

DEVELOPMENT OF A MULTIPLEX BEAD ASSAY TO DETECT EXPOSURES TO TICK-  
BORNE DISEASES IN DOGS AND A COMPARATIVE PERFORMANCE ANALYSIS

by

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## Abstract

Tick-borne bacteria, *Ehrlichia canis*, *Anaplasma platys*, and *Ehrlichia chaffeensis* are significant zoonotic pathogens of dogs and humans worldwide. In tropical regions such as Grenada, West Indies, dogs represent a major reservoir for *E. canis* and *A. platys*, and they are often co-infected. The purpose of this study was to develop a serologic, multiplex bead-based assay to detect species-specific exposures to *E. canis*, *A. platys*, and *E. chaffeensis* in dogs for purposes of surveillance and public health. Peptides from specific outer membrane proteins of P30 for *E. canis*, OMP1X of *A. platys*, and P28-19/P28-14 of *E. chaffeensis* were coupled to magnetic beads and assays were optimized using the multiplex Luminex xMAP® platform. In experimentally infected dogs, the multiplex assay successfully detected antibodies for *E. canis* and *E. chaffeensis*, but not *A. platys*. In the Grenadian population (n=104), the multiplex assay and the in-house ELISA, the SNAP® 4Dx®, detected *A. platys* antibodies as well as *Ehrlichia* spp.. Multiplex assay results were found to have “good” and “very good” agreement with the ELISA and IFA for *E. canis* antibody-positive dogs (K value of 0.73 and 0.84 respectively), while ELISA and IFA had “very good” agreement with each other (K value of 0.85). *A. platys* multiplex results had only “poor” agreement with ELISA and IFA (K value of -0.02 and 0.01, respectively), while the ELISA and IFA tests had “moderate” agreement with each other (K value of 0.5). These tests showed the prevalence of exposure to *E. canis* to be comparable with previous studies (38% in 2014), but a doubling of exposure to *A. platys* determined by IFA and 4Dx® from 9% in 2006, to 20% in 2014. Bayesian modeling (performed on *E. canis* data only) suggested conditional independence between the IFA, 4Dx®, and MAG tests using consensus priors calculated from literature, and that the bead-assay had comparable sensitivity and specificity to the IFA and ELISA tests. In conclusion, the multiplex peptide assay performed

well in detecting the seropositive status of dogs to *E. canis* and had good agreement with commercial assays; however, more work needs to be done to assess performance in populations of dogs with exposures to multiple species of *Ehrlichia*. Further, the reasons for low seroreactivity to *A. platys* need to be further investigated.

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## Abbreviations

1. OMP : Outer membrane protein
2. ELISA : Enzyme-linked immunosorbent assay
3. IFA(T) : Immunofluorescent assay (test)
4. MAG : MAGPIX<sup>®</sup>, the bead-based multiplex assay
5. IDX : an abbreviation used in the coding for the SNAP<sup>®</sup>4Dx<sup>®</sup> test
6. kDa : kilodalton
7. *E. chaff-14* : *Ehrlichia chaffeensis* OMP 28 peptide from gene14
8. *E. chaff-19* : *Ehrlichia chaffeensis* OMP 28 peptide from gene 19
9. MFI : Median fluorescent intensity
10. VBD : Vector-borne disease
11. CVBDs : Canine vector-borne diseases
12. RT : Room temperature
13. BBMA : Bead-based multiplex assays
14. CCD : Charge-coupled device camera in MAGPIX<sup>®</sup> instrument
15. OD : Optical density

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## Dedication

I dedicate this writing to Clara Marie Black, and all my family (be they biologic, unrelated, or an altogether separate species): thank you for instilling in me the thirst, tenacity, and lunacy needed to pursue my passions for veterinary research. As is characteristic of your love and unwavering support, you certainly made sure I had more than enough of aforementioned attributes needed to succeed (particularly of lunacy), and for that I am deeply grateful.

And to my fellow Millennial Generation: as we grow into our roles as leaders and guardians, may we feel compassion and empathy for all species and use an evidence-driven approach to *act* upon it. May we seek to work transparently and honestly with one another and pursue only the *right things* for the *right reasons*.

## **Chapter 1 - Literature Review**

## **The importance of studying vector-borne pathogens**

Ticks are a key vector in the spread and transmission of zoonotic and emerging infectious diseases. In 2014, the World Health Organization (WHO) estimated that tick, and all vector-borne diseases accounted for 17%, or one billion, of all cases of infectious disease worldwide. Over half of the world's population is at risk for exposure to these pathogens for many reasons: increased globalization and international travel of both humans and animals, growing resistance of vectors to insecticides, lack of timely access to antibiotics of infected reservoirs, expanded distribution of vectors due to climate change and globalization, socioeconomic disparities, dwindling research resources, and the lack of access to education in fields such as entomology and public health [1-3]. To make matters worse, it is often “resource-limited settings where many of these diseases have the highest burden” in human and animal populations, compounding the severity of endemic and epidemic disease [4]. But even in a first world country like the United States we are affected; researchers have been monitoring the ever widening distribution of tick species, and within them finding new infectious and zoonotic pathogens [5-9] that are increasingly impacting human and animal health in ways that are not fully understood [8, 10-14].

*Ehrlichia* spp. and *Anaplasma* spp. are two such agents; while these genera have a worldwide distribution, their rising presence is of particular concern to many developing countries in the tropics. It is in “resource-limited settings where many of these diseases have the highest burden... [and] here that “identification of such [public health] interventions may be particularly important for” improving the health outcomes and socio-economic prosperity [4]. Indeed, *Ehrlichia canis* and *Anaplasma platys* are endemic in developing countries near the equator, as they have been considered important emerging



zoonotic pathogens [15]. Specifically, *E. canis* has been reported in dogs in Grenada, West Indies [16], nearby islands in the Caribbean [17-19], and several countries in Central and South America [20-24]. Brazil, Argentina, Venezuela, and even the United States are finding *Ehrlichia* and *Anaplasma* infections not only in dogs, but now in humans too [12, 13, 25-32]. As the natural regions and host preferences of these pathogens continue to expand and change, it is imperative that research on these vectors and infectious agents is continued and are vigilant in their prevention and surveillance [3, 33].

### **Dogs as sentinels for human health**

The growing interest in the concept of One Health has launched investigation into the relationships between humans, companion animals, wildlife, ticks, and the epidemiology of tick-borne zoonotic disease. Such multidisciplinary endeavors have led to a large (and growing) repertoire of literature citing the need for human and veterinary medical professionals to become more collaborative, and in recent years, these professions have begun to respond [10, 33-36].

As the most common type of tick-borne disease in the United States, Lyme disease has been at the forefront of this movement. Recent analyses have suggested that canine disease prevalence mirrors that of humans to such a degree, that we might benefit from designing surveillance programs using feral or community-owned dogs to model exposure risk in human populations [37, 38]. Many dogs are fairly reasonable to handle, and live in close proximity peoples' homesteads (food source!), and they often seek out of wildlife within dense patches of forest— commonly resulting in their infection of tick-associated pathogens. In this way, feral or community-owned dogs (particularly those not on tick-preventatives) can become a persistent reservoir for the propagation of vector-borne

zoonoses [34, 38, 39]. Similar studies advocating for the surveillance of companion dogs have also come to similar conclusions [12, 29, 38, 40, 41], identifying our companion-animal reservoirs to be a “more effective... and more cost-effective” target for VBD risk assessments and interventions aimed at “reduction of human risk” as opposed to those aimed at human targets [4].

Integral to risk assessment and to design of interventions for VBDs (such as *Ehrlichia* and *Anaplasma*) is the value of testing methods used in disease surveillance[12]. Numerous journals have published surveillance studies of *Anaplasma* spp. and *Ehrlichia* spp. prevalence in dogs over the last decade, where a variety of molecular and serologic methodologies are utilized. These studies have provided the medical community with valuable information detailing the genetic profiles and antigenic variation, as well as the geographic distributions of VBD throughout the United States and the world. Unfortunately, the utility of these studies to analyze risk and prevalence suffer from the inherent limitations of their testing platforms [39].

## **Aspects of *Ehrlichia* spp. and *Anaplasma* spp.**

### **Vector competence**

The *Rhipicephalus sanguineus*—also known as the ‘brown dog tick,’ is a ubiquitous, competent vector to both *Ehrlichia canis* and *Anaplasma platys*, but not for VBDs such as *E. chaffeensis*, *E. ewingii*, *A. phagocytophilum*, and *Borrelia burgdorferi* [42-44]. As the common name suggests, *R. sanguineus* has historically been regarded as a veterinary parasite because of its feeding preference to canine hosts as opposed to humans [44]. The scarcity of *E. canis* or *A. platys* infections in humans relative to their prevalence in canines would seem to support this dogma; however, recent ecological studies suggest

that *R. sanguineus* lose this preference as environmental temperatures increase, showing a greater affinity to human hosts. Due in part to this, these classically veterinary pathogens have been discovered to be infecting humans [45, 46], elevating the concern of these organisms to par with other zoonotic VBD agents [26].

### **Grenada, West Indies**

Grenada is small, developing island-nation (120 square miles) in the Caribbean Sea, resting just off the coast of Venezuela [Figure 1-1] [47]. It is home to a resident population of roughly 100,000 humans, and a large number of (largely undescribed) feral or else community-owned dogs, referred to as ‘pothounds.’ The island’s year-round warm weather (averaging between 75°F and 85°F) and tropical climate lend itself to booming tourism and spice-cultivation industries, but also to endemic exposure of zoonotic, vector-borne disease [47]. Its canine population has little access to acaricides and those rescued by humane organizations frequently report heavy tick-infestations and coinfections of *Ehrlichia canis* and *Anaplasma platys*—both with the potential for zoonotic spread [16]. While health care resources for humans and canines has been scant in years past, there has been a significant influx of medical professionals to the area, potentially making treatment and preventive medicine more accessible to the people of this developing nation [Table 1-1].

These characteristics make Grenada an ideal site for studying zoonotic arthropod-borne diseases (VBDs). There is only one species of tick that infests dogs in Grenada, the *Rhipicephalus sanguineus* (the brown dog tick)[16]. As *E. canis* and *A. platys* are only spread by ticks, and as *R. sanguineus* is the only tick-vector present, it is the only possible origin for the island’s endemic *E. canis* and *A. platys* infections [16]. This was confirmed

by a study reported by Yabsley, et al. in 2008 that collected serum samples from 177 dogs in 2004 and 2006. All dogs were tested by the SNAP® 4Dx® canine vector-borne serologic assay and nested PCR. They found evidence of active infection or previous exposure to *E. canis*, *A. platys*, *Babesia canis vogeli*, and *Hepatozoon canis* but there was no evidence of other arthropod-borne species such as *E. chaffeensis* and *A. phagocytophilum* [16]. This is not unexpected, as these pathogens lack vector competence for *R. sanguineus*, and the tick-species that are known to possess adequate vector competence (e.g. *Amblyomma americanum* and *Ixodes* spp.) have not been reported in Grenada [16].

### **Ehrlichiosis and anaplasmosis**

*Ehrlichia* spp. and *Anaplasma* spp. are the causative agents of ehrlichiosis and anaplasmosis in dogs and humans. These organisms are gram negative, pleomorphic, obligate intracellular rickettsia transmitted by *Ixodes* spp. tick-vectors [48], and many are considered to have zoonotic potential.

Reported cases of ehrlichiosis are common in the central and southeast regions of the United States [Figure 1-2], particularly during the height of the summer months [Figure 1-3]. Dogs are infected with *Ehrlichia* spp. very quickly— in the case of nymph and adult *R. sanguineus*, *E. canis* was shown to infect dogs within 3 hours of attachment, although many variables affect the speed and efficacy of transmission [49]. *Ehrlichia* spp. incubates in the blood, spleen, liver, and lymphatics for a period of 8-20 days, after which dogs either become subacute, persistent reservoirs, or else they experience severe, acute disease [50]. Dogs with subclinical infection of *Ehrlichia* spp. are sometimes incidentally diagnosed during yearly routine bloodwork as they often times show mild thrombocytopenia and anemia detectable upon complete blood counts [51]. Clinical signs

include depression, lethargy, anorexia, pyrexia, tachypnea, petechial hemorrhage, and ecchymosis, but their relative severity varies widely for reasons yet unknown [43]. In dogs, *E. canis* tends to produce the most severe manifestations of canine monocytotropic ehrlichiosis, with *E. chaffeensis* and *E. ewingii* infections generally producing milder forms of this syndrome [52, 53]. On the other hand, human infections are most commonly due to *E. ewingii* and *E. chaffeensis* which tend to produce more severe clinical syndromes. There is also increasing recognition of subclinical or mild human cases of *E. canis* that have come to light in recent years [13, 25, 27]. Coinfections of agents within this genus and outside of it are common, and can lead to greater severity of clinical signs, stronger immunosuppression, and prolonged time to pathogen clearance [28]. As all three of these *Ehrlichia* spp. of zoonotic interest are *E. canis*, *E. chaffeensis*, and *E. ewingii*, [13, 54], causing them to become chronic reservoirs for disease spread even years later.

*Anaplasma* is very closely related to *Ehrlichia*; in fact, it had been classified under various spp. identities in the *Ehrlichia* genus until the development of genetic sequencing in the late 1990's to early 2000's [50]. Incidence of infection for *A. platys* has also increased in the last decade and is reported commonly in the Midwest and northeast, and the Atlantic and Pacific coasts [Figure 1-5]. Despite its wide prevalence [6] and ability to cause autoimmune disease, much less is known about *A. platys* than of other organisms, probably due to its inability to be grown in culture [55]. It is strongly suspected to be vectored by *Rhipicephalus sanguineus*, but lacks experimental confirmation [43]. What we do know, is that in dogs, *A. platys* causes varying severities of cyclic thrombocytopenia, a trend which is evident in blood work, lasting for 3 days and recurring every 10-14 days [56]. The differences in virulence between geographically distinct

strains of *A. platys* is notable: the strains seen in the United States tend to be more mild, and more likely to cause subclinical disease than those strains from parts of Europe and Africa [6, 55]. As all three of these *Ehrlichia* spp. of zoonotic interest are *E. canis*, *E. chaffeensis*, and *E. ewingii*, [13, 54].

## Testing methodologies

Integral to risk assessment and to design of interventions for VBDs (such as *Ehrlichia* and *Anaplasma*) is the value of testing methods used in disease surveillance. Numerous journals have published surveillance studies of *Anaplasma* spp. and *Ehrlichia* spp. prevalence in dogs over the last decade, and they utilize a variety of screening and diagnostic methodologies. They have provided the medical community with information such as the molecular origins of antigenic variation, geographic distributions of CVBDs, virulence mechanisms, etc. [12]. Unfortunately, the reliability of this information suffers from limitations intrinsic to both the methodologic pathogenicity of organisms, as well as from the limited ability of extant CVBD diagnostic assays to differentially and accurately identify organisms [57]. The advantages and disadvantages of various diagnostic platforms will be discussed.

### In vitro culture

Several *Ehrlichia* spp. have been successfully isolated and cultured, but it is a relatively recent advancement, and not one performed at veterinary clinics [58]. For years, researchers acquired *E. canis* infected monocytes from a population of infected dogs and maintained these stores via incubating blood from uninfected dogs with infected monocytes. However, these cultures had greater potential for contamination by other

biologic pathogens, and they lacked the ability to proliferate in vitro. The discovery of a dog with malignant histiocytosis in 1988 changed this practice. Its monocytes could be cleanly and continuously cultured in the laboratory for over 100 generations [59], allowing *Ehrlichia* spp. to be propagated with much more ease.

Since then, *Ehrlichia canis* and *E. chaffeensis* have been successfully cultured in a variety of cell cultures, commonly DH82 cells (canine macrophage cell line)[59], THP-1 cells (derived from the monocytes of a one year old boy suffering from leukemia)[60, 61], as well as various tick-cell lines. Our ability to culture these organisms has led to extensive research on them [62], but has left uncultured or short-term cultured pathogens, such as *E. ewingii* and *A. platys*, relatively unexplored [13, 52, 63].

### **Microscopic detection**

*Ehrlichia* and *Anaplasma* can be detected microscopically based upon their morphology and host-cell tropism in a variety of tissue samples. During the acute phase of heavily infected animals, these pathogens can be identified at point-of-care on a peripheral blood smear. However, when organisms are present in blood Giemsa stain under a light microscope, inclusion bodies (morula) of *E. canis* [Figure 1-6] and *A. platys* [Figure 1-7] are visualized in monocytes and platelets, respectively [50]. It is important to note that the cyclic nature of *Ehrlichia* spp. and *Anaplasma* spp. does not guarantee their presence in peripheral circulation at all times [64]; over the course of infection, they are commonly sequestered in the spleen, lymph, and bone marrow [65].

This method is not without shortcomings; the process is time consuming, the diagnosis is assumptive and pathogens are sparse (typically only visualized in 1% of cells [26]). Furthermore, the morulae that *are* present can be difficult to distinguish from

lymphocytic granules, phagosomes, and cellular inclusions seen normally during cellular development [15, 64]; all of which greatly reduce the sensitivity of molecular detection [64]. Despite this, morphological diagnosis remains a primary method of detection in veterinary clinics at point-of-care due to its simple and inexpensive design [43].

### **Molecular assays**

Molecular testing utilizes various forms of polymerase chain reaction tests (PCR) to identify actively infected animals by the genera, species, or strain of pathogen [Figure 1-8]. While positive PCR results are quantifiable and highly sensitive/specific in early stages of infection, they are expensive, time intensive, and are easily contaminated. Additionally, a negative PCR test result has little value; during later stages of acute disease, the organisms are sequestered to different tissues and may not be present in the sampled tissue, despite the animal being actively infected. In other words, as the infection progresses and *Ehrlichia* spp. and *Anaplasma* spp. are pulled out of peripheral blood circulation (and into spleen, lymph, bone marrow, cerebral spinal fluid, etc.) the specificity and negative predictive value of the PCR tests decrease [65-67].

A study in 2016 [66] depicts this phenomenon quite clearly using experimentally infected dogs. Researchers were able to detect *A. platys* via PCR in peripheral blood beginning 3-4 days post inoculation [Table 1-3], and parasitemia peaked at 7-10 days. Animals with a low initial burden tended to peak at day 7 and had a recurrence of DNA in circulation a week sooner (and with a much lower load) than those whose peak positive PCR test occurred later (at day 10) [66]. Before the end of the study, all of the dogs' peripheral-blood PCR results came back negative, but it was discovered that these animals



harbored organisms in spleen and bone marrow samples, instead of peripheral blood circulation [66].

A separate study (with more frequent sampling) found that *over the entire duration of infection*, experimentally infected dogs tested PCR positive 92% of the time when infected with *A. platys*, 100% of the time for *E. canis*, 50% of the time for *A. phagocytophilum*, and 75% of the time for *E. chaffeensis* [Table 1-6] [53]. This is significant because it shows the specificity and negative predictive value of PCR testing to vary widely over the course of time [65]. So while molecular assays provide highly discriminative, quantitative information on both the species and strain present, they are expensive, time-intensive to perform, and depend on individuals being *actively* infected as well as the pathogen being *actively* present in the specific tissue being tested.

Nested PCR was performed for all of the Grenadian dogs used in this thesis' research, however the scope of the results and analysis have been accepted for publication [68] and are beyond the scope of this paper.

### **Serological assays**

Antibody-based tests include a variety of immunofluorescent antibody tests (IFA or IFAT), enzyme-linked immunosorbent assays (ELISA), and occasionally Western immunoblot tests (WB), and they are widely utilized in both clinical and research environments. Many popular assays rely on peptide sequences that are highly conserved across species and between some genera, so that they can be performed for a variety of host-species [27, 43, 69]. Additionally, treatment for *Anaplasma/Ehrlichia* infections is nearly identical— even the CDC recommends starting patients on a tetracycline-type antibiotic “before diagnostic confirmation is received from the laboratory” [26].

Expedited access to treatment is crucial: in the short-term, it assists the immune system in pathogen clearance and minimizes complications, and in the long-term it reduces the risk of latent infections causing severe chronic disease, even years after the initial exposure [26]. These advantages have ultimately enabled serological assays to be more ubiquitous and versatile in international markets, and more cost effective from a clinical perspective, so more likely to be ordered by health professionals [70].

From a public health perspective, mandatory reporting of these tests has provided much information in regards to risk of exposure across the country [34]. But when considering the broad geographic diversity of *Ehrlichia*, crude serologic tests potentially fail to link the appropriate species or strain of the agent to the pathologies and risk factors [71-73]. Evidence of serologic cross-reactivity between genera, species, and geographically differentiated strains of CVBDs have been reported and identified frequently in literature. This is due in part to the close homology of *Ehrlichia* and *Anaplasma* immunodominant outer-membrane proteins [42, 74-77], and of the extensive use of the same handful of in-house and laboratory diagnostic assays throughout the international community [40, 51].

### **Enzyme-linked immunosorbent assays**

ELISAs can be performed in-house using one of many commercially available tests for *Ehrlichia* or *Anaplasma* spp., by using protein extracts or peptides to coat the plates. Each has advantages and disadvantages but both are based upon the same principles: detection of canine antibody specific to the antigen via indirect staining techniques that utilize enzyme-conjugated anti-dog antibodies.

### ***Point-of-Care commercial ELISAs***

Point-of-care ELISA tests, such as the SNAP® 4Dx® from IDEXX, are frequently used as a screening tool for exposure to *Ehrlichia* spp. and *Anaplasma* spp.. This platform uses peptides (purified, recombinant, etc.) attached to a membrane. The peptides or proteins represent major outer surface immunodominant p44 molecule from *A. phagocytophilum* to detect *A. platys* exposure [78].[79], and from the p30 and p30-1 of *E. canis* (Oklahoma strain [80]) to detect *E. canis* and *E. chaffeensis* antibodies, but it does not recognize antibodies against *E. ewingii* infected dogs [Figure 1-9].

Mass utilization of the SNAP® 4Dx® tests in both clinics and research stems from their extraordinary convenience: they are inexpensive to purchase, cheap to store, easy to perform [Figure 1-10], easy to interpret [Figure 1-11], and quick to yield results (~10 minutes) [70]. The trade-offs for this convenience are that pale spots may be deemed negative when the animal has been exposed to a closely related species, or to an antigenically distinct strain of the same pathogen. Other external factors, such as poor clinic lighting, or inconsistent storage temperatures (i.e. experiencing multiple freeze-thaw events) may decrease the relative sensitivity of SNAP® tests [70].

When using this test, it is important for clinicians to remember that the SNAP® 4Dx® is a *screening* test (as opposed to a *diagnostic* test), and its purpose is to *screen* a population); not to definitively diagnose an individual [81]. Keeping this epidemiologic context in mind, many studies recommend that veterinarians do not base treatment strictly using the SNAP® results. Rather, it is always important to consider the prevalence and predictive value within each population of animals [78, 82], and not rule-out a diagnosis in light of a positive or negative result from a screening test [43] without taking full clinical

context into consideration. For instance, for a dog that is actively exhibiting acute clinical signs, a negative serologic test should be viewed with a healthy level of skepticism (and an inclination to consider likelihood functions), as infection is likely, despite a negative test. Conversely, seropositive animals that otherwise appear to be perfectly healthy are much less likely to be actively infected and thusly not in need of treatment (low likelihood of active infection, but high likelihood of previous exposure to the agent) [43, 82].

### ***ELISAs***

ELISAs are great tools for assessing exposure, particularly because they can quantify the amount of antibody via titration, diagnose active infection, and are easily adapted to a myriad of conditions. Their accuracy and utility is directly related to the antigen coated to each well—in the case of CVBD testing, a variety of antigens are used to detect exposures in a broad, genus or family sense, or they can be highly specific to the species or strain [Figure 1-12] [77, 83]. Unlike PCR, whose sensitivity directly varies with regard to the cyclic pathology of CVBDs, ELISAs' accuracy is less dependent on the exact day of sampling, provided the animal is both immunocompetent and has had adequate time to mount an immune response [50]. While it cannot distinguish active infection from previous exposure in a single test, it is generally able to do so with two tests. It is generally accepted that a four-fold increase in titer between acute and convalescent samples (taken two weeks apart) is indicative of active infection [84]. To measure this, the test uses an enzyme-conjugated colorimetric substance that is read in a spectrophotometer based on its optical density (OD), that is capable of reading an entire 96-well plate in seconds [84].

The ELISA format has various drawbacks [85]: since they can only be run in monoplex (meaning that each well can only test the seroreactivity of antibodies to one antigen), often require large volumes of sample, which can be problematic for dehydrated or toy-breed animals. The cost of CVBD diagnostic panels is also significantly greater, as it uses more reagents and requires a much greater time commitment for processing [86].

### **Indirect fluorescent antibody testing**

Indirect fluorescent antibody tests (IFA) are generally considered the “gold standard” of detecting *Anaplasma* and *Ehrlichia* exposure in dogs. In short, cells infected with the appropriate agent are cultured (such as *E. canis*, *E. chaffeensis*, or *A. phagocytophilum*), plated onto a glass slide, and can be used or stored at -20°C until needed [Figure 1-13] [26]. Slides are allowed to thaw at room temperature and depending on the particular kit or diagnostic laboratory, one or more dilutions of suspect dog serum are incubated, tagged or stained (via indirect or direct protocols) with a fluorescent dye, and visually examined under a fluorescent microscope to detect the presence of antibodies (positive staining) at a pre-determined dilution. If an animal has had prior exposure to the pathogen, they will seroconvert, and immunoglobulins can be detected by qualitatively assessing the degree of positive staining [Figure 1-14]. To measure the animal’s titer, serial dilutions of the dog’s serum are incubated and the largest dilution deemed to be positive is reported out as the positive titer level.

This platform has many admirable attributes; the use of whole antigen or semi-purified antigen provides an array of genus and species-specific epitopes for the patients’ antibodies to bind—and antibodies are exposed to the organism in a manner that is more similar to *in situ* interactions than in a peptide-based ELISA. Because of this, the IFA has

a higher sensitivity than the ELISA, particularly where an animal has a low positive titer [78-80]. This test has limited specificity because its diverse epitopes allow high level of cross-reactivity of species within the same genus, and sometimes between genera. But typically this is seen as an advantage: cross-reactivity within genera can allow IFA using a species easily cultured, to detect a patient's exposure to a different species from the one in culture. For instance, IFA tests that are plated with *E. canis* or *E. chaffeensis* (both are easily grown in culture) can still be used to test for exposure to *E. ewingii* (only very recently has this become possible to culture, [63] even in the short term) [87]. *Anaplasma phagocytophilum* culture is also frequently used to detect *A. platys* exposure in dogs via IFA [64].

### **Bead-based multiplex assays**

The use of bead-based multiplex assays as a variant of substrate in ELISA-type assays have been used with increasing popularity in biomedical literature [86]. Numerous companies have created a wide array of bead-based platforms to optimize detection of different biomolecules. The basic concept is the use of fluorescent dyed microspheres as a highly customizable substrate for the attachment of molecules of interest—allowing the simultaneous, quantifiable detection of numerous biomolecules in the same micro-well (multiplex capabilities).

Platforms typically have two exciting light sources (lasers or LEDs) that identifies the unique spectral qualities of the bead-set and to quantify the fluorescent signal bound to the biomarker of interest. Instruments with a greater capacity for detectable bead-sets (e.g. 100 to 500 bead-sets/well) operate similarly to a miniature flow cytometer, and others (such as the instrument used in this research) with lower bead-set capabilities (e.g. <50

bead-sets/well) use magnetic beads with an LED, CCD (charge-coupled device) to position each bead separately on a plane and measure fluorescence—not unlike microarray assays [

Figure 1-15] [[86](#), [88](#)]. For example, Luminex® xMAP® technology enables users to customize their assay format, by coupling whole proteins, peptides, or nucleic acids for the detection of antibodies, cytokines, PCR products, etc. that are specific for the attached molecules.

Advantages of this technology has led to extensive modeling and use in diagnostic test design as opposed to the traditional applications of ELISAs or IFATs. Literature reviews and meta-analysis confirm the statistical benefits and further explore the impact that future implementation of BBMAs might elicit [[86](#)]. The current consensus is that this technology provides vast improvements in its high throughput of data points per sample and per study design, and furthermore, multiplex format requires a significantly smaller sample volume to be submitted due to the massive reduction in the number of microwells needed to test for multiple biomarkers at multiple dilutions each. This in-turn speeds reporting turn-around time, reduces the cost per test result, and yield a higher sensitivity than traditional assays with a similar sample size [[86](#), [89](#), [90](#)]. Development of veterinary applications have been robust in recent years, as there are now a wide variety of commercial kits available for the quantification of cytokines, immunoglobulin typing, disease testing, etc. Of particular relevance are the several publications citing the successful development and validation of BBMAs to detect another CVBD (Lyme disease) in dogs and horses [[91](#), [92](#)].

## The problems concerning serologic cross-reactivity in One Health

Considering the relevance zoonoses have with regard to societally-pervasive commensalism enjoyed by animals and humans, it is curious that few studies should design human risk assessment models using the plethora of information available from the widespread use of VBD serologic tests. There have been several, massive studies published recently that compile crude serologic SNAP® data from thousands of dogs [6, 93-96] and all assert the utility of this data in modeling human risk. However, the handful of studies that have actually designed animal-sentinel models have not utilized these resources [37, 97, 98]. Instead, they have run their own serologic and molecular assays to identify the species-specific pathogen, highlighting a need for the design and use of species-specific serologic VBD panels in veterinary clinics. Due to extensive cross-reactivity of antibodies to crude antigens, the SNAP® tests and others traditionally used by veterinarians, these massively utilized tests are ill-equipped to relay a definitive, species-specific diagnosis of *Ehrlichia/Anaplasma* that is needed by One Health surveillance efforts.

Arguably, the reason behind this dearth of species-specific serologic tests can be attributed to the scientific communities' reliance on crude, non-specific index tests. The majority of literature cites immunofluorescent assays (using *E. chaffeensis* or *E. canis* whole-organisms for Ehrlichiosis, and *A. phagocytophilum* whole-organisms for Anaplasmosis [99]) as the diagnostic “gold standard,” for exposure or active infection of CVBDs. Their very design is to utilize the extensive cross-reactivity between *Ehrlichia/Anaplasma* species [78] to their advantage, and but very little is known (or questioned) of the ability for antibodies towards one strain to recognize and adequately



bind to geographically distant strains of the same bacterial species [76]. To date, only one study has conducted experiments on this in IFA format [100], and their results suggest that *there is variation* in the antibody recognition to different strains. As such, the assumptive use of IFATs as a universal ‘gold standard’ should undergo a degree of scrutiny when interpreting serologic studies, something the human medical-community appears to be doing when designing risk assessment models of zoonotic disease.

The goal of this research was to use the Luminex® MAGPIX® testing platform to overcome the limitations of extant serologic tests for *Ehrlichia* and *Anaplasma* by designing a surveillance assay capable of differential sero-diagnosis at a single point in time. With the concerns of cross-reactivity among antigens found in immunofluorescent assays (IFA) [78] and a commercially-available ELISA [Figure 1-9], use of multiplex species-specific peptide bead-based technology was proposed as a cost-effective way to improve detection of infections and co-infections of these tick-borne agents. As discussed previously, Grenada’s isolation, and limited array of tick spp. and associated pathogens was a key advantage in our ability to assess the impact of serologic cross-reactivity in these naturally (co)infected dogs.

## **Methods of performance analysis**

Two main methodologies predominate in the performance assessment of a novel diagnostic assays: frequentist, and Bayesian analysis, and they differ fundamentally in how they frame questions of assay performance and in underlying assumptions. Frequentists do this by asking what the probability is of observing the data (i.e. an animal being test positive/negative), given that the hypothesis is true (i.e. the animal is a known disease positive/negative) [101, 102]. The underlying assumption here is that the true

disease status of an animal is known with certainty (and is constant) because an existing ‘gold standard’ reference test is able to detect the presence of disease (sensitivity = 1.0), and the absence of disease (specificity = 1.0) with 100% accuracy across all populations [82]. Unfortunately, frequentist methodology assesses the ability of a disease positive/negative animal to test disease positive/negative, and does not tell us anything about the performance of a novel diagnostic assay [101, 102].

On the other hand, Bayesian statistics *can* frame the question we’re interested in, which is to determine the ability of a test to accurately detect the presence or absence of disease. Bayesians ask what is the *defined probability* that the *hypothesis* is true (i.e. animal being disease positive/negative), given their data (i.e. animal tests positive/negative) [Figure 1-16] [103]. Instead of being hypothesis-driven (as the frequentist approach is), the Bayesian analysis is data-driven and allows researchers to model the probability of *all* possible outcome-hypotheses [104]. They seek to model and define the most likely fit (posterior) of disease prevalence, and the performance parameters of each diagnostic method (in a defined **prior** beta probability distribution derived from outside literature data) that have melded to produce the data gleaned from the study [105]. In its most basic interpretation, Bayes Theorem incorporates prior knowledge of prevalence and test sensitivity/specificity to yield the probability of positive and negative predictive values— something that we *are* interested in. In conclusion, situations where there is no true gold standard test or where prevalence is low, Bayesian inference is a valuable method of performance analysis of a novel assay [106, 107].

Bayesian analysis is commonly performed using an open-source software called OpenBUGS, which stands for ‘Bayesian Inference Using Gibbs Sampling.’ OpenBUGS

may not be quite as intuitive as other commercially available software (i.e. R, JAGS, SAS; these also offer downloadable packages to easily work a wide array of data types/models) and it does require the user to learn a small degree of programming/syntax editing to successfully input and initialize a model. However, the benefit to this is that one is required to input coding as one would set up an algebraic question—it requires that you build and assess the mathematical origin of its modeling; something that is more difficult to do with other softwares [108]. Additionally, coding can be shared (which it commonly is) in publications’ supplemental material. This offers a transparency in the statistical process; the coding and studies [91, 109-111] of which greatly benefited the writing of this thesis.

OpenBUGS uses MCMC (Markov Chain Monte Carlo) algorithms that use the probability distributions given for the model and run a pre-determined number of iterations, or Markov chains, to simulate Gibbs sampling methods (generation of data from a theoretical, and much larger sample size). A benefit to this is that the program allows the user to perform a trace function (among other types of visual representations) to watch the distributions of results as the MCMC chains are running. A good chain has tight variation across the y-axis and reaches convergence of all Markov chains to a single, small range of mean credibility [112].

# Tables and Figures



**Figure 1-1: Map of Grenada, West Indies.**

Note: Reproduced from [113]; a citation, but no written permissions needed.

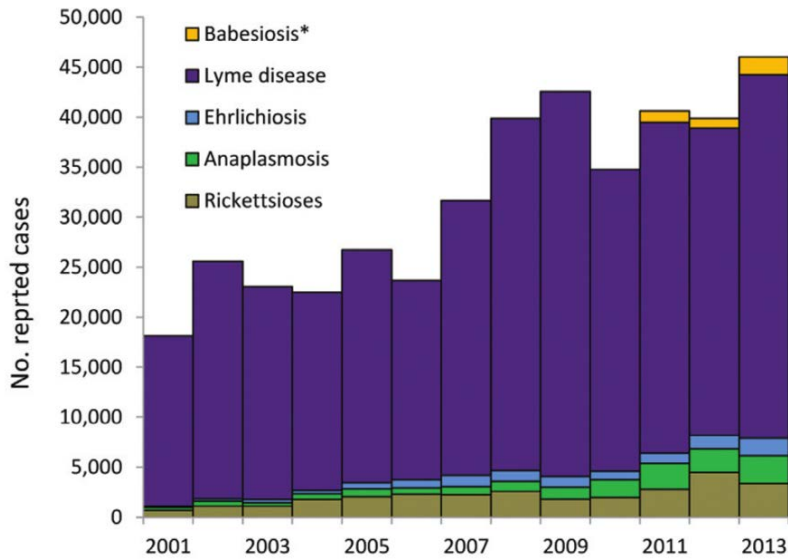
**Table 1-1: Human resources in health, by occupation and population covered per worker in Grenada in 2005 and 2010.**

<b>TABLE 5. Human resources in health, by occupation and population covered per worker, Grenada, 2003 and 2010.</b>				
Occupation	2003		2010	
	Number of workers	Population covered per worker	Number of workers	Population covered per worker
Medical doctors	58	1,769	110	1,016
Nurses and midwives	220	467	356	314
Nursing assistants	146	703	173	646
Dentists and dental assistants	13	7,894	23	4,858
Pharmacists and pharmacy assistants	22	4,577	72	1,552
Social workers	4	25,658	20	5,587
Rehabilitation workers	2	51,316	4	27,937
Technicians	20	5,137	22	5,079
Environment health officers	15	6,842	20	5,587
Nutritionists/dietitians	1	102,632	9	12,416
Mental health practitioners	—	—	11	10,159

*Source:* Reference (2).  
— Magnitude zero.

*Note:* Citation, but no written permissions needed [114]. Source reference data from Grenada, Ministry of Health, Epidemiology and Health Information Department. Unpublished Report. St. John's: Ministry of Health; 2011.

**Figure 1-2: Yearly incidence of tick-borne infections of humans in the United States as reported to the CDC.**

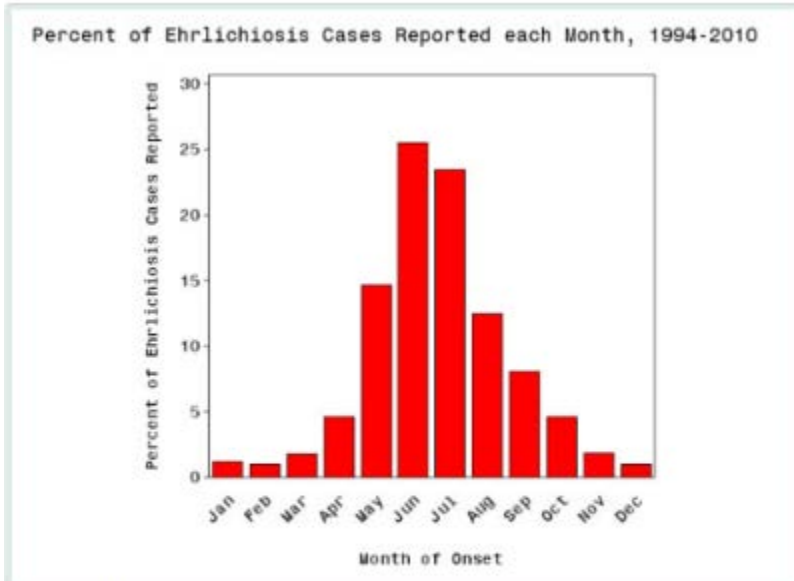


*Notes:* US cases of Lyme disease, ehrlichiosis, anaplasmosis, Babesiosis, and spotted-fever group rickettsioses reported to the CDC between the years 2001-2013. Counts include confirmed and probable cases as defined by each year's case definition. Up until 2008, anaplasmosis cases were reported as ehrlichioses, which encompassed all identifiable *Ehrlichia* spp. agents along with undetermined *Ehrlichia* spp. and *Anaplasma* spp.. Citation, but no written permissions needed [40].

**Table 1-2: Pathogens transmitted by *Rhipicephalus sanguineus* to both dogs and humans.**

<b>Pathogen</b>	<b>Disease name</b>	<b>Zoonotic potential?</b>
<i>Acanthocheilonema dracunculoides</i>	Subcutaneous filariasis	No
<b><i>Anaplasma platys</i></b>	<b>Infectious canine cyclic thrombocytopenia</b>	<b>Yes</b>
<i>Babesia gibsoni</i>	Babesiosis	No
<i>Babesia vogeli</i>	Babesiosis	No
<i>Cercopithifilaria bainaie</i>	Subcutaneous filariasis	No
<i>Cercopithifilaria grassi</i>	Subcutaneous filariasis	No
<b><i>Coxiella burnetii</i></b>	<b>Q fever</b>	<b>Yes</b>
<b><i>Ehrlichia canis</i></b>	<b>Monocytic ehrlichiosis</b>	<b>Yes</b>
<i>Hepatozoon canis</i>	Hepatozoonosis	No
<b><i>Leishmania infantum</i></b>	<b>Visceral leishmaniosis</b>	<b>Yes</b>
<i>Mycoplasma haemocanis</i>	Haemobartonellosis	No
<b><i>Rickettsia conorii</i></b>	<b>Mediterranean spotted fever (Boutonneuse fever)</b>	<b>Yes</b>
<b><i>Rickettsia massiliae</i></b>	<b>Spotted-fever</b>	<b>Yes</b>
<b><i>Rickettsia rickettsia</i></b>	<b>Rocky Mountain spotted fever</b>	<b>Yes</b>

Note: Table adapted from [115].



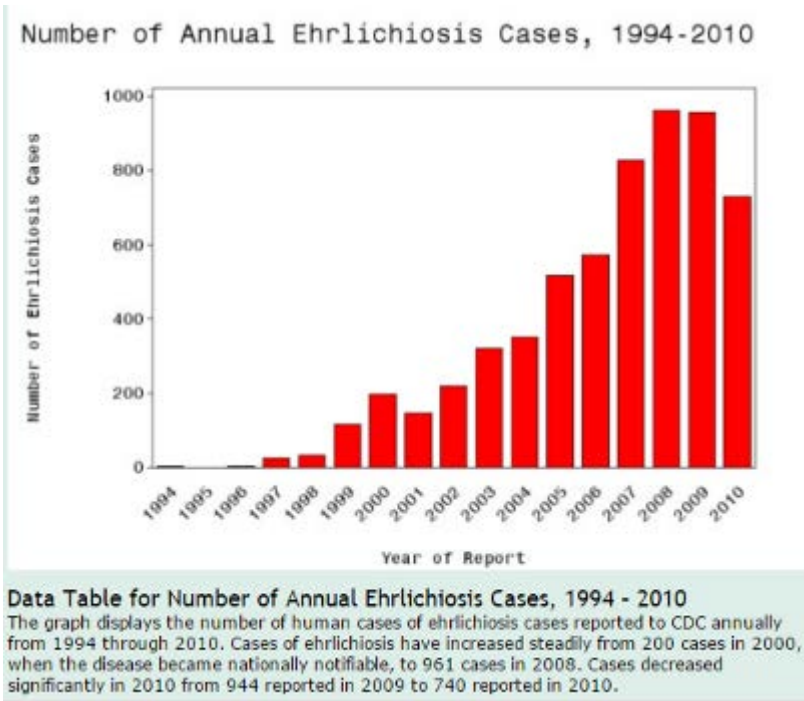
**Data Table**

This figure shows the percent of cases reported from 1994 through 2010 by month of onset to give the seasonality of cases. There are cases reported in each month of the year, however most are reported between May and August. More than 25% of cases are reported in June.

**Figure 1-3: Percent of *E. chaffeensis* cases reported to CDC by month of onset, 1994-2010.**

*Note:* Citation, but no written permissions needed [116].

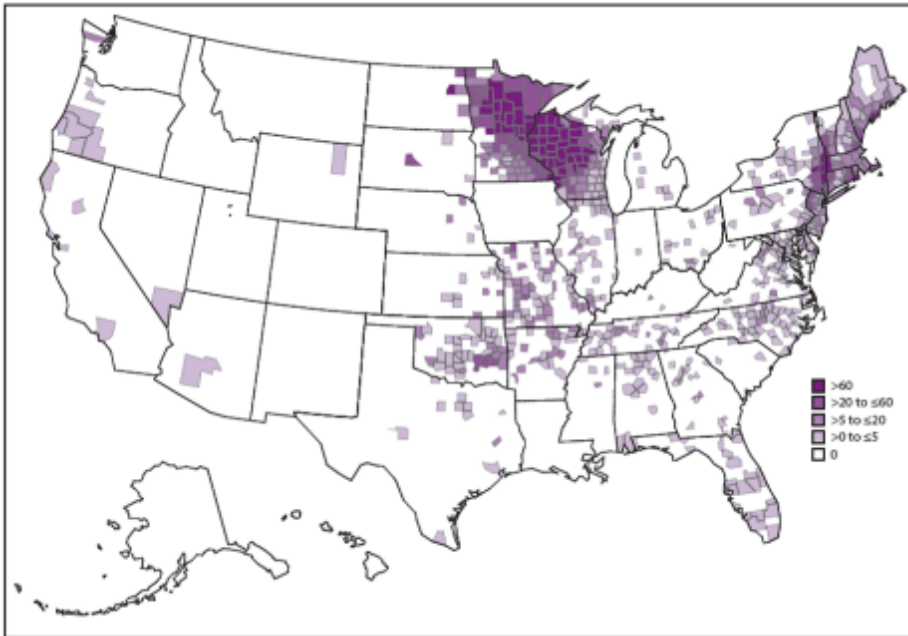




**Figure 1-4: Number of human ehrlichiosis cases reported to the CDC yearly from 1994-2010.**

*Note:* Citation, but no written permissions needed [116].

FIGURE 15. Reported incidence rate\* of anaplasmosis, by county – United States, 2000–2013



\* As reported through national surveillance, per 1,000,000 persons per year. Cases are reported by county of residence, which is not always where the infection was acquired.

**Figure 1-5: Reported incidence rate of anaplasmosis in humans, by county between 2000 - 2013.**

*Note:* Reproduced from [35]; citation, but no written permissions needed.

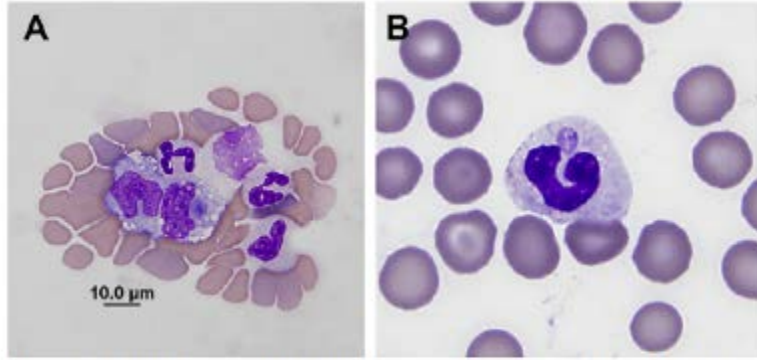
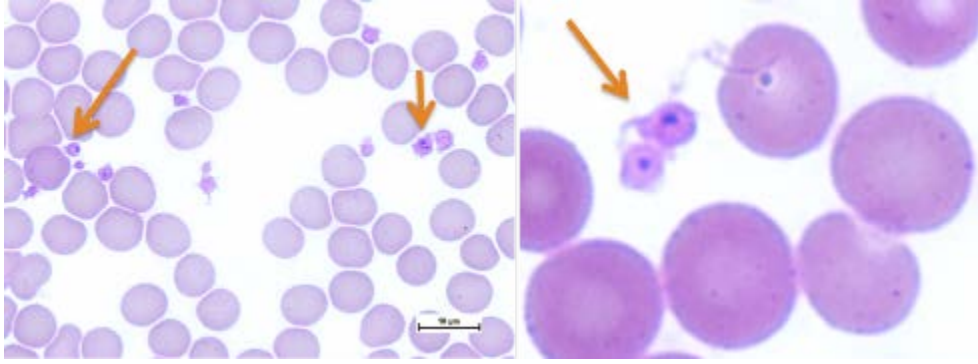


Fig. 3. Morulae of *Ehrlichia* spp. (A) *Ehrlichia canis* in a monocyte. (B) *Ehrlichia ewingii* in a neutrophil (note that *Anaplasma phagocytophilum* is morphologically identical to *E. ewingii* on blood smear). (Courtesy of Robin W. Allison, Oklahoma State University, Stillwater, OK.)

**Figure 1-6: Morphological identification of *Ehrlichia canis* and *Ehrlichia ewingii*.**

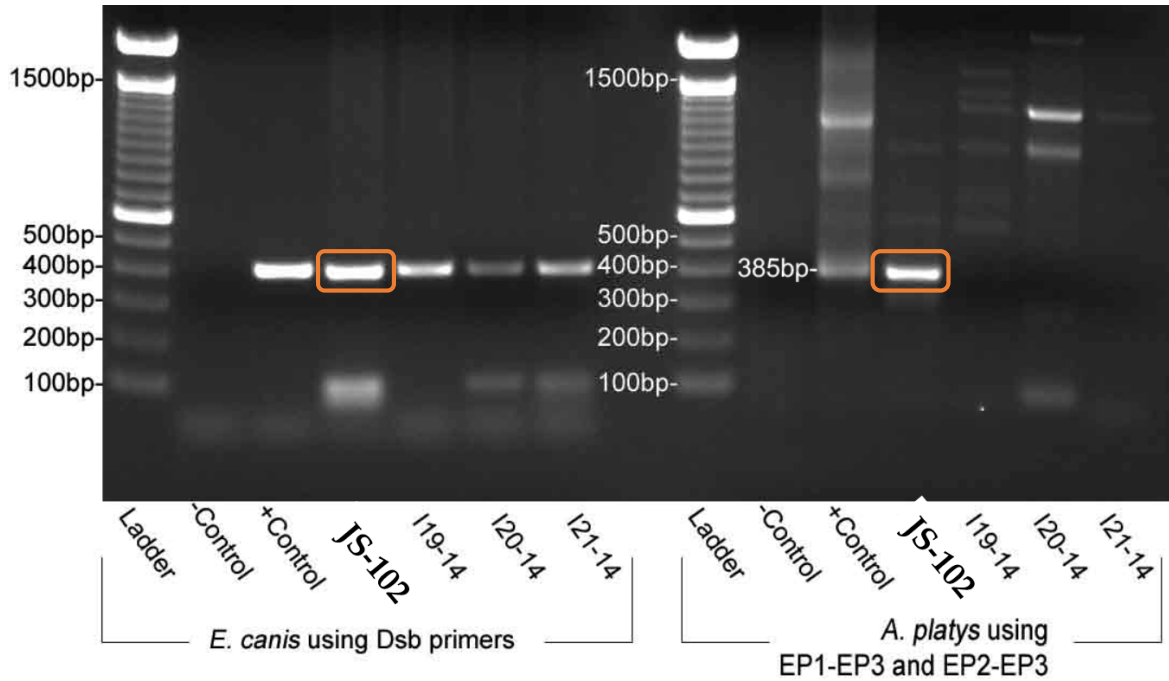
Note: Figure reprinted [56] with permissions from Elsevier. See Appendix B.



**Figure 1-7: Morphological identification of *A. platys* infecting morulae in canine platelets.**

*Note:* Blood sample from Grenadian dog, JS-102 [68]. By itself, this dog would be presumptuously identified as positive for *Anaplasma* spp., suspect *A. platys*, however a nested-PCR later confirmed the identity of *A. platys*.

**Figure 1-8: PCR confirmation of both *E. canis* and *A. platys* infection in a study dog, JS-102.**



*Note:* The presence and identity of organisms via nested-PCR was performed on all 104 Grenadian dog samples. While elaboration of mechanisms and protocols is beyond the scope of this paper, results and protocol are detailed in a manuscript submitted for publication (paper titled, 'Hematologic and serum biochemical data of dogs in Grenada naturally exposed to *Ehrlichia canis*, *Anaplasma platys*, and *Diroflaria immitis*, ' submitted for review at the Journal of Veterinary Clinical Pathology).

**Table 1-3: Level of *A. platys* DNA in buffy-coat blood cells (relative to day 0) in dogs inoculated with *A. platys*.**

Day	Dog 1	Dog 2	Dog 3	Dog 4
0	1	1	1	1
4	142	355	11,044	3754
7	5058	35,425	785,676	75,767
10	308,690	2,590,384	69,203	34,229
14	1447	20,794	85	2
17	31,350	217,874	1	1
21	1	39	1	8
24	1	249	1	736
28	6287	1	4100	1

*Note:*  $\leq 1$  = negative,  $>1$  = positive. Reproduced from [66] with permissions (see Appendix B). Values represent the number of DNA copies found in the animals' samples.

**Table 1-4: Level of *A. platys* in bone marrow and spleen (relative to day 1) in dogs inoculated with *A. platys*.**

Day	Dog 1		Dog 2		Dog 3		Dog 4	
	BM	Spleen	BM	Spleen	BM	Spleen	BM	Spleen
0	1	1	1	1	1	1	1	1
28	1	1	30	6	1	1	389	14

*Note:* Values represent the number of DNA copies found in the animals' samples. Reproduced from [66] with permissions (see Appendix B).

**Table 1-5: Serum titers of anti-*A. platys* antibody measured in serum by indirect immunofluorescence assay in dogs after inoculation with *A. platys*.**

Day	Dog 1	Dog 2	Dog 3	Dog 4
0	<40	<40	<40	<40
7	<40	<40	<40	<40
14	640	640	640	640
21	10,240	5120	5120	1280
28	5120	20,480	10,240	1280

*Note:* A titer less than 1:40 is considered a negative result. Reproduced with permissions [66] (see Appendix B).

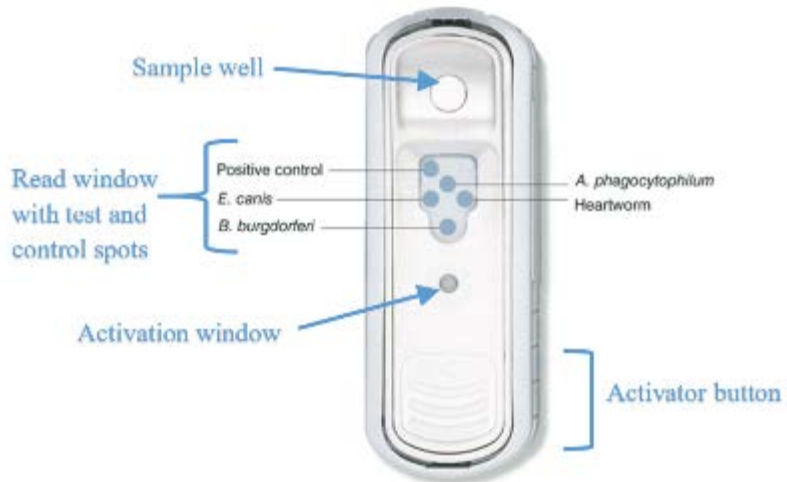


**Table 1-6: *Ehrlichia* spp. and *Anaplasma* ssp. infection progression monitored by culture isolation and PCR.**

Bacteria	Dog #	Infection status by days post infection						
		0	7	14	21	27	35	42
<i>E. canis</i>	1	-	+	+	+	+	+	+
	2	-	+	+	+	+	+	+
	3	-	+	+	+	+	+	+
	4	-	+	+	+	+	+	+
<i>E. chafeensis</i>	1	-	-	+	+	+	-	+
	2	-	+	+	-	+	+	+
	3	-	+	-	+	-	+	+
<i>A. platys</i>	1	-	+	+	+	+	+	+
	2	-	-	-	+	+	+	+
	3	-	+	+	+	+	+	+
	4	-	+	+	+	+	+	+
<i>A. phagocytophilum</i>	1	-	-	-	+	-	-	-
	2	-	-	+	+	+	+	+
	3	-	-	+	+	-	-	+
Control	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-

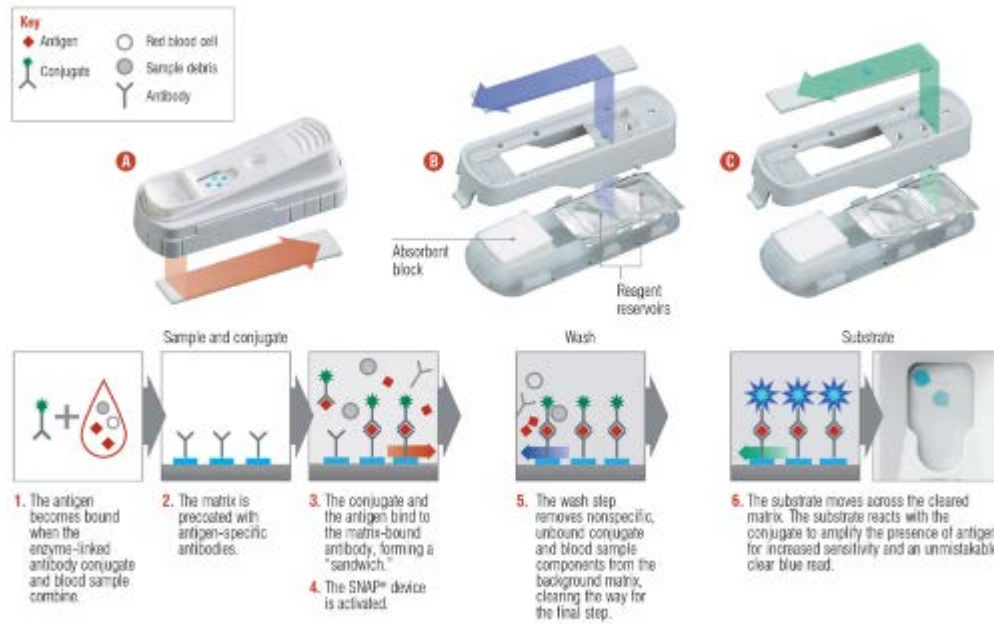
The signs – and + refer to samples tested negative or positive by culture recovery and/or by molecular method(s).

*Note:* Open access article reproduced without permissions needed [65].



**Figure 1-9: The SNAP® 4Dx® assay platform from IDEXX.**

*Note:* Image modified from [117] (open-source), using information supplemented in [70].



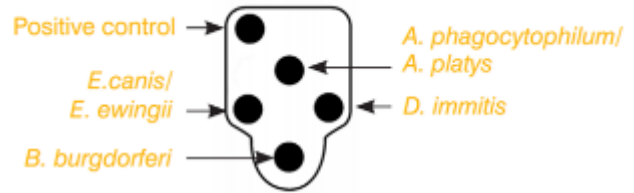
**Fig. 2.** Detailed mechanism of the SNAP device. Reaction mechanism is shown for an antigen-detection assay using immobilized antibody and an antibody-HRP conjugate. An antibody-detection assay would be similar but would use immobilized antigen and an antigen-HRP conjugate.

**Figure 1-10: Mechanism and work flow common to SNAP® 4Dx® detection assay.**

*Note:* This image displays the mechanism for detection of circulating antigens; this is the format used to screen for *Dirofilaria immitis* (representing one of the four test spots). The mechanism for detecting antibodies utilizes peptides specific to *Ehrlichia spp.*, *Anaplasma spp.*, and *Borrelia burgdorferi* (representing the remaining three of the four test spots). A citation [70], but no written permissions needed.

### Positive Result

Any color development in the sample spots indicates the presence of *Dirofilaria immitis* antigen, *A. phagocytophilum* antibody, *A. platys* antibody, *B. burgdorferi* antibody, *E. canis* antibody or *E. ewingii* antibody in the sample.



### Notes:

- The *A. phagocytophilum*/*A. platys* spot cannot differentiate between the two species: a positive result indicates presence of antibodies to *A. phagocytophilum* and/or *A. platys*.
- The *E. canis*/*E. ewingii* spot cannot differentiate between the two species: a positive result indicates presence of antibodies to *E. canis* and/or *E. ewingii*.

### Negative Result

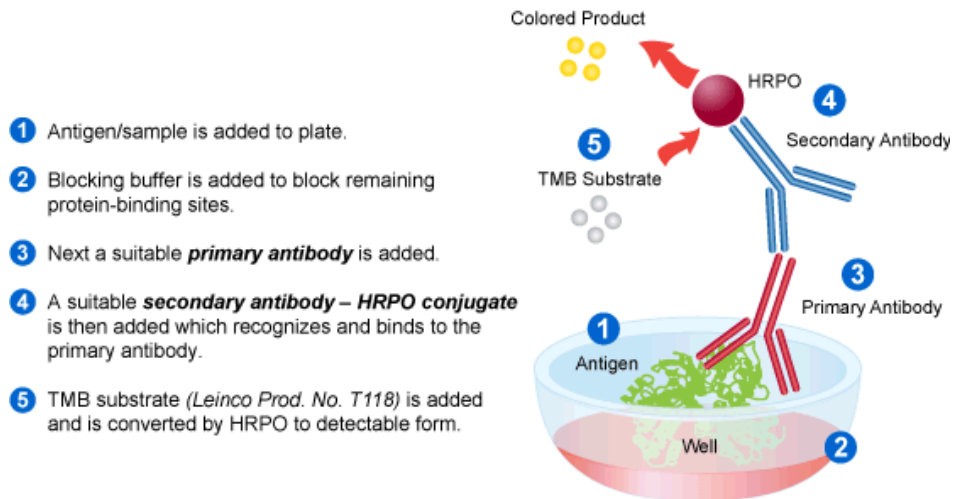
Only positive control spot develops color.



**Figure 1-11: Interpretation of SNAP® 4Dx® Plus test.**

*Note:* Image from product test-insert, IDEXX.

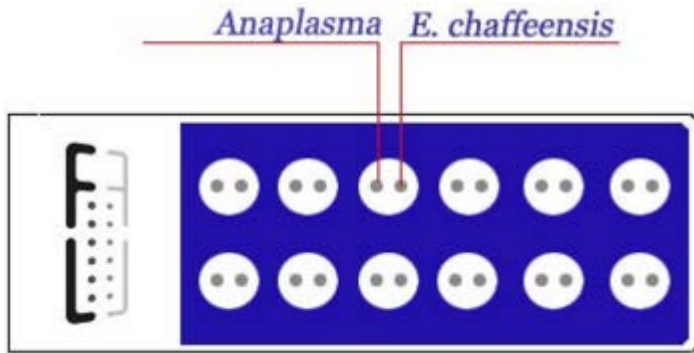
## Indirect ELISA



**Diagram 1:** Illustration of Indirect ELISA method.

### Figure 1-12: Basic mechanism behind indirect ELISA assays.

*Note:* Reproduced from [118], no permissions needed.



**Figure 1-13: Commercial micro IFA plate layout<sup>b</sup>.**

*Note:* Reproduced from [119]; citation, but no written permissions needed. This is the general setup for the commercial micro IFA tests used in this research study, although *E. canis*, and not *E. chaffeensis* organism was used to plate the slides. Test from the manufacturer’s product insert loosely describes how their modified “proprietary techniques” affect the performance of their assay. To quote:

*“We have crafted the MIF with a number of proprietary techniques intended to produce the most native antigens possible. The resulting antigens are uncommonly accurate. Benefits of the MIF include increased specificity for both antigens, significant decrease in cross-reactivity of Anaplasma with high-titer Ehrlichia reactivity, no false-positive IgM tests for Anaplasma or Ehrlichia, no non-specific reactivity against the host cells (cytoplasmic or membrane), and equivalent sensitivity with standard IFA Basically, this MIF format is either a starry-night appearance (positive) or not (negative). The trade-off is that you get the correct result in less time at the scope.” [119].*



**Figure 1-14: Interpretation of IFA test results.**

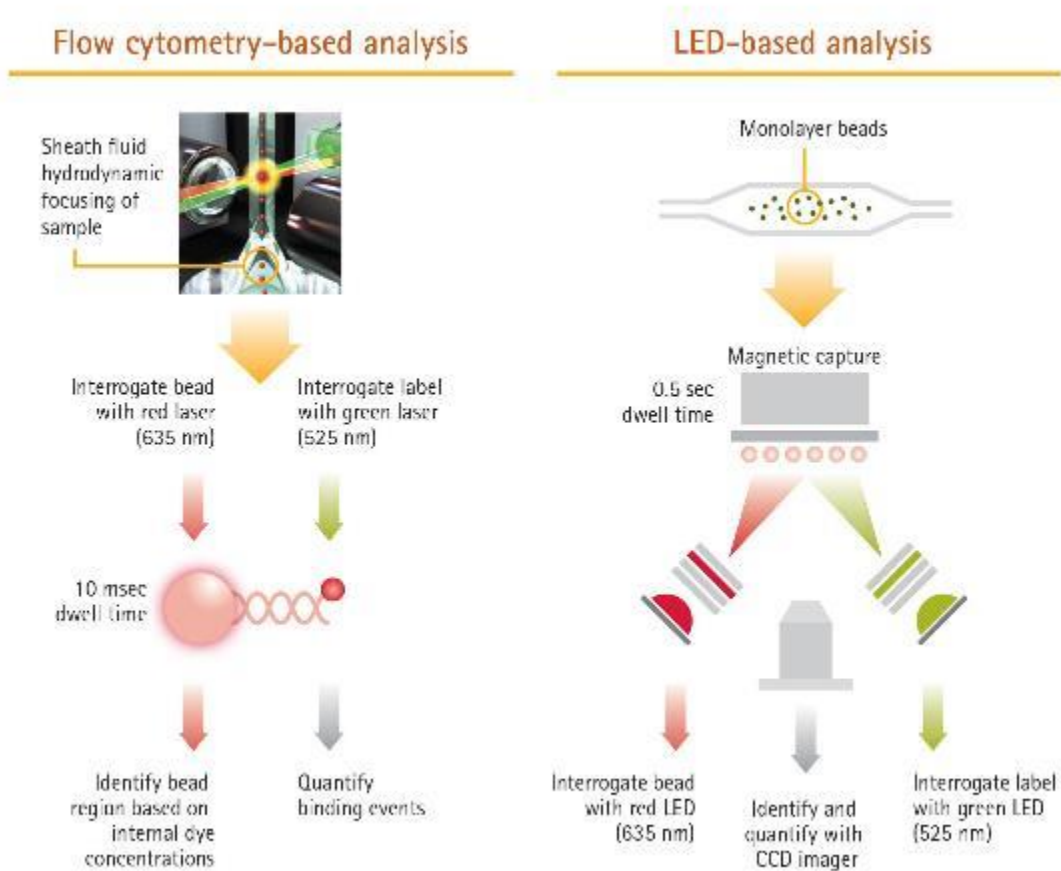
*Note:* A negative test result (left), a weakly positive result (middle), and a positive result (right) showing “starry-night” [\[119\]](#) positive staining. Images taken of Grenadian dog samples used in this study.

**Table 1-7: Serological cross-reactivity between *E. canis* whole antigen (via IFAT) and common rule-outs for canine vector-borne pathogens.**

<b>Canine vector-borne organism</b>	<b>Relative degree of positive serological cross-reactivity</b>
<i>Ehrlichia chaffeensis</i>	+ + +
<i>Ehrlichia ewingii</i> *	- / + +
<i>Anaplasma phagocytophilum</i>	- / +
<i>Anaplasma platys</i>	-
<i>Dirofilaria immitis</i>	-

Note: Table modified from [50]. -, no serologic cross-reactivity; +, weak cross-reactivity; + +, intermediary cross-reactivity; + + +, strong, high affinity cross-reactivity. \*, Uncertainty with *E. ewingii* in particular, but also in all these organisms due to their inherent antigenic variability across the globe.

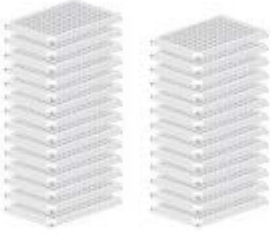





**Figure 1-15: Flow-based vs CCD fluorescent based Luminex® xMAP® instrumentation.**

*Note:* Reproduced from [120]; citation, but no written permissions needed.

**Table 1-8: Hypothetical scenario for protocol comparison between traditional ELISA assays and Luminex® xMAP® assays.**

	ELISA	Luminex® xMAP® assays
Number of cytokines	27	27
Number of samples	80	80
Total data points	2,160	2,160
Number of 96-well plates	27 	1 
Data points per plate	80	2,160
Total time required	>60 hr	3 hr
Sample volume	Serum or plasma, >1 ml* Cell culture supernatant, >1 ml*	Serum or plasma, 12.5 µl Cell culture supernatant, 50 µl
Assay range	Serum or plasma, 2–3,000 pg/ml Cell culture supernatant, 2–3,000 pg/ml	Serum or plasma, ~0.2–3,200 pg/ml Cell culture supernatant, ~2–32,000 pg/ml

*Note:* Figure modified from [88]; citation, but no written permissions needed. Values based upon the recommended sample volume of 50µl/well.

The diagram shows the mathematical formula for Bayes' theorem:  $P(H|E) = \frac{P(H) * P(E|H)}{P(E)}$ . Four orange arrows point from descriptive text to the corresponding terms in the formula: 'Prior Probability' points to  $P(H)$ , 'Likelihood of the evidence 'E' if the Hypothesis 'H' is true' points to  $P(E|H)$ , 'Posterior Probability of 'H' given the evidence' points to  $P(H|E)$ , and 'Prior probability that the evidence itself is true' points to  $P(E)$ .

$$P(H|E) = \frac{P(H) * P(E|H)}{P(E)}$$

Prior Probability

Likelihood of the evidence 'E' if the Hypothesis 'H' is true

Posterior Probability of 'H' given the evidence

Prior probability that the evidence itself is true

**Figure 1-16: The mathematical basis of Bayes' theorem.**

*Note:* Citation [[121](#)], but no written permissions needed.

## **Chapter 2 - Materials and Methods**

## Acquisition of samples

### Controls

Serum samples from three purpose bred beagle dogs were tested for antibodies to *E. chaffeensis*, *E. canis*, and *A. platys* before and after experimental infections [68]. One of the three dogs was infected with *E. chaffeensis*, one with *E. canis*, and one with *A. platys*. The infection protocol was approved by the Public Health Service (PHS) Policy on the Humane Care and Use of Laboratory Animals, the US Department of Agriculture's Animal Welfare Act & Regulations (9CFRchapter 1, 2.31), as well as the Institutional Animal Care and Use Committee at Kansas State University. The source of the infectious inoculum for each bacterium and details of the protocol has been described in previous literature [122]. Positive control serum used to test the multiplex assay and the IFA was collected from *E. chaffeensis*, *E. canis* and *A. platys* infected dogs at days 14, 27 and 35 post infection; selected at these intervals based upon their peak total IgG ELISA values. Negative control serum was obtained from these dogs prior to infection and was confirmed via nested PCR and ELISA [65].

### Test subjects

The 104 dogs from Grenada were community-owned dogs principally from St. George's parish and outlying parishes brought to the Veterinary Teaching Hospital at St. George's University School of Veterinary Medicine for assessment and blood sampling prior to spay and neuter surgeries (September through December of 2014). Collection of blood samples from these dogs was approved by the Institutional Animal Care and Use Committee at St. George's University. The population consisted of 30 male dogs and 74 female dogs, with a median age of 1.8 years (range of 2 months to 8 years). 97 were mixed

breed dogs (referred to as ‘pothounds’), 3 were allegedly pure-bred dogs, and 4 were Pomeranian/poodle or Pomeranian/Pekinese crosses. Wherever possible, animals’ full histories were recorded and later transcribed into a spreadsheet.

### **IFA, and SNAP® 4Dx® testing**

At the time of presentation, all dogs were screened for the presence of antibodies to *E. canis*, *A. platys*, *Borrelia burgdorferi*, and *Dirofilaria immitis* using the SNAP® 4Dx® test, and were processed using whole blood according to the manufacturer’s instructions<sup>a</sup>. Results were recorded, filed, and transferred to a spreadsheet at a later date.

Serum obtained from each animals’ blood sample was separated into small aliquots and stored at -20°C for the duration of the sampling period and shipped to Kansas State University on dry ice. Samples were organized and stored at -80°C until use for various testing, and were then stored in the dark at 4°C.

Commercially available micro-IFA kits<sup>b</sup> were purchased and included pre-diluted positive and negative canine control serum samples. The company reports that the 12 wells on the slide are coated with paired, elementary bodies and morulae of *E. canis* and *A. phagocytophilum* from propagated cell culture. They described their proprietary semi-purification process as “[removing] the vast majority of host cell cytoplasmic, nuclear and membrane constituents that are common sources of ‘non-specific’ fluorescence.” Serum samples of experimentally and naturally infected dogs were diluted and tested<sup>b</sup>. Samples

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<sup>a</sup> SNAP® 4Dx® Test Package Insert, IDEXX Laboratories Inc., Westbrook, ME.

<sup>b</sup> *E. canis* and *A. phagocytophilum* MIF Canine IgG Antibody Kit, Fuller Laboratories, Fullerton, CA.

were considered positive if they reacted at a dilution greater than 1:80, as per test instructions.

## Optimization of novel bead assay

### Design of detection antigens

To develop the indirect, serological multiplex bead assay, unique peptides were chosen for each of the assay's four pathogens. As many of the *Anaplasma* and *Ehrlichia* spp. share a close homology to one another, it was important for the selected peptide sequences to be short, discrete, immunodominant sequences to help minimize cross-reactivity, but not so short as to sacrifice strong and specific antibody binding. Immunogenic portions of the organisms' outer membrane proteins were selected using published sequences from the literature and are as follows: *E. chaffeensis* P28-19 [Figure 2-3] [123], *E. chaffeensis* P28-14 [Figure 2-4] [124], *E. canis* P30 [Figure 2-6] [125], and *A. platys* OMP-IX [Figure 2-5] [77]. Another argument for the use of the *A. platys* OMP-1X peptide was that it was shown not to cross-react with mouse antibodies to *A. phagocytophilum*, though it had yet to be tested with canine serum [77]. These peptide sequences were between 10 and 15 amino acids in length, and individually, their molecular mass was less than 2.0 kDa. A summary of this information as well as the peptides' specifications can be found at the end of the chapter, in Table 2-1.

Peptides were synthesized and modified<sup>c</sup> by the addition of polyethylene glycol acid (PEG 5), a 19-atom, nonimmunogenic spacer molecule. In short, the PEGylation was performed by attaching the carboxyl terminus of PEG5 to the primary amine N-terminus

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<sup>c</sup> Pierce Biotechnology, Thermo Scientific Protein Biology, Rockford, IL.

of each peptide by an amide bond [Figure 2-1Figure 2-2]. This was done to position peptides farther away from the bead surface (and other peptides) to provide abundant space for a detectable number of antibodies to bind to each of the bead units [120]. Peptide concentration was confirmed<sup>d</sup> using serial dilutions of BSA in triplicate to create a standard curve to which we comparing the OD of our peptide stock dilutions<sup>c</sup>, prior to being separated into small aliquots and stored at -80°C. To maintain the structural integrity of the peptides, each aliquot was allowed to thaw at RT only one time—immediately prior to bead conjugation.

### **Conjugation of peptides to beads**

Four separate MagPlex<sup>®</sup> magnetic bead regions<sup>e</sup> were selected [Figure 2-7] and coupled<sup>f</sup> to 20 µg/mL of NH<sub>2</sub>-PEG5 modified peptides<sup>c</sup>, using a two-step carbodiimide reaction to chemically couple the carboxylated bead regions to the primary amine N-terminus of each peptide<sup>c</sup> [Figure 2-2]. Peptide-coupled beads were resuspended to a final concentration of 2 X 10<sup>6</sup> per mL in storage buffer<sup>g</sup> and kept in the dark at 4°C, as per manufacturer's recommendations. Serum samples from experimentally-infected dogs [65] were tested against peptide-coupled beads to confirm conjugation [126].

### **Procedural optimization of bead assay**

#### **Optimizing and plating working bead-stock**

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<sup>d</sup> Pierce™ BCA Protein Assay Kit, Thermo Scientific, Rockford, IL.

<sup>e</sup> MagPlex<sup>®</sup> beads, Luminex Corporation Inc., Austin Tx.

<sup>f</sup> xMap<sup>®</sup> Antibody coupling kit, Luminex Corporation Inc., Austin, TX.

<sup>g</sup> Storage buffer: PBS 0.05% / Tween-20/0.05% sodium azide, pH 7.4, Sigma P3563, St. Louis, MO.



With the *Ehrlichia/Anaplasma* peptides successfully coupled to four distinct bead sets [Table 2-1], a working stock of the four peptide-coupled beads was prepared prior to each assay. The working stock was formulated such that the final concentration of each bead region was 100 microspheres/ $\mu\text{L}$  in filtered assay buffer<sup>h</sup> [120]. Total microsphere count per well was optimized at 1,250 beads/well, in order to balance the cost of the peptide-coupled microspheres while insuring the MagPix<sup>®</sup> probe had access to an absolute minimum of 50 microspheres per bead-set, per well [120]. Fifty  $\mu\text{L}$  of the microsphere stock was deposited into wells of a 96-well plate<sup>i</sup> containing 50  $\mu\text{L}$  of filtered assay buffer. Microsphere stock was briefly vortexed and sonicated after every 16 wells plated (two columns of wells) via a brief vortex to keep beads evenly distributed in suspension.

### **Step 1: Serum dilutions and initial plating**

To determine the optimal dilution of serum for the assay, serum samples from the three experimentally infected dogs, and twenty Grenadian dogs were serially diluted (1:100, 1:500, 1:1000 and 1:2500) in assay buffer<sup>h</sup> containing 0.1% Chemiblocker<sup>j</sup> [127], and plated with 50  $\mu\text{L}$  of diluted sample per duplicate well. Background wells, designed to measure the degree of non-specific binding, consisted of the microsphere mixture and assay buffer but no serum. They were run in duplicate (plating by a repeater pipet<sup>k</sup>) and

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<sup>h</sup> Assay buffer: PBS 0.05% Tween-20, 0.1% Chemiblocker, pH 7.4.

<sup>i</sup> Greiner black plate, 65509x, Monroe, NC.

<sup>j</sup> EMD Millipore, Billierica, MA.

<sup>k</sup> Eppendorf Repeater M4, Eppendorf, Hauppauge, NY.

incorporated into the beginning and the end of every plate. All wells were sealed using an adhesive cover film<sup>1</sup>, secured to a plate shaker (500 rpm), and incubated overnight at 4°C.

### **Step 2: Adding secondary antibody**

The following morning, plates underwent washing starting by setting plates atop a magnetic separator<sup>m</sup> for one minute to pellet the semi-metal microspheres out of suspension. The supernatant from each well was decanted by forceful manual inversion while still attached to the separator [126]. Secondary antibody, biotin-affinity purified-goat anti-dog IgG H+L<sup>n</sup>, was diluted to a concentration of 2µg/mL from stock stored at -20°C, and 50µl were added to each well. Plates were resealed, and incubated on a shaker in the dark at RT for 1 hour.

### **Step 3: Adding detection tag**

At the end of the 1-hour incubation wash steps were performed as previously described. 50µL of assay buffer was then added to microwells for resuspending beads, and a pink fluorescent tag<sup>o</sup>, phycoerythrin (PE)-labeled strep-avidin, was diluted to a final concentration of 4 µg/mL, and 50µL of the tag was added to each well. Plates were resealed and incubated at RT for 30-minutes on a shaker. A final wash sequence was performed to remove unbound PE conjugate, and microspheres were resuspended in 100

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<sup>1</sup> PlateMax™ Microplate Sealing Film, Fisher Scientific, Pittsburg, PA.

<sup>m</sup> Magnetic Plate Separator®, Luminex Corporation Inc., Austin, TX.

<sup>n</sup> Secondary antibody: biotin-affinity purified-goat anti-dog IgG H+L, at 2 µg/mL, KPL Laboratories, Gaithersburg, MD.

<sup>o</sup> Fluorescent tag: phycoerythrin (PE)-labeled strep-avidin at 4µg/mL, Life Technologies, Grand Island, NY.

$\mu$ L of Luminex<sup>®</sup> drive fluid prior to sending it through the MAGPIX<sup>®</sup> instrument for LED interrogation and CCD analysis.

### **Instrument maintenance**

Many of the settings were used as recommended by the Luminex<sup>®</sup> MAGPIX<sup>®</sup> manual, including the running of verification/calibration, and a startup cleaning-operation protocol on days that plates were to be run. On a monthly basis, the MAGPIX<sup>®</sup> probe was manually removed before being sonicated (for even several minutes at a time) and thoroughly flushed with ethanol and deionized water until all traces of debris were removed.

### **Establishing a case-definition**

Background wells, negative control serum, and known-positive control serum from experimentally-infected dogs were allocated in every plate. The mean MFI of duplicate wells were calculated using xPonent<sup>®</sup> software. The multiplex bead assay data was converted into categorical values (1= test positive, 0= test negative) using replicates of the negative control dog sera and calculating two standard deviations of the duplicate MFIs as the cutoffs. Any dog whose MFI exceed this cutoff value was defined as positive; those at or below two standard deviations was considered to be negative.

### **Statistical methods**

Tests were run to verify that the multiplex format did not have a significant effect on mean MFIs compared to when they were run in monoplex. To do this, six serum samples were selected that, as a subgroup, exemplified range values – low-range, mid-range, and high-positive range of MFIs for each of our bead-sets. These six dogs were run

in multiplex and monoplex format (in duplicate) to verify that running samples in monoplex and multiplex did not yield dissimilar results. Mean MFIs of disease positive and negative animals were calculated and compared by using Spearman rank correlations<sup>p</sup>. A Kappa test<sup>q</sup> was used to measure the degree of agreement between three possible combination of test pairings; IDX and IFA, IDX and MAG, IFA and MAG). The standard categorical ranges can be referred to in Table 2-2.

A Kruskal-Wallis ANOVA and Dunn's multiple comparisons test were performed to determine differences in the dog sera mean MFIs among the multiplex peptide-bead analytes<sup>p</sup>. Wilcoxon signed rank test (nonparametric t test) was used to compare the seroreactive and seronegative test results of the Grenadian dog samples in the multiplex assay and commercial ELISA. Significance level was set at  $< 0.05$ .

### **Bayesian priors**

In this study, Bayesian analysis was used only to evaluate each tests' performance for *Ehrlichia canis*, because frequentist analysis had shown promising results for that agent, but not for *Anaplasma platys* (additional investigation needs to be done to improve the peptide's reactivity in order to warrant the application of Bayesian methods). This was done using an open-source software<sup>r</sup> to examine the performance and degree of conditional dependence of our three assays in light of informative prior estimations.

Bayesian modeling of the *Ehrlichia canis* test performance in this study was not ideal—while we did use three tests, our sample size was relatively small for utilizing Bayesian

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<sup>p</sup> Graphpad Prism6.05, La Jolla CA.

<sup>q</sup> Analyse-it Software, Method Evaluation Edition, Version 4, Leeds, United Kingdom.

<sup>r</sup> OpenBUGS Software, Version (3.2.3), MRC Biostatistics Unit, Cambridge, United Kingdom.

analysis in one population [106]. As such, calculating appropriate priors was essential to the integrity of our Bayesian analysis. To briefly summarize—informative priors were calculated to estimate the prevalence and credible intervals of the prevalence, sensitivity, and specificity of the IFA, SNAP® 4Dx®, and Magpix bead-assay.

### **Calculation of informative priors**

#### ***Priors for prevalence***

Estimation of informative priors for the prevalence of *E. canis* exposure in Grenadian dogs was calculated from data in a study [16] using samples from 2004 and 2006 [Table 3-6]. We also took into account yearly trends in regional seroprevalence (it's increasing) and the effect of preventive care initiatives that have no doubt been employed on some level since 2006 in the general population (judging by the growing presence of the veterinary medical program on the island, and government funding of rabies prevention) [128, 129]. As a result, we assigned the prior estimation at a conservative level (42% seroprevalence of *E. canis*) with a fairly uncertain 95% confidence interval (95% sure that seroprevalence is >22%). The beta distribution is then calculated using a software<sup>s</sup> to generate beta distribution ( $\alpha$ ,  $\beta$ ) values that were used to describe the prevalence mode and credible probability distribution in the Bayesian software<sup>r</sup>.

#### ***Priors for SNAP® 4Dx®***

Reasonable estimation of the SNAP® 4Dx® test performance for *E. canis* detection was more complex; ultimately, sensitivity and specificity priors were calculated using company-advertised data<sup>a</sup>, as well as from studies using the test in naturally-infected

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<sup>s</sup> BetaBuster, software version 1.0, UC Davis, Davis, CA.

populations. Use of company-advertised data was utilized with pragmatism—in that we recognized the likelihood of bias in their reports<sup>a</sup> [94], but conceded that we lacked adequate information of the IDEXX populations and our Grenadian sample to warrant the rejection of the tests’ reported performance in the assessment of our own data. As such, the IDEXX-advertised sensitivity/specificity was included in our consensus informative prior and low-consensus informative prior modeling [Table 3-7].

Other studies using SNAP<sup>®</sup> tests were screened from published literature with an essential condition: they needed to be published within the last fifteen years (as the advent and utilization of PCR was necessary to confirm the identity of pathogens in the population). In this time frame, 18 studies were found that used SNAP<sup>®</sup> 3Dx<sup>®</sup> or 4Dx<sup>®</sup> (both tests use the same *Ehrlichia canis* p30-1 peptides which cross-react with *E. chaffeensis* and occasionally *E. ewingii*) that also performed some sort of species-specific verification in parallel. Further selection criteria were established to identify those of the 18 studies that had a similar study design to our own (surveillance sampling a naturally-infected *canine* population with a high seroprevalence, and definitive confirmation the pathogen was *E. canis*), that either presented their own sensitivity/specificity and prevalence or else provided adequate data for the SNAP<sup>®</sup> performance to be calculated. Two [78, 80] of the eighteen studies met these requirements, and their performance data (including confidence intervals) are listed in Table 3-7.

Using this frequentist data, two sets of informative priors were calculated (and later used to generate the beta distribution of *credibility*): the “IDX Consensus Prior” and the “IDX Low-Consensus Prior” [Table 3-7]. The IDX Consensus Prior was calculated by taking the mean of the three studies’ sensitivity and specificity, including a 95%

confidence interval which was calculated by taking the mean of the tests' 5% confidence interval. The other set of informative priors, the IDX Low-Consensus Prior, was calculated in a slightly different manner—the mode of sensitivity/specificity for the Low-Consensus Prior was determined using the lowest mean sensitivity/specificity of the SNAP® test as reported by the three studies. The confidence interval<sup>t</sup> for the IDX Low-Consensus Prior was determined by taking the 5% confidence parameter of the CP model, and dividing it in half [Table 3-7].

Using the performance and prevalence data from [78, 80], as well as the data provided by the IDEXX test insert, the mean sensitivity, specificity and 95% confidence intervals of each test were calculated [Table 3-7] [130]. Beta distributions of the mode and credible distribution for the prior models were calculated<sup>s</sup> as previously described.

### ***Priors for IFA testing***

Literature on IFA testing, specifically using *E. canis* to detect *E. canis* specific seroreactivity in a naturally infected population was also sought; unfortunately studies were not identified. This was due most often to studies failing to cite the species and strain of the *E. canis*-infected cells they plated, but more often than not, most studies simply used the IFA on face-value as a gold standard test. Inquiry of Fuller Laboratories (Fullerton, CA) technical department for more specifics on their *E. canis* strains used yielded an estimation of sensitivities/specificities of their assays to be >98%, but further disclosures and data were not given. IFA Consensus Priors and IFA Low-Consensus priors were calculated as described previously [Table 3-8].

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<sup>t</sup> OpenEpi: Open Source Epidemiologic Statistics for Public Health, Dean A.G. et al.,

### ***Priors for MAG testing***

Priors for the Magpix assay were estimated and given a wide probability distribution in an effort to not inject bias into the model [Table 3-9]. Non-informative priors were also used in place of low-consensus priors to assess the Magpix performance (without any prior expectations on assay performance) with the IDX and IFA tests, given the seroprevalence.

### **Sensitivity and specificity testing**

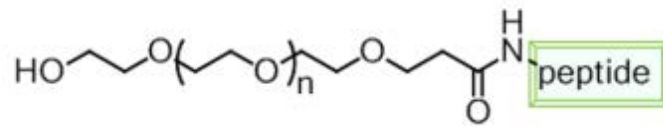
Multiple combinations of each tests' consensus priors, low-consensus priors, and two sensitivity-testing models [Table 3-11] were combined, and run in OpenBUGS with a three test one population model using test-outcomes of Grenadian dogs for the IFA, IDX, and MAG assays [Table 3-10]. The model was given a burn-in period of 20,000 iterations, and nodes were given an additional 20,000 iterations to compute the models' DIC ('deviance information criterion,' or the measure of model fit),  $pD$  ('point deviance'; the number of parameters that were estimated) to evaluate model fit, and bayesp (the Bayesian posterior probability, interpreted as a probability distribution centered around a mean estimate – 0.50 indicating acceptance of the null-hypothesis) [Table 3-12] to assess and identify the overall best model fit.



## Tables and Figures

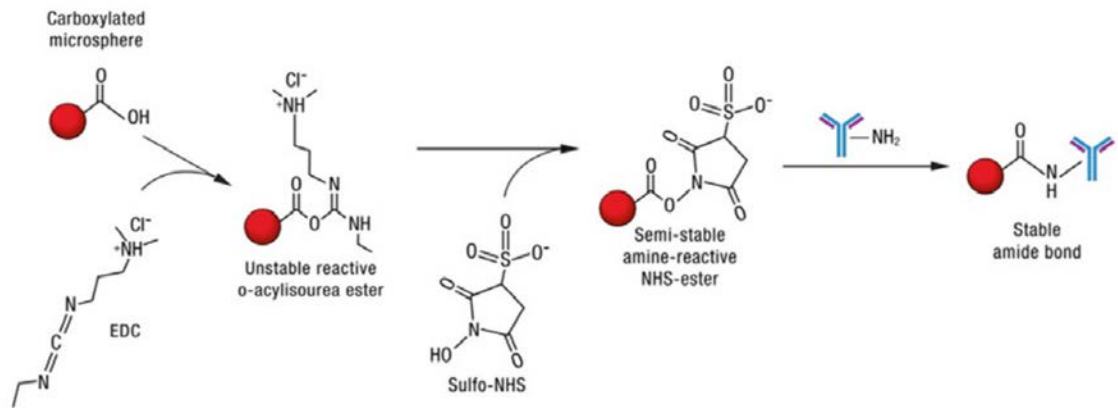
**Table 2-1:** Peptide sequence, source and bead region identifier.

Organism	OMP	GenBank ID (Isolate origin)	Peptide size	Peptide sequence with spacer and functional groups	Bead Region
<i>E. chaffeensis</i>	P28-19	AAO12932.1 (Arkansas) <a href="#">[123]</a>	15 AA	NH <sub>2</sub> -[PEG5]- VFGLKQNWDGSAISA- COOH	R15
<i>E. chaffeensis</i>	P28-14	AAO12929.1 (Arkansas) <a href="#">[124]</a>	14 AA	NH <sub>2</sub> -[PEG5]- VFGLKKDGDIAQSA- COOH	R34
<i>E. canis</i>	P30	ACC85904.1 (Jaboticabal, Brazil) <a href="#">[125]</a>	15 AA	NH <sub>2</sub> -[PEG5]- VFGLKEEWNGGTIPA- COOH	R48
<i>A. platys</i>	OMP- 1X	ADU56847.1 (Taiwan) <a href="#">[77]</a>	10 AA	NH <sub>2</sub> -[PEG5]- AVQEKKPPEA-COOH	R53



**Figure 2-1: Generic formula for variable length PEG spacer-molecule with respective peptide and MagPlex® binding sites.**

## Method



**Figure 2-2: Two-step carboimide reaction used to couple peptides to MagPlex® beads**

*Note:* Image reproduced and modified from [131], no permissions needed.

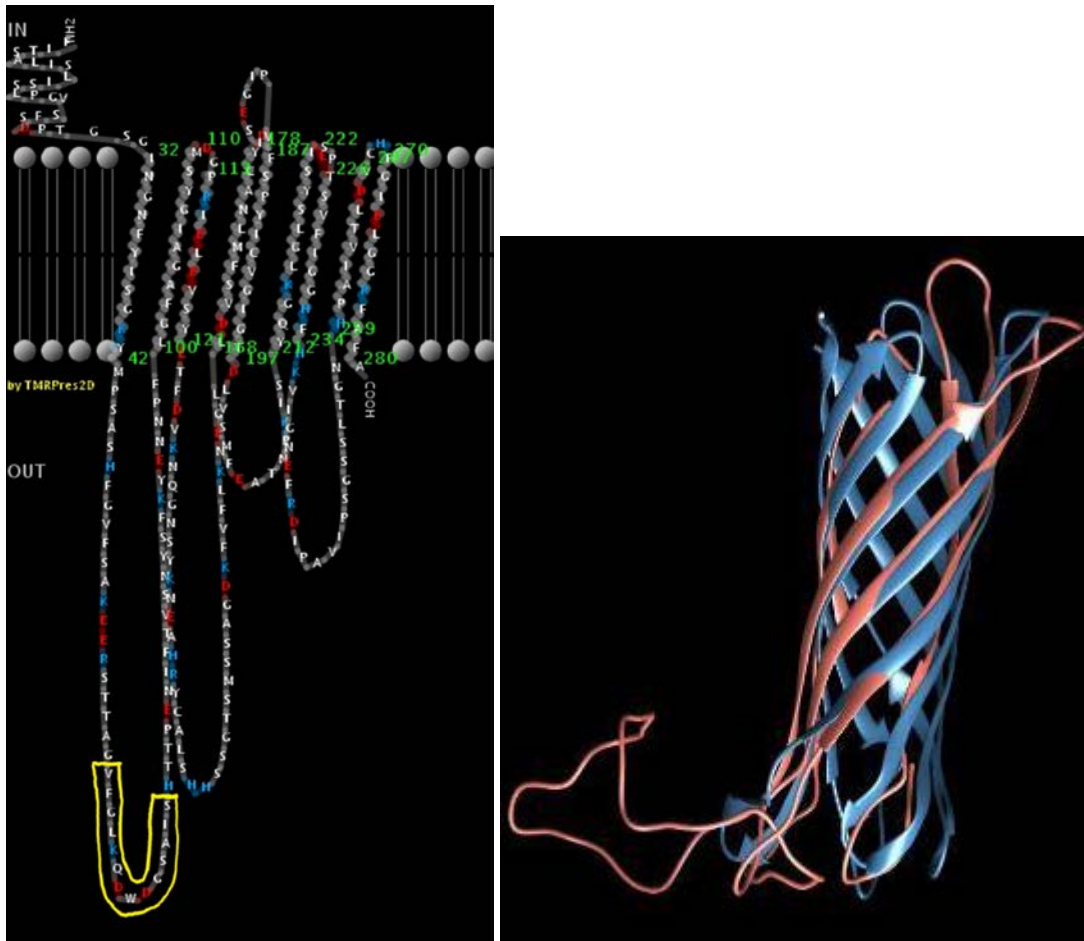


Figure 2-3: *E. chaffeensis* P28-gene 19 peptide sequence highlighted in its native protein in two dimensions [132] and three dimensions [133].

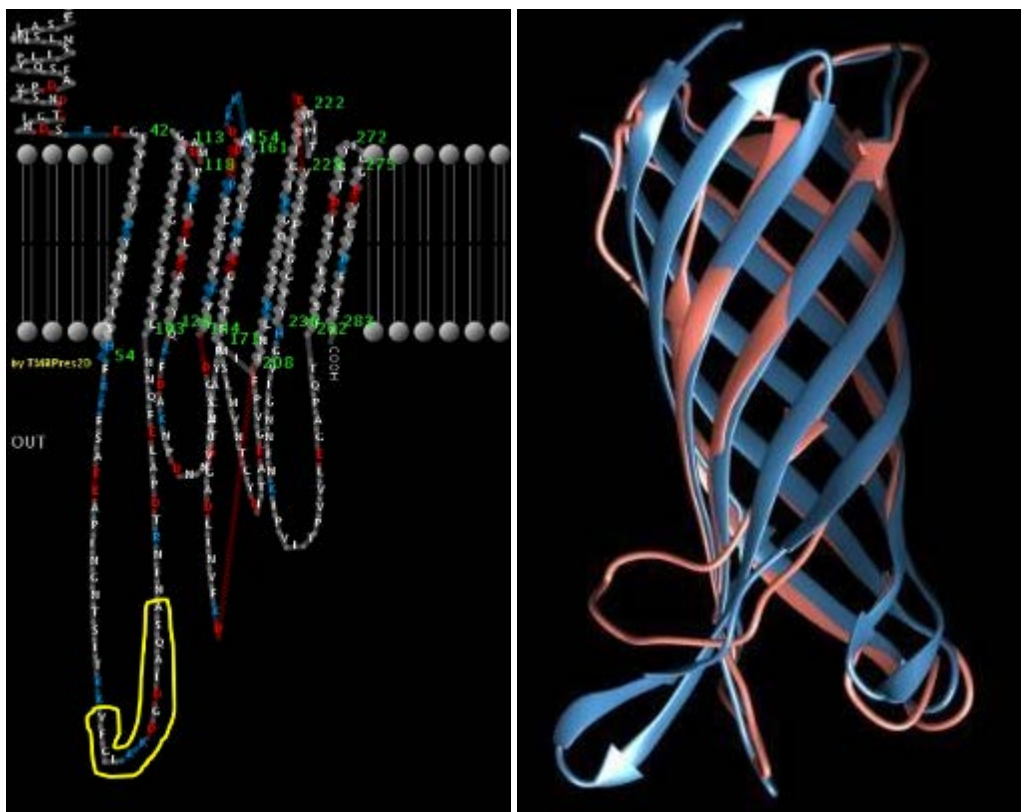


Figure 2-4: *E. chaffeensis* P28-14 detection peptide sequence identified within its native OMP structure in two dimensions [132], and three dimensions [133].

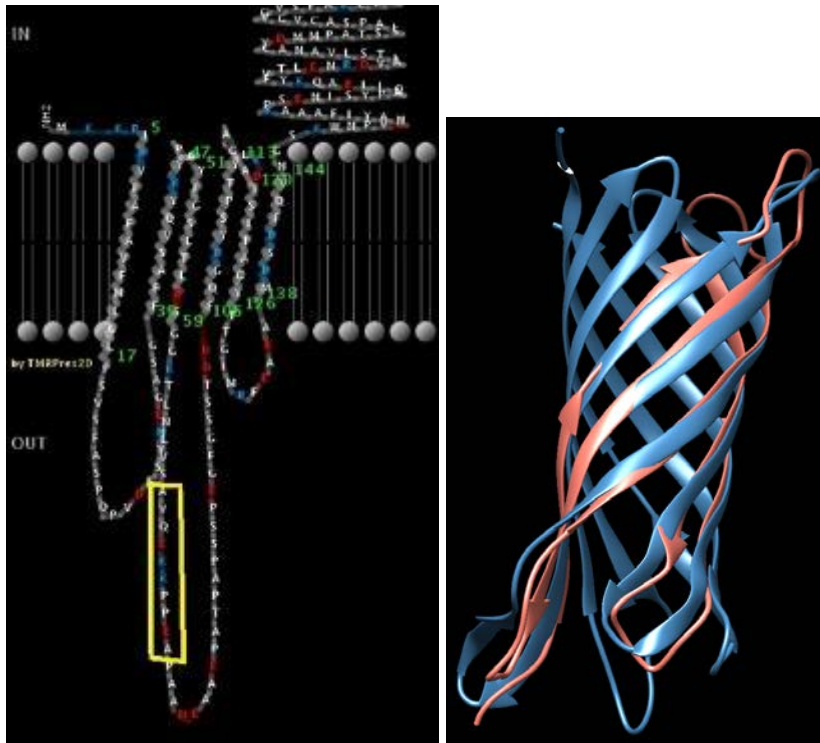
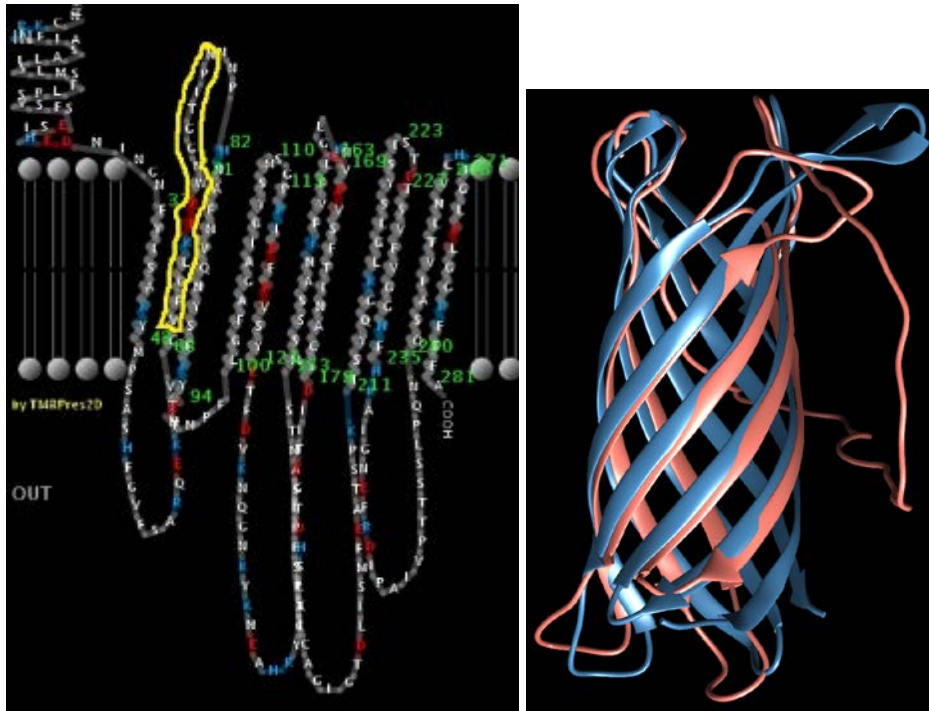
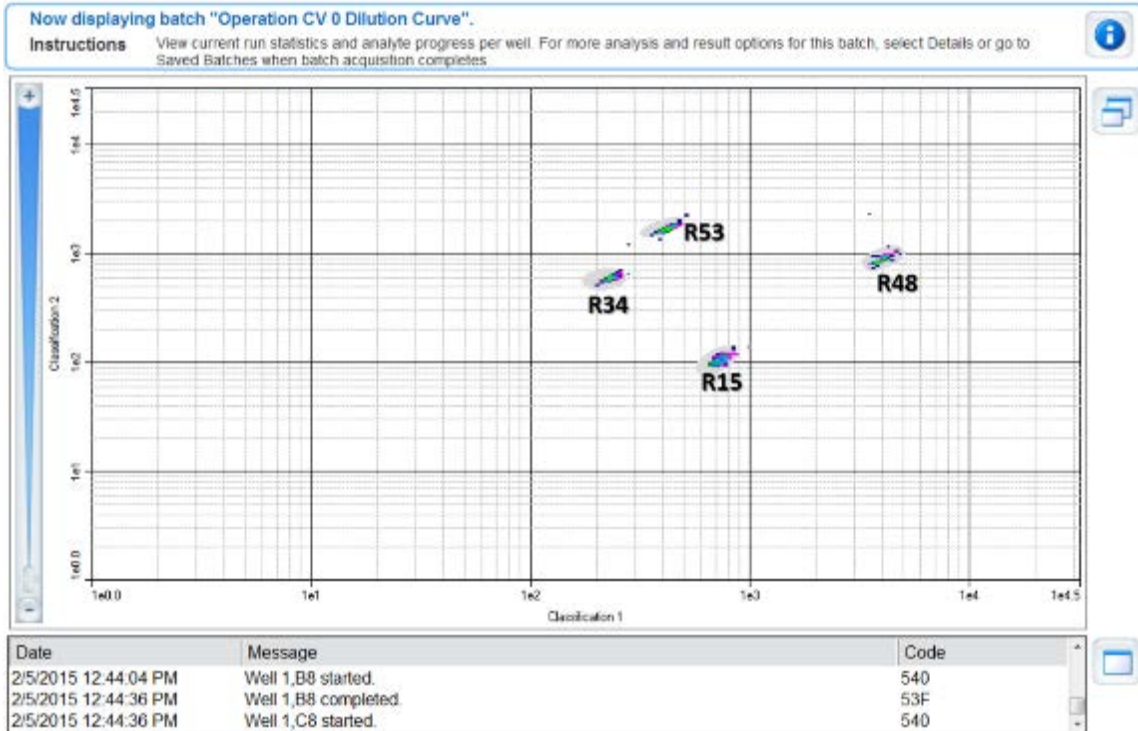


Figure 2-5: Location of *A. platys* peptide sequence identified in its native structure in two dimensions [122] and three dimensions [133].



**Figure 2-6: *E. canis* P30 sequence as identified in its native porin structure.**

*Note:* Schematic created using an online program, [132], and three dimensions [133].



**Figure 2-7: MAGPIX® map of MagPlex® fluorescent-bead regions.**

*Note:* This is a screenshot of our actual bead-regions used.



**Table 2-2: Interpretation of Kappa statistic for assessing test agreement.**

<b>Kappa statistic</b>	<b>Agreement</b>
< 0.20	Poor
< 0.40	Fair
< 0.60	Moderate
< 0.80	Good
to 1	Very good

*Note:* Adapted from [\[82\]](#).

## **Chapter 3 - Results and Discussion**

## Frequentist Results

### Controls

The results of the multiplex assay using serum collected from the experimentally infected dogs are shown in Table 3-1. Cut offs for positive samples for each bead set were determined by calculating 2 standard deviations of the mean MFIs of the negative control samples [Table 3-1]. Dogs whose serum MFI was equal to, or higher than that cut-off, were classified as positive for that pathogen.

As anticipated, there was some cross reactivity among sera from *E. canis*, *E. chaffeensis*, and *A. platys* experimentally infected dogs and in the naturally infected Grenadian dogs. However, the highest MFIs were *always* detected in dogs exposed to the same antigen as on the bead (bolded numbers with asterisk in Table 3-1) as opposed to the cross-reactive species. Serum from the *A. platys* experimentally infected dog did not competently seroreact with the *A. platys* peptide, as the MFI was below the negative control cut off.

### In-house ELISA

Since the positive control used to establish cutoffs for *A. platys* microspheres failed to react, we translated the monoplex version of our bead assay into a laboratory type ELISA [Figure 3-1] [65]. The in-house ELISA performed similarly to the bead-assay, as the ODs detected by the ELISA were higher for *E. chaff-19* and *E. canis* p30, and lower for *E. chaff-14* and *A. platys* OMP1X. Furthermore, serum from a Grenadian dog reacted to *A. platys*-coated wells with a higher OD than the *A. platys* experimentally infected dog [Figure 3-2]. This was performed to assess whether the low seroreactivity to *A. platys* OMP1X peptides was due to the change in assay platform (i.e. unexpected binding

interactions of peptides to microspheres vs ELISAs, causing an adulteration in peptide conformation and interfering with the formation of antibody-peptide immunocomplexes) or if further studies were needed to explore a possible antigenic drift of OMPIX peptides derived from Taiwanese strains of *A. platys* from the Floridian strain used to experimentally infect our positive control dog.

### **Test Precision**

The measurement of analytical specificity of serum antibody to *E. chaffeensis*, *E. canis*, and *A. platys* peptide-coated beads was compared by running bead assays on individual bead sets (monoplex) and by a multiplex format (i.e. all bead sets were placed in a single well and incubated with serum) in the same plate. The monoplex bead-assay results correlated well with the multiplex bead assay results for each bead set. The intra-assay precision determined by the coefficient of variation (CV) calculation was < 18% for all bead sets. The inter-assay precision was < 15% for all bead sets except *A. platys* which was < 20%.

### **Patterns in MFI and Cross-reactivity**

The multiplex bead assay results for the Grenadian dog sera are presented in [Figure 3-2]. The maximum MFI's for *E. chaff*-19 and *E. canis* p30 were of similar range, whereas the maximum MFI's for *E. chaff*-14 and *A. platys* were significantly lower ( $p < 0.05$ ). All serum that reacted with *E. chaff*-19 peptide also cross-reacted with the *E. canis* peptide; however, there were nine dogs whose sera reacted positively with the *E. canis* peptide and *not* the *E. chaff*-19 peptide. There were a separate nine dogs that reacted to *E. chaff*-14, and of those, four samples also reacted with *E. chaff*-19 and/or *E. canis*. Four

other samples seroreacted with all bead sets with low to mid-range MFIs. An ANOVA test detected significant differences among the MFI's of the dog sera against each peptide coated bead ( $p= 0.0019$ ). Dunn's multiple comparisons test identified significantly higher MFI's for *E. chaff*-19 and *E. canis* p30 than for *E. chaff*-14 and *A. platys*. There was no statistically significant difference between the MFI's for *E. chaff*-19 and of *E. canis* [Figure 3-2].

### **Results of Grenadian Dogs**

All 104 Grenadian dogs were tested by the SNAP® 4Dx® at point-of-care for antibodies to *Ehrlichia* spp., *Anaplasma* spp., and *B. burgdorferi* from September, 2014 through December, 2014. At this time, all feeding ticks removed were identified as *Rhipicephalus sanguineus*. Consistent with the prior observation that the vector (*Ixodes* spp.) is not found on the island [16], all dogs tested negative for the Lyme disease agent using the SNAP® 4Dx®. Additional serum from each dog was aliquoted, frozen at  $-20^{\circ}\text{C}$ , and shipped at one time to Kansas State University, where the multiplex assays were performed.

The multiplex peptide bead assay results of the 104 Grenadian dog sera for *E. canis* and *A. platys* were compared to the SNAP® 4Dx® results [Table 3-4][Table 3-5]. Based on the analyte used for antibody detection more dogs were detected as antibody positive for *E. canis* in the multiplex assay than the commercial assay (37 vs 21); Wilcoxon signed rank test indicated significant differences ( $p=0.0225$ ). The commercial assay detected more *A. platys* positive-only dogs (5 dogs for SNAP® 4Dx® vs. 1 dog for multiplex assay) and more dogs that were antibody positive for both *E. canis* and *A. platys* than the multiplex assay (13 vs 6) [Figure 3-3]; Wilcoxon signed rank test was  $p = 0.0347$

and 0.0156, respectively. Dogs antibody negative for both were not significantly different (65 for ELISA and 60 for multiplex assay,  $P=0.1250$ ). The MFI range of the *E. canis* seropositive samples was 10-fold higher than that of the *A. platys* positive samples

### **Test Agreement**

A Kappa value (0.73) indicated good agreement between the SNAP® 4Dx® and the multiplex bead assay when naturally exposed *E. canis* antibody positive Grenadian dogs were compared [Figure 3-2]. In contrast, the agreement for *A. platys* antibody detection between the SNAP® 4Dx® and the multiplex bead assay was poor (-0.21) [Table 3-3], and the agreement between the IFA and the multiplex bead assay was also poor (0.01). The agreement improved when comparing the *A. platys* IFA with the SNAP® 4Dx® rated in the (0.55) moderate category. The scale used to determine agreement categorical classifications can be found in Table 2-2.

### **Bayesian Results**

Two types of prior beta distributions with a most credible, and a 5<sup>th</sup> percentile credibility interval were calculated<sup>s</sup> for test parameters (derived from literature): a Consensus Prior and a Low-Consensus Prior, respectively [Table 3-7, Table 3-8, and Table 3-9]. Next, these priors were input into OpenBUGS and assessed the degree of conditional dependence [Table 3-13] as opposed to the degree of conditional independence [Table 3-12]. The conditionally dependent model was rejected because it was determined to have slightly poorer fit and less agreement than the conditionally independent model, with the dependence model estimating a DIC of 32.11 and pD of 3.83 [Table 3-13], as compared to

the independence model with a DIC of 30.69 (not significantly different, but close) and a  $pD$  of 3.12 [Table 3-12].

Sensitivity and specificity modeling ensued using the assumption of conditional independence. This was done by running a Consensus Prior Model A [most likely priors using non-informative prior with a (1,1) beta distribution for the Magpix bead-assay), Consensus Model B (most likely priors using informative priors for the Magpix assay). Neither of these models were significantly different from one another with regard to DIC and  $pD$  (Model A= 30.95 and 3.23 respectively, and Model B= 30.69 and 3.12 respectively) [Table 3-12]. Because they produced nearly identical prevalence, and sensitivity/specificity for the IDEXX test, IFA, and the multiplex bead assays, the Low-Consensus Prior Model was calculated using informed priors for the Magpix assay, which was not found to be significantly different from the Consensus Prior Models. Sensitivity and specificity modeling was performed as is described and depicted in table Table 3-11.

## **Discussion**

### **Frequentist analysis**

In this study, we developed and tested a multiplex bead assay to detect antibodies in dogs exposed to tick borne pathogens (*E. chaffeensis*, *E. canis*, and *A. platys*). We designed a peptide-based bead assay for the purpose of distinguishing antibody reactivity to species-specific separate infections simultaneously. The advantages to the multiplex assay is that it can detect antibodies to more than one protein or peptide simultaneously, has a high throughput, less sample volume is required due to the smaller surface area of the beads, and it can detect antibodies in the pg/mL range compared to ng/mL for ELISA assays [126, 134]. Many applications can be adapted to this technology and it has become

more widely used in veterinary species [86]. Similar multiplex assays have been developed to detect serum antibodies to outer surface proteins of *B. burgdorferi* in dogs, horses, and deer [92, 135, 136]. To test the implementation of the canine *Ehrlichia/Anaplasma* assay developed here and to compare the performance with a commonly used in-house commercial ELISA (uses *E. canis* and *A. phagocytophilum* peptides), and the gold standard IFA test. Samples from Grenadian dogs were selected because this population is exposed to only one tick species, *Rhipicephalus sanguineus* that carries both *E. canis* and *A. platys*. *E. chaffeensis*, the causative agent of human and canine monocytic ehrlichiosis, is transmitted by the *Amblyomma americanum* tick [42], a vector not found on the island of Grenada [16]. Serum samples from dogs experimentally infected with *E. chaffeensis*, *E. canis*, and *A. platys* were used to test the specificity of serum antibodies to each peptide in the multiplex bead assay.

The multiplex bead assay had good agreement with the SNAP® 4Dx® when antibody reactivity to *E. canis* was compared. These findings are not unexpected because the commercial assay uses peptides from the p30 and p30-1 OMP immunodominant proteins of *E. canis* and the multiplex bead assay uses a similar peptide to p30 of *E. canis*. In addition, the prevalence of Grenadian dogs seropositive for *E. canis* but not *A. platys* by the multiplex assay was 37%, which is close to an island-wide survey that recently reported 34% *E. canis* seroreactive dogs collected in 2012 [137]. Dogs co-exposed to both pathogens were 6% for the multiplex assay and 13% for the commercial assay, results similar to a prior report of 8% [16].

Since the p28/p30 OMP gene clusters are highly conserved between *E. chaffeensis* and *E. canis*, similar immune responses are expected in the host infected with the paralog



of p30 gene of *E. canis*. The majority of *E. canis* reactive sera also reacted with *E. chaffeensis* p30 coated beads, with four samples reacting with all peptides. These findings indicate conservation of antibody binding epitopes among the various peptides. *E. chaffeensis* p28 OMP consists of a multigene locus of 22 arranged genes that encode for immunodominant 28 kDa outer membrane proteins and form porin-like structures on the membrane of the organism [61]. The p28 OMP from gene 14 is an outer membrane protein expressed in tick infected cells and is a paralog to the p30-10 gene in *E. canis*. In contrast, the p28 gene 19 peptides of *E. chaffeensis* are expressed in infected canine macrophages. In this study, very few serum samples had antibodies that reacted with the peptide of *E. chaffeensis* p28 OMP gene 14. The biological significance of *E. chaffeensis* p28 OMP gene 14 is not completely known, however, prior studies in mice infected with bacteria grown in tick cells indicates the immune response is less effective in clearing the organism grown in tick cells compared to the organism grown in dog macrophages (i.e. there is lower cytokine responses and higher antibody responses in mice exposed to p38 gene 14) [138].

Although the Kappa agreement for *A. platys* seroreactive between the SNAP® 4Dx® and multiplex assay was classified as “poor”, we hypothesized this was a consequence of the low prevalence of *A. platys* detected by the multiplex assay, potentially due to antigenic drift seen in the Grenadian population as compared to the Floridian strain of *A. phagocytophilum* used in the tests. Agreement between the SNAP® 4Dx® and the IFA for *A. platys* seroreactivity was “moderate.” The prevalence of *A. platys* infected dogs for the SNAP® 4Dx® was found to be 17.3%, which was double the serologic prevalence reported in 2006 (9%), but roughly equal to the PCR prevalence of

19% reported in that year as well [16]. Interestingly, PCR results of the Grenadian dogs used in this study were found to be even higher (35% from September 2014 through December 2014), suggesting either a change in the *A. platys* infection rate on the island over a 9-year period, or as a difference between collection periods (2006 study collected results from January 2006 through February 2006).

Although the serum from the dog experimentally infected with *A. platys* did not react well with the *A. platys* peptide coated bead, several Grenadian dog samples reacted in mid MFI range compared to negative control serum. This suggests the sera from the naturally infected Grenadian dogs may be a better positive control for the *A. platys* peptide than the experimentally infected dog, although more work needs to be done to understand why the *A. platys* beads did not react as strongly, relative to the other beads. According to the previous report, specific antibody was detected in an ELISA format from serum samples of the dog experimentally infected with a blood inoculum of *A. platys*; however, in that report the ELISA plate was coated with heterologous whole cell antigens from *A. phagocytophilum* [122]. Contributions to the low antibody reactivity in the Grenadian dog samples may be a consequence of the small size of the OMP IX peptide; however, the *Ehrlichial* peptides were not much larger. Another consideration is that the commercial ELISA uses a synthetic peptide from the major outer surface immunodominant protein p44 of *A. phagocytophilum*, which even though it has been reported to have cross-reacting epitopes to *A. platys*, may not react as well as reported with the serum of *A. platys* exposed Grenadian dogs. As a result of these findings and the changes in prevalence, we hypothesize that genetic changes in the *A. platys* strain have occurred in Grenada. Future

studies are being planned that will look more in depth at *A. platys* nucleotide and amino acid sequences for the OMP IX gene from Grenadian *A. platys* strains.

### **Bayesian analysis**

In this study, we used OpenBUGS software to build a 3 test 1 population formula to assess the prevalence, sensitivity and specificity of the IFA test, the SNAP® 4Dx® test, and our novel Magpix bead-based assay to detect species-specific exposures to *Ehrlichia canis* in dogs. The Consensus Prior and Low-Consensus Prior modeling [Table 3-11] showed little to no difference between each other; for one, prevalence did not significantly change (it remained at 37% and the credible intervals were all between 30%-45%). Furthermore, the sensitivity and specificity credible intervals for the tests remained the same: across all models IFA testing had a mean sensitivity range of 97-98% (credible intervals from 91-99%) and 97-99% specificity (with a credible interval from 92-100%). The IDEXX test had a mean sensitivity range from 82-83% (credible interval from 71-92%) and specificity was 94-95% (with a credible interval of 89-97%) – a slightly wider posterior than the IFA. And lastly, all the models found the mean sensitivity of the Magpix assay to be 94-95% (credible interval of 86-99%), and the specificities had a mean range of 88-90% (credible interval ranging from 80-95%) [Table 3-12]. The Magpix assay was found to be comparable, and perhaps better than the IDEXX test performance.

Sensitivity analysis models using alternate priors all converged and the estimates of sensitivity, specificity, and prevalence were not sensitive to priors, [Figure 3-4] and [Figure 3-5], as the plots showed decently tight credible interval about the y-axis for all performance and prevalence priors used in modeling. Autocorrelation generated by OpenBUGS was also good [Figure 3-6], showing the posterior distributions steadily

normalizing, or converging, with iterations over time within this population of Grenadian dogs.

## Concluding Remarks

This study successfully developed a serologic, multiplex bead-based assay for detection of species-specific exposures to *E. canis* (using peptides from specific outer membrane proteins of P30) as opposed to *E. chaffeensis* (using P28- gene 19/P28- gene 14) in dogs. The seroprevalence of *E. canis* in this subpopulation of Grenadian dogs and the sensitivity, specificity, agreement and goodness of fit were also estimated using frequentist and Bayesian analytic methods. The multiplex bead assay was found to be comparable to, and in some cases better than traditional testing methods but not significantly so. Modeling conditional dependence and independence, consensus prior probabilities and low-consensus prior probabilities, and sensitivity/specificity in OpenBUGS produced robust Bayesian models with a high degree of convergence in all cases—indicating the goodness of fit and appropriateness of the posterior assumptions to the prior assumptions.

For *Ehrlichia canis* detection, further work needs to be done to optimize the coupling concentration of peptides onto *Ehrlichia* beads, as well as investigation into methods of preserving peptide integrity while being stored for weeks to months between assay runs. Additional populations of dogs need to be sampled from regions with different prevalence characteristics and more *Ehrlichia* spp. in order to further model Bayes' inference to apply to a wider variety of dog populations.

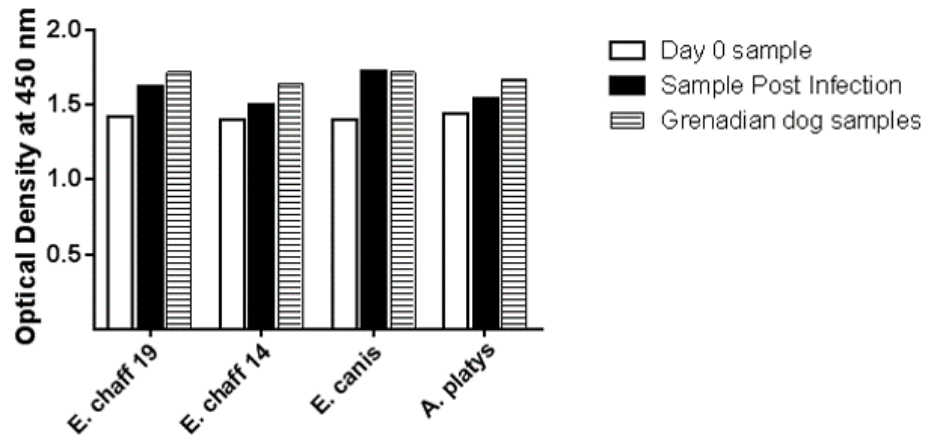
On the other hand, initial optimizations for detecting species-specific antibodies to *A. platys* (using OMP1X a peptide sequence from Taiwanese strain) was not successful, and thusly only analyzed using frequentist methods that confirmed this. Further

investigations are in the planning stages to sequence the strain of *Anaplasma platys* present in Grenada, as it is suspected to be antigenically dissimilar to the Taiwan strain.

## Tables and Figures

**Table 3-1: Multiplex results (MFIs) for experimentally infected dogs and establishment of cut-offs.**

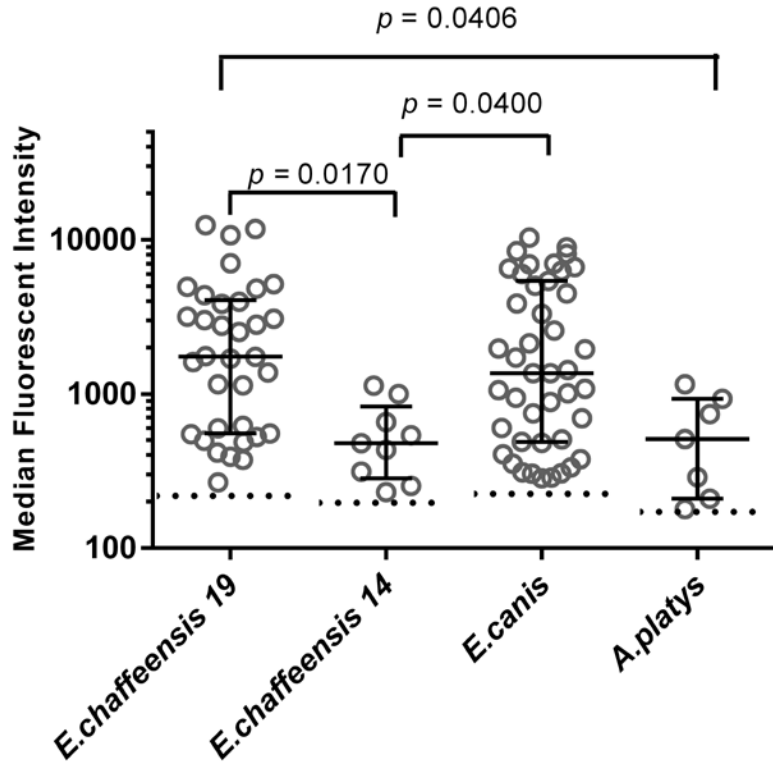
<b>Exp. Control Dogs</b>	<b>Infection Status (day drawn)</b>	<b>R15 MFI E. chaff-19</b>	<b>R34 MFI E. chaff-14</b>	<b>R43 MFI E. canis p30</b>	<b>R58 MFI A.platys OMP IX</b>
<b>Negative Control</b>	—  (day 0)	Mean ± 2SD (cut off)	Mean ± 2SD (cut off)	Mean ± 2SD (cut off)	Mean ± 2SD (cut off)
		<b>185 ± 73 (&gt;258)</b>	<b>179 ± 51 (&gt;230)</b>	<b>181 ± 66 (&gt;247)</b>	<b>156 ± 22 (&gt;178)</b>
<b>Dog 1</b>	<i>E. chaff</i> (day )	<b>439</b>	381	382	327
<b>Dog 2</b>	<i>E. canis</i> (day )	298	287	<b>302</b>	257
<b>Dog 3</b>	<i>A. platys</i> (day )	174	162	159	146



**Figure 3-1: Comparison of monoplex ELISA OD values for controls and sample dogs to validate appropriate binding in the multiplex bead-assay.**



Figure 3-2: MFI range and distribution of seroreactive Grenadian dogs.



*Note:* Circles in this chart represent the MFI (median fluorescence intensity) of all seropositive animals for that analyte.

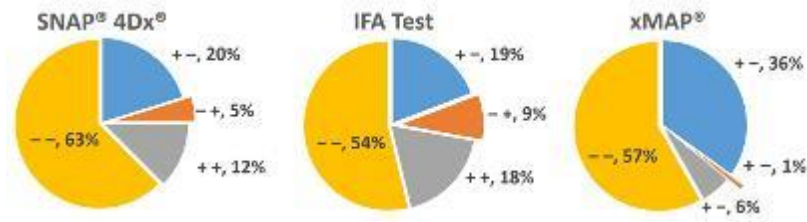
**Table 3-2: Kappa statistic for test agreement of *Ehrlichia* assays.**

	<i>Ehrlichia</i> SNAP® 4Dx® (n= 34)	<i>E. canis</i> MAG Bead-Assay (n=43)
<i>Ehrlichia</i> IFA (n= 39)	0.85 (very good)	0.84 (very good)
<i>Ehrlichia</i> SNAP® 4Dx®	—	0.73 (good)

**Table 3-3: Kappa statistic for test agreement of *Anaplasma* assays.**

	<i>Anaplasma</i> SNAP® 4Dx® (n=18)	<i>A. platys</i> MAG Bead-Assay (n=6)
<i>Anaplasma</i> IFA (n=28)	0.50 (moderate)	0.01 (poor)
<i>Anaplasma</i> SNAP® 4Dx®	—	-0.02 (poor)

**Figure 3-3: Percent of dogs classifying into the four possible *Ehrlichia* and *Anaplasma* diagnosis combinations, by testing modality.**



Note: **Yellow:** *E. canis* (-), *A. platys* (-); **Blue:** *E. canis* (+), *A. platys* (-); **Orange:** *E. canis* (-), *A. platys*(+); **Gray:** *E. canis* (+), *A. platys* (+)

**Table 3-4: Two-by-two contingency tables of IFA, IDX, and MAG testing outcomes.**

IFA				IFA				MAG					
		+	-			+	-			+	-		
IDX	+	33	1	MAG	+	37	6	IDX	+	32	2		
	-	6	64		-	2	59		-	11	59		
			104				104				104		

**Table 3-5: Two-by-two comparison of paired-tests' agreement.**

	1) IFA and IDX	2) IFA and MAG	3) MAG and IDX
<b>Cohen's Kappa</b>	0.85	0.84	0.73
(Confidence Interval)	(0.66 – 1.00)	(0.65-1.00)	(0.55-0.92)

*Note:* Values calculated using [\[130\]](#).

**Table 3-6: Historical seroprevalence of *E. canis* in Grenadian dogs used to generate priors, juxtaposed with current seroprevalence values (not used for priors).**

Test Platform	2004 Data [16] (95% CI)	2006 Data [16] (95% CI)	2014 Data [68] (95% CI)
<b>IFA</b>	-	-	<b>37.5%</b> (29-47%)
<b>IDX</b>	<b>42.3%</b> (33-52%)	<b>49.3%</b> (38-61)	<b>32.7%</b> (24-42%)
<b>PCR</b>	-	<b>24.7%</b> (16-36%)	<b>26%</b> (19-35%)
<b>Magpix</b>	-	-	<b>41.3%</b> (32-51%)
<b>Sample size</b>	n= 104	n= 73	n= 104
<b>Coding input to OpenBUGS</b>	pi ~ dbeta(5.009, 6.5362) ## Mode=0.42, 95% sure > 0.22		

*Note:* SNAP® 4Dx® results from 2006 study are somewhat contradictory in the paper; in the abstract, the author reports their 4Dx results for *E. canis* to be 43%, whereas the result analysis section and a figure identify the prevalence as 49.3%. In calculating the beta distribution ( $\alpha$ ,  $\beta$ ), the mode was set to 42% with a 95% CI that prevalence was greater than 22%. These values were decided upon by taking into account both the posterior prevalence findings as well as attempting to factor in prevalence trend over time. Because the PCR prevalence in 2006 was nearly identical to PCR prevalence of the present study, it was estimated that the animals' relatively lower seroprevalence in our study's data could reasonably put the prevalence mode between 49.3% and 32.7% with a broad CI (95% sure >22%).

**Table 3-7: Sensitivity and specificity of SNAP® 4Dx® for *E. canis* as reported from literature and company documentation, and calculated Consensus Prior and Low-Consensus Prior for Bayesian priors.**

Test name	Source	Source's gold standard IFA strain	Sensitivity (95% CI)	Specificity (95% CI)
3Dx®	[78]	Florida strain	<b>75.93%</b> (63.1, 85.4)	<b>62.5%</b> (38.6, 81.5)
3Dx®	[80]	Israeli strain	<b>71.4%</b> (50.0, 86.2%)	<b>100%</b> (92.3, 100%)
4Dx®	IDEXX reported [94]	Florida strain	<b>98.8%</b> (90.1, 99.9%)	<b>100%</b> (98-100%)
<b>IDX Consensus Prior (CP)</b>			Mean of mean: <b>0.820</b> Mean of 5% CI: (0.677)	<b>0.875</b> (0.763)
<b>IDX Low-Consensus Prior (Low-CP)</b>			Lowest mean: <b>0.714</b> (Mean of 5% CI) /2: (0.339)	<b>0.625</b> (0.382)

Note: CI: confidence interval, set at 95%; Both the SNAP®3Dx® and the SNAP® 4Dx® utilize the same peptides (*E. canis* p30, p30-1) and are thusly comparable directly.

- 1) IDX consensus 5% CI: mean of tests' 5% CI of sensitivity and specificity;
- 2) IDX Low-Consensus Prior Mean: lowest parameter estimate of the two 3Dx® tests and one 4Dx® test;
- 3) IDX Low-Consensus Prior: (CP 5% CI)/2 = calculated by taking the mean of 5% CI (listed in the CP row) and dividing it in half.



**Table 3-8: Calculation of priors used in the Bayesian estimation of IFA characteristics for the detection of *E. canis* specific antibodies.**

<b>Test ID</b>	<b>Variable</b>	<b>Mean</b>	<b>5% CI</b>
<b>IFA = CP</b> (Consensus Prior)	<b>Se</b>	0.98	.95
	<b>Sp</b>	1.00	0.98
<b>IFA = Low-CP</b> (Low Consensus Prior)	<b>Se</b>	0.98	0.475
	<b>Sp</b>	1.00	0.49

*Note:* At the time of writing the author is unaware of any studies comparing the performance of various IFA tests and the sensitivity and specificity were chosen given literature from Fuller Laboratories, Fullerton, CA presented in test inserts;

- 1) Animals were considered positive with titers at or above 1:80 serum dilution;
- 2) Low-Consensus Prior estimates were calculated by taking the 5<sup>th</sup> percentile CP and dividing it in half.

**Table 3-9: Values for priors used in the estimation of MAG (bead-assay) characteristics for the detection of *E. canis*-specific antibodies.**

<b>Source</b>	<b>Variable</b>	<b>Most likely</b>	<b>5% CI</b>
<b>MAG estimate</b>	Se	0.95	0.50
	Sp	0.90	0.45
<b>MAG n/i priors</b>	Se	n/i	n/i
	Sp	n/i	n/i

*Note:* n/i: Non-informative prior; MAG: MagPix Multiplex bead assay; CI= Credible interval. Non-informative priors were entered into OpenBUGS as having a beta distribution of (1,1).

**Table 3-10: All possible testing outcomes of *E. canis* with number of dogs classified in each category for Bayesian modeling.**

<b>Outcome</b>	<b>IFA</b>	<b>IDX</b>	<b>MAG</b>	<b>Number of dogs</b>
p[1]	+	+	+	32
p[2]	+	+	-	1
p[3]	-	+	+	0
p[4]	-	+	-	1
p[5]	+	-	+	5
p[6]	+	-	-	1
p[7]	-	-	+	6
p[8]	-	-	-	58
				n=104

*Note:* IFA: Indirect fluorescent assay; IDX: IDEXX, SNAP<sup>®</sup>4Dx<sup>®</sup> test<sup>u</sup>; MAG: MAGPIX<sup>®</sup> Multiplex bead assay; +: Test positive; -: Test negative.

**Table 3-11: Use of Consensus Priors to and Low-Consensus Priors to evaluate sensitivity models for *E. canis* assays.**

<b>Model</b>	<b>Consensus Prior</b>	<b>Low-Consensus Prior</b>
<b>(CM)</b> Consensus Prior Model  Model A= non-informative MAG prior Model B= informative MAG prior	<u>Se</u> : IFA; IDX; MAG <u>Sp</u> : IFA; IDX; MAG	–
<b>(Low-CM)</b> Low-Consensus Prior Model  Model A= non-informative MAG prior Model B= informative MAG prior	–	<u>Se</u> : IFA; IDX; MAG <u>Sp</u> : IFA; IDX; MAG
<b>(SeM-1)</b> Sensitivity Model 1	<u>Se</u> : IFA; IDX; MAG	<u>Sp</u> : IFA; IDX; MAG
<b>(SeM-2)</b> Sensitivity Model 2	<u>Sp</u> : IFA; IDX; MAG	<u>Se</u> : IFA; IDX; MAG

*Note:* Refer to previous tables for the corresponding values used: Table 3-7 for IDX values, Table 3-8 for IFA values, and Table 3-9 for MAG values used.

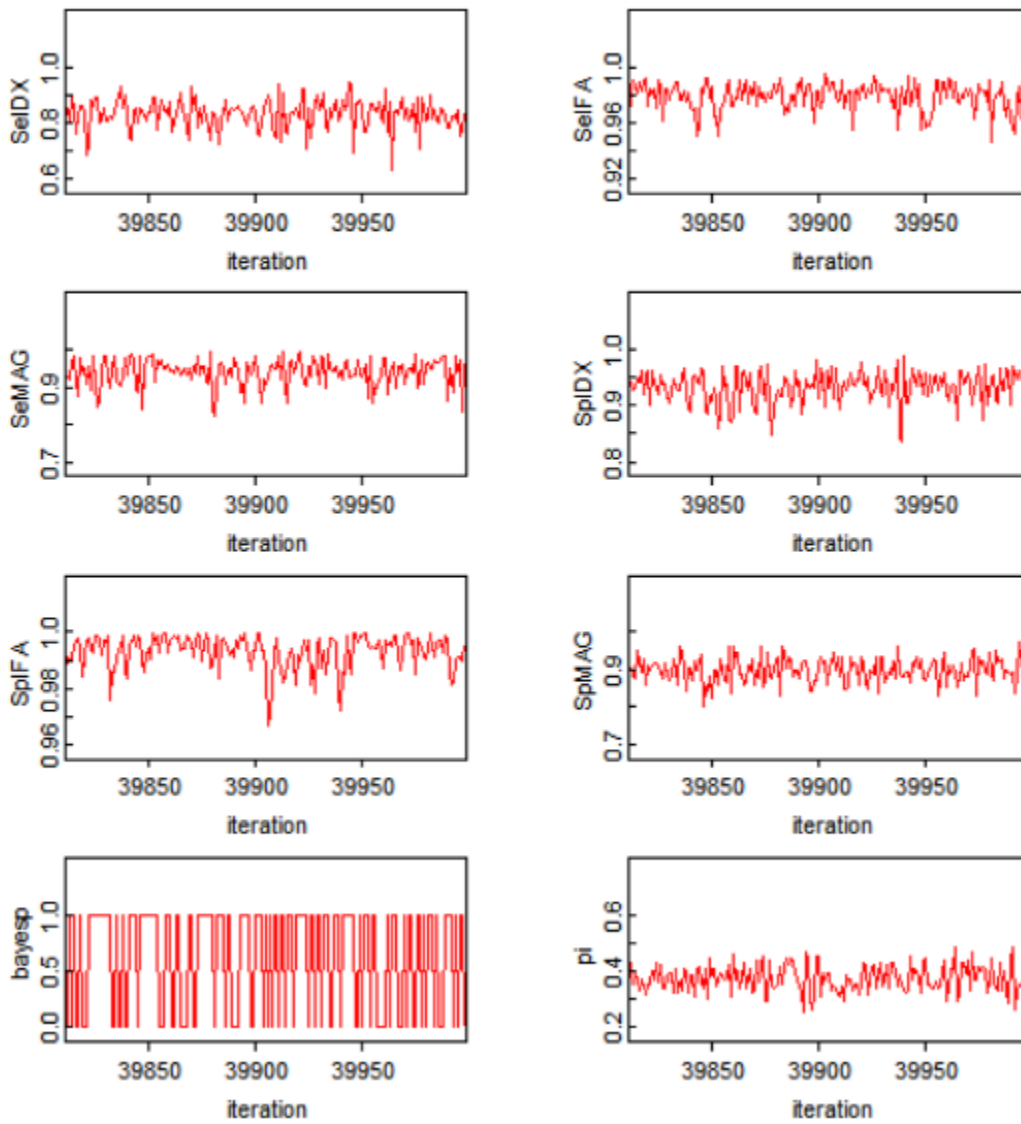
**Table 3-12: Conditional independence model using Consensus Priors, Low-Consensus Priors, Sensitivity Models 1 and 2.**

Model	Priors	Bayesp	pD	DIC	Prevalence (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
<b>CM-A</b> (Consensus Model-A)	CP for Se and Sp	0.60	3.23	30.95	37.7 (30.5-45.4)		
	IFA					98.0 (96.0-99.2)	99.5 (98.1-1.0)
	IDX					83.2 (75.1-89.8)	93.6 (89.1-96.8)
	MAG <b>non-informative</b>					94.5 (86.0-98.8)	90.2 (83.0-95.2)
<b>CM-B</b> (Consensus Model-B)	CP for Se and Sp	<b>0.59</b>	<b>3.12</b>	<b>30.69</b>	<b>37.7 (30.4-45.4)</b>		
	IFA					<b>98.0 (95.5-99.2)</b>	<b>99.5 (98.0-1.0)</b>
	IDX					<b>83.3 (75.1-89.8)</b>	<b>93.6 (89.1-96.8)</b>
	MAG					<b>94.5 (86.7-98.7)</b>	<b>90.1 (83.2-95.0)</b>
<b>Low-CM-A</b>	LP for Se and Sp	0.66	3.67	32.75	37.4 (30.0-45.1)		
	IFA					97.8 (91.5-99.8)	97.7 (92.8-99.7)
	IDX					83.4 (72.6-91.5)	93.6 (89.0-96.8)
	MAG <b>non-informative</b>					95.4 (87.4-99.2)	90.1 (82.9-95.3)
<b>Low-CM-B</b>	LP for Se and Sp	0.67	3.40	32.87	37.3 (30.0-45.0)		
	IFA					98.0 (91.7-99.8)	97.6 (92.7-99.7)
	IDX					83.5 (72.9-91.6)	93.6 (88.9-96.8)
	MAG					95.3 (87.8-99.1)	87.7 (80.3-93.2)
<b>SeM-1</b> (Sensitivity Model-1)	LP for Se; CP for Sp	0.64	3.39	31.9	37.8 (30.4-45.5)		
	IFA					97.8 (91.3-99.8)	99.5 (98.1-1.0)
	IDX					82.6 (71.8-90.7)	93.6 (89.1-96.8)
	MAG					94.5 (86.8-98.5)	90.3 (83.3-95.2)
<b>SeM-2</b> (Sensitivity Model-2)	CP for Se; LP for Sp	0.56	3.36	30.75	37.3 (30.3-44.9)		
	IFA					98.0 (95.9-99.2)	97.7 (92.9-99.7)
	IDX					83.7 (75.5-90.2)	94.5 (89.0-97.8)
	MAG					95.3 (87.8-99.0)	90.0 (83.1-95.0)

Note: These values were generated using open-access OpenBUGS software [108]. Consensus Model-2 was deemed the most robust, though not significantly different from other models.

**Table 3-13: Results of Consensus-Prior models with and without MAG priors using a Conditional Dependence 3-Test Model.**

		CONDITIONAL DEPENDENCE MODEL – 3 TEST							
Prior test	Priors	Bayesian posterior probabilities							
		covDn/ covDp	rhoD/ rhoDc	Bayesp	pD	DIC	Prevalence (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
<b>Dep. CML-A</b>	CP for Se and Sp	0.0137	0.192	0.62	3.93	32.41	37.9 (30.5-45.5)		
	IFA	/	/					98.0 (95.8-99.2)	99.5 (98.2-1.0)
	IDX	0.0188	0.168					82.5 (74.3-89.1)	93.6 (88.9-96.8)
	MAG non-informative priors							94.1 (85.8-98.6)	88.6 (81.1-94.4)
<b>Dep. CML-B</b>	CP for Se and Sp	0.0136	0.194	0.61	3.83	32.11	38.9 (30.6-45.6)		
	IFA	/	/					98.0 (95.8-99.2)	99.5 (98.1-1.0)
	IDX	0.187	0.168					82.6 (74.4-89.1)	93.6 (89.0-96.8)
	MAG							94.2 (86.5-98.4)	88.7 (81.5-94.2)



**Figure 3-4: Dynamic Trace plot using Consensus Model-2 with informative MAG priors.**

*Note:* Trace plots had decently tight ranges for all nodes of informative prior parameters. Note that y-axes in the charts are not scaled identically. Running this model with multiple initial priors yielded trace plots nearly identical to these, in that they had good convergence, and no significant fluctuations in trend were observed.

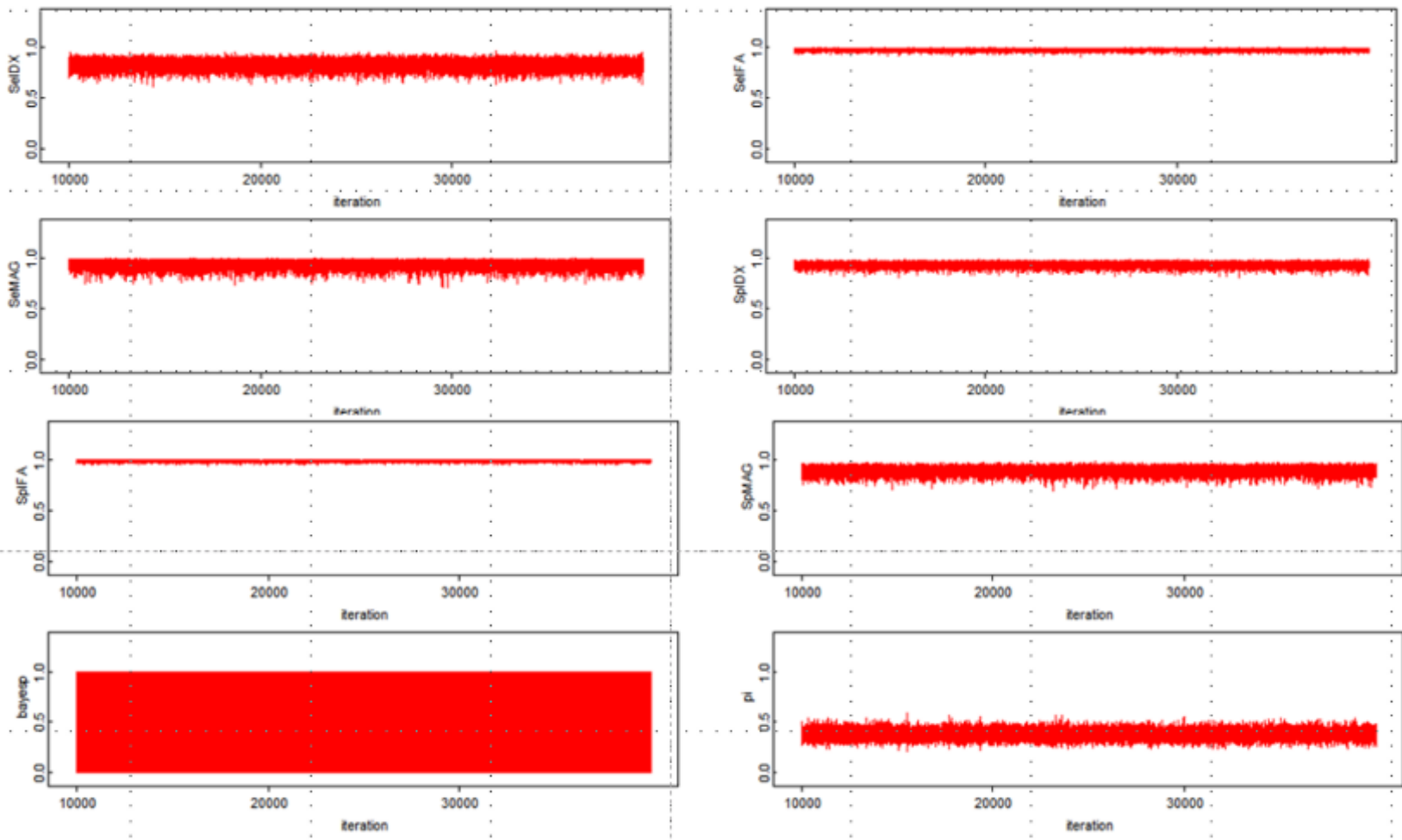
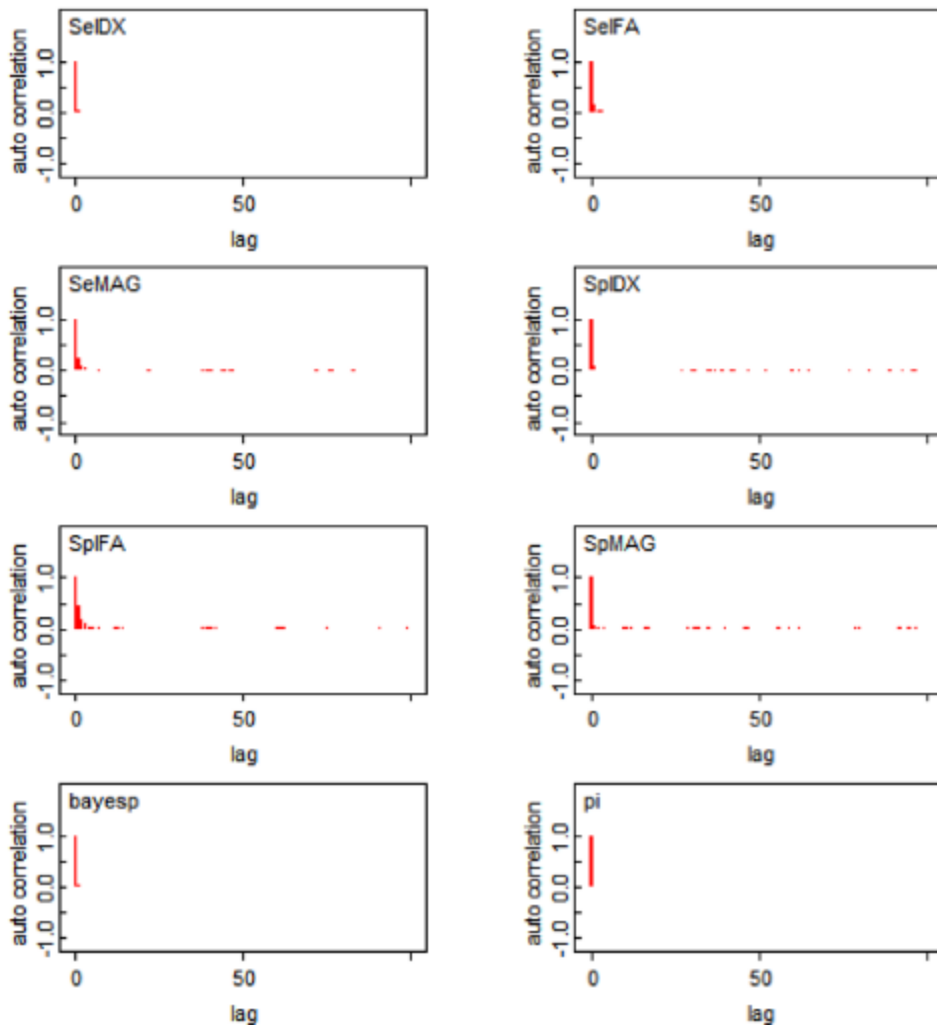
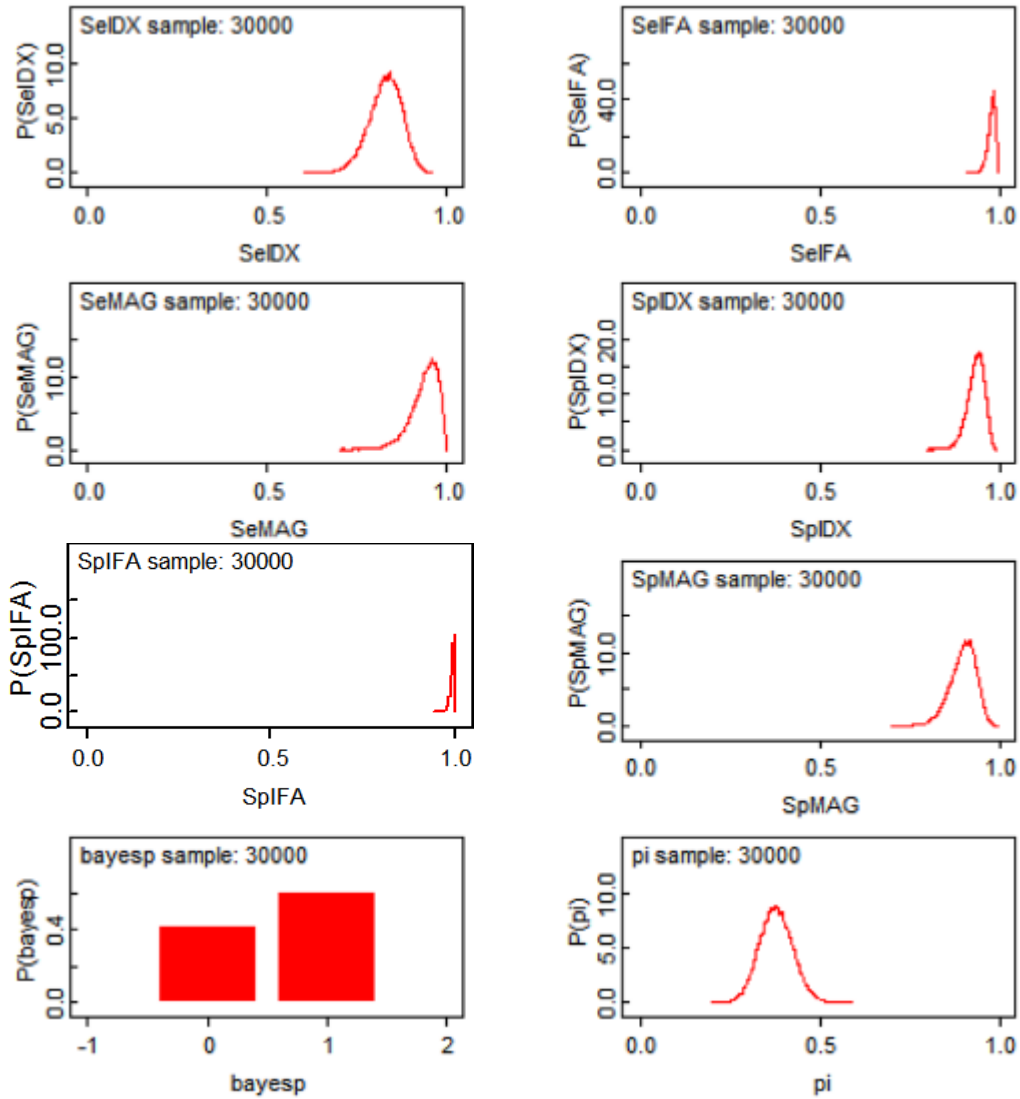


Figure 3-5: History plot function as generated in OpenBUGS using Consensus Model-2 with informative MAG priors.





**Figure 3-6: Auto-correlation generated from OpenBUGS using Consensus Model-2 with informative MAG priors.**



**Figure 3-7: Posterior density plots generated from OpenBUGS using Consensus Model-2 with MAG informative priors.**

## Appendix A: Sources and Manufacturers

- a. SNAP® 4Dx® Test Package Insert, IDEXX Laboratories Inc., Westbrook, ME.
- b. *E. canis* and *A. phagocytophilum* MIF Canine IgG Antibody Kit, Fuller Laboratories, Fullerton, CA.
- c. Pierce Biotechnology, Thermo Scientific Protein Biology, Rockford, IL.
- d. Pierce™ BCA Protein Assay Kit, Thermo Scientific, Rockford, IL.
- e. MagPlex® beads, Luminex Corporation Inc., Austin Tx.
- f. xMap® Antibody coupling kit, Luminex Corporation Inc., Austin, TX.
- g. Storage buffer: PBS 0.05% / Tween-20/0.05% sodium azide, pH 7.4, Sigma P3563, St. Louis, MO.
- h. Assay buffer: PBS 0.05% Tween-20, 0.1% Chemiblocker, pH 7.4.
- i. Greiner black plate, 65509x, Monroe, NC.
- j. MD Millipore, Billierica, MA.
- k. Eppendorf Repeater M4, Eppendorf, Hauppauge, NY.
- l. PlateMax™ Microplate Sealing Film, Fisher Scientific, Pittsburg, PA.
- m. Magnetic Plate Separator®, Luminex Corporation Inc., Austin, TX.
- n. Secondary antibody: biotin-affinity purified-goat anti-dog IgG H+L, at 2 µg/mL, KPL Laboratories, Gaithersburg, MD.
- o. Fluorescent tag: phycoerythrin (PE)-labeled strep-avidin at 4µg/mL, Life Technologies, Grand Island, NY.
- p. Graphpad Prism6.05, La Jolla CA.
- q. Analyse-it Software, Method Evaluation Edition, Version 4, Leeds, United Kingdom.
- r. OpenBUGS Software, Version (3.2.3), MRC Biostatistics Unit, Cambridge, United Kingdom.
- s. BetaBuster, software version 1.0, UC Davis, Davis, CA.
- t. OpenEpi: Open Source Epidemiologic Statistics for Public Health, Dean A.G. et al.,

## **Appendix B: Permissions**

See supplemental documents.

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