UNIVERSITI PUTRA MALAYSIA

SEROPREVALENCE OF JOHNE’S DISEASE AMONG DEER LIVESTOCK IN UPM DEER FARM (PPP UPM): PRELIMINARY STUDY

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CERTIFICATION

It is hereby certified that I have read this project paper entitled "Seroprevalance of Johne's Disease Among Deer Livestock In UPM Deer Farm (PPP UPM): Preliminary Study", by Shivnraj A/L Sundar, and in my opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999 – Final Year Project.

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DEDICATION

This thesis is especially dedicated to:

My beloved parents (Sundar and Mageswary)

My respected supervisor (Professor Ts. Dr. Faez Firdaus Jesse Abdullah)

My fellow DVM friends

ACKNOWLEDGMENT

I would like to extend my gratitude to the Almighty. It is the blessings from the Almighty that helped me obtain the guidance I needed throughout my Final Year Project.

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LIST OF ABBREVIATIONS

UPM	Universiti Putra Malaysia
FYP	Final Year Project
OIE	World Organisation for Animal Health
MAP	*Mycobacterium avium* subspecies. *paratuberculosis*
JD	Johne’s disease

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek Tahun Akhir.

Seroprevalensi Penyakit Johne dalam Kalangan Rusa Ternakan di Ladang Rusa UPM (PPP UPM): Kajian Awal

Oleh

Shivnraj A/L Sundar

2023

Penyelia: Professor Ts Dr Faez Firdaus Jesse Abdullah

Penyakit Johne (juga dikenali sebagai paratuberkulosis) disebabkan oleh bakteria *Mycobacterium avium subspesies paratuberculosis (MAP)* merupakan penyakit bakteria yang berkait rapat dengan penyakit pada sistem gastrousus ternakan ruminan dan ruminan liar. Penyakit ini akan menyebabkan kerugian ekonomi yang signifikan pada ladang ternakan disebabkan oleh penurunan produktiviti dan produksi ternakan, sistem pembiakan terjejas dan kesuburan rendah, dan kematian mengejut. Penyakit ini telah diketahui bahawa akan mengakibatkan penyakit gastroenterik yang kronik dan tidak boleh dipulihkan dan mempunyaitempoh inkubasi yang panjang dan jangkitan ini akan berterusan seumur hidup bagi ternakan yang dijangkiti. Walaupun terdapat banyak kajian seroprevalens yang diterbitkan mengenai MAP dalam ternakan ruminan termasuk rusa, akan tetapi sehingga kini, tiada data seroprevalens mengenai jangkitan MAP di kalangan rusa di Malaysia yang telah dilaporkan atau direkodkan. Maka kajian awal ini dirancang untuk dijalankan bagi menentukan kadar seroprevalens dan faktor risiko yang menyumbang kepada jangkitan MAP di kalangan ternakan rusa (*Cervus timorensis*) di Ladang Rusa UPM. Sejumlah sembilan puluh dua sampel serum rusa digunakan dalam kajian ini mana kesemua sampel ini diperolehi dari Ladang Ternakan Rusa UPM mana sejumlah tiga puluh enam sampel serum rusa dikumpul pada tahun 2023 (kawanan baru) dan sejumlah lima puluh enam sampel serum rusa daripada arkib pada tahun 2017 (kawanan lama) digunakan dalam kajian ini. Kesemua sampel serum ini tealah disaring bagi penyakit MAP dengan menggunakan ujian saringan ELISA tidak langsung {ID Screen komersial (PARAS Ver0516, ID.VET, Perancis)} untuk mengesan antibodi serum yang ditujukan terhadap MAP. Pakej Statistik Perniagaan Antarabangsa (IBM) telah digunakan bagi analisis statistik untuk kajian ini dan nilai *p* telah dihitung menggunakan *Pearson Chi-square*. Hasil keputusan kajian ini menunjukkan kadar prevalens ketara keseluruhan sebanyak 2.2% (95% CI= 1.00 – 8.00) dan prevalens sebenar sebanyak 1.3% (95% CI= -0.45 – 7.40) untuk MAP di kalangan rusa yang disampel.Analisis univariabel dalam kajian ini menunjukkan bahawa faktor usia ($\chi^2 = 3.655$: $p=0.161$), jantina ($\chi^2 = 0.643$: $p=0.423$), dan kumpulan kawanan ($\chi^2 = 1.314$: $p=0.252$) rusa tidak berkait rapat ($p>0.05$) dengan seropositiviti kepada MAP. Secara kesimpulan, berdasarkan pengetahuan kami, kajian ini merupakan kajian pertama yang mendokumentasikan bukti kehadiran antibodi serum terhadap MAP di kalangan *Cervus timorensis* di Malaysia. Kajian lanjut diperlukan untuk mengasingkan dan mencirikan strain MAP yang wujud di kalangan rusa di Malaysia.

Kata kunci: Seroprevalensi; Penyakit Johne; Rusa; Ladang & UPM.

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4999 – Final Year Project.

Seroprevalance of Johne's Disease Among Deer Livestock In UPM Deer Farm (PPP UPM): Preliminary Study

by

Shivnraj A/L Sundar

2023

Supervisor: Professor Dr Faez Firdaus Jesse Abdullah

Johne's disease (also known as paratuberculosis) caused by *Mycobacterium avium* subspecies. *paratuberculosis* (MAP) is an underdiagnosed bacterial disease associated with gastrointestinal systems of domestic and wild ruminants, causing significant economic losses through decreased productivity, low fertility and mortality with worldwide occurrence. It is known to affect the animal with a chronic irreversible wasting gastro-enteric disease featured by a long incubation period and a lifelong persistent infection. Despite extensive published seroprevalence studies on MAP in ruminant livestock including deer and to date, no seroprevalence data on MAP infection among deer in Malaysia has been reported or recorded. Therefore this preliminary study was conducted to determine the seroprevalence and risk factors associated with MAP among deer (*Cervus timorensis*) livestock at the UPM Deer Farm. A total of ninety-two samples were sourced from UPM Deer Farm comprising thirty-six serum samples collected in 2023 (new herd) and an additional fifty-six archived samples from 2017 (old herd) were included in this study. All the samples

were screened using a commercial using ID Screen (PARAS Ver0516, ID.VET, France) indirect ELISA screening test to detect serum antibody directed against MAP. The International Business Machine (IBM) Statistical Package was used to perform statistical analysis and *p*-value was calculated using the Pearson Chi-square. The result of this study has revealed an overall apparent prevalence of 2.2% (95% CI= 1.00 – 8.00) and a true prevalence of 1.3% (95% CI= -0.45 – 7.40) for MAP among deer. However, the univariable analysis revealed that age ($\chi2 = 3.655$: p=0.161), gender ($\chi2 = 0.643$: p=0.423) and herd group ($\chi2 = 1.314$: p=0.252) of deer were not significantly associated ($p>0.05$) with seropositivity to MAP. To the best of our knowledge, this study is the first documented evidence of serum antibodies towards MAP among *Cervus timorensis* in Malaysia. Further studies are needed to isolate and characterise the strain of MAP circulating among deer in Malaysia.

Keywords: Seroprevalence; Johne’s Disease; Deer; Farm & UPM

Chapter 1.0

INTRODUCTION

1.1 BACKGROUND OF STUDY

Johne's disease (JD) or paratuberculosis is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), which is a chronic irreversible wasting gastro-enteric disease of ruminants (Geraghty *et al.*, 2014). The significance of MAP as a potential zoonotic disease has become progressively apparent since Johne and Frothingham originally identified it as the bacterial pathogen responsible for chronic gastroenteritis in bovine species in 1895 (Mary Garvey, 2018). It is an acid fast, short rod and obligate intracellular parasite of ruminant (Grant, 2005), which means it can only reproduce within an animal host, specifically macrophage and is unable to reproduce if the conditions are not met. This disease has a global distribution and is observed in all ruminants (Jesse *et al.*, 2013) including farmed deer.

Infected livestock regularly shed the organism in their faeces, milk, and colostrum, resulting in faecal–oral transmission which is considered the primary route of infection due to the disseminated nature of the pathogen (Khol *et al.*, 2017). Some asymptomatic and ill animals shed large numbers of bacteria in their faeces. Others may shed organisms intermittently, transiently or never during the subclinical stage. Susceptible species are most likely to become infected with MAP in the first few months after birth although young animals can be born infected. This could happen through colostrum, milk, or faecal contamination of the udder, or through direct-contact with contaminated pens. Animals can also become infected later in life, but they are less likely to do so and may be better at handling the infection (Spickler, 2007). Incubation period for paratuberculosis can take months or even years. In cattle, the incubation period is typically 2-5 years, and animals rarely show signs before they are 2 years old. In rare cases, the incubation period can be as long as 15 years. Small ruminants, deer, and some camelids have a shorter incubation period for paratuberculosis than cattle. This means that they can become sick sooner, sometimes as early as 5-8 months old in deer (Spickler, 2007).

The classical clinical findings in MAP infected deer include emaciation, minimal body fat, chronic diarrhea which supports the term wasting disease (Sleeman et al., 2009). MAP infection initially develops in the small intestine which progressively spreads to the lymph nodes which will ultimately interfere with the digestion and absorption of nutrients. The most common and routinely used diagnostic tests for JD are bacterial examination in feces and ELISA (Espejo et al., 2015).

Harris and Barletta (2001) stated that JD is an infectious bacterial disease of ruminants of economic importance to a variety of agricultural industries, such as dairies and meat producers. Harris and Barletta (2001) also stated that limited research was conducted and published on the seroprevalence of MAP infection (JD) in farmed deer compared to sheep and dairy cattle which has been comprehensively reviewed at herd-level. The initial infection to advanced clinical disease stages of JD have been clearly outlined by Whitlock and Buergelt (1996). Knowledge in this field is still scarce in Malaysia. Thus, the aim of this planned study will determine the seroprevalence rate of JD among deer livestock and identify its contributing risk factors.

1.2 PROBLEM STATEMENT

JD is an infectious bacterial disease of ruminants of economic importance to a variety of agricultural industries. Despite numerous study and research being done worldwide regarding seroprevalence of MAP in ruminant livestock that includes deers but there is no seroprevalence study on MAP infection (JD) among deer livestock has been conducted and recorded in Malaysia till date. Therefore this preliminary study was planned to determine the seroprevalence of JD among deer livestock in UPM Deer Farm (PPP UPM). The outcome of this study will aid in narrowing the gap of knowledge in this field.

1.3 HYPOTHESIS OF THE STUDY

H_0: There will be low or no seroprevalence of *Mycobacterium avium* subspecies *paratuberculosis* or Johne's Disease infection among deer from UPM Deer Farm (PPP UPM).

H_A: There will be high seroprevalence of *Mycobacterium avium* subspecies *paratuberculosis* or Johne's Disease infection among deer from UPM Deer Farm (PPP UPM).

H_0: There is no association between the seroprevalence of *Mycobacterium avium* subspecies *paratuberculosis* and the age, gender, or herd of deer.

H_A: There is association between the seroprevalence of *Mycobacterium avium* subspecies *paratuberculosis* and the age, gender, or herd of deer.

1.4 OBJECTIVES OF THE STUDY

The objectives of this study were:

1. To determine the seroprevalence rate of *Mycobacterium avium* subspecies *paratuberculosis* infection among deer from UPM Deer Farm (PPP UPM).

2. To identify the contributing risk factors towards *Mycobacterium avium* subspecies *paratuberculosis* infection and its correlation on deer from UPM Deer Farm (PPP UPM).

Chapter 2.0

LITERATURE REVIEW

2.1 Aetiology and transmission of *Mycobacterium avium* subsp. *paratuberculosis* (MAP)

Johne's disease, also known as paratuberculosis, is a chronic, progressive disease of domestic and wild ruminants triggered by infection with the bacterium *Mycobacterium avium* subsp. *paratuberculosis* (MAP) previously referred to as *M. paratuberculosis* or *M. johnei* (Chiodini et al. 1984). The disease is characterized by the formation of granulomas, or small lumps of inflammatory tissue, in the intestines and mesenteric lymph nodes (Brown et al. 2007). MAP is a small (0.5 to 1.5 µm), slow-growing, Gram-positive, acid-fast rod bacillus that belongs to the *M. avium* complex of organisms within the *Mycobacteriaceae* family, falling under the order Actinomycetales (Chiodini et al. 1984).

Multiple methodologies have been employed to investigate the genetic diversity of MAP. According to Collins et al. (1990), classification of MAP strains into three primary groups has been determined based on patterns obtained through IS900 restriction fragment length polymorphism (RFLP), pulsed field gel electrophoresis (PFGE), growth characteristics, and pigmentation. These groups appear to exhibit some degrees of host preference. Type II strains, also referred to as type C, were initially identified in cattle, yet they exhibit a broad host range encompassing sheep, goats, camelids, and various ruminant and non-ruminant wildlife species. Type I (type S) strains are most commonly found in small ruminants but have increasingly been reported in other species, including cervids, South American camelids, camels, and some cattle in close proximity to sheep. Formerly considered a separate "intermediate" group, Type III strains are now regarded as a subtype of Type I. Notably, there are two distinct bison Type II (B) strains, one in North America and another in Asia, the latter being a sub-lineage of Type II known as the "Indian Bison type." The Indian Bison type has been observed in cattle, water buffalo, sheep, goats, deer, bison, rabbits, wild boar, and other animals and predominates in some Asian regions. Collins et al. (1990) further noted that most isolates from goats and deer in New Zealand and Australia belong to the Type C strain. Interestingly, the Type S strain has been found to be less virulent for deer than the C strain in experimental exposures (Mackintosh et al. 2007). It is believed that domesticated ruminants serve as the primary reservoirs for this organism, with wildlife mostly contracting the infection from domestic herds. However, certain wild cervid populations may maintain the organism in localized settings. Although the potential connection between MAP zoonosis and Crohn's disease has been a matter of debate for over a century (Sechi and Dow, 2015), supporting evidence for this association has emerged through studies demonstrating the resolution of Crohn's disease with anti-mycobacterial therapy specifically targeting MAP (Qasem et al. 2020).

M. avium subsp. *paratuberculosis* is primarily transmitted via the faecal-oral route. Clinically affected animals can shed substantial quantities of bacilli in their feces, numbering in the billions per day. In contrast, other animals may shed bacteria intermittently, transiently, or not at all during the subclinical phase (Spickler et al. 2007). The presence of nucleic acids in the saliva of cattle has been detected, although the significance of this observation remains unclear. Offspring from various species, including cattle, sheep, red deer, and chamois, can be born infected. In cattle and sheep, this occurrence is more likely if the dam is in an advanced stage of paratuberculosis. However, prenatal transmission is reported to be highly prevalent in both symptomatic and asymptomatic red deer (Spickler et al. 2007). Majeed et al. (2007) further support this, stating that intrauterine infection of the foetus has been observed in goats, cattle, sheep, and deer. Animals may also shed MAP, to varying extents, in colostrum and milk, with shedding more common in clinically affected animals than asymptomatic ones, and bacterial quantities appearing highest within the initial two months of lactation. It is presumed that newborn and young animals become infected by ingesting bacilli from a manure-contaminated udder shortly after birth or from pasture (Whittington et al. 2001). Notably, an oral dose of 1,000 organisms (colony-forming units) has been found to induce intestinal infection in lambs and young deer (Mackintosh et al. 2007). Resistance to MAP infection has long been established to increase with age (Sweeney, 2011). Additionally, MAP has been detected in semen. It should be noted that MAP is common in the environment, particularly on contaminated farms, but it does not propagate outside of a living host (Spickler et al. 2007). The organism can endure for a year or more in some pasture settings, although its infectivity diminishes significantly within a few months. The persistence of MAP in the environment is favoured by stable low-temperature conditions and protection from direct solar radiation (Whittington et al. 2001). Further research indicates that MAP can endure in some water sources and faecal slurry for extended periods, ranging from 9 months to nearly 2 years, as well as in bovine faeces for up to 8-11 months (Whittington et al. 2005). Certain data suggests that the organism can enter a dormant state in the environment for a limited duration. In this context, it has been reported that MAP can form spore-like structures that may contribute to its survival under adverse conditions (Eamens et al. 2015).

2.2 Pathogenesis and clinical signs of *Mycobacterium avium* subsp. *paratuberculosis* (MAP)

In accordance with the guidelines provided by the United States Department of Agriculture (USDA) in 2023, Johne's disease is generally categorized into four distinct stages, namely Stage I (Silent, subclinical, nondetectable infection), Stage II (Subclinical shedders), Stage III (Clinical Johne's disease), and Stage IV (Emaciated animals with watery diarrhea).

The pathogenesis initiates with the animal's oral exposure to *Mycobacterium avium* subsp. *paratuberculosis* (MAP), whereby these microorganisms may enter the host through one of two distinct entry points, as noted by Payne and Rankin in 1961. Early investigations involving experimental infections in calves with substantial MAP dosages suggested that MAP likely entered via the tonsils, as evidenced by the presence of MAP organisms in retropharyngeal lymphoid tissue shortly after exposure. Subsequently, these MAP organisms can disseminate through the hematogenous route or via the lymphatic system to reach mesenteric lymph nodes and the ileum. However, most evidence from studies utilizing more realistic MAP doses or homogenates of tissues obtained from naturally infected cows suggests that the ileum serves as the primary point of entry, as documented by Sweeney et al. in 2006. Within the small intestine's Peyer's patches, specialized non-villous epithelial cells known as M cells play a crucial role in facilitating the translocation of MAP across the intestinal epithelium (Bermudez *et al*. 2010). These M cells serve as carriers for the MAP organisms, releasing them on the submucosal layer of the intestinal epithelium unchanged, as reported by Patel et al. in 2006. Subsequently, these MAP organisms are phagocytosed by macrophages, entering these immune cells via phagocytosis (Bharath et al. 2023). The ultimate fate of the infection hinges on the macrophages' ability to eliminate the phagocytosed MAP organisms. If the macrophages prove successful in this regard, it may prevent the progression of the infection. However, MAP organisms possess a distinctive capacity to endure within macrophages by altering their lipid content, and it is this unique characteristic that underpins the chronic and progressive nature of paratuberculosis, as highlighted by Bharath et al. in 2023.

As is the case with most intracellular pathogens, the initial stage of defence against the establishment of MAP infection hinges on the effective elimination of ingested MAP organisms by macrophages. The activation of macrophages through cytokines such as interferon-gamma, which are produced by Th1-type T-helper lymphocytes, enhances the intracellular killing of MAP organisms, as discussed by Sweeney et al. in 2011. It is probable that some exposed animals successfully eliminate the MAP infection through this mechanism, thus averting progression to clinical Johne's disease. Nevertheless, many exposed animals prove unsuccessful in eradicating MAP, and the pathogen persists within macrophages in these cases (Sweeney *et al*. 2011). Although the precise mechanism behind intracellular survival is not completely understood, MAP appears to prevent the maturation and acidification of the phagocytic vacuole within macrophages. This action prevents the exposure of MAP organisms to the bactericidal effects of lysosomal enzymes and oxygen-derived radicals (Hostetter *et al*. 2003). Typically, in the early stages of infection, the host's defenses are able to contain the MAP infection, permitting only gradual proliferation and spread of MAP within the gut and gut-associated lymphoid tissue. This early, controlled infection results in an extended incubation period, which may persist for two or more years (Spickler, 2007). During this period, the infected animal exhibits no clinical signs of the disease, and there is no appreciable impact on production or weight gain. In addition, faecal shedding of MAP and serum antibodies are typically undetectable, as indicated by Sweeney et al. in 2011.

The presence of MAP antigens within the intestinal submucosa and mesenteric lymph nodes triggers an inflammatory response, representing the host's attempt to confine the infection (Everman *et al*. 2015). This response attracts additional macrophages and lymphocytes to the affected area, leading to the formation of granulomas with multinucleated giant cells, epithelioid cells, lymphocytes, and macrophages. During the initial phase of infection, these lesions are limited in severity and localized, and, in fact, this response aids in containing MAP to the initial sites of infection (Gonzalez *et al*. 2005). Macrophages containing acid-fast organisms are relatively sparse in these early, focal lesions. However, as the infection gradually progresses, the organism disseminates, and the lesions become more severe, eventually manifesting as clinical disease. Thus, the granulomatous response initially confines the MAP infection, but the damage caused by the granulomatous inflammation ultimately leads to the clinical signs of Johne's disease (Sweeney *et al*. 2011).

The granulomatous inflammatory response, while disrupting mucosal structure and function, especially in the small intestine and associated lymph nodes, serves to confine MAP-laden macrophages to the gut and gut-associated lymphoid tissue (Sweeney *et al.* 2011). In deer, the presence of tuberculoid granulomas in the intestines, lymphatic vessels, and lymph nodes has been observed, as documented by Gelberg in 2017. Through this process, MAP and its host may coexist for many years without clinical signs of disease in the host. However, during this time, despite being contained, MAP remains viable. Over time, and for reasons that are poorly understood, the infection begins to gain the upper hand. The cell-mediated immunity (CMI) responsible for containment wanes, and the infection starts to progress more rapidly, as noted by Mercier in 2021. With the loss of immune control, the infected animal begins to shed MAP in increasing quantities in the faeces, and MAP organisms spread to other tissues, including the uterus, causing in utero transmission, the mammary gland, other internal organs, and muscle tissue, as reported by Streeter in 1995. At this stage, the animal may still not exhibit obvious clinical signs of disease. According to Mercier in 2021, serum antibodies are detectable later than CMI. Subtly, animals in this subclinical stage of the infection may experience reduced reproductive efficiency.

Subsequently, after the detection of faecal shedding of MAP and serum antibodies, apparent clinical manifestations of Johne's disease become evident, as noted by Mercier in 2021. In certain instances, asymptomatic faecal shedding persists for several years without cattle advancing to the clinical disease stage. As the MAP infection continues to develop, the lesions observed in the intestines and mesenteric lymph nodes intensify (Sweeney *et al.* 2011). Instead of focal or multifocal lesions, the granulomatous infiltrate becomes diffused, impacting the jejunum, ileum, cecum, and, to a lesser extent, the colon, as documented (Eamens *et al.* 2015). The lining of the small intestine, particularly the ileum, undergoes thickening due to extensive cellular infiltration, and the intestinal villi take on a "clubbed" appearance, becoming shorter and thicker, diminishing their absorptive efficacy (Sweeney *et al*. 2011). Granulomatous lymphadenitis leads to lymphangiectasia and the rupture of lacteals with fistulation into the bowel lumen. Additionally, mucosal hyperaemia, erosions, and petechiae have been macroscopically observed in deer (Eamens *et al.* 2015).

The granulomatous inflammation, primarily centred in the ileum but also involving various segments of the small and large intestine, induces malabsorption, diarrhoea, and protein-losing enteropathy characterized by hypoproteinemia, as elucidated by Mercier in 2021. The hallmark clinical sign in cattle is watery diarrhoea (USDA, 2023). The faeces do not exhibit grossly visible blood or mucus, and the animals do not experience tenesmus. Vital signs remain within normal limits, and the animals' appetite remains good until the terminal stages of the disease (Sweeney *et al.* 2011). Anecdotally, it has been noted that the clinical symptoms of Johne's disease may exacerbate following the stresses associated with parturition and early lactation. Weight loss usually accompanies the diarrhoea, and as the concentration of plasma proteins decreases, subcutaneous oedema may develop, particularly in dependent areas such as the brisket or submandibular region, a condition often referred to as "bottle jaw," in accordance with Spickler et al. in 2007. In this advanced stage of the disease, the metastasis of MAP organisms to extraintestinal sites occurs frequently, and in over 40% of these cases, foetal infection in utero takes place (Sweeney *et al*.

2011). Profuse faecal shedding of MAP by clinically affected animals results in substantial environmental contamination (Mercier, 2021). Most animals are culled once the typical clinical signs are recognized; however, if left within the herd, they will inevitably progress to a moribund state, with death ensuing due to cachexia and dehydration (Spickler, 2007).

2.3 Prevalence of *Mycobacterium avium* subsp *paratuberculosis* (MAP)

Paratuberculosis was first described in Germany over 100 years ago, and is a worldwide disease affecting developing as well as developed countries in Europe, North America, South America, Asia, Australia, and Africa (WOAH, 2022).

In Malaysia, no study has been conducted to determine the prevalence of MAP in deer till date. However, a report by Roseliza *et al.* (2009) documented suspected cases of Johne's disease in ruminants based on microbiological culture submitted to Veterinary Research Institute, Ipoh from year 2001 to 2018. Based on the report, throughout the 17 years, only 168 suspected Johne's infection samples were received for culturing. The highest number of suspected cases, 94, was recorded in 2001. Later in 2002 and 2003, 42 and 15 suspected cases were received respectively. Between the years 2005 to 2018, a significant drop in the number of suspected MAP infections was observed averaging one case per year. In 2001, a case of MAP was isolated in cattle from Sabah, however no other detail was recorded. Another case, in 2018, was isolated in cattle in Kulim District, Kedah. Most of the suspected Johne's samples sent to VRI had requested for testing without providing full history and information pertaining to the cases.

Meanwhile, Whittington *et al*. (2019) has conducted a study and reported the prevalence rate of MAP among farmed deer in 48 countries, where only 20 countries had data on their deer population. Whittington *et al*. (2019) recorded that the herd-level prevalence of MAP among deer in Argentina, Japan, New Zealand and Thailand were 1-10%, <1%, >40% and <1% respectively. Whereas, the animal-level prevalence within the herd was also able to be retrieved from Argentina with 5-10% prevalence, New Zealand and Thailand with >15% and <1% prevalence respectively.

As per a report from the National Academic Press, United States (2003), it is notable that a comprehensive global survey for Johne's disease (JD) or the presence of MAP in domesticated animals has not been systematically conducted. However, JD has been documented on every continent across the globe, with the exception of Antarctica. It is important to acknowledge that some nations, primarily those of island status, have reported no incidence of JD, while others have confined JD to specific geographic regions. Additionally, the determination of global prevalence, with a high level of confidence, is rendered intricate due to the absence of international consensus regarding population-testing protocols. Moreover, there exists no international scientific consensus concerning the precise definition and verification of MAP-free zones or regions.

2.4 Diagnosis of *Mycobacterium avium* subsp *paratuberculosis* (MAP)

The choice of optimal tests for the identification of infected animals is contingent upon the particular stage of the disease under consideration. It is noteworthy that animals in the early phases of infection may not be recognizable through the utilization of any of the existing tests, as articulated by Spickler in 2007. The sensitivity of diagnostic tests exhibits an upward trend as the mycobacterial load escalates, thereby generally achieving notable accuracy in advanced stages of infection. The precision of diagnostic tests is further influenced by various host factors and the extent of exposure to *Mycobacterium avium* subsp. *paratuberculosis* (MAP) and other closely related bacteria within the environment (Eamens *et al*. 2015).

The isolation of MAP from faecal culture of infected live animal, together with intestinal tract and associated lymph node (mesenteric and ileocecal lymph node) at necropsy is widely considered to be the gold standard for the diagnosis of paratuberculosis (Spickler, 2007). Notably, during necropsy examinations, the posterior jejunum and the ileocecal lymph nodes have proven to be the most valuable samples for detecting mild lesions in sub-clinically infected red deer through culture (Spickler, 2007). Furthermore, the dependence of MAP on mycobactin J for growth in specialized laboratory media offers a distinguishing feature that can be employed to discriminate MAP from other acid-fast bacilli. Recent advancements have led to the development of an improved growth medium, which has been reported to enhance MAP recoverability and sensitivity by a factor of 1,000 (Bull *et al*. 2017). It is worth mentioning that the culture of faecal samples on solid media detects approximately 30–40% of infected cattle, whereas the utilization of liquid media has demonstrated superior sensitivity compared to solid culture (Eamens *et al*. 2015). Despite the commendable sensitivity achieved by faecal culture in clinically affected animals (100%), it is vital to acknowledge its limitations in identifying animals in the early stages of infection, specifically those in Stages I and II. This limitation arises from the fact that some infected animals either do not shed the agent in their manure or exhibit intermittent shedding, rendering them susceptible to being overlooked during testing (Nielsen & Toft, 2008). Nonetheless, it is important to note that the isolation of MAP from faecal or tissue samples is regarded as achieving 100% specificity, according to Whitlock *et al*. in 2000. Moreover, the culture of environmental samples from areas where a significant proportion of cows defecate provides a convenient and unobtrusive alternative strategy in monitoring the status of Johne's disease in dairy cattle herds (Berghaus *et al*. 2006).

Recent advancements in polymerase chain reaction (PCR) technology and DNA extraction techniques have paved the way for the development of novel diagnostic approaches to address Johne's disease rapidly with greater sensitivity, and specificity (Clark *et al.* 2008). An insightful comparative study conducted by Clark *et al.* (2008) revealed that direct faecal PCR exhibited a sensitivity of 70.2% and a specificity of 85.3% when contrasted with culture, whereas the enzyme-linked immunosorbent assay (ELISA) method exhibited a sensitivity of 31.3% and a specificity of 97.8% for the diagnosis of MAP. Furthermore, the identification of novel DNA sequences that are regarded as unique to MAP, such as ISMav2, f57, and ISMap02 sequences, has introduced supplementary tools for swift and precise detection of this pathogen through PCR technology, as articulated by Stabel and Bannantine in 2005. Notably, the restriction enzyme analysis of IS1311, an insertion sequence that is common to both *M. avium* subsp. *avium* and MAP, can be harnessed for distinguishing between these species and for the typing of ovine, bovine, and bison strains of MAP (Sevilla *et al*. 2005).

The predominant serological test employed for the diagnosis of paratuberculosis in animals is the enzyme-linked immunosorbent assay (ELISA). It is relevant to highlight that the complement fixation test (CFT) and agar gel immunodiffusion (AGID) exhibit diminished sensitivity and specificity, and therefore, they are no longer recommended, in accordance with Mercier's findings in 2021. Analogous to culture methods, the sensitivity of ELISA depends upon the level of MAP shedding in faeces and the age of the animals (Mercier, 2021). Conversely, animals that have successfully cleared the infection may still test seropositive. Moreover, it is important to consider that detectable titters in infected animals tend to develop at a gradual pace, with serum titters typically becoming evident 10-17 months after infection, and occasionally, the timeframe may extend beyond this range, as documented by Spickler in 2007. To improve the specificity of ELISA, the absorbed ELISA method combines the sensitivity of ELISA with an additional absorption step. In this procedure, sera designated for testing are diluted with a buffer containing soluble *M. phlei* antigen prior to testing in an indirect ELISA. This approach serves to eliminate non-specific cross-reacting antibodies (Mercier, 2021). In the context of small ruminants, the sensitivities of ELISA typically fall within the range of 16–100%, while specificities range from 79–100% (Nielsen & Toft, 2008).

The microscopic examination of clinical samples has been done using Ziehl–Neelsen or acid-fast staining. Acid-fast staining is a fastest, cost-effective, and straightforward method for the diagnosis of MAP, yet it has limitations in terms of specificity and sensitivity, primarily due to challenges in distinguishing MAP from other acid-fast bacilli (Manning *et al.* 2001). Although Ziehl–Neelsen staining serves as a preliminary screening tool for MAP, it necessitates supplementary confirmation through more specific assays such as PCR and/or immunoassays (Manning *et al*. 2001).

Tests focusing on the assessment of cell-mediated immune responses (CMI), including the gamma interferon assay and skin tests, are not conventionally employed in the diagnosis of Johne's disease in domestic ruminants. CMI tests exhibit the ability to identify animals in the early phases of infection, particularly those that yield negative results in serological tests (Eamens *et al.* 2015). Intradermal testing with johnin or avian purified protein derivative tuberculin has the capacity to detect delayed-type hypersensitivity (DTH) reactions to MAP, yet it is considered to be an insensitive approach, frequently associated with nonspecific reactions. Furthermore, the potential for cross-reactivity with other organisms introduces the risk of false-positive reactions in both tests (Spickler, 2007).

Chapter 3.0

MATERIALS AND METHODS

3.1 ETHICS STATEMENT

All samples and data in this study were collected according to the approved guidelines of the Institutional Animal Care and Use Committee of Universiti Putra Malaysia (UPM/IACUC/AUP-U042/2023). The blood samples were collected by trained Veterinarians and personnel of UPM Deer Farm, Universiti Putra Malaysia. All the laboratory protocols were also conducted under controlled conditions and global best practices in the Clinical Research Laboratory, University Veterinary Hospital, Universiti Putra Malaysia.

3.2 METHODOLOGY

For this study, UPM Deer Farm (PPP UPM), located at Serdang, Selangor, Malaysia within the geographical coordinates of approximately 2.982962 North latiitude and 101.729190 East longitude, was enrolled as the study site. The study population consisted of *Cervus timorensis* deer and a total of 36 deer were selected via convenient sampling method from the farm together with 56 archived serum samples of deer from UPM Deer Farm that were collected during routine test for Brucellosis from year 2017 were used in this study. Risk factors such as age, breed, gender, animal source and origin, sanitation practise, and farm biosecurity management practices were recorded and questionnaire was used to interview the farmer manager for detailed information.

3.3 BLOOD COLLECTION

Blood samples were collected via jugular venipuncture using 21 G vacutainer using plain tubes (red tube) and were transported in an ice box to the laboratory for serological analysis.

3.4 SERUM SAMPLE PREPARATION

Blood samples in the plain tube were brought back to the clinical lab and centrifuged at 3000 rpm for 5 minutes. The resulting supernatant is the designated serum. The serum was extracted and transferred to the eppendorf tube using a clean pipette after the centrifugation process was completed. The desired volume of serum for each eppendorf tube is 1.5 ml. The serum samples were stored at -20°C to be preserved before subjected for commercial ELISA to determine the seropositivity towards Johne's disease.

3.5 SEROLOGY TESTING

3.5.1 ELISA TEST KIT PROCEDURE

The ID Screen (ID.VET, France) indirect enzyme-linked immunosorbent assay (ELISA) screening tests were used to detect serum antibodies directed against MAP (PARAS Ver 0516) in the selected samples with 99% specificity and 90% sensitivity. The test will detect antibodies by forming an antigen-antibody complex with the purified extract of MAP coated on the microplates. The description and procedure of the test is as below:Firstly, serum samples, reagents and plates were brought to room temperature about one hour before the test was conducted. Single and multichannel adjustable-volume pipettes and disposable plastic tips, ELISA microplate absorbance spectrophotometer with 450 nm filter, distilled water, paper towels and wash bottle, pre-dilution microplate were prepared. The materials supplied were checked before the test was conducted, which includes reagents, microplates coated with purified MAP extract, 10X concentrated conjugate, 20X wash concentrate, substrate solution (TMB), and stop solution. Other than that, 20X wash concentrate was diluted using distilled water with the ratio of 1:20 to prepare 1x wash solution. The serum samples and reagents were homogenized by inversion before every use.

Samples and controls were diluted 1:12 in a predilution microplate with dilution buffer 6, which is a neutralizing buffer containing *Mycobacterium phlei* to avoid cross-reaction before transferred to coated plates. Well A1 and B1 were added with 10µl of negative control while, well C1 and D1 were added with 10µl of positive control. The remaining 92 wells were added with 10µl serum samples each. All 96 wells were then added with 110µl of dilution buffer 6. These procedures were all done separately in additional pre-dilution plate to be used as duplicates in the test. The plates were then covered and incubated at 21°C for 45 minutes. Once the incubation ends, 100µl of the neutralized samples and controls were each transferred to both coated ELISA microplates accordingly. The plates were then covered and incubated at 21°C for 45 minutes. At the end of incubation, the plates were emptied and each well was washed 3 times with at least 300µl of wash solution prepared earlier. Meanwhile, concentrated conjugate (horseradish peroxidase) was diluted 1:10 with dilution buffer 3 to prepare conjugate. 100µl of the conjugate were added to each well and incubated at 21°C for 30 minutes. Once the incubation ends, all wells were emptied and washed similarly as previous washing. Next, 100µl of substrate solution is added into each well and incubated at 21°C for 15 minutes. Finally, once the incubation ends, 100µl of stop solution was added into each well in the same order as the addition of substrate was done to stop the reaction where the colour changes from blue to yellow was detected immediately.

Optical densities (OD) of samples were measured and standard at 450 nm using an ELISA Microplate Reader (Tecan Infinite 200 Pro®). The percentage of inhibition was calculated as 100 x [1- (Sample optical density/Negative control OD)], and OD values ≥ 70% were regarded as a positive result. In contrast, OD values ≤ 60% were considered a negative outcome.

3.6 RISK FACTOR DATA

The risk factors investigated in this study are the deer's age, breed, gender, source of the deer and origin, sanitation practise, vaccination and deworming status, farm biosecurity management practise, and Body Condition Score (BCS). UPM Deer Farm staff were interviewed regarding the management and history of previous diseases in the farm.

3.7 STATISTICAL METHODS

All data were summarized in Microsoft Excel Professional Plus 2019 spreadsheet programme. Statistical analysis was performed using IBM Statistical Package for Social Sciences (SPSS) Software version 25.0. Chi-square test was performed to determine the relationships between the seroprevalence of *Mycobacterium avium* subspecies *paratuberculosis* (dependent variable) and the age, gender, or herd of deer (independent variable). A P-value ≤0.05 was considered significant.

Chapter 4.0

RESULTS

Table 1 The Estimated Apparent and True prevalence of MAP among Deer

Variable	Categories	Tested	Positive	Apparent prevalence		True prevalence	
				Estimate (%)	95% CI	Estimate (%)	95% CI
Age	Young	22	0	0.0	0.00 -15.00	-1.1	-1.12 – 15.58
	Young adult	37	0	0.0	0.00 – 9.00	-1.1	-1.12 – 9.44
	Adult	33	2	6.1	2.00 –20.00	5.7	0.76 – 20.91
Gender	Male	22	0	0.0	0.00 –15.00	-1.1	-1.12 – 15.58
	Female	70	2	2.9	1.00 –10.00	2.1	-0.24 – 9.92
Herd	New herd	36	0	0.0	0.00 –10.00	-1.1	-1.12 – 9.71
	Old herd	56	2	3.6	1.00 –12.00	2.89	-0.41 – 12.49
Total	All	92	2	2.2	1.00 – 8.00	1.3	-0.45 -7.40

Table 2 The Overall Seroprevalence of MAP among Deer

Variable	Categories	Number Examined	Number Negative (%)	Number Positive (%)	χ2	*P*
Age	Young	22	22 (100)	0 (0.0)	3.655	0.161
	Young adult	37	37 (100)	0 (0.0)	3.655	0.161
	Adult	33	31 (93.9)	2 (6.1)	3.655	0.161
Gender	Male	22	22 (100)	0 (0.0)	0.643	0.423
	Female	70	68 (97.1)	2 (2.9)	0.643	0.423
Herd	New herd	36	36 (100)	0 (0.0)	1.314	0.252
	Old herd	56	54 (96.4)	2 (3.6)	1.314	0.252
Total	**All**	**92**	**90 (97.8)**	**2 (2.2)**	**-**	**-**

4.1 SEROPREVALENCE AND RISK FACTORS OF MAP

Out of a total 92 serum samples tested using antibody ELISA screening test against MAP, 2 (2.2%) samples were identified positive, representing a true and apparent seroprevalence of 1.3% (95% CI= -0.45 – 7.40) and 2.2% (95% CI= 1.00 – 8.00).

The seroprevalence of MAP according to age shows that adult group deer have a higher prevalence rate of 6.1% (95% CI= 2.00 – 20.00) compared to the young with 0.0% (95% CI= 0.00 – 15.00) and young adult with 0.0% (95% CI = 0.00 – 9.00). The seroprevalence of MAP according to the gender shows that female deer have a higher prevalence rate of 2.9% (95% CI= 1.00 – 10.00) compared to males with 0.0% (95% CI = 0.00 – 15.00) prevalence. The seroprevalence of MAP according to the herd shows that the old herd has a higher prevalence rate of 3.6% (95% CI= 1.00 –12.00) compared to the new herd with 0.0% (95% CI= 0.00 – 10.00) prevalence.

There was no association between the seroprevalence of MAP and age ($\chi2$= 3.655, P= 0.161), gender ($\chi2$= 0.643, P= 0.423), and herd ($\chi2$= 1.314, P= 0.252) of deer examined in this study (Table 2).

Chapter 5.0
DISCUSSION

5.1 THE SEROPREVALENCE OF MAP AMONG DEER

In this study, it was hypothesized that there was no association between the seroprevalence of *Mycobacterium avium* subspecies paratuberculosis (MAP) and the risk factors of age, gender and herd of the deer. The result of the serum antibody ELISA screening test revealed an apparent prevalence of 2.2% and true prevalence of 1.3% among sampled deer that tested positive for serum antibodies against MAP. This is the first study reporting the prevalence of MAP infection among deer in Malaysia, where the overall seroprevalence recorded in this study is lower than the previous reports from Europe.

The results of this study showed that deer which were not vaccinated against MAP and expressed no clinical signs, had antibodies against MAP. This may be due to probably the infected deer not reaching the clinical stage of the disease. Therefore it can be concluded that this present risk to domestic animals and other wildlife as they have the potential to harbour the disease without eliciting any clinical signs. There is increased chance of MAP infection spilling from deer to domestic animals and wildlife as they may act as carrier for the disease transmission, especially on animals with semi-intensive or extensive management system as practised in Malaysia. Since MAP is a chronic and persistent disease (Geraghty *et al*., 2014), animals are infected for life and become permanent carriers who continuously shed organisms in their faecal material. Therefore, deer play a crucial role as a potential source of infection to other animals. So, in order to control the disease we need to consider the disease and carrier status of MAP among deer in Malaysia holistically. Although this study has identified the seroprevalence of MAP among deer in UPM Deer Farm, it was noted that there was no association found between the risk factor and seropositivity and this may be due to small sample size and low number of deer tested positive. The result from this study is similar and comparable to the previously reported study by Whittington *et al.* (2019) across 48 countries and documented the prevalence of MAP in Thailand was <1% among farmed deer. Besides that, study by Jori *et al.* (2014) in Mauritius revealed prevalence rate of 1.7% (6/351) while, Sleeman *et al.* (2009), Davidson *et al.* (2004) and Raizmen *et al.* (2005) in United States revealed 0.5% (1/216), 0.3% (1/313) and 0.3% (1/309) prevalence rate of MAP respectively. The study in Mauritius was conducted with Rusa deer, *Cervus timorensis rusa*, which is similar to this current study. However, the other 3 studies were on white-tailed deer, *Odocoileus virginianus*. All these studies used the ELISA test for specific antibody detection against MAP.Meanwhile, Whittington *et al.* (2019) reported that Sweden and Norway are the 2 countries that claimed to be free of MAP. This was achievable as they were the only two countries which set their objective towards national eradication of Johne's disease. They have remained MAP free countries since 2015 through stamping out and active surveillance programmes which were also financially supported by their respective governments to increase farmers' participation. In addition, Sweden and Norway had legislations that made reporting of any suspicion of MAP mandatory and compulsory eradication if MAP is detected regardless of species. However, in Malaysia, we practice test and cull systems as control measures instead of eradication and active surveillance programmes for this disease in Malaysia are limited.In contrast, result of this study was lower than previous study by Meng *et al.* (2015) in China, who reported a seroprevalence rate of 17.64% (247/1400) in Sika deer, *Cervus nippon*. Elsewhere, in Spain, 30.16% (257/852) seroprevalence of MAP was reported among red deer, *Cervus elaphus* (Reyes Gracia *et al.* 2008); In United States, Palmer *et al.* (2019) reported 37.5% seroprevalence of MAP in white-tailed deer while Stinger *et al.* (2013) reported 45.0% (98/251) seroprevalence of MAP in New Zealand among red deer.

According to Meng et al. (2015), the high seroprevalence rate from ELISA antibody test in their study was majorly due to low emphasis and care given towards disease prevention and control work in deer farms by farmers and related government departments which is on the contrary to UPM Deer Farm where their farm management practice have emphasized on good biosecurity and minimal contamination to protect deer from infection. Besides that, Reyes Gracia *et al*. (2008) proved that high prevalence of MAP among red deer in Spain was not due to animals come in-contact with livestock, instead it is mainly the ability of MAP to maintain epidemiological cycle within the red deer. This could be highly related to the ability of MAP to remain dormant in the environment, especially in water bodies and sediments for a period around 48 weeks (Whittington *et al.* 2005). But, in this study the environmental sampling was not done to isolate MAP.Moreover, a report by Palmer *et al*. (2019) detected a high seroprevalence rate in the United States as the study was conducted on a herd in which MAP infection was endemic, which was in contrast with our study as the spread of MAP in Malaysia is still unclear. In addition, results of Palmer *et al*. (2019) were also obtained through isolation of MAP from fecal and tissue culture followed by Polymerase chain reaction (PCR), a molecular test which provides better sensitivity and specificity than ELISA screening test, because initial identification of MAP in infected animal is through fecal and tissue culture compared to serology test (USDA, 2023). The tissue culture sampled and used in this study was mesenteric lymph node, which is a common site for MAP colonization in deer, especially in subclinical or mild clinical cases (Clark *et al.* 2010). Report from Stinger *et al*. (2013) has recorded a significantly higher seroprevalence rate of MAP in New Zealand compared to this study. The study was done using mesenteric lymph node samples that were collected from deer slaughter premises and followed by tissue culture for MAP, a direct method to detect pathogens instead of targeting the antibody which is produced at a later stage of infection. Furthermore, Stinger *et al*. also mentioned that the highest number of positive samples were retrieved during summer and winter season, which explains the seasonal/environmental stress could be a potential risk factor for MAP infection as the animals undergo stress-induced immunosuppression. Compared to New Zealand that has four seasons, Malaysia's environment is more stable and does not undergo drastic or extreme seasonal changes, which helps in keeping the immune system to function in a relatively normal state.In conclusion, the lower prevalence rate of MAP in this study may be associated with the fact that the samples were only collected and tested from a single farm and due to the low number of deer samples which couldn't be associated with any risk factor related to this disease. Despite that, the data provided in this serological survey supports the infection of MAP in deer. Thus, further studies are needed to identify the risk factors at both individual and herd level in deer livestock to assess the impact of Johne's disease in Malaysia.

Chapter 6.0

CONCLUSION & RECOMMENDATION

This preliminary study has recorded an overall apparent prevalence of 2.2% (95% CI= 1.00 – 8.00) and a true prevalence of 1.3% (95% CI= -0.45 – 7.40) for MAP among deer from UPM Deer Farm (PPP UPM), Serdang, Selangor, Malaysia. However, there was no significant association found between seropositivity and the risk factors. Nevertheless, to the best of our knowledge, this study is the first documented evidence of serum antibodies towards MAP among *Cervus timorensis* in Malaysia. Thus, further studies are needed to isolate and characterise the strain of MAP circulating among deer in Malaysia.

For future studies, it is best to have a larger sample size of deer samples in Peninsular Malaysia and more risk factors can be studied.

Chapter 7.0

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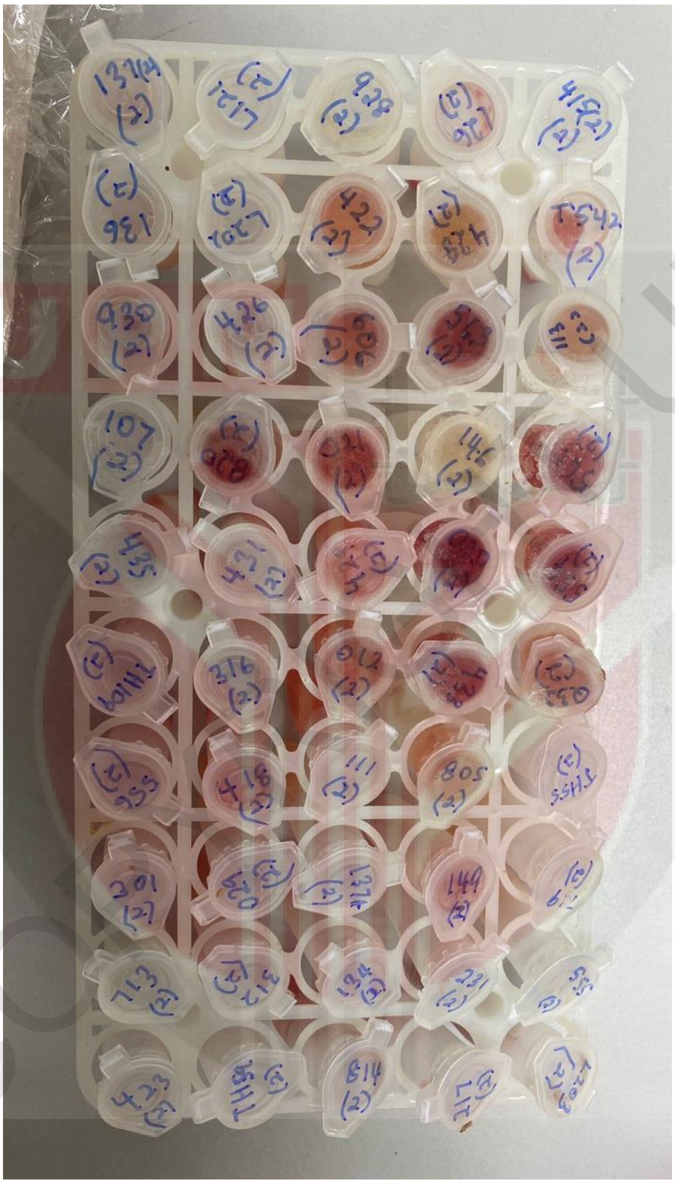
Chapter 8.0

APPENDIX

Appendix 1:

Blood collection from *Rusa timorensis* deer via jugular venipuncture technique

Appendix 2:



56 archived serum samples from year 2017 collected during Brucellosis screening

Appendix 3:

Paratuberculosis Indirect - Screening test

20230913-GB-PARAS/0516-000001-2

	1	2	3	4	5	6	7	8	9	10	11	12
A	ODnc 0.190	20230913-2 0.209 05 Negative	20230913-2 0.163 13 Negative	20230913-2 0.271 21 Negative	20230913-2 0.162 29 Negative	20230913-2 0.148 37 Negative	20230913-2 0.207 45 Negative	20230913-2 0.234 53 Negative	20230913-2 0.198 61 Negative	20230913-2 0.160 69 Negative	20230913-2 0.136 77 Negative	20230913-2 0.200 85 Negative
B	ODnc 0.150	20230913-2 0.267 06 Negative	20230913-2 0.214 14 Negative	20230913-2 0.209 22 Negative	20230913-2 0.195 30 Negative	20230913-2 0.196 38 Negative	20230913-2 0.174 46 Negative	20230913-2 0.834 54 Negative	20230913-2 0.218 62 Negative	20230913-2 0.146 70 Negative	20230913-2 0.229 78 Negative	20230913-2 0.130 86 Negative
C	ODpc 2.508	20230913-2 0.311 07 Negative	20230913-2 0.242 15 Negative	20230913-2 0.227 23 Negative	20230913-2 0.629 31 Negative	20230913-2 0.205 39 Negative	20230913-2 0.178 47 Negative	20230913-2 2.637 55 Positive	20230913-2 0.167 63 Negative	20230913-2 0.140 71 Negative	20230913-2 0.123 79 Negative	20230913-2 0.179 87 Negative
D	ODpc 2.452	20230913-2 0.221 08 Negative	20230913-2 0.598 16 Negative	20230913-2 0.177 24 Negative	20230913-2 0.137 32 Negative	20230913-2 0.126 40 Negative	20230913-2 0.264 48 Negative	20230913-2 0.176 56 Negative	20230913-2 0.210 64 Negative	20230913-2 0.156 72 Negative	20230913-2 0.151 80 Negative	20230913-2 0.111 88 Negative
E	20230913-2 0.376 01 Negative	20230913-2 0.170 09 Negative	20230913-2 0.145 17 Negative	20230913-2 0.147 25 Negative	20230913-2 0.261 33 Negative	20230913-2 0.206 41 Negative	20230913-2 0.376 49 Negative	20230913-2 0.145 57 Negative	20230913-2 0.176 65 Negative	20230913-2 0.216 73 Negative	20230913-2 0.121 81 Negative	20230913-2 0.214 89 Negative
F	20230913-2 0.166 02 Negative	20230913-2 0.160 10 Negative	20230913-2 0.125 18 Negative	20230913-2 0.157 26 Negative	20230913-2 0.162 34 Negative	20230913-2 0.166 42 Negative	20230913-2 0.587 50 Negative	20230913-2 0.173 58 Negative	20230913-2 0.242 66 Negative	20230913-2 0.232 74 Negative	20230913-2 1.464 82 Negative	20230913-2 0.199 90 Negative
G	20230913-2 0.234 03 Negative	20230913-2 0.183 11 Negative	20230913-2 0.106 19 Negative	20230913-2 0.140 27 Negative	20230913-2 0.236 35 Negative	20230913-2 0.179 43 Negative	20230913-2 0.177 51 Negative	20230913-2 0.200 59 Negative	20230913-2 0.152 67 Negative	20230913-2 0.189 75 Negative	20230913-2 2.642 83 Positive	20230913-2 0.177 91 Negative
H	20230913-2 0.389 04 Negative	20230913-2 0.238 12 Negative	20230913-2 0.095 20 Negative	20230913-2 0.206 28 Negative	20230913-2 0.178 36 Negative	20230913-2 0.966 44 Negative	20230913-2 0.200 52 Negative	20230913-2 0.179 60 Negative	20230913-2 0.142 68 Negative	20230913-2 0.184 76 Negative	20230913-2 0.167 84 Negative	20230913-2 0.230 92 Negative

Results obtained through ID Soft program using readings from ELISA microplate reader Tecan Infinite 200 Pro