APPARENT PREVALENCE OF HEMOTROPIC MYCOPLASMA INFECTING MICHIGAN DAIRY CALVES

By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Comparative Medicine and Integrative Biology – Master of Science

2023

ABSTRACT

Hemotropic mycoplasma is increasingly being recognized as an infectious agent by dairy practitioners, but little is known about this organism or its pathogenic mechanisms, raising concern about the potential effects of the infection on dairy animal health and productivity. *Mycoplasma wenyonii* and *Candidatus Mycoplasma haemobos* are parasitic bacteria that infect red blood cells of cattle. Until recently, very little was known about the prevalence of infection with these organisms in U.S. dairy cattle. Schambow et al (2021) reported a 100% herd-level apparent prevalence in adult cows tested in 82 herds located in Wisconsin and Michigan. In that study, >70% of first lactation cows were infected, which suggested that infection occurred prior the first calving *(*Schambow et al., 2021). The overall hypothesis of this thesis is that infection with hemoplasmas primarily occur prior to first calving, vary with age, impact some hematological values, and that transplacental transmission is uncommon. The aims of this thesis are to: 1) determine the prevalence and dynamics of infection of with *M. wenyonii* and *C.M. haemobos* infections in calves and replacement heifers on Michigan dairy farms and assess potential associations between infection status and altered hematological values; and 2) determine if infection with *M. wenyonii* or *C. M. haemobos* occurs prior to birth (vertical transmission), and to evaluate the presence of bovine hemoplasma DNA in the colostrum.

This thesis is dedicated to my family and many friends. A special feeling of gratitude to my parents, Débora and Luis de Souza who have been my source of support and guidance. To my grandmother Maria, for her love, encouragement, and prayers. Lastly, I express my gratitude to my partner, Steven, who has supported me throughout the process.

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INTRODUCTION

Mycoplasma wenyonii (previously known as *Eperythrozoon wenyonii*) and *Candidatus Mycoplasma haemobos* are uncultivable and cell wall-less bacteria known to parasitize the red blood cells of cattle. Both bovine hemoplasmas have been reported in many countries worldwide, however, the consequences of these infections for animal health and the dairy industry remain unknown. Previous research has primarily focused on the prevalence of these infections in adult cattle. Limited information is available regarding infections in calves and replacement heifers, as well as the dynamics of infection. Determining the timing of infection will provide the foundation for developing interventions and management programs aimed at reducing the risk of infection.

The epidemiology of bovine hemoplasma remains poorly understood, but possible transmission routes may include blood sucking insects and direct contact with infected blood via fomites. Transplacental transmission has been demonstrated in a few studies, but its overall prevalence is unknown. To date, only a single study, which focused on beef cattle, explored colostrum as a potential source of infection with *M. wenyonii.* There is a very limited amount of research about age-specific prevalence in dairy cattle and about the presence of bovine hemoplasmas in colostrum. Understanding possible routes and age of transmission of hemoplasma organisms in cattle can contribute to development of management practices that mitigate risk of transmission.

The aim of this thesis is to determine the prevalence and dynamics of *M. wenyonii* and *C.M. haemobos* infections in calves and replacement heifers on Michigan dairy farms, assess the potential impact of these infections on hematological values, investigate the possibility of transplacental transmission , and evaluate the presence of bovine hemoplasma DNA in colostrum.

CHAPTER 1: LITERATURE REVIEW

1.1 ABSTRACT

The objective of this literature review is to better understand bovine hemoplasmosis, an emerging disease that threatens dairy animal health. Several species of hemotropic mycoplasma are known to infect both animals and humans and *Mycoplasma wenyonii* and *Candidatus Mycoplasma haemobos* are the species that infect red blood cells of cattle. These microorganisms are increasingly associated with clinical signs of disease in dairy cattle, but the impacts of infection on health and productivity of dairy cows are poorly understood. In chapter 1 we review information about the epidemiology of bovine hemoplasmosis in different countries, including clinical signs associated with hemoplasmosis in cattle, methods of diagnosis, treatment, possible routes of transmission, risk factors for infection, and disease progression. Although hemoplasmas have been reported to infect cattle in many countries, and methods used to detect these organisms have improved, there remain numerous gaps in knowledge of the epizootiology of bovine hemoplasmas. The pathogenesis of the disease, and potential impact on animal health and productivity remain unclear. With this review we seek to contribute to the understanding of hemoplasmosis in cattle and provide insights for further research to improve disease management strategies and overall animal health in the dairy industry.

1.2 INTRODUCTION

Hemotropic mycoplasmas, which are collectively referred to as" hemoplasmas," are increasingly detected in cattle, but the impact of these organisms on health and productivity of dairy cattle remains poorly understood. Changes in nomenclature and difficulties in accurate diagnosis have contributed to a general lack of knowledge about these organisms. Hemoplasmas are composed of a group of Gram-negative bacteria that infect red blood cells of various animals (Tyzzer, 1942; Kreier J P and Ristic, 1984; Kreier, 1992). These organisms were previously classified as *Haemobartonella* and *Eperythrozoon* spp but the taxonomy has evolved. Based on similar characteristics, hemoplasmas were originally classified

as rickettsiae (Kreier & Ristic, 1984). Later they were described as related to *Anaplasma* (Neimark et al., 2001). In 2001, based on molecular characterization using 16S rRNA gene sequencing, the organisms were reclassified as *Mycoplasma*, with the Candidatus designation applied to new species that do not have enough information to support their classification (Neimark et al., 2001).

Understanding the dynamics of hemoplasma infection in dairy cattle is necessary to develop management practices that minimize potential consequences of infection on animal health. A few studies have described the regional prevalence of hemoplasmas in cattle (Schambow et al., 2021; Tagawa et al., 2013; Tatsukawa et al., 2021), providing insight into the potential burden of this relatively unknown disease that may pose a threat to the health and productivity of dairy cattle. The purpose of this review is to consolidate current research about hemoplasmas in cattle and to review the epidemiology, clinical signs, diagnosis, treatment, and risk factors associated with infection.

1.3 HEMOPLASMA SPECIES OF CATTLE

Several species of hemotropic mycoplasma have been reported to affect animals and most cause species specific infections. Historically, cattle were reported as infected with *Mycoplasma wenyonii* (previously known as *Eperythrozoon wenyonii) (M. wenyonii)* (Adler and Ellenbogen, 1934); *Eperythrozoon teganodes* (Hoyte, 1962); and *Eperythrozoon tuomii* (Uilenberg, 1967). Species differentiation between *E. wenyonii* and *E. teganodes* was based on morphological and immunological characteristics (Uilenberg, 2009). A further specie, *E. tuomii* was described as attached to platelets from splenectomized calves in Finland, Holland, and Madagascar. Currently, isolates of *E. teganodes* or *E. Tuomii* are not available to analyze their 16S rDNA sequences (Hoelzle et al., 2011) and only *M. wenyonii* was included in the 1980 approved list of bacterial names (Hoelzle et al., 2011).

In the United States, *M. wenyonii* was first identified in cattle by Lotze and Yiengst (1941) about 7 years after the first reported infection in a splenectomized calf in Palestine by Adler and Ellenbogen (1934). Since then, this organism has been reported in cattle worldwide (Montes et al., 1994; Neimark &

Kocan, 1997; Smith A. et al., 1990; Sutton& Collins, 1977).

A new species of hemoplasma, *Candidatus Mycoplasma haemobos*, was reported in Japan and northern Germany (Tagawa et al., 2008; Hoelzle et al., 2011). Unlike other species that seemed to be related to *M. wenyonii*, 16S rRNA gene sequencing demonstrated that *C. M. haemobos* is similar to *Mycoplasma haemofelis*, which infects cats and typically leads to infectious anemia (Tagawa et al., 2008). The pathogenesis of *M. wenyonii* and *C. M. haemobos* remains unclear, however, some researchers have reported that *C. M. haemobos* seems more pathogenic than *M. wenyonii* (Tagawa et al., 2010). It is possible that clinical signs of infection in cattle may be more severe when both organisms are present (Meli et al., 2010; Hornok et al., 2012), but more evidence is needed to support this theory.

1.4 CLINICAL SIGNS AND LABORATORY FINDINGS OF INFECTED CATTLE

Hemoplasmas have been detected in blood of both ill and apparently healthy cattle. In most infected cattle, the disease is reported as subclinical and, in some cases, as chronic (Messick, 2004; Montes et al., 1994; Smith A. et al., 1990). There is some thought that clinical signs occur in stressed or compromised cattle (for example when cattle are co-infected with immunosuppressive organisms (Hofmann-Lehmann et al., 2004)). In cattle, many nonspecific signs have been attributed to hemoplasma infection including immune-mediated anemia, anorexia, edema of the mammary gland, edema of rear legs, fever, lymphadenopathy, reduced milk yield, weight loss, and infertility. (Genova et al., 2011; Gladden et al., 2016; Hoelzle et al., 2011; Montes et al., 1994; Smith A. et al., 1990; Strugnell et al., 2011). Risk factors associated with progression from subclinical to a clinical state are unknown.

The effects of hemoplasma infections on reproductive performance reportedly include abortion, infertility, and delayed estrus (Smith A. et al., 1990). The main consequences of *M. wenyonii* infection in bulls include swelling of the scrotal wall and poor semen quality, which may result in a transient infertility (Montes et al., 1994; Welles et al., 1995). Recently, infections with C. *M. haemobos* and *M. wenyonii* have been detected in newborn calves and in aborted fetuses of infected cows, suggesting

transplacental infection (Hornok et al., 2011; Girotto-Soares et al., 2016), but causal relationships between infection and reproductive outcomes of cows have not been established.

Hematological values of cattle infected with bovine hemoplasmas have been described in only a few studies (Tagawa et al., 2010; Hornok et al., 2012; Niethammer et al., 2018; Tatsukawa et al., 2021). When compared with herdmates infected with *C. M. haemobos* or with non-infected herdmates, cattle infected with *M. wenyonii* had elevated white blood cells (WBC) counts (Tagawa et al., 2010; Congli et al., 2011). In a study that enrolled 41 herds containing Simmental cattle, blood of animals that were infected with *C. M. haemobos* or were co-infected with both bovine mycoplasmas, contained more WBC as compared to cows that were infected solely with *M. wenyonii* (Niethammer et al., 2018). Similarly, in a Japanese study that enrolled 400 beef cows, greater numbers of WBC were reported in cows that were positive for hemoplasmas as compared to non-infected cows (Tatsukawa et al., 2021). Taken together, these studies suggest that immune stimulation in cows infected with *C. M. haemobos* may increase WBC as infected cows attempt to clear the infections (Niethammer et al., 2018). However, controlled experimental infection studies are needed to elucidate the impact of infection on immune responses.

The impact of hemoplasma infection on red blood count **(RBC)** has been the subject of limited investigation, with only a few studies available (Tagawa et al., 2010; Hoelzle et al., 2011; Hofmann-Lehmann et al., 2004; Su et al., 2010). Tagawa et al. (2012) reported that cattle with hemoplasma infections exhibit a decrease in anemia indicators (packed cell volume **(PCV)**, RBC and concentration of hemoglobin accompanied by an increase in mean corpuscular volume **(MCV)** (Tagawa et al., 2012). In comparison to PCR negative cattle and cattle infected with *M. wenyonii*, PCV and the concentration of RBC and HB were less in cattle infected with *C. M. haemobos,* suggesting a stronger effect of *C. M. haemobos* infection on hematological values compared to *M. wenyonii* (Tagawa et al., 2010). Later, the same group of investigators reported that the pathogenicity of co-infection with *M. wenyonii* and *C. M.*

haemobos was either similar or slightly weaker compared to *C. M. haemobos* alone. The changes observed in RBC among animals infected with hemoplasmas have been attributed to some level of hemolytic anemia (Tagawa et al., 2012).

Evidence of anemia subsequent to *M. wenyonii* infection is variable. When anemia is present, it is most commonly mild to moderate in severity and accompanied by other clinical signs such as fever, malaise, or edema of the hind limbs, udder, or scrotum (Smith A. et al., 1990; Montes et al., 1994; Genova et al., 2011; Strugnell et al., 2011). Severe anemia has been reported in young animals and in splenectomised calves that were experimentally infected with *M. wenyonii* (Purnell et al., 1976). In addition, anemia has been reported in mature cows that were naturally infected with *M. wenyonii* regardless of occurrence of clinical signs (Gladden et al., 2016). While *M. wenyonii* directly infect RBC, and has been associated with anemia, the prevalence of anemia in infected cattle and risk factors associated with occurrence of anemia have not been determined and should be the focus of future research.

Acute infection with hemoplasmas can affect productivity and has been described as leading to a sudden drop in milk yield (Sutton and Collins, 1977). In severe cases, other clinical signs such as fever, anemia, fatigue, and hind limbs edema have been reported (Sutton and Collins, 1977; Smith A. et al., 1990). In chronically infected cows, reduced productivity has been reported even in cows without clinical signs (Tagawa et al., 2013). In one study, clinically normal cows that were PCR-positive for *M. wenyonii*, *C. M. haemobos* or co-infected with both organisms had reduced milk yield as compared to PCR-negative cows (Tagawa et al., 2013). Based on the limited amount of published research, both acute and chronic infection with hemoplasmas have been associated with decreased milk yield, but the overall relevance of infection with these organisms on dairy cow productivity remains largely unknown.

1.5 TREATMENT OF HEMOPLASMA INFECTIONS

No treatment protocols for cattle have been conclusively shown to eliminate hemoplasma infections in cattle (Strugnell et al., 2011). Despite the lack of data demonstrating efficacy, treatment of symptomatic cattle is based on administration of tetracyclines for a prolonged duration with the goal of reducing bacterial load and resolution of clinical signs (Genova et al., 2011). Clinical responses after treatment with oxytetracycline are variable (Montes et al., 1994; Genova et al., 2011; Strugnell et al., 2011). Clinical signs of some affected cattle have been reported to resolve after treatment (Genova et al., 2011), while others have reported that treatment did not affect the duration of clinical signs. (Strugnell et al., 2011). These findings have been based on observations made by farmers and controlled trials are lacking. Thus, there is a need for well-designed clinical trials to evaluate the efficacy of treatments used in symptomatic cattle.

1.6 TESTS USED TO DIAGNOSE INFECTIONS WITH HEMOPLASMAS

1.6.1 Blood smears

Over the years, a variety of diagnostic tests have been used to detect hemoplasma infections. In the past, diagnosis of infection was usually based on cytological identification of the organisms using light microscopy of blood smears stained using acridine orange or Giemsa dyes or by use of electron microscopy (Sutton and Collins, 1977; Smith A. et al., 1990; Welles et al., 1994). In recent years, those methods have been shown have low diagnostic sensitivity and specificity (Messick, 2004; Ritzmann et al., 2009). Molecular techniques such as conventional polymerase chain reaction (PCR) and real-time PCR (rt-PCR) are now the methods of choice for diagnosing hemoplasma infection (Tagawa et al., 2008; Ritzmann et al., 2009; Hoelzle et al., 2011; Girotto-Soares et al., 2016; Niethammer et al., 2018).

1.6.2 PCR

Conventional PCR and real time PCR are both laboratory techniques used to amplify and detect specific DNA sequences. The main difference between the two lies in the way the amplification products

is detected. While standard PCR provides qualitative information about the presence or absence of the target DNA sequence (Leontis and Westhof, 1998), rt-PCR allows for quantification of DNA amplification in real time (Willi et al., 2009). Both PCR and rt-PCR, targeting the 16S rRNA region, are commonly used to detect hemoplasmas in cattle (Nishizawa et al., 2009; Willi et al., 2009; Meli et al., 2010; Ade et al., 2018; Schambow et al., 2021).

Real time PCR is faster and more specific then conventional PCR and offers the advantage of quantification (if performed with a standard curve), allowing for accurate estimation of the pathogen load (Willi et al., 2009). However, rt-PCR is relatively expensive thus potentially limiting use and may miss hemoplasma species that have not been previously characterized (Willi et al., 2009). A variety of PCR assays have been used to detect hemoplasmas in cattle (Sasaoka et al., 2015; Ade et al., 2018; McAuliffe et al., 2005; Nishizawa et al., 2009). There are no known differences in accuracy among the PCR assays and the choice of which technique to use depends on availability, objectives, and costs. While significant progress has been made in detecting hemoplasmas in cattle, associations between test outcomes and the occurrence of clinical signs or impact on productivity have not been reported. Future research is needed to better relate test results to disease progression, occurrence of clinically or economically relevant outcomes and favorable results of treatments.

1.7 EPIDEMIOLOGY OF HEMOPLASMAS IN CATTLE

An increase in recent reports of hemotropic mycoplasmas in cattle may indicate a newly emerging threat to animal health or could be a result of improved detection. Both *M. wenyonii* and *C.M. haemobos* have been detected in cattle located in many countries (Table 1.1). Infection seems to be more commonly reported in countries in the northern hemisphere with the greatest number of infections reported in Japan (Nishizawa et al., 2010; Tagawa et al., 2010, 2012, 2013; Fujihara et al., 2011). However, this finding could be a result of detection bias because more studies have been conducted in northern countries. Studies including a greater geographic area are needed to better

understand potential differences in geographic distribution of infection.

As several studies have been conducted in different regions of Japan, comparison of the geographical distribution of hemoplasmas may provide clues about risk factors for infection. In Japanese studies, the greatest prevalence of hemoplasma infections in cattle has been reported in the western (93.8%) and southern (91,5%) regions (Fujihara et al., 2011; Tatsukawa et al., 2021), as compared to the northern part of Japan (71.6%, 64.7%, 22.3%) (Tagawa et al., 2010, 2012; Sasaoka et al., 2015). As arthropod vectors are more abundant in lower latitudes, differences in prevalence might be due to climatic conditions that influence the distribution of insects that may transmit hemoplasmas (Fujihara et al., 2011; Reisen, 2010). Different breeds of cattle were sampled in several studies, and some researchers have suggested that some breeds might be more susceptible to infection (Tatsukawa et al., 2021). However, researchers have not confirmed that breed is a risk factor for infection and interpretation of such results should be made cautiously.

Until recently, the prevalence of infection with hemotropic mycoplasmas in US dairy cattle was unknown. In a recent study, blood samples (n = 2,521) were collected from adult cows in 64 and 18 dairy herds in Wisconsin and Michigan, respectively, and demonstrated 100% herd-level prevalence and >70% within herd prevalence of cows infected with *M. wenyonii* and *C. M. haemobos* (Schambow et al., 2021). In the same study, the seroprevalence of bovine leukemia virus (BLV) was compared to the prevalence of hemoplasmas as the mode of transmission is thought to be similar. Surprising, the overall prevalence for BLV was less (40%) than for hemoplasmas (Schambow et al., 2021). These findings highlight the greater prevalence of hemoplasmas in comparison to a disease like BLV, which is well-known in the dairy industry. However, it is important to interpret these results cautiously due to the different detection methods employed for each pathogen (PCR for hemoplasmas and serum antibodies for BLV) (Schambow et al., 2021). The difference in test methods can affect comparison of results, as PCR allows for detection of an organism's presence, while serological testing reflects past exposure (Lee et al., 2016).

Currently, there is insufficient evidence to determine differences in prevalence of the two bovine hemoplasmas. The proportion of infected cattle has varied among studies depending on country, breed, and age of the cattle that were sampled. Recently, Tatsukawa et al (2021), sampled 400 beef cattle in Japan ranging in age 1 -16 years old and reported that 40.3% and 9.5% of cattle tested positive for *M. wenyonii* and *C.M. haemobos*, respectively. Similar results were reported in an earlier Japanese study that sampled mature Holstein dairy cows (Tagawa et al., 2012). In that study, 25.5% of cows were infected solely with *M. Wenyonii*, 19.4% were infected solely with *C. M. haemobos*, and 34.4% were co-infected (Tagawa et al., 2012). In Germany, researchers sampled beef cows and reported that infection with *C. M. haemobos* was more common than infection with *M. wenyonii*. Niethammer et al. (2018) sampled Simmental cows in 41 herds and reported that 57% were positive for *C. M. haemobos*, while 9% and 5% were positive for *M. wenyonii* or co-infected, respectively. Infection with *C. M. haemobos* was reported to be 41.7% in a small Chinese study that enrolled 42 dairy cows and 12 beef cows (Su et al., 2010). No difference in prevalence of *C. M. haemobos* (77.3%) or *M. wenyonii (*71.1%) was reported in an observational study of dairy cows located in the upper Midwest of the US (Schambow et al., 2021). Most studies that have determined prevalence have enrolled relatively few herds and were not designed to identify differences among hemoplasma species. Prevalence estimates could be influenced by geographic location, breed of animals samples, housing conditions, and age of the sampled cattle. To establish meaningful associations between risk factors and determine whether these factors are truly associated with the prevalence of hemoplasmas, surveys designed with sufficient statistical power and large sample sizes are necessary.

Little is known about the prevalence of hemoplasma infection in calves. A recent study of dairy animals in MI and WI, reported that >70% of first lactation cows were already infected, inferring that infection occurred prior to calving (Schambow et al., 2021). Only a few researchers have reported the prevalence of hemoplasma infection in young stock (Table 1.2). Prevalence studies in calves have not

tested animals older than 7 days of age and none have been conducted in North America (Table 1.2). Research investigating age-specific prevalence is urgently needed to identify risk factors and health outcomes that may be associated with hemoplasma infections. Understanding the dynamic of hemoplasma infection in cattle is the first step for the development of effective management strategies to prevent and control the disease.

Overall, numerous researchers have documented the presence of hemoplasmas in both dairy and beef cattle located in more than 10 countries, although the majority of investigations have focused on dairy herds (Table 1.1). Between studies, animal level prevalence of each hemoplasma organism has ranged from <5% to >95% (Table 1.1), but comparisons are difficult due to differences in locations, objectives, study design, testing methodology, breeds and ages of cattle that were tested. Many studies are simple reports of prevalence and did not measure potential confounding characteristics. While there are a limited number of studies, results have consistently indicated widespread infection of cattle with hemoplasmas but properly designed larger studies are needed to identify risk factors associated with infection.

Table 1. 1 – Summary of prevalence of hemoplasma in adult cattle from 19 studies conducted in 12 countries during 2004 – 2022.

						Animal Level Prevalence[1](%)		
Authors	Location	Herds/animals n	Type of animal	Breed	Type of Test	*M. wenyonii*	*C.M. haemobos*	Co-infection
Tatsukawa et al. 2021	Japan	80/ 400	Not stated	Japanese Black	Direct PCR	40.3	9.5	41.8
Fujihara et al. 2011	Japan	68	Dairy	Not stated	rt-PCR	Hiroshima: 14.0 Miyazaki: 6.0	Hiroshima: 42.0 Miyazaki: 63.0	Hiroshima: 14.0 Miyazaki: 25.0
Nishizawa et al. 2010	Japan	1/ 109	Dairy & Beef	Not stated	rt-PCR	61.5	22.9	12.8
Tagawa et al. 2010	Japan	1/ 103	Dairy	Holstein Friesian	PCR	13.5	6.7	1.9
Tagawa et al. 2012	Japan	1/ 49	Not stated	Not stated	PCR	36.7	22.4	12.2
		3/ 343			Direct PCR	38.5	39.1	12.8
Tagawa et al. 2013	Japan	1/ 93	Dairy	Holstein	PCR	35.5	19.4	34.4
Girotto et al. 2012	Brazil	433	Dairy	Holstein & Jersey	PCR	–	61.0	–
Girotto et al. 2016	Brazil	1 abattoir/ 22	Dairy	Holstein & Jersey	PCR	–	40.9	–
Schambow et al. 2021	US	82/ 2,521	Dairy	Holstein	PCR	72.0	78.0	–
Su et al. 2010	China	12 beef & 42 dairy	Beef & Dairy	Yellow cattle	PCR	–	Beef: 41.7 Dairy: 14.3	–
Hornok et al. 2012	Hungary	1/ 24	Beef	Limousine	rt-PCR	91.7	–	–
Hornok et al. 2011	Hungary	1/ 38	Beef	Limousine	rt-PCR	94.7	97.3	–

[1] – indicate that the corresponding type of organism was not tested/reported in those studies.

Table 1. 1 (cont'd)

Authors	Location	Herds/animals n	Type of animal	Breed	Type of Test	Animal Level Prevalence[1](%)		
						M. wenyonii	*C. M. haemobos*	Co-infection
Hasan et al. 2017	Malaysia	5/ 100	Not stated	10 different breeds	PCR	50.0	2.0	17.0
Díaz-Sánchez et al. 2019	Cuba	41	Dairy	Not stated	rt-PCR	63.4	63.4	63.4
Hofman-Lehmann et al. 2004	Switzerland	1/ 58	Dairy	Not stated	PCR	Group 1: 78.0 Group 2: 16.0 Group 3: 0.0	–	–
Byamukama et al. 2020	Uganda	16/ 208	Not stated	Not stated	PCR	32.2	–	–
Niethammer et al. 2018	German	41/ 410	Beef	Simental	rt-PCR	56.5	8.5	4.8
McFadden et al. 2016	New Zealand	1/ 47	Dairy	Not stated	PCR	13.0	28.0	–

[1] – indicate that the corresponding type of organism was not tested/reported in those studies

Table 1.2 – Summary of prevalence of hemoplasmas in calves from 6 studies conducted in 5 countries during 2010 – 2018.

						Animal Level Prevalence[1] (%)		
Authors	Location	Farms/animals n	Age sampled	Type of animal	Type of test	*M. wenyonii*	*C.M. haemobos*	Co-infection
Tagawa et al. 2013	Japan	1/ 71 calves	1 – 7 days old	Dairy	PCR	7.0	2.8	4.2
Sasaoka et al. 2015	Japan	1/ 17 calves	Newborn	Beef	rt-PCR	23.5	–	–
Girotto et al. 2016	Brazil	22 aborted fetuses	–	Dairy	PCR	–	18.2	–
Hornok et al. 2011	Hungary	1/ 38 calves	Newborn	Beef	rt-PCR	18.1	27.2	–
Niethammer et. al 2018	Germany	41/ 25 calves	Newborn	Beef	rt-PCR	0.0	8.0	–
Meli et al. 2010	Switzerland	21/ 47 calves	Not cited	Dairy	rt-PCR	4.0	2.0	–

[1] – indicate that the corresponding type of organism was not tested/reported in those studies.

1.8 TRANSMISSION OF *M. WENYONII* AND *C. M. HAEMOBOS*

The epidemiology of hemotropic mycoplasmas in bovines is poorly understood, and there are many questions about possible mechanisms of transmission. Transmission of hemoplasmas can occur through contact with infected blood and there are several potential vectors that are considered potential sources of transmission (Smith A. et al., 1990). Several researchers have suggested ticks as vector for transmission of hemoplasmas, but no studies have proven that the hemoplasma detected in ticks can be transmitted to cattle (Hofmann-Lehmann et al., 2004; Mohd Hasan et al., 2017; Shi et al., 2022).

While researchers have not proven that detection of *M. wenyonii* or *C. M. haemobos* in ticks results in transmission to cattle, (Hofmann-Lehmann et al., 2004), DNA of hemotropic mycoplasmas has been found in several tick species, including *M. Wenyonii* in *Dermacentor andersoni*, *Rhipicephalus microplus and H. bispinosa*. Neimark et al (2001) first documented *M. wenyonii* infecting the *Dermacentor andersoni* tick. Later, the same organism was also reported in *Rhipicephalus microplus* and *Haemaphysalis bispinosa* ticks (Mohd Hasan et al., 2017). Recently, *Rhipicephalus microplus* was reported as carrying *C.M. haemobos* (Shi et al., 2019). An indirect association between the prevalence of *C. M. haemobos* in water buffalo and tick-infestation was reported in Cuba (Díaz-Sánchez et al., 2019). In one study, researchers reported that buffalo free of ticks were more frequently infected with *C. M. haemobos* than buffalo infested with ticks (Díaz-Sánchez et al., 2019). It is possible that this observation was confounded by the age of the animals, as only younger buffalo were found to be tick infested.

Additional blood-sucking insects such as horn flies (*Haematobia irritans*), stable flies (*Stomoxys calcitrans*), horse flies (*Tabanus bovinus*, *T. bromius*), blood-sucking lice (*Hematopinus eurysternus*), and house flies (*Musca domestica*) have been suggested as potential vectors for the transmission of hemotropic mycoplasmas (Hornok et al., 2011; Hofmann-Lehmann et al., 2004). Some researchers have documented mechanical transmission of these pathogens by these insects (Hornok et al., 2011; Song et

al., 2012). Based on Hornok et al, (2011), *M. wenyonii* was detected in blood-sucking insects more frequently than *C. M. haemobos*. In the same study, cattle infected with *M. wenyonii* had greater bacteremia than cattle infected with *C. M. haemobos*, which the authors attributed to *M. wenyonii* being more available to ticks or in greater concentration in the blood (Hornok et al., 2011). Further research is needed to evaluate the transmission capability of insects including experimental infection of natural hosts. Understanding the role of vectors in transmission of hemoplasmas is crucial in developing practices to reduce exposure of cattle

Vertical transmission is thought to be rare but is considered as a possible route of infection for both bovine hemoplasmas (Fujihara et al., 2011). Researchers have demonstrated that 10.5 % of neonatal beef calves born to infected dams were infected with hemoplasma (Hornok et al., 2011). Later studies also suggested vertical transmission as an alternative route of hemoplasma infection (Sasaoka et al., 2012; Niethammer et al., 2018). In addition to possible vertical transmission, an association between hemoplasma infection of the dam and calf birth weights has been reported. Japanese researchers examined 71 dairy calves and their dams and reported that calves born to cows infected with hemoplasmas had lower birth weights as compared to PCR-negative calves (Tagawa et al., 2013). However, in this study, the route of transmission was uncharacterized because newborn animals were not sampled and the blood sampling was done several days after delivery (Tagawa et al., 2013).

The role of ingestion of infected colostrum in transmission of hemoplasmas to calves is not known. To date, no studies have documented transmission through ingestion of colostrum from infected dairy cows. In beef cattle, researchers did not detect DNA of *M. wenyonii* in 17 colostrum samples collected from infected dams (Sasaoka et al., 2015). However, it is important to acknowledge certain limitations, such as the small sample size and resulting low power to detect infection. Moreover, this study focused on detecting *M. wenyonii*, and further investigations with larger sample sizes and exploring the presence of both *M. wenyonii*, and *C. M. haemobos,* are needed to provide a

comprehensive understanding of transmission dynamics associated with colostrum.

Some transmission mechanisms of hemoplasmas may be similar to BLV as both are blood borne pathogens. It is thought that BLV is primarily transmitted through infected blood (Kuczewski et al., 2021). Several management practices have been related to transmission of BLV, including frequent re-use of needles and rectal palpation sleeves as well as failure to adequately remove blood from instruments used for dehorning and hoof-trimming (Divers et al., 1995; Kuczewski et al., 2021). Based on extrapolation from BLV, practices likely to result in exposure to blood from infected animals are potential routes of infection with hemoplasmas (Strugnell & McAuliffe, 2012) but transmission of hemoplasmas by fomites contaminated with blood has not been confirmed. On many dairy herds, there are numerous opportunities for exposure to infected blood from another animal. In a survey provided during a cross-sectional study in 82 dairy herds in Wisconsin and Michigan, producers estimated that from birth to maturity cows had received a total of 65 injections (Schambow et al., 2021). These producers estimated that each needle used for injection was used for 15.1 ± 2.6 animals before replacement. The use of palpation sleeves on multiple animals was also reported (Schambow et al., 2021). In that study, association between shared needles or palpation sleeves and hemoplasma infection was not possible as the herd-level prevalence was 100%. Thus, while management practices that can potentially transmit hemoplasmas are widespread, additional studies are needed to better define risk factors for transmission.

Better knowledge of potential routes of transmission (including vectors, fomites, vertical transmission, or consumption of colostrum) would help farmers make decisions about using management practices that may reduce transmission of these organisms. For example, the risk of BLV infection was 2.8-fold higher in cows palpated without changing rectal palpation sleeves as compared to cows that the sleeves were changed between animals (Divers et al., 1995). Management changes have been shown to be effective in reducing transmission of BLV. In one dairy herd with high prevalence of

BLV, use of a control program that included single use needles and obstetrical sleeves, disinfection of tattoo equipment, use of electrical dehorning, and feeding milk replacer or heat-treated colostrum, the prevalence of BLV was reduced from 44 to 17% in two years without culling or segregation of infected animals (Sprecher et al., 1991). As has been demonstrated for BLV, use of management practices such as control of insects, use of sterile needles, disinfection of instruments used for disbudding and dehorning and single use of rectal sleeves between animals, could potentially reduce transmission of hemoplasma in dairy herds.

1.9 RISK FACTORS FOR INFECTION AND DISEASE

Several potential risk factors for transmission of hemoplasmas have been reported in observational studies and risk analysis models including: age (Congli et al., 2011; Tagawa et al., 2012), gender (Byamukama et al., 2020), and living conditions (Tatsukawa et al., 2021). Age is a common risk factor for many diseases, and the prevalence of hemoplasmas has been observed to vary among animals at different age groups. In one study, cattle from 1 – 3 years of age had greater prevalence of hemoplasma infection as compared to younger animals (Tagawa et al., 2012). For infection with *C. M. haemobos* only, the proportion of infected cattle was greater in animals older than 2 years as compared to younger animals (Girotto et al., 2012). It is difficult to determine when animals become infected because after infections occur, the animals generally remain positive for the rest of their life (Messick, 2004). In addition, overall prevalence is determined by the incidence rate and duration of the infection, thus the proportion of hemoplasmas infections generally increases with age, as new cases become chronic.

Living conditions have been suggested as risk factors for hemoplasma infection (Messick, 2004; Tagawa et al., 2012). In one study, confined cattle had a greater prevalence of infection as compared to pastured cattle (Tagawa et al., 2012). Similar results were reported in a study that compared 138 grazing cows to 262 cows that were confined. In that study, the proportion of cows infected with *C. M.*

haemobos was greater in the confined group as compared to the grazing group (Tatsukawa et al., 2021). These results seem contradictory as it would be expected that pastured cattle would have greater exposure to vectors (such as ticks and flies) that could contribute to greater transmission. More research with a greater variety of herds with different management practices and associated risk factors would help to determine if housing and environmental conditions are associated with infection or occurrence of clinical signs.

Similar to mature cattle, a potential association between infection with hemoplasmas and housing of calves has also been reported (Schambow et al., 2021). In this study, herds that housed calves in outdoor hutches had greater prevalence of *C. M. haemobos* infection in adult cows as compared to herds that used only indoor calf housing. No association was observed for infection with *M. wenyonii* (Schambow et al., 2021). These preliminary associations demonstrate the need for additional research to determine if housing of calves and replacement heifers contribute to transmission of hemoplasmas.

Gender is another potential risk factor for hemoplasma infection, but conflicting conclusions have been made in different studies (Girotto et al., 2012; Byamukama et al., 2020). One researcher reported a greater proportion of female infected with *C. M. haemobos* than males (Girotto et al., 2012), while in a small Ugandan study the opposite was reported (Byamukama et al., 2020). A possible explanation for gender bias could be differences in stress experienced by females during pregnancy and lactation, which could lead to immunosuppression and increased susceptibility to infectious diseases (Evermann, 1994). However, these findings are not consistent with those of other studies that failed to find an association between gender and infection with hemoplasmas (Díaz-Sánchez et al., 2019; Mohd Hasan et al., 2017). Like other risk factors, additional research is needed to determine the role of gender in risk of hemoplasma infection.

Observational studies have been commonly used to describe hemoplasma prevalence and

potential risk factors (Schambow et al., 2021; Niethammer et al., 2018; Fujihara et al., 2011) . These studies have provided valuable insights into relationships between hemoplasma infection and various potential risk factors, including age, gender, and living conditions. However, it is important to acknowledge that observational studies are unable to establish causal inference due to the potential influence of unmeasured or unknown confounding variables that may impact the observed associations and selection bias can restrict the generalizability of the findings (Shott S 2011). To establish causal relationships, experimental studies are required. Experimental studies offer a more controlled research approach (Shott S 2011), allowing researchers to better isolate the true causal factors contributing to hemoplasma infection and provide stronger evidence for causal relationships.

1.10 RISK FACTORS FOR OCCURRENCE OF CLINICAL SIGNS

Little is known about risk factors that contribute to the occurrence of clinical signs in cattle infected with hemoplasmas. Previous studies suggest that infection with *M. wenyonii* is a necessary but not sufficient cause of clinical signs, which requires other conditions to manifest (Smith et al., 1990; Strugnell et al., 2011). In a specific instance, four lactating cows within a 40-cow dairy herd that were apparently healthy and tested positive for hemoplasmas, developed acute symptoms related to hemoplasma infection within six days of receiving a herd-wide bluetongue vaccine (Strugnell et al., 2011). In that case, hemoplasma infection was insufficient for the development of clinical signs. However, it appeared that the bluetongue vaccine might have played a role in suppressing the cows' immune systems, thereby triggering the manifestation of the signs. In summary, the occurrence of clinical signs in some cattle infected with hemoplasmas appears to require additional stressors, (such as vaccine-induced immune suppression), as observed in the four lactating cows in the dairy herd. Additionally, co-infection of hemoplasmas with other infections has also been suggested to contribute to the development of clinical signs (Hofman-Lehmann et al. 2004, Hornok et al., 2012).

Infection with *M. wenyonii* and *C. M. haemobos* usually only results in mild anemia due to the

attachment and damage of the erythrocytes (Smith et al., 1990). When animals are also infected with other hemotropic bacteria, the pathogenic effect of each may be enhanced and result in more severe clinical signs (Hornok et al., 2012). Concurrent infection with *Anaplasma marginale*, *Anaplasma phagocytophilum*, *Babesia*, *Theileria* spp and *M. wenyonii* was reported in a fatal outbreak in a dairy herd in Switzeland *(*Hofman-Lehmann et al. 2004). Another outbreak with fatal bovine anaplasmosis was later reported in beef cattle in Hungary, (cattle infected with *Anaplasma marginale* were also positive for *M. wenyonii* and *C. M. haemobos)* (Hornok et al., 2012). In both cases, although infection with *M. wenyonii* and *C. M. haemobos* were not considered to be the primary causative agents, they may have contributed to the morbidity and mortality experienced in the herd (Hofman-Lehmann et al. 2004, Hornok et al., 2012).

The pathogenesis of hemoplasmas in cattle remains poorly understood. Additional knowledge of risk factors that can trigger clinical signs, (including stress, vaccines, and coinfections) is needed to better understand the disease process.

1.11 CONCLUSIONS

This review highlights the significance of infection with *M. wenyonii* and *C. M. haemobos* in dairy cattle. While research about the burden of the disease in several countries has increased and methods of diagnosis have improved, the impact of hemoplasma infections on cattle health and productivity, as well as associated risk factors, requires more systematic research. Understanding the pathogenesis and epidemiology of hemotropic mycoplasma in dairy cows is crucial for developing effective prevention, control, and management strategies. Future research should focus on filling the knowledge gaps, identifying transmission pathways, and investigating the potential impact on dairy industry sustainability and animal welfare. By enhancing our understanding of these organisms, we can work towards minimizing the consequences of hemotropic mycoplasma infections on dairy animal health and the industry as a whole.

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CHAPTER 2: APPARENT PREVALENCE OF HEMOTROPIC MYCOPLASMA IN DAIRY CALVES ON MICHIGAN FARMS

2.1 ABSTRACT

The bovine hemoplasmas include *Mycoplasma wenyonii* and *Candidatus Mycoplasma haemobos* which are increasingly recognized as infecting cattle throughout the world. Infection with hemotropic mycoplasma has been reported to be widespread in mature dairy cows, but little is known about prevalence in calves and heifers. The objective of this study to investigate the prevalence and dynamics of infection with *M. wenyonii* and *C.M. haemobos* infections in calves and replacement heifers on Michigan dairy farms and assess potential associations between infection status and hematological values. The study was designed as a prospective cross-sectional study with a longitudinal component. A convenience sample of 11 farms agreed to participate and were visited twice between March and September 2022. During the first farm visit, researchers collected blood samples from up to 94 animals per farm distributed among newborn and pre-weaned calves ($n \leq 31$), weaned calves ($n = 21$), pre-breeding heifers ($n = 21$), and pregnant heifers ($n = 21$). During the first visit, blood samples ($n = 174$) were also collected from a convenience sample of mature cows to confirm their infection status. The same calves were sampled during the second visit which occurred 95 d (±3.01) later. During the first visit, blood samples were collected from 797 calves and replacement heifers, while 675 samples were collected during the second visit due to inability to locate some animals. Detection of *M. wenyonii* and *C. M. haemobos* were based on results of real time PCR. Hematocrit was determined using microcentrifugation and the concentration of the leukocytes using an automated cell counter. The herd-level apparent prevalence of infection in mature cows was 100% and the within herd apparent prevalence in mature cows was 95.1 ± 1.8%. Herd-level apparent prevalence for calves and replacement heifers was also 100% for both *M. wenyonii* and *C. M. haemobos*. The apparent prevalence of hemoplasma in calves was associated with age. In calves that were 1 to 6 months old, the prevalence of

infection was about 6-8% but sharply increased to 31% by 8 months of age. In older animals, the prevalence remained high, and was almost 100% in animals that were greater than 17 months of age. Based on animals that were sampled twice, the cumulative incidence varied widely among herds ranging from 3.7% to 96.0% and increased with age of animals. There was no difference in hematocrit or number of lymphocytes, monocytes, neutrophils, or total leukocytes based on infection status. The number of eosinophils was greater in infected animals. This is the first study to report the prevalence of hemoplasmas in calves and replacement heifers in the U.S. It indicates that young calves can be infected with hemoplasmas, but the rate of infection is low. The likelihood of infection increases as animals age, with a notable rise in the proportion of infected heifers occurring by 8 months old and the prevalence eventually reaching nearly 100% of infection in older animals. Once infected, heifers appear to remain chronic carriers for life. Hemoplasma infection alone does not usually lead to the development of clinical signs and most of the animals remain apparently healthy.

2.2 INTRODUCTION

Hemotropic mycoplasma (hemoplasma) are Gram-negative bacteria known to infect red blood cells of mammals including cats and dogs (Messick, 2003), swine (Gatto et al., 2019), cattle (Strugnell and McAuliffe, 2012; de Mello et al., 2019), and humans (Ristic M, 1979). The organisms are generally considered to be species specific and both *Mycoplasma wenyonii* (previously known as *Eperythrozoon wenyonii*) (Neimark et al., 2002) and *Candidatus Mycoplasma haemobos (C. M. haemobos)* (Tagawa et al., 2008*)* are described as infectious for cattle. *Mycoplasma wenyonii* was first identified in splenectomised calf in 1934 in Palestine (Adler and Ellenbogen, 1934) and later, detected in a splenectomized Holstein-Friesian bull in the United States (Lotze and Yiengst, 1941). Infections with hemoplasmas have been detected in both ill and apparently healthy cattle and in most infected cattle the disease remains subclinical and persistent (Messick, 2003; Montes et al., 1994; Smith A. et al., 1990). Many nonspecific signs have been attributed to hemoplasma infection, including immune-mediated

anemia (Gladden et al., 2016), anorexia (Genova et al., 2011), edema of the mammary gland (Smith A. et al., 1990), edema of rear legs (Genova et al., 2011), fever (Smith A. et al., 1990), prefemoral lymphadenopathy (Smith A. et al., 1990), reduced milk yield (Strugnell and McAuliffe, 2012; Gladden et al., 2016; Hoelzle et al., 2011), weight loss (Genova et al., 2011), and infertility (Montes et al., 1994). Decreased milk production has been observed in cows that are chronically infected with *M. wenyonii* and *C. M. haemobos* (Tagawa et al., 2013). In cattle presenting clinical signs, treatment using oxytetracycline has reportedly resulted in resolution of signs (Montes et al., 1994; Genova et al., 2011). Hemoplasmas are thought to be primarily transmitted through contact with infected blood, and some transmission has been attributed to vectors as well transplacental infection (Smith A. et al., 1990; Fujihara et al., 2011).

Although hemoplasmas have been reported to infect cattle in several countries (dos Santos et al., 2012; Girotto et al.2012; Tatsukawa et al 2021; Díaz-Sánchez et al., 2019), changes in the nomenclature and diagnostic methods have contributed to the lack of knowledge about the epidemiology and pathogenesis of these organisms. In recent years, there has been growing recognition of the scope of hemoplasma infections in cattle, particularly in countries in the northern hemisphere (Nishizawa et al., 2010; Tagawa et al., 2010, 2012, 2013; Fujihara et al., 2011; Schambow et al., 2021). These studies have documented widespread prevalence of infection and highlighted their significance as an emerging pathogen in cattle populations. While previous research has focused on the prevalence of these infections in adult cattle, limited information is available about infection in calves and replacement heifers. In a recent study that tested adult cows in 82 herds located in Wisconsin or Michigan, they reported 100% herd-level apparent prevalence for both *M. wenyonii* and *C. M. haemobos (*Schambow et al., 2021). The prevalence of infection was > 70% in first lactation cows, which suggested that infection occurred prior to initial calving *(*Schambow et al., 2021). Unfortunately, no studies have been conducted to investigate the rate of hemoplasma infection in calves and replacement

heifers. Given the limited understanding of hemoplasma epidemiology in cattle, understanding the rate of infection in calves can provide insights on the timing of infection, which is essential for the development of effective preventive measures.

The objective of this study was to determine the prevalence and dynamics of infection of with *M. wenyonii* and *C.M. haemobos* in calves and replacement heifers on Michigan dairy farms and assess potential associations between infection status and hematological values. We hypothesized that infections occurred prior to first calving, varied with age, and would impact some hematological values.

2.3 MATERIALS AND METHODS

2.3.1 Farm Eligibility and Recruitment

Based on 100% herd-level prevalence and >75% within-herd apparent prevalence of adult cows reported in a previous study of herds in this region (Schambow, et al 2021), a convenience sample of herds was used in this study. Herds that previously participated in a prevalence study of adult cows in Michigan (n=18) (Schambow et al., 2021) were mailed a recruitment letter and then contacted by phone to invite them to participate in this study. Additional herds in Michigan were recruited through announcements at regional conferences. All herds volunteered to participate and were chosen based on their location in Mid-Michigan, as well as having a sufficient number of animals, including pre-weaned calves to pregnant heifers. Farmers that expressed interest in the study were contacted by phone to schedule a sampling visit.

2.3.2 Animal Eligibility and Enrollment Criteria

All calves and replacement heifers between 1 day old and 27 months old were eligible to be tested. To detect at least 5% prevalence of infection with 95% confidence we estimated that we needed to sample $\geq$ 15 calves within each age group.

Based on the sample size estimate we planned to collect blood samples from up to 94 calves and replacement heifers per farm distributed as: newborn and pre-weaned calves <60 d of age (n < 31),

weaned calves (2-8 mo of age, n = 21), pre-breeding heifers (8-12 mo of age; n = 21), and pregnant heifers (>12 mo of age; n = 21). When herds contained less than our target number of animals in an age group, all animals in that group were sampled. Based on logistical challenges of identification and restraint of group housed replacement animals, when greater than the target number of animals were present in a group, convenience sampling was used to select animals. While our primary objective was to estimate prevalence in calves and replacement heifers, to verify that all enrolled farms contained infected mature cows, during the first farm visit blood samples were also collected from a convenience sample of about 15 mature cows.

2.3.3 Sample Collection and Questionnaire

Each farm was visited twice. During the first visit, researchers collected 2, 5 mL vials of blood into EDTA tubes using venipuncture of the jugular veins or coccygeal vessels. A unique ear tag was attached to each animal to facilitate identification during the second farm visit and demographic data (age, breed, and housing) was collected. During the second visit, a brief questionnaire (available from the authors) was used to collect information about housing, husbandry, vaccination protocols, treatment protocols, and reproductive management. During the second farm visit, blood samples were collected from animals that had been tested previously and were easily able to be located. All blood samples were promptly cooled to 4°C. One of the duplicate samples was sent to the Michigan State University's Veterinary Diagnostic Laboratory (located in Lansing, MI) to undergo Real Time PCR (rt-PCR) testing, while the other was used to determine hematocrit and leukocyte differential count in the Ruegg Laboratory in the College of Veterinary Medicine at Michigan State University.

2.3.4 Real Time PCR Detection (rt-PCR)

Whole blood in evacuated tubes containing EDTA were stored at 4° C for 24 to 48 hours before DNA extraction. Immediately before extraction, the tubes of blood were inverted several times to mix. A magnetic bead assisted DNA extraction method was performed using the KingFisher Flex Purification

System (Thermo Fisher Scientific, Waltham MA) with the MagMAX Core Nucleic Acid Purification Kit (Thermo Fisher Scientific, Waltham, MA), following the manufacturer's instructions. Briefly, 200 µl whole blood from a sample was added to a well of King Fisher™ Flex Deep Well 96 Plate which contained 20 ul of MagMAX™ CORE magnetic bead solution and 10 ul of MagMAX™ CORE proteinase. This was mixed gently by pipetting up and down several times and was followed by a 2-minute incubation at room temperature. Then 700 µl of a 1:1 mixture of MagMAX™ Core Lysis Solution with MagMAX™ CORE Binding Solution was added to the well. The plate of blood samples in extraction reagents were loaded onto the King Fisher™ Flex platform that had been preloaded with a Pharma KingFisher™ Flex 96 Deep-Well Tip Comb plate, appropriate plates of wash solution, and a plate of elution solution. The wash and elution solutions were supplied in the kit. The MagMAX Core Flex program was used for automated DNA extraction and the extracted DNA was eluted into 90 µl of elution solution. Plates of eluted DNA were stored frozen at -20° until use.

The PCR reaction mixture was 10 µl of 2X PerfeCTa® qPCR ToughMix® Low Rox™ (Quanta Bio, Beverly, MA), 400 nmol each of forward and reverse PCR primers, and 250 nmol of hydrolysis probe. Molecular grade water was added to the reaction mix to bring the reaction mix volume to 18 µl. Finally, 2 µl of extracted sample DNA or positive control DNA was added to the reaction mixture. The negative control was 2 µl of molecular grade water added to the reaction mixture. To detect and quantitate copies of DNA from *M. wenyonii* or *C. M. haemobos*, real-time qPCR assays specific to each organism were used. The PCR primers and probes used targeted the 16S ribosomal RNA gene. A common set of PCR primers was used for both *M. wenyonii* and *C. M. haemobos*. The forward primer, 5'-GAAAGYCTGATGGAGCAATA-3', had a predicted melting temperature (Tm) range of 56/59° C and the reverse primer, 5'-SCTTTACGCCCAATAAATC-3', had a predicted Tm range of 55/56°C. The hydrolysis probe for *C. M. haemobos*, FAM-TGAGGTACT/ZEN/ATCAGTTGTTATCCCTC-3IABkFq), had a predicted Tm of 65°C. The hydrolysis probe for *M. wenyonii*, JOEN-CGCGCCTTG/ZEN/ATGGTACTAATTGA-3IABkFq, had

a Tm of 66°C. The PCR primers and probes were obtained from Integrated DNA Technologies, Coralville, IA. Reaction conditions were optimized using a temperature gradient to determine optimal annealing temperature(s) for the reaction.

The PCR reaction used 2x PerfeCTa qPCR ToughMix Low Rox (Quanta Bio, Beverly, MA). The positive controls for the PCR assays were separate synthetic DNAs that included the PCR primers and probe for *M. wenyonii* or for *C. M. haemobos* (Integrated DNA Technologies, Coralville IA). Those positive controls were included in duplicates of 10-fold dilutions of known concentration of synthetic DNA to produce a standard curve for quantitation of organism DNA copy number. Two negative controls included for each assay consisted of reagent mix plus molecular grade water. The PCR assays for detection of *M. wenyonii* or *C. M. haemobos* consisted of a single initial polymerase enzyme activation step of 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 55°C for 1 min. The target amplification was monitored in real time using an ABI 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA) and software that was supplied by the manufacturer. To reduce risk of cross-contamination during the PCR essays, reagent preparation, nucleic acid extraction, addition of sample DNA to the PCR reactions tubes, and the PCR essay all were done in separate rooms with dedicated supplies and laboratory gowns.

The criteria for the standard curves representing copy number of DNA from *M. wenyonii* or from *C. M. haemobos* were defined by 5 data points of a 10-fold serial dilutions synthetic DNA of known copy number, each dilution was tested in duplicate. The R^2 value to assess linearity of the standard curve was required to be greater than or equal to 0.980. If the R^2 value fell below 0.980, one datum point (typically the lowest or highest concentration or an outlier) could be removed, changed from a standard to an unknown, and the data reanalyzed. If the R^2 value remained below 0.980 even after the adjustment, one more datum point could be removed if there were three consecutive linear duplicate datum points. If the R^2 value still did not meet the threshold, the standard curve was considered a failure, and the run

was repeated with a new set of 10-fold dilutions of the synthetic DNA. Threshold setting was described, with both auto threshold and manual options (*C. M. haemobos*: 0.2 +/- 0.05 and *M. wenyonii*: 0.03 +/- 0.05). Threshold crossing (Ct value) ranges were provided to interpret the PCR results, classifying positive as Ct < 35.4, suspect as Ct 35.5 – 37.0, or negative as Ct > 37.0.

Samples falling within the suspect range were confirmed as positive or negative using an in-house diagnostic gel-based PCR assay targeting the 16S ribosomal RNA gene. The forward PCR primer for the assay was 5´-ACGAAAGTCTGATGGAGCAATAC-3´ and the reverse PCR primer was 5´-ACGCCCAATAAATCCGRATAATG-3´. The reaction mixture concentration was 12.5 µl AmpliTaq Gold 360 Master Mix (Thermo Fisher Scientific, Waltham MA), 400 nmol each for the forward and reverse PCR primer, and molecular biology grade water added to a final volume of 18 µl. A 2 µl aliquot of either, sample DNA, positive control DNA, or negative control molecular biology grade water were added to complete the reaction mixture. The positive control DNA was the same as that used for the qPCR assay. The reaction conditions were 1 cycle of 95° C for 5 min then 40 cycles of 56° C for 30 sec and 72° C for 30 sec, followed by a final extension step of 72° C for 5 minutes. The PCR product was electrophoresed through a 1.5% agarose gel and visualized with ethidium bromide staining. Detection of a PCR amplicons of 170 bp and/or 193 bp indicated the sample was positive.

2.3.5 Hematological Analysis

The hematocrit of each blood sample was determined using microcentrifugation (Hawksley and Sons, Ltd, Sussex, UK) at 18,600×g for 5 minutes. The hematocrit value was measured using a micro-capillary reader (Damon/IEC Division, Needham Heights, MA, USA). To ensure precision and accuracy, triplicate capillary tubes were used for each blood sample, and the mean value was calculated. The leukocyte differential (count of eosinophils, lymphocytes, monocytes, neutrophils, and total leukocytes) was measured using an automated cell counter (QScout, Advanced Animal Diagnostics, Morrisville, NC) according to the manufacturer's protocol.

2.3.6 Study Approval

This study was approved by the Institutional Animal care and Use Committee at Michigan State University (PROTO202100154) and was deemed exempt from human subjects' review by the Institutional Review Board at Michigan State University (Study ID 0008451).

2.3.7 Statistical Procedures

Animals were the unit of analysis. Statistical analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC) and significance was defined as $P \leq 0.05$. Normality of the data was evaluated using normal probability and box plots with PROC UNIVARIATE. Descriptive statistics were performed using PROC MEANS and used to characterize the participating herds, animals, and hematological findings. For categorical variables the x^2 test or Fisher's exact test (cell frequencies of ≤5) was used. The prevalence of hemoplasmas was calculated as the number of PCR positive animals divided by the total number of animals sampled on the first visit. The age of the animals was analyzed as a categorical variable with animals classified in groups (group 1: 1-3 months of age, group 2: 4-6, group 3: 7-9, group 4: 10-12, group 5: 13 -15, group 6: 16-18, group 7:19 -21, group 8: 22 – 28 months of age). The association between the age group categories and the likelihood of hemoplasma infection was evaluated using a Logistic regression model (PROC LOGISTIC). Cumulative incidence rates were calculated as follows:

Cumulative incidence = number of new cases occurred during observation period / total calves at risk

The numerator was the number of animals that were PCR negative at first visit and become positive at the second test, and the denominator was the number of calves that were PCR negative on the first sampling. Animals that were negative during the first sampling but were lost to follow up, were excluded from the denominator. As the time to follow up varied among farms, the incidence density rate was used for comparing incidence among different age groups and farms. Incidence density was calculated as the number of new PCR positive cases divided by the number of calf-days at risk. Calf-days

at risk were calculated by multiplying the number of calves that tested PCR negative during the first sampling by the number of days between the two visits. Incidence density was expressed as the number of new cases per 100 calf-months at risk. PROC GLIMIXX was used to compare the incidence density rate among the different age groups, including farm as a random effect. Group comparisons were conducted using the Tukey adjustment for multiple comparisons. The normality of residuals was assessed by employing PROC UNIVARIATE and visually explored through residual plots.

Separate analyses were performed to examine the association between infection status for *M. wenyonii*, *C. M. haemobos* or co-infection, and dependent hematological variables. PROC GLIMMIX was used to test the hypothesis that each dependent variable (PCV, Monocytes count, Neutrophil count, Lymphocytes count, or Total Leukocyte count) was associated with infection status (0 = PCR negative, 1 = PCR positive) and each model included herd as random effect and age as a fixed effect. Neutrophil count was $\log_{10}$ transformed to achieve normality. Because the eosinophil count was not normally distributed, the Mann–Whitney U test was used to assess the hypothesis that eosinophil count was associated with infection status.

To compare the hematological values, animals were classified into two groups based on infection status during each of the two visits. The NEG group consisted of animals that tested negative on both visits, while NEWINF comprised animals that tested negative initially but became positive in the subsequent visit. An independent two-sample t-test was employed to test the hypothesis that there was an association between the mean of each dependent variable (PCV, LC, MC, NC, and TLC) and infection status (NEG, NEWINF) groups. To ensure normality, the NC and MC variable were $\log_{10}$ transformed. As the EC was not normally distributed, the Mann-Whitney U test was performed to test the hypothesis that there was a difference between the distribution of the EC among the two groups (0 = NEG, 1 = NEWINF). PROC MIXED was used to test the hypothesis that the dependent variable PCV was associated with the nucleic acid load (cycle threshold (CT)) of the rt-PCR for each hemoplasma infection (CT for *M.*

wenyonii and CT for *C. M. haemobos*) and the model included age as fixed effect.

2.4 RESULTS

2.4.1 Herd Characteristics and General Management Practices

Of 18 farms that had participated in the previous study, 5 (27.2%) were enrolled in this study. An additional 6 farms that had not been previously sampled were recruited based on contacts at industry meetings. The herds were located in 9 counties in central Michigan (Ingham, Ionia, Isabella, Barry, Washtenaw, Osceola, Gratiot, Clinton, and Montcalm). Enrolled herds together contained a total of about 6,850 lactating and dry cows, 773 pre-weaned calves, 1,254 weaned calves, 1,448 pre-breeding heifers and 2,401 pregnant heifers. The median number of mature cows per herd was 260 but ranged in size from 108 to 3,9000 mature cows (mean = 622 ± 1102 SE). The average bulk tank SCC was 140,000 cells/mL ranging from 60,000 to 275,000 calls/mL. All mature cows were housed in freestalls while pre-weaned calves were mostly housed in individual hutches (73.7% of farms), and 26.2% of farms used group housing for this group. All farms housed weaned, pre-breeding and pregnant heifers in groups. Typical of dairy farms in Michigan, animals were predominantly Holstein (98.9%). About 45% of farms provided occasional access to pasture throughout the year for some age groups. Fly control measures were used on 81.8% of the farms. Approximately 50% of farmers stated that they used needles on more than one heifer, with an estimated average of 6.5 heifers per needle. Furthermore, 36% reported reusing palpation sleeves on multiple animals.

Each herd was visited twice, with an average interval of 95 d (SE = 3.01) between visits ranging from 85 to 120 d. Farms were visited for the first time between March and June 2022 and second visits occurred between June and September 2022. During the first visit, blood samples (n = 797) were collected from calves and replacement heifers with about 72. 5 ± 5.78 animals per farm tested (range was 41 to 95 animals per farm). Not all farms contained all age groups of calves. During the second visit, 122 (15%) animals could not be located resulting in collection of blood from of 675 calves and

replacement heifers. The number of animals per farm tested during the second visit was 61.4 (±5.5) and ranged from 32 to 81 animals. During the first herd visit, blood samples (n = 174) were also collected from mature cows (median of 15 per farm, ranging from 8 to 30).

2.4.2 Apparent Prevalence

The herd-level apparent prevalence of infection of calves and heifers was 100% for both hemoplasmas (Table 1). The first herd tested only contained calves ≤ 4 months of age (pre-weaned and weaned) and had an apparent prevalence of 7.1% for *M. wenyonii* and 4.7% for *C. M. haemobos* (Table 2.1). For the remaining 10 herds, within-herd prevalence for calves and replacement heifers was associated with farm and ranged from 28 to 68% (Table 1; $P < 0.001$). Within-herd apparent prevalence of the 10 herds that included all age groups was 10.3% ± 1.2% (77/755) for infection with *M. wenyonii* only (ranging from 3.6 to 17.8%), 7.5% ± 1.3% (56/755) for *C. M. haemobos* only (ranging from 1.2% to 15.2%), and 23.5% ± 3.7% (172/755) for co-infection with both bovine hemoplasmas (ranging from 10.5% to 43.5%) (Table 2.1).

Mycoplasma wenyonii and *C. M. haemobos* were identified by rt-PCR in blood samples obtained from at least 8 mature cows in all herds (herd-level apparent prevalence in cows of 100%). Of the 11 herds, 5 herds had a 100% apparent prevalence of infection with *M. wenyonii,* or *C. M. haemobos* as none of the mature cows tested negative (Table 2.2). Among all farms only 4.8% ± 1.8% of the cows tested negative. Within-herd apparent prevalence of *M. wenyonii* in mature cows differed among farms ($P = 0.01$), but no difference was observed for infection with *C. M. haemobos* only ($P = 0.25$), or co-infection with both hemoplasmas ($P = 0.12$).

The apparent prevalence and odds of infection with hemoplasmas increased as calves aged (Table 2.3). The infection rate remained relatively stable at around 6-8% during the first six months of life. A sharp increase occurred by 8 mo, and thereafter continued increasing to almost 100% prevalence after 17 mo of age (Figure 1). Among all farms, the least proportion of positive tests were observed in

calves aged 1 to 3 mo, with proportions of positive tests ranging from 0.0% to 18.1% (Table 2.3). For calves aged 4 to 6 mo positive animals ranged from 0.0% to 25.0%. As compared to prevalence in the first 3 mo of age, the likelihood of infection was similar 1.0 (95% CI: 0.3 - 2.7) for animals 4-6 mo of age. The proportion of infection increased for animals aged 7 – 9 mo ranging from 0% to 66.6% and the likelihood of infection was 4.7 (95% CI: 2.3 - 9.7) times greater as compared to animals in the first 3 mo of age. Both proportion of infection and the likelihood of being infected increased considerably in animals > 10 mo. As compared to animals in the group 1 (age 1-3 mo), the likelihood of infection increased dramatically during the second year of life (Table 2.3).

The apparent prevalence showed a positive association with age group for infection with *C. M. haemobos* only ($P < 0.01$), *M. wenyonii* only ($P < 0.01$), and co-infection with both hemoplasmas ($P < 0.01$) (Table 2.4). Notably, only one calf in the 1 – 3 mo age group was co-infected with both hemoplasmas, while 6 (1.6%) tested positive for *C. M. haemobos* only, 18 (5.0%) for *M. wenyonii* only and 334 (93.0%) were negative for hemoplasmas (Table 2.4). The proportion of single infections for animals aged 4-6 months was <5% for both organisms (Table 2.4) and no calves were co-infected. Co-infection became more prevalent in calves aged 7-9 mo as compared to younger animals (Table 2.4), but the proportion of single infections did not vary between *C. M. haemobos* and *M. wenyonii* (Table 2.4). For animals > 10 mo of age, co-infection was more common as compared to single infections and most calves were positive (Table 2.4).

Table 2.1 – Within herd apparent prevalence of infection with *Candidatus Mycoplasma haemobos* and *Mycoplasma wenyonii* using rt-PCR for 797 calves and heifers aged between 1 day and 27 months old, tested on 11 farms in Michigan in March – June 2022 (initial sampling).

Farm	Month of Initial visit	Number of lactating cows	Number of calves and replacement heifers	Number of calves and replacement heifers tested	Positive rt-PCR result (%)			
					C. M. haemobos	*M. wenyonii*	Co-infection	Negative
1	March	260	70	42[a]	2 (4.7)	3 (7.1)	0 (0.0)	37 (88.1)
2	March	120	99	58	2 (3.4)	4 (6.9)	25 (43.1)	27 (46.5)
3	March	108	69	41	4 (9.7)	5 (12.2)	6 (14.6)	26 (63.4)
4	April	698	581	85	13(15.2)	8 (9.4)	9 (10.5)	55 (64.7)
5	April	130	105	62	7 (11.2)	8 (12.9)	27 (43.5)	20 (32.2)
6	April	400	549	94	4 (4.2)	9 (9.5)	20 (21.2)	61 (64.8)
7	May	480	390	84	7 (8.3)	15 (17.8)	9 (10.7)	53 (63.1)
8	May	375	350	83	8 (9.6)	3 (3.6)	12 (14.4)	60 (72.2)
9	May	3900	3327	95	6 (6.3)	7 (7.3)	24 (25.2)	58 (61.0)
10	May	160	196	81	1 (1.2)	8 (9.8)	21 (25.9)	51 (62.9)
11	June	220	230	72	4 (5.5)	10 (13.8)	19 (26.3)	39 (54.1)

[a]Only pre-weaned and weaned calves ≤ 4 months of age were present on this farm.

Table 2.2 – Within herd apparent prevalence of infection with *Candidatus Mycoplasma haemobos* or *Mycoplasma wenyonii* using rt-PCR for 174 mature cows tested on 11 farms in Michigan in March – June 2022 (initial sampling).

		PCR (%)			
Farm	Number of cows tested	*C.M. haemobos*	*M. wenyonii*[1]	Co-infection	Negative
1	30	6 (20.0)	5 (16.6)	18 (60.0)	1 (3.3)
2	11	5 (45.4)	1 (9.0)	3 (27.2)	2 (18.1)
3	8	0 (0.0)	3 (37.5)	5 (62.5)	0 (0.0)
4	16	8 (50.0)	0 (0.0)	8 (50.0)	0 (0.0)
5	15	3 (20.0)	3 (20.0)	9 (60.0)	0 (0.0)
6	15	4 (26.6)	3 (20.0)	8 (53.3)	0 (0.0)
7	15	5 (33.3)	3 (20.0)	6 (40.0)	1 (6.6)
8	17	2 (11.7)	0 (0.0)	15 (88.2)	0 (0.0)
9	16	2 (12.5)	3 (18.7)	9 (56.2)	2 (12.5)
10	15	0 (0.0)	3 (20.0)	11 (73.3)	1 (6.6)
11	16	5 (31.2)	2 (12.5)	8 (50.0)	1 (6.2)

[1] Prevalence differed among farms (P = 0.01).

Table 2.3 - Apparent prevalence of infection with either *Mycoplasma wenyonii* or *Candidatus Mycoplasma haemobos* in calves and replacement heifers by farm and months of age at the first sampling visit detected using real-time PCR on 11 farms in Michigan.

	AGE GROUPS (%)							
	1	2	3	4	5	6	7	8
FARMS	Mo. 1-3	4-6	7-9	10-12	13-15	16-18	19-21	22-28
1 (n=42)[a]	5/40 (12.5)	0/2 (0.0)	-	-	-	-	-	-
2 (n=58)	0/12 (0.0)	0/12 (0.0)	3/6 (50.0)	8/8 (100)	5/5 (100)	4/4 (100)	6/6 (100)	5/5 (100)
3 (n=41)	0/6 (0.0)	0/10 (0.0)	2/3 (66.6)	6/11 (54.5)	3/6 (50.0)	1/2 (50.0)	-	3/3 (100)
4 (n=85)	2/40 (5.0)	0/3 (0.0)	3/14 (21.4)	6/7 (85.7)	11/13 (84.6)	7/7 (100)	-	1/1 (100)
5 (n=62)	1/13 (7.6)	1/4 (25.0)	2/5 (40.0)	12/12 (100)	8/9 (88.8)	2/3 (66.6)	12/12 (100)	4/4 (100)
6 (n=94)	1/52 (1.9)	-	0/1 (0.0)	9/18 (50.0)	18/18 (100)	5/5 (100)	-	-
7 (n=84)	1/21 (4.7)	4/21 (19.0)	-	1/2 (50.0)	9/15 (60.0)	11/17 (64.7)	5/7 (71.4)	0/1 (0.0)
8 (n=83)	3/39 (7.6)	0/16 (0.0)	0/7 (0.0)	-	14/15 (93.3)	5/5 (100)	1/1 (100)	
9 (n=95)	0/53 (0.0)	-	-	8/12 (66.6)	8/9 (88.8)	-	11/11 (100)	10/10 (100)
10 (n=81)	4/39 (10.2)	-	5/21 (23.8)	-	-	1/1 (100)	20/20 (100)	-
11 (n=72)	8/44 (18.1)	0/2 (0.0)	-	1/1 (100)	9/10 (90.0)	12/12 (100)	3/3 (100)	-
Overall Prev.	25/359 (6.9)	5/70 (7.1)	15/57 (26.3)	51/71 (71.8)	85/100 (85.0)	48/56 (85.7)	58/60 (96.6)	23/24 (95.8)
Odds Ratio[b]	1.00	1.02	4.77	34.06	75.70	80.16	387.43	307.43
95% CI	1.00	0.37 - 2.78	2.33 - 9.76	17.4 - 65.76	38.2 - 149.88	34.20 - 187.86	89.34 - >999.99	39.83 - > 999.99

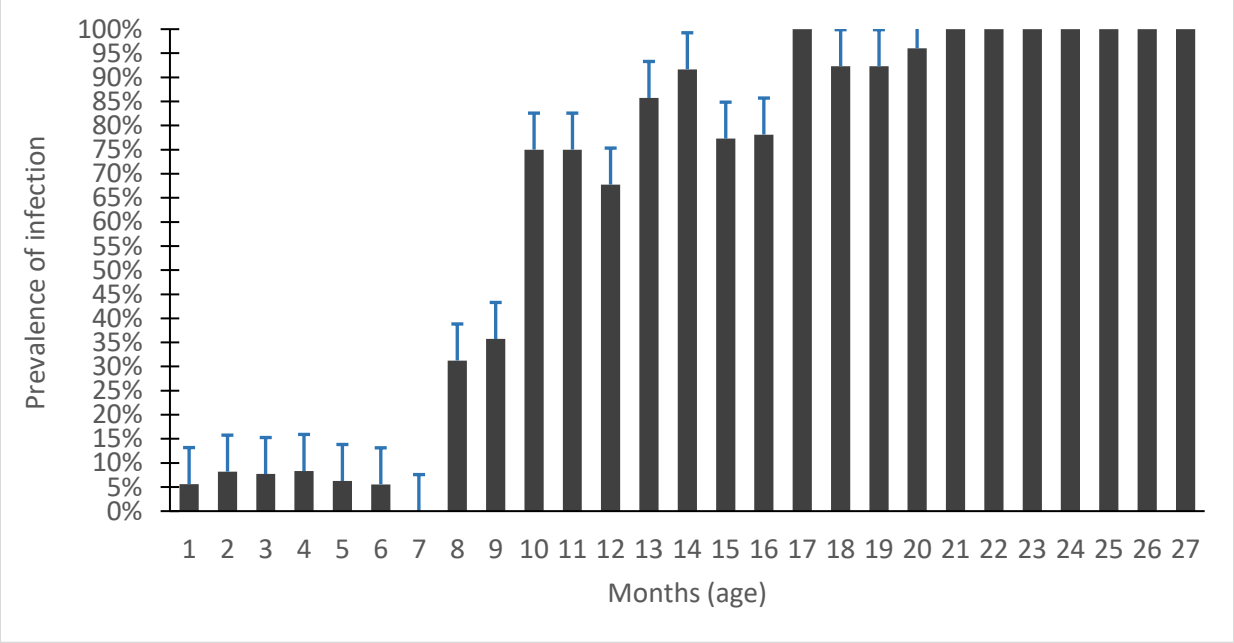
[a]Only pre-weaned and weaned calves ≤ 4 months of age were present on this farm; [b]odds of age group testing positive as compared to group 1.

Table 2.4 – Apparent prevalence of infection with *Mycoplasma wenyonii*, *Candidatus Mycoplasma haemobos* and co-infection by months of age at the first sampling visit tested using rt-PCR on blood samples collected from calves and replacement heifers on 11 farms in central Michigan during March-June 2022 .

Groups	Months of age	PCR (%)			
		C. M. haemobos n (%)	*M. wenyonii* n (%)	Co-infection n (%)	Negative n (%)
1 (n=359)	1-3	6 (1.6)	18 (5.0)	1 (0.2)	334 (93.0)
2 (n=70)	4-6	2 (2.8)	3 (4.2)	0 (0.0)	65(92.8)
3 (n=57)	7-9	5 (8.7)	5 (8.7)	5 (8.7)	42(73.6)
4 (n=71)	10-12	9 (12.6)	18 (25.3)	24 (33.8)	20(28.1)
5 (n=100)	13-15	21 (21.0)	20 (20.0)	44 (44.0)	15(15.0)
6 (n=56)	16-18	11 (19.6)	7 (12.0)	30 (53.5)	8 (14.2)
7 (n=60)	19-21	2 (3.3)	8 (13.3)	48 (80.0)	2 (3.3)
8 (n=24)	>22	2 (8.3)	1 (4.1)	20 (83.3)	1 (4.17)
P-value [1]		P<0.01	P<0.01	P<0.01	

[1] Prevalence was associated with age groups. P-values were derived from x^2 test.

Figure 2.1 – Apparent prevalence of infection with either *Mycoplasma wenyonii* or *Candidatus Mycoplasma haemobos* by month of age at the first sampling visit for calves and replacement heifers on 11 farms in central Michigan during March-June 2022.



Bars represent SE.

2.4.3 Incidence and Persistence of Infection

Among herds, the cumulative incidence of infection during the 95 day period between sampling visits varied from 3.7% to 96.0% (36.0 ± 9.7) (Table 2.5). A greater cumulative incidence was observed for farms 10 and 11 when compared to the other farms (P<0.001; Table 2.5). Most of the calves that were positive on the first visit remained positive for hemoplasmas at the second visit, (89.6% ± 3.6). Apparent clearance (indicating animals were initially PCR positive but became negative when tested at the second visit), varied from 0% to 40.0% (10.18 ± 3.6).

The incidence density rate differed among groups of age for infection with both *M. wenyonii* and *C. M. haemobos* (Table 2.6). The incidence density rate for animals in group 5 (13 -15 mo) was greater compared with animals in group 1 (1 – 3 mo) (P = 0.008) and group 2 (4 – 6) (P = 0.02). A greater incidence density rate was also observed in group 4 (10 – 12 mo) compared to group 1 (1 – 3 mo) (Table 2.6; P = 0.04).

Table 2.5 – Herd-level apparent prevalence, persistence infection, apparent absence, and cumulative incidence of *Candidatus Mycoplasma haemobos* and *Mycoplasma wenyonii* tested using rt-PCR on blood samples collected twice from calves and replacement heifers on 11 farms in central Michigan during March-September 2022.

						Both visits[1]				
Farm	N sampled visit 1	Apparent prev. visit 1 (%)	Neg visit 1 (%)	N sampled both visits (%)	Days between visits (%)	Prevalence (%)	Persistence[2] (%)	Apparent clearance[3] (%)	Neg visit1 sampled twice (%)	Incidence[4] (%)
1	42[a]	5/42 (11.9)	37/42 (88.0)	42	92	5/42 (11.9)	3/5 (60.0)	2/5 (40.0)	37	4/37 (10.8)
2	58	31/58 (53.4)	27/58 (46.5)	58	89	31/58 (53.4)	31/31 (100.0)	0/31 (0.0)	27	1/27 (3.7)
3	41	15/41 (36.5)	26/41 (63.4)	32	91	7/32 (21.8)	6/7 (85.7)	1/7 (14.2)	25	8/25 (32.0)
4	85	30/85 (35.2)	55/58 (64.7)	47	90	11/47 (23.4)	9/11 (81.8)	2/11 (18.1)	36	4/36 (11.1)
5	62	42/62 (67.7)	20/62 (32.2)	39	106	21/39 (53.8)	20/21 (95.2)	1/21 (4.7)	18	5/18 (27.0)
6	94	33/94 (35.1)	61/94 (64.8)	73	92	14/73 (19.1)	13/14 (92.8)	1/14 (7.1)	59	20/59 (33.8)
7	84	31/84 (36.9)	53/84 (63.1)	75	92	26/75 (34.6)	24/26 (92.3)	2/26 (7.6)	49	10/49 (20.4)
8	83	23/83 (27.7)	60/83 (72.2)	76	91	18/76 (23.6)	18/18 (100.0)	0/18 (0.0)	58	21/58 (36.2)
9	95	37/95 (38.9)	58/95 (61.0)	81	85	33/81 (40.7)	27/33 (81.8)	6/33 (18.1)	48	17/48 (35.4)
10	81	30/81 (37.0)	51/81 (62.9)	81	101	30/81 (37.0)	30/30 (100.0)	0/30 (0.0)	51	49/51 (96.0)
11	72	33/72 (45.8)	39/72 (54.1)	71	120	32/71 (45.0)	31/32 (96.8)	1/32 (3.1)	39	35/39 (89.7)

[a]Only pre-weaned and weaned calves ≤ 4 months of age were present on this farm;[1]rt-PCR results from animals that were sampled twice, excluding the animals lost to follow up;[2]Persistence apparent prevalence, represents animals that tested positive on both samplings;[3]Apparent clearance represents animals that were PCR positive at the first sampling and became negative on the second sampling;[4]Cumulative incidence differed by farm (P <.0001); P-value was derived from fisher exact test.

Table 2.6 – Incidence Density Rate (per 100 animal-months)[1] of infection with either *Mycoplasma wenyonii* and or *Candidatus Mycoplasma haemobos* by age groups recorded at the first sampling visit tested using rt-PCR on blood samples collected two times from calves and replacement heifers on 11 farms in central Michigan during March-September 2022.

		FARMS												
Groups	Age Months	1	2	3	4	5	6	7	8	9	10	11	Mean	SE
1 (n=332)	1-3	3.73	0.00	5.49	3.03	0.00	8.95	-	2.83	10.67	10.93	27.78	7.34[a]	2.59
2 (n=69)	4-6	0.00	2.81	3.30	0.00	18.90	-	6.11	22.66	-	-	15.15	8.61[ab]	3.16
3 (n=52)	7-9	-	0.00	0.00	6.67	28.30	-	-	32.97	-	-	-	13.58[abc]	7.10
4 (n=67)	10-12	-	-	19.78	0.00	-	24.46	32.61	-	26.47	31.94	-	22.54[b]	4.91
5 (n=59)	13- 15	-	-	32.97	-	-	-	19.57	32.97	35.29	-	30.30	30.22[bc]	2.77
6 (n=38)	16 – 18	-	-	32.97	-	-	-	6.52	-	-	-	-	19.74[abc]	13.22
7 (n=58)	>19	-	-		-	-	-	32.61	-	-	-	-	-	-

[a,b]Mean values within the same column with different superscripts differ from each other ($P < 0.05$).

[1]Incidence density rate was calculated as the number of new PCR positive cases on the second sampling by the number of calf-days at risk and expressed as the number of new cases per 100 calf-months at risk.

2.4.4 Hematological Findings

On the first visit, of 797 blood samples, leukocyte counts (including eosinophils, lymphocytes, monocytes, neutrophils, and total leukocytes), were performed on 728 and hematocrit were performed on 789 samples. On the second visit, of 675 animals sampled, leukocyte differentials were processed for 627 samples, while hematocrits were performed on 672 samples (Table 2.7). The mean hematocrit and leukocytes counts were within normal reference ranges on both visits. There were no differences observed in PCV, LC, MC, NC, and TLC based on rt-PCR test results for hemotropic mycoplasma ($P>0.06$). Greater EC ($P < 0.001$) was observed on both visits for PCR-positive animals compared to PCR-negative.

The mean value of all blood tests were within their reference ranges for animals that were negative at both visits (NEG) and for animals that became positive at the second visit (NEWINF). Except for greater eosinophil counts ($P <0.01$) and lower monocytes counts ($P = 0.01$), animals that developed new infections did not have differences in hematological values as compared to animals that remained PCR-negative (Table 2.8).

There were no associations between PCV and CT values of animals infected either with *M. wenyonii* (*P >0.08*) or *C. M. haemobos (P >* 0.16) on either the first or second sampling visits.

Table 2.7 – Hematological findings in calves and replacement heifers tested for infection with *Mycoplasma wenyonii* or *Candidatus Mycoplasma haemobos* tested using rt-PCR at 2 sampling visits on 11 farms in central Michigan during March-September 2022.

		PCR-positive				PCR-negative				
Variables	N analyzed	Mean ± SE	Median	Q1[1]	Q3[2]	Mean ± SE	Median	Q1	Q3	P-value[3]
PCV (%) (24.0 – 46.0)										
V 1[4]	789	31.41 ± 0.18	31.16	29	33.66	32.14 ± 0.19	32.33	29.50	35.00	0.83
V 2[5]	672	30.89 ± 0.15	30.50	29.50	33.00	31.24 ± 0.16	31.00	29.50	33.50	0.06
Eosinophil (10^3/µL) (0.1- 1.2)										
V 1	728	0.31 ± 0.02	0.23	0.12	0.38	0.11 ± 0.00	0.06	0.02	0.15	**<0.01**
V 2	627	0.35 ± 0.01	0.29	0.20	0.43	0.21 ± 0.01	0.16	0.09	0.25	**<0.01**
Lymphocyte (10^3/µL) (1.8- 8.1)										
V 1	728	6.39 ± 0.09	6.33	5.35	7.26	6.03 ± 0.07	5.88	4.93	6.88	0.68
V 2	627	6.63 ± 0.09	6.55	5.35	7.58	7.09 ± 0.10	6.97	5.90	8.00	0.20
Monocyte (10^3 /µL) (0.1- 0.7)										
V 1	728	0.59 ± 0.01	0.57	0.45	0.72	0.79 ± 0.01	0.74	0.56	0.97	0.74
V 2	627	0.59 ± 0.01	0.54	0.41	0.72	0.75 ± 0.01	0.74	0.57	0.88	0.20
Neutrophile (10^3/µL) (1.7- 6.0)										
V 1	728	3.02 ± 0.06	2.85	2.29	3.6	3.28 ± 0.08	2.94	2.14	3.98	0.25
V 2	627	3.23 ± 0.06	3.05	2.32	3.85	3.47 ± 0.09	3.11	2.44	4.30	0.26
Total Leukocyte (10^3/µL) (5.1- 13.3)										
V 1	728	10.38 ± 0.13	10.21	8.97	11.85	10.35 ± 0.13	9.91	8.39	11.9	0.51
V 2	627	10.87 ± 0.14	10.70	8.92	12.45	11.64 ± 0.17	11.31	9.84	13.28	0.16

[1]Q1= lower quartile; [2]Q2= upper quartile; [3] P-value in bold is statistically significant (P<0.05); ; [4]V1 = visit 1, [5]V2= visit2; The statistics of the hematological parameters of hemoplasma-positive group were compared with those of the negative group by a Generalized Linear Mixed Model including herd as random effect and age as fixed effect.

Table 2.8 – T-test results comparing hematological parameters in calves and replacement heifers negative for infection with *M. wenyonii* or *C. M. haemobos* at both sampling visits (NEG) or negative at the first sampling visit but PCR positive on the second sampling visit (NEWINF) for *M. wenyonii* and/or *C. M. haemobos* tested using rt-PCR on 11 farms in central Michigan during March-September 2022.

	Remained Negative "NEG"[1]				New Infection (NEWINF)[2]				
Variables – Reference range	n	Mean	95% CL Mean	SE	n	Mean	95% CL Mean	SE	t-test[3]
PCV (%) - (24.0 - 46.0)	273	31.25	(30.9-31.39)	0.17	174	30.86	(30.45-31.28)	0.20	0.66
Lymphocyte (10^3/µL) - (1.8 - 8.1)	258	7.10	(6.88-7.33)	0.11	167	6.89	(6.63-7.14)	0.12	0.27
Monocytes (10^3/µL) - (0.1 - 0.7)	258	0.76	(0.73-0.79)	0.01	167	0.64	(0.59-0.68)	0.02	0.01
Neutrophil (10^3/µL) - (1.7 - 6.0)	258	1.16	(1.10-1.21)	0.02	167	1.08	(1.01-1.15)	0.04	0.99
Total Leukocytes (10^3/µL) - (5.1 - 13.3)	258	11.69	(11.32-12.05)	0.18	167	11.14	(10.72-11.59)	0.22	0.52
Eosinophil (10^3/µL) - (0.1 - 1.2)	258	0.20	-	0.01		0.30	-	0.01	<0.01[a]

[a] Eosinophile non-parametric, compared by Mann-Whitney U test.

[1]Group of heifers that tested negative for hemotropic mycoplasma on both visits.

[2]Group of heifers that tested negative for hemotropic mycoplasma on the first and became PCR positive on the second visit.

[3]The statistics of the hematological parameters of hemoplasma-positive group were compared with those of the negative group by t-test.

2.5 DISCUSSION

To the best of our knowledge, this study is the first to report the prevalence of hemoplasma infection in U.S. dairy calves. In agreement with Schambow et al. (2021) herd-level apparent prevalence of infection in mature cows was 100% for both hemoplasmas. Similar to mature cows, all farms contained calves and replacement heifers that tested positive for hemoplasmas although the apparent prevalence and incidence appeared to vary among farms. Taken together, the results of Schambow et al. (2021) and this study suggest that hemoplasma infection is common in dairy cattle in Michigan. While some of the herds in our study were the same as those in the previous report (Schambow et al., 2021), we included six previously untested herds in this study. The consistent prevalence of hemoplasma infection in these new herds further supports that hemoplasma infections are widespread among dairy farms in Michigan. Most reports on hemoplasma infection in cattle have focused on assessing prevalence at a specific point in time, using a cross-sectional study design (Fujihara et al., 2011; Congli et al., 2011; Schambow et al., 2021). Consequently, little is known about the rate at which new cases of hemoplasmas emerge. Understanding the incidence is crucial, as it provides insights into the dynamics of the affected population and helps identify individuals at risk of contracting the disease (Smith 2012). While our study also has a cross-sectional approach, we incorporated a longitudinal component to examine the occurrence of new cases, particularly in younger animals.

The overall animal-level prevalence of single hemoplasma species in our study was similar to the proportions reported in a Japanese study that tested 124 beef cattle aged 1-4 yr (Tatsukawa et al., 2021). They reported that 25.8% were infected with *M. wenyonii* only, 8.1% tested positive for *C. M. haemobos*, and 62.1% had co-infections with both hemoplasmas (Tatsukawa et al., 2021). In another study that tested 19 animals < 1 yr of age in Malaysia, 31.5% were infected with *M. wenyonii*, 10.5% were positive for *C. M. haemobos*, and 36.8% had co-infections with both hemoplasmas (Mohd Hasan et al., 2017). In contrast, another study conducted in Japan that tested 128 animals < 1 yr old found that

infection with *M. wenyonii* was more prevalent (27.3%) compared to co-infection with both hemoplasmas (2.3%) (Tagawa et al., 2012). It has been suggested that differences in cattle breeds and geographical variations may influence the prevalence of each hemoplasma (Tatsukawa et al., 2021). Interestingly, our findings suggest that as animals age, co-infection with both bovine hemoplasmas becomes more common than single infections. This could be a cumulative result of the prolonged exposure to both pathogens. As older animals spend more time within the herd compared to younger animals, they accumulate contact with potential sources of hemoplasmas infection. However, the prevalence of each hemoplasma infection among older animals has varied among different studies. Some researchers have reported a higher prevalence of single *M. wenyonii* infections (Hasan et al., 2011; Niethammer et al., 2018; Erol et al., 2022), while others have observed a greater prevalence of single *C. M. haemobos* infection (Fujihara et al., 2011) (as compared to co-infections). Currently, there is not enough evidence to determine potential risk factors associated with infection for individual hemoplasmas, as most studies that have assessed prevalence have included relatively few herds and were not designed to identify differences among hemoplasma species.

The variation in apparent prevalence and cumulative incidence we observed among herds may be partially attributed to the time of the year the animals were sampled. In agreement with Strugnell and McAuliffe, (2012) we observed greater cumulative incidence on farms that were sampled in late summer as compared to farms we sampled in earlier months. Although potential modes of transmission of hemoplasmas in cattle remain poorly understood, mechanical transmission through blood sucking insects has been reported as a possible route (Hofmann-Lehmann et al., 2004). Increased activity of arthropod vectors during warmer months might account for our results, suggesting that vectors may play a role in transmission. Nevertheless, we also observed new infections during colder months which is unfavorable for arthropod vectors in our region . This suggests that other transmission mechanisms may be involved. Transplacental transmission has been reported but its overall significance is unknown

(Fujihara et al., 2011; Sasaoka et al., 2015). In a prevalence study conducted in Japan, dairy calves were tested using PCR during their first week of their life. Of 71 calves, 14% were infected with hemoplasmas, while 86% tested negative (Tagawa et al., 2013). Our results were similar as few young calves were infected. We hypothesize that transplacental transmission may be another route of infection. However, as reported by Tagawa et al. (2013), we could not differentiate between transplacental or transmission in early life as calves were not tested immediately after delivery. Further studies should focus on sampling newborn calves to better characterize this route of infection.

Given that age is a common risk factor for many diseases, determining the timing of infection with hemoplasmas can contribute to a better understanding of risk factors and aid development of interventions to reduce the risk of infection. Our results indicate that the apparent prevalence of infection with hemoplasmas remains relatively stable within the first months of life, with a sharp increase at 8 months, and a consistently high prevalence thereafter. While we did not evaluate modes of transmission, possible risk factors could be related to routine practices that facilitate exposure. Transmission of Bovine Leukemia Virus (BLV) has been associated with exposure to infected blood by re-use of needles and rectal palpation sleeves as well as failure to adequately remove blood from instruments used for dehorning and hoof-trimming (Divers et al., 1995; Kuczewski et al., 2021). Re-use of needles for multiple injections is common in Michigan and likely in other regions. In 2014, the National Animal Health Monitoring System (NAHMS) reported that > 51% of operations administrated 2 to 10 injections pre needle (NAHMS 2014). Producers interviewed by Schambow et al. (2021) estimated that a typical mature cow had received approximately 65 injections from birth and the same needle was used to inject about 15 animals. In our current study, about half of farmers indicated that they used the same needle to inject about 7 animals . Reuse of needles is an obvious risk factor for transmission of blood borne organism. Adoption of practices that reduce potential exposure to blood borne pathogens (use of single-use needles and obstetrical sleeves) has been shown to reduce the prevalence of BLV in a

dairy herd from 44% to 17% over a two-year period, (Sprecher et al., 1991). Similar practices could reduce transmission of hemoplasmas.

There is limited knowledge about variation in bacterial load of hemoplasmas in the bloodstream of cows (Keeton and Jain, 1973; Strugnell et al., 2011). In an older study that tested blood obtained from a splenectomized bull and used scanning electron microscopy for diagnosis, many RBC were initially infected with *E. wenyonii* but were not diagnosed in blood samples collected 10 d later (Keeton and Jain, 1973). Apparent clearance of infection with *M. wenyonii* was reported in 9 of 12 cows that were tested 40 days apart using PCR and Denaturing Gradient Gel Electrophoresis (DGGE) (Strugnell et al., 2011). In contrast, in our study very few test-positive animals later tested negative. Molecular diagnostics are preferred for diagnosing hemoplasma infection (Tagawa et al., 2008; Ritzmann et al., 2009; Hoelzle et al., 2011; Girotto-Soares et al., 2016; Niethammer et al., 2018). We used rt-PCR for testing but could not determine if animals that initially tested positive and then became negative achieved bacteriological clearance or if infection was not detected due to a low bacterial load. However, most animals that tested positive remained positive and our results similar to previous observations that cats infected with hemoplasmas become persistent carriers (Messick, 2003). Our results suggest that infections occur during the first year of life and remain persistent, thus potentially providing a reservoir for infections of herdmates.

Hemotropic *Mycoplasmas* attach to the surface of red blood cells and can cause hemolysis, resulting in anemia (Gladden et al., 2016). Although infection with *C. M. haemobos* and *M. wenyonii* have been associated with this condition (Hoelzle et al., 2011; Hofmann-Lehmann et al., 2004; Purnell et al., 1976; Gladden et al., 2016), the evidence of bovine hemoplasmas leading to anemia is contradictory (Gladden et al., 2016). Unlike Tagawa et al., (2010, 2012), we were not able to identify an association between infection status and anemia. However, our results were consistent with other researchers who used PCR for testing (Tatsukawa et al., 2021; McFadden et al., 2015). Animals that we sampled

appeared clinically healthy, and previous researchers have reported that when anemia is present in infected animals it is usually accompanied by other clinical signs such as fever (Smith et al., 1990), malaise, or edema of the hind limbs, udder (Genova et al., 2011), or scrotum (Montes et al., 1994). At least one researcher has reported the presence of anemia in the absence of clinical signs in a mature cow infected with *M. wenyonii* and bovine hemoplasmas should be considered in the differential diagnosis for any cow presenting with anemia (Gladden et al., 2016).

Only a few researchers have investigated the impact of hemoplasma infection on WBC of cattle (Tagawa et al., 2010; Niethammer et al., 2018; Tatsukawa et al., 2021). In one study, greater WBC was reported for cows co-infected with both bovine hemoplasmas as compared to negative cows (Tatsukawa et al., 2021). Infection with *M. wenyonii* (Tagawa et al., 2010) or *C. M. haemobos* in cows (Niethammer et al., 2018) have both been associated with greater WBC as compared to negative cows. Although our results did not agree with these studies, we did observe an interaction between hemoplasma infection and eosinophils. While the eosinophils were within the normal range, animals infected with hemoplasmas had a greater concentration of eosinophils than non-infected animals. The differences in EC were unexpected, as an increase in eosinophils is typically associated with parasitic infections (Kramer 2000). We also compared leukocyte counts of animals that remained negative on both visits with animals that changed from negative to positive. While an increase in monocytes could be anticipated due to the involvement of monocytes in immune defense against bacterial pathogens, the observed trend was rather unexpected. Animals that tested positive for the infection exhibited slightly lower mean MC values compared to those that tested negative. While relatively few differences were seen in hematological values, previous researchers have reported that findings that hemoplasmas may stimulate the immune system resulting in changes in leukocyte counts (Niethammer et al., 2018; Tatsukawa et al., 2021).

2.6 CONCLUSIONS

Our results provide novel information about the prevalence and incidence of infection with hemotropic mycoplasmas in dairy calves and heifers. The increase in prevalence as the heifers age may be attributed to the chronic nature of hemoplasma infections, since it appears that most of the heifers do not eliminate the organism following infection. The greater cumulative incidence observed on farms sampled during the summer may further support the hypothesis that vectors may play a significant role in hemoplasmas transmission. Our findings agree with previous observations that infection with hemoplasmas alone does not usually lead to anemia and the vast majority of animals chronically infected with hemoplasmas remained apparently healthy. The association between infectious status and levels of eosinophils and monocytes may potentially indicate an attempt of the immune system to combat the infection. However, further studies are necessary to gain a better understanding of this association and its underlying mechanisms.

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CHAPTER 3: SHORT COMMUNICATION: APPARENT PREVALENCE OF TRANSPLACENTAL TRANSMISSION OF HEMOTROPIC MYCOPLASMAS IN HOLSTEIN DAIRY CALVES

3.1 ABSTRACT

Hemotropic mycoplasmas are unculturable bacteria that infect the surface of red blood cells of several mammalian species including cattle. The significance of hemoplasmas in cattle remains unclear and limited information is available on biological routes of transmission. The objective of this cross-sectional study was to determine the prevalence of transplacental transmission of hemoplasmas and to determine if colostrum of infected cows contained DNA from hemoplasma organisms. In March 2023, researchers collected colostrum and peripheral blood samples from 39 dairy cows and their newborn calves (before ingestion of colostrum) at a single dairy farm in Michigan. Detection of *M. wenyonii* and *C. M. haemobos* was performed using real-time PCR. The apparent prevalence of hemoplasma infection in dams was 100%, with 84.6% (33/39) co-infected with both *M. wenyonii* and *C. M. haemobos*, while the remaining 15.3% (6/39) were infected solely with C. M. *haemobos*. The prevalence of newborn calves infected with *C. M. haemobos* was 15.3% (4/39) and none were infected with *M. wenyonii*. No colostrum samples tested positive for either *M. wenyonii* or *C. M. haemobos*. This is the first report of vertical transmission of hemoplasmas in a dairy herd located in the United States. However, the relatively low prevalence of infected newborn calves suggests that transplacental transmission is a minor pathway of infection. The absence of positive tests in colostrum indicate that ingestion of colostrum is unlikely to contribute to infection with hemoplasmas. The clinical significance of fetal infection remains unknown.

This study was funded by Michigan Alliance for Animal Agriculture project #AA-21-142

3.2 INTRODUCTION

Mycoplasma wenyonii and *Candidatus Mycoplasma haemobos* are gram-negative, wall-less bacterial parasites, that have been described as major hemotropic mycoplasmas (hemoplasma) in cattle (Neimark et al., 2001; Tagawa et al., 2008). Hemoplasmas have been identified in blood from both symptomatic and apparently healthy cattle. Clinical signs of hemoplasma infection have included immune-mediated anemia (Gladden et al., 2016), anorexia (Genova et al., 2011), mammary gland edema (Smith A. et al., 1990), edema of rear legs (Genova et al., 2011), fever (Smith A. et al., 1990), prefemoral lymphadenopathy (Smith A. et al., 1990), reduced milk yield (Strugnell and McAuliffe, 2012; Gladden et al., 2016; Hoelzle et al., 2011), weight loss (Genova et al., 2011), and infertility (Montes et al., 1994). While most infected cattle appear apparently healthy, the factors that trigger manifestation of clinical signs are not yet fully understood. When animals exhibit clinical signs, oxytetracycline has been reported to result in resolution of signs in some cattle (Montes et al., 1994; Genova et al., 2011). In the past, hemoplasma infections were diagnosed with observation of organisms on Giemsa-stained blood smears, but this method has low diagnostic accuracy and PCR testing is the preferred method of diagnosis (Hofman-Lehmann et al., 2004).

There is very little information about potential routes of transmission and risk factors for infection with hemoplasma in cattle. It is thought that transmission occurs through direct contact with infected blood or through blood-sucking insects (Smith A. et al., 1990; Fujihara et al., 2011). Biological modes of transmission, such as transplacental transmission, have been demonstrated in a few studies conducted in Hungary, Japan, Brazil, and Bavaria (Hornok et al., 2011; Girotto-Soares et al., 2016; Niethammer et al., 2018; Sasaoka et al., 2015). However, variations in the study population, such as differences in animal breeds, location, and the selective detection of organisms in certain studies (focusing on either *M. wenyonii* or *C. M. haemobos* instead of both organisms) make it challenging to fully characterize this transmission route. Ingestion of colostrum is another potential route of infection.

To date, only a single study which focused on beef cattle, explored colostrum as a potential source of infection. In that study, DNA of *M. wenyonii* was not detected in any of 17 colostrum samples assessed. To date, there have been no studies examining the occurrence of both *M. wenyonii* and *C. M. haemobos* in colostrum collected from dairy cows.

The objective of our study was to determine if infection with *M. wenyonii* or *C.M. haemobos* occurs prior to birth (vertical transmission), and to evaluate the presence of bovine hemoplasma DNA in the colostrum. We hypothesized that most infections with these organisms occur after birth and that transplacental transmission is uncommon.

3.3 MATERIALS AND METHODS

The study was designed as a prospective cross-sectional study conducted in a single herd, with animals as the experimental unit. Prevalence of hemoplasma infection in youngstock of the herd used in this study had been previously determined to be >50% *(*Chapter 2) and the herd was expected to have at least 35 calves born during the period selected for sampling. To detect at least 5% prevalence of infection with 95% confidence we estimated that we needed to sample $\geq$ 15 calves Researchers visited the farm during weekdays from 8:00 am to 5:00 pm during two weeks in March 2023. All cows that calved during that period and their newborn calves were eligible for sampling, regardless of parity, breed, or gender. Study personnel observed parturition and collected whole blood samples from the coccygeal vessels of cows, and from jugular vein of their calves immediately after birth and prior to ingestion of colostrum using 10-mL serum-separator and EDTA tubes. Colostrum samples were collected from cows immediately after calving, following guidelines for aseptic sampling (NMC 2017). After sampling, blood and colostrum samples were immediately cooled to 4°C for transport to the Michigan State University's Veterinary Diagnostic Laboratory (Lansing, MI) to undergo Real Time PCR (rt-PCR) testing. The age and parity of each cow were obtained from herd records. This study was approved by the Institutional Animal care and Use Committee at Michigan State University (Study ID 0008451).

Whole blood harvested into evacuated tubes containing EDTA were stored at 4°C for 24 to 48 hours before DNA extraction. A magnetic bead assisted DNA extraction method was performed from a 200 µl whole blood using the KingFisher Flex Purification System (Thermo Fisher Scientific, Waltham MA) with the MagMAX Core Nucleic Acid Purification Kit following the manufacturer's instructions. Real-time qPCR assays were employed to detect and quantify DNA from *Mycoplasma wenyonii* and *Candidatus Mycoplasma haemobos* using specific primers and probes targeting the 16S ribosomal RNA gene. The PCR reaction mixture for both organisms included 10 µl of 2X PerfeCTa qPCR ToughMix Low Rox (Quanta Bio, Beverly, MA), 400 nmol each of forward and reverse PCR primers, and 250 nmol of hydrolysis probe. For *M. wenyonii*, the forward primer was 5'-GAAAGYCTGATGGAGCAATA-3', with a predicted melting temperature (Tm) range of 56/59°C, and the reverse primer was 5'-SCTTTACGCCCAATAAATC-3', with a predicted Tm range of 55/56°C. The hydrolysis probe for *M. wenyonii* was JOEN-CGCGCCTTG/ZEN/ATGGTACTAATTGA-3IABkFq, with a Tm of 66°C. For *Candidatus Mycoplasma haemobos*, the forward primer was 5´-ACGAAAGTCTGATGGAGCAATAC-3´, and the reverse primer was 5´-ACGCCCAATAAATCCGRATAATG-3´. The hydrolysis probe for *C. M. haemobos* was FAM-TGAGGTACT/ZEN/ATCAGTTGTTATCCCTC-3IABkFq, with a predicted Tm of 65°C. Molecular grade water was added to the reaction mix to bring the reaction mix volume to 18 µl. Positive controls for PCR assays were synthetic DNAs, and standard curves were generated from 10-fold dilutions of known DNA concentrations ranging from 10^2 to 10^7 copies/µl, tested in duplicate. The threshold for positive results was set at Ct < 35.4. The negative control was reagent mix without any additions. To confirm suspect results, an in-house diagnostic gel-based PCR assay was used. Precautions were taken to prevent cross-contamination during the PCR assays including use of separate rooms for making reagent mixes, extraction of DNA, loading extracted DNA into reagent mix, and conducting the PCR process. Each work area had dedicated laboratory gowns, gloves, pipets, and other supplies.

Colostrum samples were tested using similar methods as whole blood, with two exceptions. An

initial centrifugation step of 10 minutes at 1730 rcf and 4° C to pellet cells and separate whey from lipid was done. Then 200 µl of whey and 200 µl of cells suspended in phosphate buffered saline solution were extracted separately to obtain DNA. The extraction methos was as described above using the KingFisher Flex Purification System (Thermo Fisher Scientific, Waltham MA) with the MagMAX Core Nucleic Acid Purification Kit (Thermo Fisher Scientific, Waltham, MA), following the manufacturer's instructions. An additional extraction method with silica columns and centrifugation (DNeasy blood and tissue kit, Qiagen, Germantown, MD) was used following the manufacturer's instructions to obtain DNA from pelleted cells or whey. Because colostrum was a seldom used sample type, this second extraction process was done to determine if extraction method affected results of the PCR assays. Positive controls for the PCR assays used with colostrum included cattle blood known to be positive for bovine hemoplasma and 2 ml of colostrum spiked with 100 µl of cattle blood known to be positive for bovine hemoplasma. The negative control was reagent mix without any additions.

3.4 STATISTICAL ANALYSIS

Statistical analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC) and statistical significance was defined as $P \leq 0.05$. Descriptive statistics were performed using PROC MEANS and used to characterize the participating animals. Normality of the data was evaluated using normal probability and box plots with PROC UNIVARIATE. The Mann–Whitney U test was used to evaluate the hypothesis that the age of the cow was associated with transplacental transmission.

3.5 RESULTS AND DISCUSSION

The enrolled herd contained about 3,900 lactating cows, 474 preweaning calves, and 1,376 replacement heifers. All cows were housed in freestalls. Blood and colostrum samples were collected from Holstein cows (n = 39) and blood was collected from each calf (n = 39) immediately after birth, before ingestion of colostrum. The overall prevalence of hemoplasma infection in cows was 100%. More specifically, 6 (15.3%) cows were positive for *C. M. haemobos* only, while co-infection with *M.*

wenyonii and *C. M haemobos* was detected in 33 (84.6%) cows (Table 3.1). Interestingly, none of the cows were infected solely with *M. wenyonii*. Of 39 calves, 4 (10.2%) were positive only for *C. M. haemobos* and all positive calves were born to a dam co-infected with both bovine hemoplasmas. No calves were positive for *M. wenyonii* (either single or as a co-infection). The mean age of cows was 2.9 ± 0.1 years (SE) and ranged from 1.8 to 5.7 years. The mean age of cows delivering infected calf was numerically younger (2.4 ± 0.3 years) than the age of cows that did not deliver an infected calf (3.0 ± 0.2 years), but this difference was not significant (P = 0.48). The DNA of neither *M. wenyonii* or *C. M. haemobos* was detected in any colostrum samples.

Table 3.1 – Apparent prevalence of infection with *M. wenyonii*, *C. M. haemobos* and co-infection tested using rt-PCR on blood samples collected from cows and their corresponding calves (prior to ingestion of colostrum) on 1 farm in Michigan in March 2023.

	Calves[1]		
Cows[2]	*C. M. haemobos* (%)	Negative (%)	Total (%)
C. M. haemobos (%)	0 (0.0)	6 (100.0)	6 (15.3)
Co-infection (%)	4 (12.2)	29 (87.8)	33 (84.6)
Total (%)	4 (10.2)	35 (89.7)	39 (100.0)

[1]There were no calves positive for *M. wenyonii* only or co-infected with both organisms.
[2]There were no cows positive for *M. wenyonii* only and all cows were positive for some organism.

Infections in calves that were sampled immediately after birth were likely a result of transplacental transmission. Parturition was closely observed by researchers who collected blood immediately after birth (before ingestion of colostrum), thereby eliminating the possibility of other potential routes of infection. Transplacental transmission of hemoplasmas has been suggested for only a few species, including dogs (Lashnits et al., 2019), swine (Guimaraes et al., 2007), and alpacas (Almy et al., 2006). In cattle, several hemotropic organisms such as *Anaplasma marginale, Babesia bovis* and T*heileria orientalis* are known to cause transplacental infection (Costa et al., 2016; Swilks et al., 2017), however, only limited research has been performed about the potential for vertical transmission of *M.*

wenyonii and *C. M. haemobos.* The first evidence of transplacental transmission of *C. M haemobos* was reported in Hungary by Hornok et al. (2011) and subsequent studies reported vertical transmission of *M. wenyonii* or *C. M. haemobos* (Sasaoka et al., 2015; Girotto-Soares et al., 2016; Niethammer et al., 2018). Our results support transplacental transmission of bovine hemoplasmas. The incidence of PCR-positive calves at birth in our investigation was similar to rates reported in Bavaria (8.0%) and Switzerland (10.5%) (Niethammer et al., 2018; Hornock et al., 2011), but less than those reported in Brazil (18.2%) or Japan (23.5%) (Girotto-Soares et al., 2016; Sasaoka et al., 2015). Although we observed a high prevalence of hemoplasma infection in cows, the low proportion of calves born infected suggests that infection of cows is a minor pathway of infection. While the mechanisms by which hemoplasmas are transmitted through the placenta in cows are not known, for cattle infected with *A. marginale*, *B. bovis* and *B. bigemina*, researchers have suggested that intrinsic characteristics of the organisms, (such as their size and strain), as well as periparturient immunosuppression in dams, can increase subclinical infection, and might affect transmission through the placenta (Costa et al., 2016). Although the association between immunosuppression during the peripartum period and transplacental transmission of hemoplasmas requires further investigation, the adoption of management practices such as providing appropriate nutrition and effective stress management should be investigated as management strategies to mitigate potential risk associated with this mode of transmission.

Unlike our previous prevalence study on the same farm (Chapter 2), where 3(*M. wenyonii)*, 2 (*C. M. haemobos)* and 9 (co-infection) of 16 randomly sampled adult cows were infected, none of the cows in this study were infected solely with *M. wenyonii.* In our previous investigation, most heifers seemed to be persistent carriers, but a small proportion of animals appeared to have cleared the infection. Although the reasons for which animals appeared to clear the infection are not apparent, variations in the bacterial load of each hemoplasma may result in negative tests in previously positive animals. Despite variation in the prevalence of each hemoplasma, hemoplasma infection is highly prevalent in

this herd. In our previous study (chapter 2), we tested pre-weaned calves (from 1 to 60 days of age) for hemoplasmas, but none of the 31 calves tested on this farm were positive for infection. This contrasts with our current study, where some newborn calves tested positive for hemoplasma. Based on our observation of transplacental transmission in this study, it is puzzling that all pre-weaned calves were negative in the earlier study. The two studies were conducted several months apart, and it is possible that a low prevalence of infection in preweaned calves reduced our ability to detect infection. However, the dynamics of infection with hemoplasmas in early life of calves is unknown and requires further investigation.

Similar to Hornock et al., (2021), all positive calves were born from a dam co-infected with both bovine hemoplasmas. Interestingly, all calves were solely infected with *C. M. haemobos* and infection with *M. wenyonii* was not detected. In previous studies, infection with *M. wenyonii* (Sasaoka et al., 2015) and co-infection with both organisms (Hornok et al., 2011) were reported in newborn calves. It is possible that variations in prevalence of hemoplasma organisms among dams could account for differences in hemoplasma infections in calves, but additional studies are needed to explore this possibility. Additional risk factors such as differences in breeds, (Sasaoka et al., 2015, Niethammer et al., 2018), method of diagnosis, and geographic locations may affect the prevalence of hemoplasmas. While our small study was not designed to establish associations between risk factors (such as age of the dam) and transplacental transmission the numerical difference in age suggests that more research about age and risk of transplacental transmission should be performed. In a previous study that sampled mature cows (n = 2,521) (Schambow et al. 2021), the apparent prevalence of hemoplasma infection was less in cows that were in parity 3+ as compared to primiparous cows but was not associated with stage of lactation. This observation may suggest that the concentration of bacteria in blood is greater in younger cows thus facilitating transplacental transmission. However, a larger study that enrolled cows from multiple herds is needed to better characterize potential relationships between age and risk of

transplacental transmission of hemoplasmas. The gender of the infected calves (two females, and two males) did not appear to influence the risk of infection. Similar findings related to the age of the cow and the gender of the calves have been previously reported (Hornock et al., 2011).

In addition to transplacental transmission of hemoplasmas, other modes of transmission to calves should be considered. During the pre-partum period, there is an increased permeability of the mammary gland, which potentially allows erythrocytes to be secreted into colostrum (McGrath et al., 2016). The ingestion of colostrum containing infected red blood cells could be a possible route of transmission from dams to calves, however, this route has not been confirmed. Our study is the first to investigate the presence of DNA of both *C. M. haemobos* and *M. wenyonii* in colostrum of dairy cows, but we failed to detect hemoplasmas in colostrum using analysis based on 16S rRNA gene. A study using colostrum from beef cattle analyzed ribonuclease P RNA gene for *M. wenyonii* using 17 colostrum samples from infected dams but did not detect any infected colostrum (Sasaoka et al., 2015). In our previous prevalence study (Chapter 2), only a small proportion of pre-weaned calves were positive for hemoplasmas. This suggests that even if hemoplasmas are present in the colostrum and can potentially serve as a source of infection for calves, their contribution to infections in calves appears to be very low. Detecting such a small prevalence would require a larger sample size. Another possible explanation for the absence of hemoplasmas in the colostrum tested could be related to negative samples not having any blood from the dams. Hammer et al., (2016) reported that colostrum can contain significant quantities of *T. orientalis* as determined by qPCR. Moreover, other factors such as the bacterial load of hemoplasmas in colostrum may have been too low to be detected, or it is possible that red blood cells infected with bovine hemoplasmas cannot be transmitted to colostrum. Additional research is needed to investigate the role of colostrum in transmission of hemoplasmas, but our result suggests that colostrum might be not involved in the transmission of hemoplasmas.

Our results suggest that transplacental transmission could serve as an alternative route of

bovine hemoplasma transmission. However, the low prevalence of infected calves at birth indicates that this pathway plays a minor role in infection. Additionally, hemoplasma do not appear to be present in colostrum.

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SUMMARY

Understanding the prevalence and dynamics of hemoplasma infection in dairy cattle is the first step in the development of interventions and management programs to reduce the risk of infection and provide the foundation for further research in determining the impact of infection on animal health and productivity. The objective of this thesis was to assess the prevalence of *Mycoplasma wenyonii* and Candidatus *Mycoplasma haemobos* infections in calves and replacement heifers on Michigan dairy farms, investigate the potential for vertical transmission of hemoplasmas, and analyze the presence of bovine hemoplasma DNA in colostrum.

Chapter 2 assessed the apparent prevalence, incidence, and the association between hemotropic mycoplasma infection and hematological values in calves and replacement heifers aged 1 to 27 months located in 11 dairy herds in Michigan. The results of this chapter demonstrate that hemoplasmas infection is widespread in Michigan, and that infections can occur in young calves, but the prevalence increases significantly as heifers age. Furthermore, once animals are infected, few test negative and most infected heifers appear clinically healthy.

Chapter 3 explored the possibility of hemoplasma infection occurring through transplacental transmission and investigated the presence of hemoplasma DNA in colostrum on 39 dams and their calves in a single farm in Michigan. Results from this study suggest that transplacental transmission could serve as an alternative route of bovine hemoplasma transmission. However, the low prevalence of infected calves at birth indicates that this pathway plays a minor role in infection. Additionally, hemoplasmas do not appear to be present in colostrum.

Based on results from these studies, hemoplasma is a chronic infection that can occur in young calves, but the proportion of animals infected tends to increase as heifers age. While further research is needed to understand the mechanisms involved in hemoplasmas transmission, adopting management practices that have been shown to reduce potential exposure to other blood-borne pathogens, such as

the use of single-use needles and obstetrical sleeves, could have a positive impact on reducing the transmission of hemoplasmas. Although most hemoplasma infected animals appear to remain healthy, previous researchers have indicated illness associated with these organisms. Further studies should focus on exploring factors contributing to the development of clinical signs to determine the impact of these infections on animal health and productivity in order to develop more effective prevention and management strategies.