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Abstract of Thesis/Dissertation

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Title: Molecular epidemiology and genotypic diversity of equine piroplasma parasites (馬ピロプラズマの分子疫学調査と遺伝子型の多様性)

Abstract

Equine piroplasmosis (EP) is an infectious disease caused by two parasite species, *Theileria equi* and *Babesia caballi*, in equines. In the infected equines, the proliferation of the two parasites species within the red blood cells lead to intravascular haemolysis, resulting in anaemia and other clinical signs such as jaundice, haemoglobinuria, and sometimes death. EP causes economic losses not only due to treatment costs and production losses, but also due to restrictions on the international movement of horses. Therefore, controlling EP is essential to maintain the benefits of the equine industry and ensure its socio-economic viability.

EP is globally distributed and is particularly common in equines in tropical, subtropical, and some temperate regions. Infected equines remain chronic carriers, with *T. equi* persisting for a lifetime and *B. caballi* persisting for up to four years. These carriers act as a source of infections for ticks, which subsequently transmit the infections to other equines. As such, countries that are free of EP have stringent regulations on the importation

of equines to prevent the entry of *T. equi* and *B. caballi*. Only a few countries have been confirmed to be EP-free, while the status of EP in several countries remains unknown due to a lack of epidemiological surveys. Therefore, it is crucial to survey *T. equi* and *B. caballi* infections in countries with an unknown EP status. The epidemiological surveys should typically be followed by studies to investigate the genotypic diversity, as previous studies have demonstrated that the genotypes of *T. equi* and *B. caballi* have implications for the control of EP.

Sri Lanka and Paraguay, both agricultural countries, are home to large populations of livestock. Because the livestock in these countries are managed by an extensive system, tick infestation and associated tick-borne diseases are common among them. However, the epidemiological status of EP in Sri Lanka and Paraguay is unknown. Therefore, the objectives of my study were to survey *T. equi* and *B. caballi* infections in donkeys in Sri Lanka and horses in Paraguay and to determine the genotypes of the detected parasite species.

For the donkey survey, thin blood smears and DNAs were prepared from blood samples collected from 111 randomly selected donkeys in Sri Lanka and examined for *T. equi* and *B. caballi* infections. I analysed the thin blood smears with microscopy and DNA samples with *T. equi*- and *B. caballi*-specific PCR assays. The results showed that 57.7% and 85.6% of the surveyed donkeys were positive for *T. equi* by microscopy and PCR, respectively. However, none of the donkeys were positive for *B. caballi* in either method. Sequencing analysis of 18S rRNA from the *T. equi*-positive samples revealed the presence of genotypes C and D, with genotype C being reported for the first time in donkeys. My findings highlighted the importance of addressing EP, in the efforts to conserve the shrinking donkey population in Sri Lanka.

Similar to Sri Lanka, the status of EP in the Paraguayan horse population is unknown. Therefore, I surveyed *T. equi* and *B. caballi* infections in horses in Paraguay. For this study, blood DNA samples were prepared from 545 horses in 16 of the 17 departments of the country and then tested for *T. equi* and *B. caballi* infections using specific PCR assays. I found that 32.7% and 1.5% of the surveyed horses were infected with *T. equi* and *B. caballi*, respectively. Co-infections with both the parasite species were detected in 0.4% of horses. I also observed that the infection rates were comparable between different horse breeds, sexes, and age groups. The haematological parameters of the infected horses were within the normal range, except for the two co-infected horses, which were anaemic. Phylogenetic analysis revealed that the Paraguayan horses were infected with *T. equi* 18S rRNA genotypes A and C and *B. caballi rap-1* genotype B. The present study, which is the first to report of *T. equi* and *B. caballi* infections in Paraguayan horses, underscores the need for EP management strategies in this country.

A comprehensive understanding of the *T. equi* genotypes prevalent in endemic countries is of paramount importance, given their diagnostic, clinical and therapeutic implications for managing EP. At present, PCR-sequencing is the most commonly used technique to determine *T. equi* genotypes, but it lacks sensitivity. Therefore, a simple, sensitive, and cost-effective genotyping assays are essential to determine the true extent of the genotypic diversity of *T. equi*. To address this issue, I have developed highly specific conventional PCR assays for the differential detection of each *T. equi* genotype (A – E). These assays were then evaluated on 270 *T. equi*-positive DNA samples from donkeys in Sri Lanka and horses in Paraguay. I found that the genotype-specific PCR assays were highly sensitive, as they detected four genotypes (A, C, D, and E) in the Sri Lankan samples and all five genotypes in the Paraguayan samples. All of the Sri Lankan samples and 93.3%

of the Paraguayan samples tested positive for at least one genotype, further emphasising the sensitivity of the assays. The PCR assays also detected co-infections with various combinations of genotypes in 90.2% and 22.5% of the Sri Lankan and Paraguayan samples, respectively. In addition, the sequences of amplicons from the genotype-specific PCR assays clustered with their respective phylogenetic clades, validating the specificity of the PCR assays. These findings highlighted that the newly developed genotype-specific PCR assays are reliable tools for the detection of *T. equi* genotypes.

The findings of my study demonstrated that the equines in countries with unknown EP status are likely to be endemic for *T. equi* and *B. caballi* if competent tick vectors are prevalent. As evident from my studies, the majority of equines infected with *T. equi* and *B. caballi* remains asymptomatic in endemic countries. However, the parasites can still be transmitted from these carriers to susceptible equines, where the infections may result in clinical EP. Therefore, equine populations in endemic countries, including Sri Lanka and Paraguay, should be monitored for clinical EP and treated promptly.

The risk factors for clinical EP are not well defined. However, recent studies have shown that clinical cases of EP are often associated with *T. equi* genotype A. Therefore, equines in countries with *T. equi* genotype A, such as Sri Lanka and Paraguay, are at risk for clinical EP. The genotypic diversity of *T. equi* may also have diagnostic and therapeutic implications in Sri Lanka and Paraguay. For instance, the cELISA may produce false-negative results in equines infected with *T. equi* genotype C, as it lacks the EMA-1, based on which the assay was developed. As a result, infected equines may be cleared for export, leading to potential disease outbreaks in EP-free countries. Similarly, the drug-induced complete clearance of *T. equi* from infected equines may sometimes be challenging in these two countries due to the prevalence of genotype C, against which the

therapeutic regimens are ineffective. Therefore, EP control strategies in endemic countries, including Sri Lanka and Paraguay, should be carefully designed based on the genotypic diversity.

Taken together, my findings highlight the importance of surveying *T. equi* and *B. caballi* in equines in countries where the status of EP is unknown. For designing effective EP control programs, the epidemiological investigations should be followed by studies to identify parasite genotypes using appropriate assays, such as the genotype-specific PCR assays developed in this study. In short, the series of studies I have conducted and the findings will provide a framework for conducting epidemiological surveys in countries where the disease is unknown, and will ultimately be useful in the management of EP both locally and globally.