and group the species accordingly. More recently, the understanding of *Brucella*'s host preferences has been debated due to the bacteria's ability to readily adapt and evade host immunity, along with a broad number of case reports indicating much variability in each *Brucella* specie's ability to infect hosts outside of the traditional host-preference classifications.

The causative agent of brucellosis infection in the domestic dog has historically been recognized as a rough strain of the bacteria, *Brucella canis* (Wanke, 2004). The seroprevalence of *B. canis* among dogs in the United States is difficult to estimate, and varies depending on the subpopulation of dogs screened with an estimated nation-wide prevalence of approximately 5.6% (Santos et al., 2021). Interestingly, recent studies have estimated higher seroprevalence ranges, eluding to a growing concern that this disease could be on the rise in the canine population within the United States. One study conducted between 2007-2016 showed *B. canis* prevalence ranged from 0.4% in non-commercial breeding facilities, up to 83% in commercial breeding facilities in the United States (Johnson et al., 2018). Another recent seroprevalence study of dogs rescued from South Dakota Indian reservations from 2015-2019 revealed an overall apparent *B. canis* seroprevalence rate of 6.8%, or adjusted true prevalence estimated at 29.4% (Daly et al., 2020). More recently, canine infection with smooth *Brucella* strains outside

of the traditional host range, i.e., *B. suis*, *B. melitensis* and *B. abortus*, have been evidenced by many case reports (Taylor et al., 1975; Barr et al., 1986; Baek et al., 2003; Hinic et al., 2010; Ramamoorthy et al., 2011; Mor et al., 2016; Wareth et al., 2018). Smooth strains of *Brucella* are, in general, considered more virulent due to the presence of a non-endotoxic lipopolysaccharide (LPS) coating which allows the bacteria to evade the host's innate immune response (Lapaque et al., 2005; María Pía et al., 2007). Therefore, in general, the smooth *Brucella* species are known to cause more significant disease and carry higher zoonotic potential. Thus, cause for concern has arisen from the scientific community regarding canine infection with the smooth strains of *Brucella*, and the risk of transmission to humans.

Although at present, the United States is considered a “brucellosis free” country, the smooth strains of the disease still exists in several wildlife reservoirs including bison and elk within the Greater Yellowstone Area, and within the feral swine population across the country (Rhyan et al., 2013). Canine infection with the smooth strains of *Brucella* has been well documented in multiple case reports, with the most notable risk factors being: contact with infected livestock or hoof stock (Baek et al., 2003), contact with infected feral swine (Ramamoorthy et al., 2011; Mor et al., 2016), and the consumption of infected raw meat and/or unpasteurized dairy products (Frost, 2017; van Dijk et al., 2018). At present, the estimated incidence and prevalence of dogs infected with the smooth strains of *Brucella* is unknown. To the authors' knowledge, only one prevalence study has been conducted on a cohort of 571 Mississippi shelter dogs utilizing the Buffered Acidified Plate Antigen (BAPA) test, in which no serologically positive dogs were identified (Hubbard et al., 2018). However, study limitations including geographical limitations, unknown history and therefore unknown risk factors for smooth brucella strain infection, and choice/lack of validation of the serologic (BAPA) test

utilized. Currently, the most commonly utilized serologic tests to diagnose brucellosis infection in dogs are targeted to only recognize antibodies against the rough canine-specific strain of brucellosis, i.e., *B. canis*, and will not detect antibodies to any of the smooth strains of *Brucella* (i.e. *B. suis*, *B. abortus* or *B. melitensis*) (Hensel et al., 2018). This is due to differences between the LPS cell wall morphologies, with smooth strains possessing an O-antigen side chain on the LPS and rough strains lacking an O-antigen side chain on the LPS (Mancilla, 2016; Hensel et al., 2018). It is these differences between cell-wall morphologies that comprise the differences between serologic tests, as these tests evaluate the host's antibody response against *Brucella* cell wall surface antigens (Hensel et al., 2018). Therefore, serologic tests designed to detect smooth *Brucella* species will not cross react with the rough strains of the bacteria (Hensel et al., 2018).

Currently, there is no serologic test validated for the detection of antibodies to the smooth strains of *Brucella* in canines. Rather, previous reports have used a number of different serologic tests that have been validated for the detection of smooth *Brucella* infection in livestock species. A list of some of the different serologic tests utilized include, but are not limited to, the *Brucella* Card Agglutination Test, Rose-Bengal Test, BAPA Test, and Serum Tube Agglutination Test. In livestock species, the prevalence of false positive results with many of these serologic tests requires that all positive serology results be verified with additional follow-up confirmatory testing modalities. The gold standard for the confirmation of brucellosis infection in any species is via positive culture result from either blood or tissue. Unfortunately, false negative culture results are very common, with low and highly variable published sensitivity rates ranging from 10-90% (Yagupsky, 2015). Because the bacteria is so fastidious and slow growing, negative culture results can be due to a number of reasons, including: overgrowth of contaminate bacteria, absence of the bacteria in the cultured specimen, and inappropriate culture conditions (Mantur et

al., 2008). Recently, the United States Department of Agriculture (USDA) added the Fluorescent Polarization Assay (FPA) as an approved test for the confirmation of brucellosis infection in cattle, bison and swine (2004). The FPA test has been repeatedly shown to be an efficient, economical, and highly accurate serum test validated for the confirmatory diagnosis of brucellosis in cattle, swine, sheep, goats, bison and cervids due to sufficient cross reactivity between the three common smooth *Brucella* strains, i.e., *B. abortus*, *B. melitensis* and *B. suis* (Nielsen and Gall, 2001), and has also been utilized to confirm human brucellosis cases due to zoonotic infection for years. Therefore, given the FPA test's successful validation across many different species and common smooth *Brucella* strain types, it is plausible to consider that this test could also be successful in detecting smooth strain *Brucella* infection in canine sera.

There is much debate on the validity and reliability of serologic tests in the diagnosis of the smooth strains of brucellosis in canines, as there is a significant need for validation studies in canines. Clinically, many veterinarians utilize the commercially available *Brucella abortus* Card Agglutination Test (BCA) to aid in the diagnosis of smooth *Brucella* strain infection in canine cases where there is a high index of suspicion. However, limited research has been conducted to support the decision to utilize this test. Additionally, although feral swine hunting dogs are considered to be at increased risk of smooth *Brucella* strain infection (Ramamoorthy et al., 2011), it is unknown how common infection is within this population in the United States. With these facts in mind, our study was divided into two parts, prospective and retrospective. The goal of the prospective aspect of our study was twofold. First, to detect antibodies to the smooth strains of *Brucella* in sera of clinically healthy dogs with (i.e. feral swine hunting dogs) and without (i.e. dogs presenting for routine spay/neuter) known risk factors for infection. Second, as no agreement exists on which serologic test should be utilized for the diagnosis of the smooth

strains of brucellosis in canines, we chose to utilize and report the results of the commonly used BCA test as well as the broadly validated confirmatory FPA tests for comparison. Lastly, the goal of the retrospective aspect of this study was to detect antibodies and evaluate the prevalence of seropositivity to smooth *Brucella* strains via FPA testing of banked canine sera from dogs that were previously screened for *B. canis*. In doing so, our hope was to shed light on if and how the veterinary community should be screening dogs for smooth *Brucella* strain infection. Particularly dogs with clinical evidence of brucellosis infection, an unknown background, or known risk factors for smooth *Brucella* strain infection.

Materials and Methods

Prospective Study Samples

Ninety-five dogs of various ages (12 weeks to 9 years of age), sex (males n= 57, females n= 38), spay/neuter status, parities and breeds were included in this part of the study. All animals were apparently healthy at the time of sampling with inclusion criteria being limited to facility accessibility and owner consent. Reporting of active feral swine hunting history was determined via self-reporting by the owner. Blood samples were collected from all dogs using standard venipuncture protocols approved by the Virginia Tech Institutional Animal Care and Use Committee (IACUC Protocol Number: 18-220). The blood was placed into a sterile, 5 mL serum tube and allowed to clot at room temperature for 30 minutes before centrifugation at 1500g for ten to fifteen minutes. After centrifugation, the serum was removed, transferred to a sterile 5 mL red top tube, and stored in a freezer at -20°C until analysis.

Serologic testing was performed at Animal Health Diagnostic Center at the Cornell University College of Veterinary Medicine, which is the reference laboratory for canine brucellosis testing. Testing for *B. canis* included the Canine *Brucella* Slide Agglutination Test

(CBSA) and *B. canis* Agar Gel Immunodiffusion II (AGIDII). . The CBSA is performed using an in-house produced killed whole-cell antigen made from an M- strain of *B. canis* stained with Rose Bengal. Equal volumes of each patient serum and 0.2M 2-Mercaptoethanol (2-ME) are mixed together on an agglutination plate and allowed to sit for 30 seconds. The slide antigen is then mixed in with each treated sample and rocked for 3 minutes before being evaluated on an inverted microscope at 4X for graded levels of agglutination and clearing of the antigen. Samples that exhibit 3-4+ agglutination are considered positive and 0-2+ agglutination are considered negative. The AGIDII is performed using an in-house prepared cytoplasmic antigen made from an M- strain of *B. canis* and using an AGID agar prepared at the AHDC Media Processing Center. A template is used to punch a seven-well pattern (a center well with six surrounding wells) in the AGID agar. Antigen is loaded into the center well and the outer wells are loaded with equal volumes of patient serum and positive control in alternating fashion. The plate is incubated in a humid box at 25-29°C in ambient air for 18 to 24 hours on a level surface. After incubation, precipitin lines are observed using an illuminating device. Lines of identity or weak positive reactions between the sample well and the antigen well are determined to be AGIDII Positive. Samples that form no precipitin lines are called AGIDII Negative. Lines of non-identity are reported as AGIDII Suspect and samples exhibiting lines of partial identity may be reported as AGIDII Positive (if CBSA positive) or AGIDII Suspect (if CBSA negative). Final results are interpreted in conjunction with the CBSA as either Positive, Negative or Inconclusive. Controls using well-characterized positive stock sera and a negative serum pool are used in both of these tests on every testing day to confirm the test is working properly.

For detecting antibodies against smooth strains of *Brucella*, the *B. abortus* Card

Agglutination Test (BCA) and the *B. abortus* Fluorescence Polarization Assay (FPA) were run concurrently. The BCA is performed using *B. abortus* buffered *Brucella* antigen prepared at the National Veterinary Services Laboratory (NVSL) in Ames, Iowa and Becton Dickinson Brucellosis Testing Cards. The patient serum and *Brucella* antigen are each loaded onto the marked teardrop-shaped well in 30 µl volumes. The serum and antigen are mixed together using a stirrer and then the card is placed on a rocker for 4 minutes. Reactions are observed promptly at the end of the rocking period using an illuminating device. Samples showing no characteristic clumping and having a grainy appearance with dispersal of particles are considered negative. Samples showing characteristic macroscopic agglutination with moderate to large clumps are considered positive. Some species other than bovine (such as cervids, equine, porcine and canine) will often exhibit a different clumping due to non-specific cross-reaction. All BCA positive samples are then run on FPA tube for confirmation. Positive and negative controls from NVSL are run alongside patient samples on every testing day to ensure the test is performing properly. The FPA is a test that uses an O-polysaccharide (OPS) extracted from *B. abortus* and labeled with fluorescein. The fluorescence polarization instrument is used to measure the polarization state of the light emitted by the OPS conjugate and reports the values in millipolarization units (mP). When no antibodies are present, the polarization is low. Polarization increases when antibodies bind to the conjugate. The FPA test is performed either in microwells (strips) as a screening test or in tubes as a confirmation test using the FPA test kit and instrumentation purchased from Ellie Lab. The FPA microwell test is run with the Sentry® 2000S™ strip reader and 8-well strips. 20 µl of the patient sample is pipetted into a well followed by 180 µl of reaction buffer. This strip is loaded into the machine where the wells are agitated and incubated for 2 minutes and then the mP is read. 10 µl of conjugate is added to each

well with the machine agitating the wells again, incubating for an additional 2 minutes and the mP is read a second time. The machine calculates the delta mP for all controls (three negatives and one positive) and each sample, comparing the results of the sample to the controls to determine the final result. Samples with a delta mP value less than 10 above the mean negative control value are considered negative. Samples with a delta mP value greater than or equal to 10 above the mean negative control value must be retested in duplicate for confirmation using the FPA tube test. For the FPA tube test, 20 µl of patient sample is pipetted into each of two test tubes followed by 1 ml of reaction buffer and vortexed gently to mix. The tubes are incubated at room temperature for 3 to 30 minutes and read in a Sentry ® 200™ tube reader. 10 µl of conjugate is added to each tube and vortexed gently to mix. The tubes are incubated again at room temperature for 2 to 5 minutes and read a second time in the tube reader, with the machine calculating the delta mP values. Samples with both tests having a delta mP value of less than 10 above the mean negative control value are considered negative. If one or both tests have a delta mP value of 10-20, the sample is considered suspect. If both tests have a delta mP value greater than 20, the sample is considered positive.

Additionally, for participating dogs that were presenting for routine spay and neuter, discarded epididymal and uterine tissue samples were sterilely collected, sectioned and stored in a freezer at -80°C following acquisition. Alternatively, when available, intact male dogs were manually ejaculated to obtain a semen and prostatic sample into a sterile bag, and vaginal swabs were collected from intact female dogs by passing a sterile, guarded Kalayjian swab into the vagina. Both semen samples and vaginal swabs were stored in a freezer at -80°C following acquisition. Tissue, sperm or vaginal swab samples from dogs that tested serologically positive on *B. canis* serology or the *B. abortus* FPA were transferred to a BSL3 lab and subjected to

culture. Clinical samples were routinely grown in brucella broth (BD, Sparks, MD) at 37°C with constant shaking or on Schaedler blood agar (SBA), composed of Schaedler agar (Acumedia, Burton, MI) containing 5% defibrinated bovine blood (Quad Five, Ryegate, MT) at 37°C with 5% CO₂.

Retrospective Study Samples

Seven hundred and sixty-nine serum samples, frozen and stored at -20°C, submitted to Cornell's Veterinary Diagnostic Laboratory for *B. canis* screening between 2018 and 2019 were subjected to antibody testing for smooth strains of *Brucella* via *B. abortus* Fluorescence Polarization Assay (FPA). All frozen-thawed serum samples were from canines of various ages, sex, spay/neuter status, and geographical locations that had been previously screened for *B. canis* via CBSA and AGIDII. Inclusion criteria included a test interpretation result of either “positive” or “inconclusive” following CBSA and AGIDII screening (Figure 1). Serum samples were initially tested by *Brucella* FPA microtiter strip test, which utilizes a small test volume in a format which allows for the testing of eight samples at a time. Results are measured in milipolarization units (mP) above the mean negative control value of 10-20, which is referred to as the delta mP. Any *Brucella* FPA microtiter strip test interpreted as “suspect” (delta mP of 10-20) or “positive” (delta mP>20) was retested by FPA tube in duplicate. The tube tests allow for a higher dilution of the serum, thereby decreasing components that can cause a non-specific polarization reaction. The FPA tube results were considered “negative” if both tubes had a delta mP of <10, “suspect” if either tube has a delta mP of 10-20 and “positive” if both tubes have a delta mP of >20. All FPA microtiter strip test “negative” samples were considered truly negative and did not undergo FPA tube testing. FPA results on all canine samples were reported with a

disclaimer as the test is not validated for this species. Finalized FPA interpretations for the combined strip and tube test results are listed in Figure 2.

Test Interpretation

Test interpretation for *B. canis* serology (AGIDII and CBSA) results were determined by the Animal Health Diagnostic Center at the Cornell University College of Veterinary Medicine and are listed in Figure 1. Suggested test interpretation for the serologic tests (BCA and FPA tests) utilized for the detection of smooth *Brucella* strain antibodies were determined by expert opinion and are outlined in Figure 2. It is important to reiterate that the test interpretation utilized for the FPA and BCA tests in this study are based on a culmination of current knowledge, expert opinion, clinical suspicion, and interpretation utilized by previous case reports, as currently no validated serologic test for the smooth strains of brucellosis exists for canines.

Data Analysis

For both prospective and retrospective data, all data was entered and maintained in an electronic spreadsheet, which was then used for statistical analysis. Animal gender, breed and geographic location were determined by the reporting veterinary practitioner, reference laboratory, or labeled as “not specified” (Nos) when descriptive data was not provided. Associations between categorical variables were assessed using Fisher’s exact tests. A p-value of < 0.05 was utilized to determine statistical significance. All analyses were performed using SAS version 9.4 (Cary, NC, USA). When analyzing results based on breed or sex, samples where descriptive data was not provided (i.e. Nos) were excluded from statistical analysis.

Results

Prospective Study Samples

Of the 95 samples collected, seventy-six were from client owned dogs residing in the following states: Tennessee, Louisiana, Kentucky, South Carolina, Massachusetts, Texas, New York, Florida, Georgia and Virginia. Forty-five of the client owned dogs were classified as active feral swine hunting dogs. Additionally, nineteen shelter owned dogs were enrolled from shelters in Georgia, Florida, and Virginia. Serologic testing for *B. canis* resulted in 98% negative samples (93/95) and 2% inconclusive samples (2/95) [Table 1]. Of the inconclusive results, one test result (a male, intact, shelter dog from Florida) was “suspicious” on AGIDII and the other (a male, intact, feral swine hunting dog from Tennessee) was “positive” on CBSA but negative on AGIDII. Three culture attempts were made on the epididymal tissue of the shelter dog from Florida which yielded contaminant growth only. Test results for the smooth strains of brucellosis, including the BCA and the FPA tests, for all 95 samples are reported in Table 2. The overall interpretation of the smooth strain serology results was 0% positive, with 38/95 (40%) BCA tests yielding a positive result, and 0/95 (0%) FPA tests yielding a positive result [Table 2].

Retrospective Study Samples

Of the 769 canine serum samples tested, most (n= 424, 55%) were from intact animals (33%, n= 253 females; and 22%, n= 171 males) with altered animals (n= 300, 39%) encompassing both spayed females (n= 154, 20%) and castrated males (n= 146, 19%). For 45 samples (6%), the sex of the animal was not specified. Samples were submitted from many different referral laboratories and referral veterinarians across the United States, including the

following states: Alabama (2), Arkansas (79), Arizona (2), California (138), Colorado (31), Connecticut (1), Florida (8), Iowa (13), Idaho (1), Illinois (IL), Indiana (1), Kentucky (4), Louisiana (61), Massachusetts (50), Maryland (4), Maine (2), Michigan (7), Minnesota (8), Missouri (1), Mississippi (5), Montana (6), North Carolina (7), North Dakota (1), New Hampshire (5), New Mexico (4), New York (148), Ohio (2), Oklahoma (9), Oregon (4), Pennsylvania (9), Rhode Island (1), Tennessee (34), Texas (19), Virginia (6), Washington (13), Wisconsin (15). Additionally, serum samples from Ontario, Canada (n=42), Prince Edward Island, Canada (n=2) and Brazil (n=17) were utilized. Regarding breed, the majority (n=502, 65.3%) were reported as an American Kennel Club recognized purebred canine, with mixed breed (n=156, 20.3%), and breed not specified (n=111, 14.4%) also reported.

Brucella serology results for *B. canis* (including CBSA and AGIDII) screening for the 769 samples were as follows: 1% (4/769) yielded invalid test results, 63% (489/769) yielded a positive result, and 36% (276/769) yielded an inconclusive result [Table 3]. Test results for the smooth strains of brucellosis utilizing FPA are reported in Table 4, including suggested test interpretations. Of the 769 sera tested, 13/769 (1.7%) yielded an inconclusive result, 725/769 (94.2%) were negative, 30/769 (4%) yielded a positive FPA test result, and 1/769 (0.1%) yielded an invalid test result [Table 4]. Invalid test results were due to not enough sample remaining to perform the diagnostic test.

Statistical analysis regarding categorical variables of smooth strain FPA interpretation results were as follows. No statistically significant pattern was identified between the outcome of the FPA interpretation and the AGIDII result (p-value= 0.3). However, there was a statistically significant pattern (p-value= 0.029) between the outcome of the FPA interpretation and the

CBSA result, with 97% (29/30) of FPA interpretation “positive” samples also testing positive on the CBSA test. Serum from both spayed and castrated gender classifications were more likely (pvalue: <0.0003) to be positive on FPA test interpretation than the intact dogs (Table 5). A significant association was also identified between breed and outcome of the FPA test interpretation, with mixed breed dogs being more likely to have a positive FPA test interpretation result than purebred dogs (p-value= <0.0001) (Table 6). Forty-one and one-hundred and eleven samples were excluded due to the absence of reported patient gender and breed respectively.

Geographic information regarding the locality of the 30 FPA test interpretation “positive” samples are listed in Table 7.

Of the 763 *B. canis* serology interpretation results, no statistically significant pattern was identified between *Brucella canis* serology interpretation by gender or breed.

Discussion

Brucellosis is a calamitous bacterial disease that is considered under-recognized by the CDC in canines (Hensel et al., 2018). The bacteria carry high zoonotic potential as it is readily transmitted through ingestion, inhalation, and mucous membrane contact with milk and reproductive secretions, as well as saliva, feces, urine, raw meat and respiratory secretions to a lesser degree (Moore, 1969; Moore and Gupta, 1970; Carmichael and Joubert, 1988; Hensel et al., 2018). Once infected, the disease is associated with significant long term and often lifethreatening health implications in both dogs and humans alike, as therapy is often ineffective at clearing the organism completely (Roushan et al., 2016). Although much effort has been given to the control of the disease within the United States livestock population, the surveillance and

control of brucellosis infection in canines and the potential for more widespread human infection with the disease due to close contact with canine companions has been vastly overlooked.

Although brucellosis in dogs remains endemic in many parts of the world, including some breeding facilities within the United States, there are currently no mandatory testing regulations (nor regulatory guidelines) for dogs prior to interstate travel, international travel, or adoption (Hensel et al., 2018; Johnson et al., 2018). At present, the only control strategy regularly practiced within the veterinary community is *B. canis* screening of breeding bitches and stud dogs prior to mating, due to the well-known risk of venereal transmission. Although less well-recognized, brucellosis infection within the spayed and neutered canine population has been increasingly recognized, with a recent abstract published by Cheong et al. revealing a proportionally higher number of spayed (33%) and castrated (28%) dogs testing positive on serology for *B. canis* compared to intact males (8%) and females (9%) (Cheong, 2020). Thus, it is possible that the current exclusive strategy of surveillance testing breeding dogs prior to mating may be vastly under-estimating the current seroprevalence of brucellosis within this canine population in the United States.

In addition to a growing awareness of the disease within the castrated dog population, an increasing number of reports have been published regarding the expanding concern of smooth *Brucella* strain (*B. abortus*, *B. suis*, and *B. melitensis*) infections in dogs, especially within the feral swine hunting canine population. Surprisingly, the prospective portion of our study failed to detect any positive smooth *Brucella* strain interpretation results within the feral swine hunting dogs that were sampled (seroprevalence reported at 0%). This could be due to study limitations including small sample size, lack of test validation in canines, and geographic sampling limitations. Therefore, the overall prevalence of smooth *Brucella* strain infection (i.e. *B. suis*) in

feral swine hunting dogs remains ultimately unknown, as a much larger study population would be needed to better estimate true seroprevalence.

Interestingly, in agreement with Cheong et al. (Cheong, 2020), our study identified that mixed breed and spayed/neutered canines were much more likely to be interpreted as positive on FPA for smooth *Brucella* strain antibody detection than intact or purebred canines.

Unfortunately, due to the retrospective study design, previously reported patient history was limited. Therefore, other potential risk factors which have been previously identified in the literature such as contact with infected livestock or hoof stock (Baek et al., 2003), contact with infected feral swine (Ramamoorthy et al., 2011; Mor et al., 2016), the consumption of infected raw meat and/or unpasteurized dairy products (Frost, 2017; van Dijk et al., 2018), importation from a *Brucella* endemic country, or the presence of clinical signs related to brucellosis could not be assessed. Nonetheless, our results suggest that *Brucella* screening recommendations should not be limited to intact animals prior to breeding and should instead include any canine that has a history of livestock or feral swine exposure, importation from a *Brucella* endemic country, and/or a history of consuming raw meat (especially raw wild game) diets.

All samples in the retrospective portion of our study tested either positive or suspicious on CBSA or AGIDII for *B. canis*, which was the prerequisite for inclusion. Interestingly, we found a significant association between canine serum that was interpreted as positive on the FPA test also tested positive on CBSA. It is the authors' opinion that this finding is probably largely due to observed cross-reaction on the CBSA test. Cross reactivity as evidenced by non-specific agglutination is a common finding with CBSA testing that inherently lowers the test's [specificity](#) for *B. canis* when interpreted alone. This is why the CBSA test is used and interpreted jointly with the AGIDII test for the serologic diagnosis of *B. canis*. Therefore, our findings suggest that

for clinical cases with known risk factors or a high index of suspicion for clinical disease, dogs testing positive on CBSA and negative AGIDII test should undergo follow-up testing for a smooth strain of brucellosis.

In regards to the debate of which serologic test should be utilized for the detection of canine antibodies to the smooth strains of *Brucella* (*B. abortus*, *B. suis*, and *B. melitensis*), there is still a considerable amount of research that needs to be conducted, including validation studies. Our finding of 40% BCA positive and 0% FPA positive samples in the prospective portion of the study suggest that the *B. abortus* card agglutination test alone should not be utilized for the diagnosis of smooth *Brucella* strain infection in the canine. Given the high number of positive BCA results noted within a clinically healthy population of dogs, the lack of accompanying FPA positivity, and the low specificity of agglutination tests due to non-specific agglutination, the BCA positive results from this study were considered false positive results. Rather, future research efforts should likely focus on the validation of the *B. abortus* Fluorescent Polarization Assay for the diagnosis of smooth *Brucella* strain infection in the canines.

In conclusion, our study supports the finding that brucellosis infection is not exclusive to the intact canine breeding population and serves as a reminder that spayed and neutered dogs are also at risk of harboring and spreading this zoonotic disease. Additionally, spayed and neutered mixed breed dogs may be more likely to harbor smooth *Brucella* strain infection than the intact and purebred canine populations. Though speculative, we hypothesize that this could be explained by the recent influx in the importation of canines into the United States from *Brucella* endemic countries (Patti Strand, 2019). There is significant need for future research efforts involving the validation of a serologic test for the detection of smooth *Brucella* strain infection in canines. Our results suggest that research efforts should likely focus on validating the *Brucella*

abortus Fluorescent Polarization Assay, and until further research is conducted, the *Brucella abortus* card agglutination test should not be used as a diagnostic tool in canines. Lastly, once a validated test is made available, regulatory testing recommendations to include both rough and smooth *Brucella* strain serologic testing should be instituted. This should include both intact and spayed/neutered canines with known risk factors for infection including: breeding, intimate contact with livestock or hoof stock, contact with feral swine, the consumption of unpasteurized dairy products, the consumption of raw wild game meat, and importation from a *Brucella* endemic country.

Supplementary Material

Tables

Table 1. Test Results and Interpretation of rough strain *Brucella canis* tests for 95 prospective samples.

Test Result	AGIDII	CBSA	Interpretation	% Samples
Positive	0	1	0	0%
Negative	94	94	0	97%
Inconclusive/ Suspect	1	0	2	2%

Table 2. Test results and suggested interpretation of smooth strain *Brucella* tests for 95 prospective samples.

Test Result	BCA	FPA strip	Suggested Interpretation	% Samples
Positive	38	0	0	0%
Negative	57	95	95	100%
Inconclusive/ Suspect	N/A	0	0	0%

Table 3. Test results and interpretation of rough strain *Brucella canis* tests for 769 retrospective samples.

Test Result	AGIDII	CBSA	Interpretation	% Samples
Positive	489	619	489	63%
Negative	81	146	0	0%
Inconclusive/ Suspect	195	0	276	36%
Invalid	4	4	4	1%

Table 4. Test results and suggested interpretation of smooth strain *Brucella* tests for 769 retrospective samples.

Test Result	FPA strip	FPA tube	Suggested Interpretation	% Samples
Positive	138	30	30	4%
Negative	594	131	725	94.2%
Inconclusive/Suspect	36	13	13	1.7%
Invalid	1	0	1	0.1%

Table 5. Breakdown of smooth strain interpretation by patient gender (retrospective samples).

	Smooth <i>Brucella</i> strain interpretation			
Gender	Inconclusive	Negative	Positive	Total
Castrated Male	3	129	13	145
Spayed Female	3	139	12	154
Intact Female	3	249	1	253
Intact Male	3	164	4	171
Total	12	681	30	723

Samples with gender not specified were excluded from the analysis.

Table 6. Breakdown of smooth strain interpretation by breed (retrospective samples).

	Smooth <i>Brucella</i> strain interpretation			
Breed	Inconclusive	Negative	Positive	Total
Mixed Breed	2	137	17	156
Purebred	5	487	10	502
Total	7	624	27	658

Samples with breed (purebred or mixed breed) not specified were excluded from the analysis.

Table 7. Geographic location of the 30 FPA test interpretation “positive” samples.

State	Number of FPA “positive” results
Arkansas	1
California	7
Colorado	2
Idaho	1
Massachusetts	4
Mississippi	1
North Carolina	1
New Hampshire	1
New York	3
Tennessee	4
Texas	2
Wisconsin	3

Figures

Figure 1. Test interpretation utilized for serologic screening for the rough strain of brucellosis (*Brucella canis*).

Negative Interpretation AGIDII <u>and</u> CBSA: Negative
Inconclusive Interpretation AGIDII <u>or</u> CBSA: Positive <u>or</u> Suspicious
Positive Interpretation AGID <u>and</u> CBSA: Positive

Figure 2. Test interpretation utilized for serologic screening for the smooth strains of brucellosis (*Brucella abortus, suis, melitensis*).

<p style="text-align: center;">Negative Interpretation</p> <p style="text-align: center;">FPA strip Negative</p> <p style="text-align: center;">FPA strip Positive <u>and</u> FPA tube Negative</p> <p style="text-align: center;">FPA strip Suspect and FPA tube Negative</p> <p style="text-align: center;">BCA Positive <u>and</u> FPA tube Negative</p>
<p style="text-align: center;">Inconclusive Interpretation</p> <p style="text-align: center;">FPA strip Positive <u>and</u> FPA tube Suspect</p> <p style="text-align: center;">FPA strip Suspect <u>and</u> FPA tube Suspect</p>
<p style="text-align: center;">Positive Interpretation</p> <p style="text-align: center;">FPA strip Positive <u>and</u> FPA tube Positive</p> <p style="text-align: center;">FPA strip Suspect <u>and</u> FPA tube Positive</p>

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

AH, CC, KL and JC contributed to conception and design of the study. AH and JF-G organized the data. AH wrote the first draft of the manuscript. AH, JC, OB, JF-G wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Chapter 4: Final Comments

The results from this study demonstrate the assumed identification of dogs with circulating antibodies to both the rough and smooth strains of *Brucella* in the United States. Therefore, both smooth and rough strain *Brucella* infection should be considered, especially when clinical disease with canine brucellosis is suspected, or when risk factors for infection are reported. Additionally, given the widespread popularity of canine pet ownership and close contact between humans with their canine companions, it is important to consider that canines can serve as a nidus for human exposure to both the rough and the more virulent smooth strains of brucellosis.

A significant link was identified between samples testing positive for smooth *Brucella* strain antibodies on the *B. abortus* Fluorescent Polarization Assay also testing positive on the rough *Brucella* strain Canine *Brucella* Slide Agglutination Test. It is hypothesized that this is in large part due to non-specific agglutination that is often noted on the Canine *Brucella* Slide Agglutination Test, which inherently lowers the test's specificity. Nonetheless, this finding also implies clinical utility in that canines testing positive on the Canine *Brucella* Slide Agglutination Test, but negative on the *B. canis* Agar Gel Immunodiffusion II test should be screened for smooth *Brucella* strain infection. Especially when such cases carry a high degree of clinical suspicion for brucellosis infection, or a history of risk factors for smooth *Brucella* strain infection.

Interestingly, the results of the study also demonstrated a higher likelihood of antibody presence to the smooth strains of *Brucella* in the spayed and neutered canine population in comparison to the intact population. This information is novel to what is traditionally taught and

considered among veterinarians. Therefore, the spayed and neutered canine population should not be underestimated as potential vectors for disease transmission. Although the reasoning behind this finding is yet to be fully elucidated, one possible hypothesis includes the mass importation of dogs from *Brucella* endemic countries into the United States rescue and spay/neuter adoption agencies.

Further research is needed regarding the validation of smooth *Brucella* strain serologic testing in canines. This study supports the need of the development of a commercially available, reliable screening test for smooth *Brucella* strain infection in canines. Furthermore, the results of this study revealed that the *B. abortus* card agglutination test exhibits a large degree of nonspecific agglutination with canine serum leading to a high number of false positive results and should therefore not be utilized for smooth *Brucella* strain infection screening in dogs at this time. On the contrary, minimal to no false positive results were suspected with the use of the *Brucella abortus* Fluorescent Polarization Assay in this study. Therefore, given its widespread clinical use among other species, ease of sample handling, availability, and likely utility demonstrated in this study, future research efforts surrounding the validation of the *B. abortus* Fluorescent Polarization Assay for smooth *Brucella* strain infection in canines should be pursued.