Prevalence of *Babesia* Infection in Cattle from Jiri and Bardaghat, Nepal

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A dissertation submitted
In partial fulfilment of the requirements for the award of the degree of Master of Science in Zoology with special paper Parasitology

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Recommendation

This is to recommend that the dissertation entitled **"Prevalence of *Babesia* infection in cattle from Jiri and Bardaghat, Nepal"** has been carried out by Madhav Pandey for the partial fulfilment of Master's Degree of Science in Zoology with special paper Parasitology . This is his original work and has been carried out under my supervision. To the best of my knowledge, this dissertation work has not been submitted for any other degree in any institutions.

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Letter of approval

On the recommendation of supervisor "Dr. Kishor Pandey" this dissertation submitted by Madhav Pandey entitled **"Prevalence of *Babesia* infection in cattle from Jiri and Bardaghat, Nepal"** is approved for the examination in partial fulfilment of the requirements for Master's Degree of Science in Zoology with special paper Parasitology.

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Certificate of acceptance

This dissertation work submitted by Madhav Pandey entitled **"Prevalence of *Babesia* infection in cattle from Jiri and Bardaghat, Nepal"** has been accepted as a partial fulfilment for the requirements of Master's Degree of Science in Zoology with special paper Parasitology.

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Abstract

Babesia infection is a significant tick-borne disease affecting cattle worldwide, leading to substantial economic losses in the livestock industry. This study aimed to determine the prevalence of *Babesia* infection in two different regions of Nepal: Jiri and Bardaghat Municipalities of Dolakha and Nawalparasi-west district respectively using both microscopy and PCR techniques. A total of 190 cattle were examined, with 90 from Jiri and 100 from Bardaghat. Sample from Jiri were from a single farm with open grazing system, while those from Bardaghat were from multiple farms and confined. Blood samples were collected in EDTA tubes . Thin blood smears were prepared for microscopic examination. Parasite's DNA was extracted from blood sample. PCR test was performed to identify *Babesia* present in cattle. Microscopy detected *Babesia* in 11.1% cattle from Jiri and 7% from Bardaghat, while PCR demonstrated higher detection rates, identifying infection in 56% and 50% of tested samples from Jiri and Bardaghat, respectively. The findings highlight the sensitivity of PCR compared to microscopy for diagnosing *Babesia* infection. Upon DNA sequencing, 6 piroplasm species were recorded among which *Babesia bovis* and *Theileria orintalis* were most prevalent. The localized transmission of ticks within Jiri farm influenced by farm management practices and open grazing contributed for higher prevalence. The hematological analysis revealed significant ($P \leq 0.05$) reduction in Hb concentration, RBCs and increase in WBC count in infected cattle. This molecular level research provides new data on the wide distribution of *Babesia* infection across diverse geographical zones, which could be important for implementing effective disease control strategies in both the Terai and Himalayan regions.

शोध सारांश

बेबेसिया संक्रमण विश्वव्यापी रूपमा गाईवस्तुहरूलाई असर गर्ने एक किर्नाबाट सर्ने रोग हो । यसले पशुपालनको क्षेत्रमा ठूलो आर्थिक नोक्सान निम्त्याउँछ । यस अध्ययनले माइक्रोस्कोपी र पीसीआर दुवै प्रविधिको प्रयोग गरी नेपालका दुई क्षेत्रः दोलखा जिल्लाको जिरी र नवलपरासी पश्चिम जिल्लाको बर्दघाट नगरपालिकामा *बेबेसिया* संक्रमणको व्यापकता पत्ता लगाउने उद्देश्य राखेको छ । यस अध्ययनमा जिरीका ९० र बर्दघाटका १०० गरी जम्मा १९० गाईहरुको रगत परीक्षण गरिएको थियो । जिरीका नमुनाहरू खुला चरन प्रणाली भएको एउटै फार्मबाट लिइएको थियो भने बर्दघाटका नमुनाहरू खुला चरन प्रणाली नभएका धेरै फार्मबाट लिइएको थियो । रगतको नमूनाहरू इडीटिए ट्युबहरूमा सङ्कलन गरिएको थियो । त्यसपछि माइक्रोस्कोपिक परीक्षणको लागि पातलो रगतको स्मियरहरू तयार गरियो । त्यस्तै, रगतको नमूनाबाट परजीवीको डीएनए निकालिएको थियो भने त्यसमा *बेबेसिया* पहिचान गर्न पीसीआर परीक्षण गरिएको थियो । माइक्रोस्कोपीले जिरीका ११.१ प्रतिशत र बर्दघाटका ७ प्रतिशत गाईमा बेबेसिया पत्ता लगाएको छ भने पीसीआरले जिरी र बर्दघाटका क्रमशः ५६ प्रतिशत र ५० प्रतिशतमा संक्रमण पहिचान गरेको छ । बेबेसिया संक्रमणको परीक्षणको लागि माइक्रोस्कोपीको तुलनामा पीसीआरको संवेदनशीलतालाई यस अध्ययनका निष्कर्षहरूले उजागर गर्दछ । डिएनए अनुक्रमण गर्दा ६ वटा पाइरोप्लाज्म प्रजातिहरू रेकर्ड गरिएको छ जसमध्ये *बेबेसिया बोभिस* र *थेइलेरिया ओरिन्टालिस* सबैभन्दा प्रचलित देखिए । जिरी फार्म भित्र किर्नाको स्थानीय प्रसारण, फार्म व्यवस्थापन अभ्यासहरू र खुला चरनले पाइरोप्लाज्मको उच्च प्रसारमा योगदान पुऱ्याएको हुन सक्ने यस अध्ययनले अनुमान गरेको छ । हेमाटोलोजिकल विश्लेषणले संक्रमित गाईवस्तुहरूमा हेमोग्लोबिन र रातो रक्त कोशिका गणनामा उल्लेखनीय ($P \leq 0.05$) कमी भएको खुलासा गरेको छ । यी भौगोलिक रुपमा फरक क्षेत्रहरूमा बेबेसिया संक्रमणको नयाँ डाटा उपलब्ध गराउने मोलिक्युलर स्तरको यस अनुसन्धानले तराई र हिमाली क्षेत्रमा रोग नियन्त्रण गर्न प्रभावकारी रणनीतिहरू लागू गर्न महत्त्वपूर्ण हुन सक्ने छ ।

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List of abbreviations

Abbreviated form	Details of abbreviations
GDP	Gross Domestic Product
TBBPs	Tick Borne Blood Protozoans
µl	Micro litre World Health Organization
CRGC	Cattle Genetic Research Center
µm	Micro meter
DNA	Deriboneucleic Acid
RBC	Red blood Cell
CBC	Complete Blood Count
PCR	Polymerase Chain Reaction
nPCR	Nested Polymerase Chain Reaction
ELISA	Enzyme-linked Immunosorbent Assay
ICTs	Immunochromatography Tests
IFAT	Indirect Fluroscent Antibody Technique
MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Corpuscular Volume
PCV	Packed Cell Volume
rRNA	ribosomal Ribonucleic Acid
TEC	Total Erythrocyte Count
TLC	Total Leucocytes Count

1. Introduction

1.1 Background

Agriculture in Nepal holds huge cultural and economic significance. Deeply embedded in the daily lives of its people, agriculture serves as the backbone of Nepal's rural economy. The diverse geography of the country, ranging from the Himalayas to the plains of the Terai, creates a rich environment for a variety of farming practices. By 2023, more than half of the population (50.4 %) was directly engaged in agricultural activities, supporting livelihoods and ensuring food security (MOF, 2023). The agricultural sector not only supports rural families but also significantly contributes to the country's economic output, accounting for approximately 24.1 % of the national GDP (MOF, 2023). Among the key components of agriculture, livestock plays a vital role in the subsistence farming model, where families raise animals for both income and nutrition. Particularly in rural areas, livestock such as cattle and buffaloes are central to agricultural practices, contributing to nearly 80% of the total livestock population (MoALD, 2023) . This integration of crop and livestock production is essential for meeting nutritional needs, ensuring economic stability, and maintaining the cultural ties that bind the Nepalese people to their land.

1.1.1 Overview of Livestock Farming in Nepal

Livestock farming is vital to Nepal's economy, including animals like buffaloes, cattle, pigs, sheep, goats, fowl, and ducks. These animals provide milk, egg, wool, manure, meat, and also serve for various agricultural works. Cattle and buffaloes are the primary livestock farmed in Nepal . with cattle encompassing cows, calves, bulls, and oxen. It is believed that more than 1.5 billion of cattle are present globally (FAO, 2021). According to the Livestock census (2022/23) 4,750,329 cattle are present in Nepal which is the main source of traction and manure. Among them milking cow population is 916,579 producing 1.21 milliom metric tons of milk (MoALD, 2022/2023) . In Nepal, cattle are raised solely for milk production, as their slaughter is prohibited by law due to the sacred status of cow in Hinduism as representations of the goddess Lakshmi (Dhakal et al., 2023).

Buffaloes are the primary contributors to milk and meat. As of 2022/2023, Nepal had 3.08 million buffaloes, with 851272 million being milking buffaloes that produced 1.40 million metric tons of milk (MoALD, 2022/2023). Although there has been significant decrease in the number of cattle and buffaloes in 2022/2023 (-35.93% and -39.96%), the number of cow

buffaloes of advanced breed and higher productivity has increased compared to indigenous cattle. As a result, milk production has also increased during 2022/23 by 1.84 % (MOF 2022/23) (Economic Survey, 2023).

Among other livestock, chickens are especially favored for meat production due to their high nutritional and economic value. In 2022/2023, approximately 200,658 metric tons of chicken meat were produced. Goats and pigs are another key contributor, producing about 77162 and 32533 metric tons of meat in 2022/2023 respectively, where the goat meat production showed a positive growth rate of 3.93% while pork production slightly declined reflecting growth rate of -9.78%. Ducks, numbering 1,325,999, produced 60,229 eggs and 1,355 metric tons of meat in the same year. Sheep, primarily valued for wool, had a population of 501,849 and contributed 381,106 kg of wool in 2022/2023. This diverse range of livestock highlights the important role they play in meeting the country's food, economic, and agricultural needs.

1.1.2 Livestock diseases

Livestock production is an important source of income for many people in developing countries. However, inadequate control of livestock diseases reduces productivity, putting farmers' well-being and food security at risk (Nuvey et al., 2023). The majority (61%) of human diseases are zoonotic, meaning they can be transmitted between animals and humans, and include many of the leading causes of illness and death. Livestock play a direct role in health burden by transmitting foodborne diseases through animal-based foods, zoonoses that spread between animals and humans, and the emergence of human diseases originating from livestock (Herrero et al., 2013). Vector-borne diseases, previously a concern in tropical and subtropical regions, are now emerging as a threat in temperate countries as well (Savić et al., 2014). Ticks and tick-borne diseases (TBDs) reduce bovine productivity, affecting 80% of the world cattle population and severely impairing the livelihoods of resource-poor farmers particularly in tropical and subtropical countries including India, Nepal, Pakistan, and Bangladesh (Jabbar et al., 2015), (Ghosh et al., 2007). In Nepal, during the fiscal year 2079/80 (2022/23), a total of 53,091 cattle and 1,051 buffaloes died due to a disease outbreak. (MOF 2022/23).

The International Organization of Animal Health (IOE) has identified a list of fatal cattle diseases, including lumpy skin disease, foot and mouth disease, hemorrhagic septicemia,

trypanosomiasis, vesicular stomatitis, anthrax, rinderpest, bovine brucellosis, cysticercosis, contagious bovine pleuropneumonia, bovine tuberculosis, Rift Valley fever, enzootic bovine leukosis, bovine anaplasmosis, bovine babesiosis, theileriosis, hydatidosis, trichomoniasis, and heartwater (Dhakal et al., 2023). Protozoan parasites are a major cause of severe infections in humans and animals globally. These infections are primarily spread through arthropod vectors or via blood transfusions (Maharana et al., 2016). Important hemoprotozoan diseases of veterinary significance include trypanosomiasis, theileriosis, babesiosis, and anaplasmosis. These diseases are caused by various species of *Trypanosoma*, *Theileria*, *Babesia*, and *Anaplasma*, respectively, affecting multiple livestock species (Tewari et al., 2001). The hemoparasitic diseases threatens an estimated 250 million cattle and acts as a major constraint on livestock production and improvement in many developing countries (Durrani & Kamal, 2008).

1.1.3 Babesia

Piroplasms, primarily consisting of the genera *Theileria* and *Babesia*, are protozoan parasites that pose a significant threat to cattle, sheep, goats, and occasionally humans (Mehlhorn & Schein, 1985). *Babesia* are tick-borne protozoa belonging to the phylum Apicomplexa and are responsible for causing babesiosis (Chauvin et al., 2009). *Babesia* are the second most prevalent blood-borne parasites in mammals, following trypanosomes (Hunfeld et al., 2008) . *Babesia* species cause tick-borne splenic fever in various vertebrate hosts, with significant effects on cattle and horses, placing over half of the global population at risk. The disease's spread is restricted to regions where tick hosts are found, primarily in tropical and subtropical areas (Lau, 2009). The *Babesia* genus comprises over 100 identified species (Chauvin et al., 2009) .

Babesia was first identified by the Russian researcher Victor Babes in 1888 during the period when babesiosis posed a significant economic threat due to decreased meat and beef production, along with high animal mortality rates (Dhakal et al., 2023) . The primary tick vectors of *Babesia* include various Ixodid ticks such as *Boophilus* spp., *Rhipicephalus* spp., *Ixodes* spp., *Haemaphysalis* spp., *Dermacentor* spp., and *Hyalomma* spp (Bock et al., 2004). *Babesia bovis* and *Babesia bigemina* are the most clinically and economically significant species that infect cattle and are extensively found in tropical and subtropical regions (Bock et al., 2004) .

1.2 Statement of problem

Babesiosis, caused by protozoan parasites of the genus *Babesia*, is a significant tick-borne disease affecting cattle worldwide. It leads to severe health complications such as anemia, fever, weight loss, and, in some cases, death. The economic impact of these parasites is substantial, as infected cattle suffer from reduced productivity, lower milk yield, and increased veterinary costs. In Nepal, where livestock plays an important role in the nation's economy and rural livelihoods, the burden of babesiosis remains largely underexplored. Despite reports of tick infestations in various regions, there is limited scientific data on the prevalence of *Babesia* in cattle. The lack of such information hampers early diagnosis, disease control strategies, and effective treatment protocols. This study aims to address this gap by determining the prevalence of *Babesia* in cattle from two geographically different districts. The findings will contribute to a better understanding of the disease's distribution, helping veterinarians, farmers, and policymakers implement preventive and control measures to safeguard livestock health and economic stability of the nation.

1.3 Objectives

1.3.1 General objective

To determine the prevalence of *Babesia* infection in cattle from Jiri and Bardaghat, Nepal

1.3.2 Specific objectives

- To find the prevalence of *Babesia* in cattle using microscopy and molecular diagnostic methods.
- To sequence the 18S rRNA gene for the identification of piroplasm species.
- To find the differences in prevalence of *Babesia* in cattle from a single, open grazing farm and multiple, confined farms.
- To analyze the alteration in Hematological parameters in cattle infected with *Babesia.*

1.4 Research questions

- What is the prevalence of *Babesia* infection in cattle from Jiri and Bardaghat ?
- How does the prevalence of *Babesia* vary between these two rearing strstegies ?
- What is the better way to diagnose *Babesia* infection ?
- How does *Babesia* infection affect the hematological parameters of infected cattle compared to healthy ones?

1.5 Significance of the study

Livestock plays a vital role in the livelihoods of rural communities of Nepal, contributing significantly to agriculture, food security, and income generation. However, various livestock diseases, including *Babesia*, pose a serious threat to animal health, leading to reduced productivity and economic losses. This study is significant as it aims to explore the prevalence of *Babesia* in cattle across two different rearing system i.e. Single and open grazing farm in Jiri located in the Himalayan region and Multiple confined farms in Bardaghat in the Terai region. This research examines the variations in infection rates through microscopy as well as the modern diagnostic techniques such as PCR and associated hematological changes in the infected and non infected cattle. Thus, this research will provide valuable data to improve disease management strategies, enhance cattle health, and ultimately support the well-being of farmers and the agricultural economy in Nepal.

2. Literature review

2.1 History of *Babesia*

The microorganisms now identified as *Babesia* were first discovered by Babes in 1888 during his investigation into the cause of hemoglobinuria in cattle with fever (Vannier et al., 2015). In 1893, Smith and Kilbourne determined that *Babesia bigemina*, the causative agent of Texas cattle fever, was transmitted by ticks. This discovery was the first to demonstrate that an arthropod could serve as a carrier for an infectious disease (Vannier et al., 2015) . The parasites initially described by Babeş, Smith and Kilbourne, were later designated as *Babesia bovis*, *B. ovis*, and *B. bigemina*, respectively.

Shortly after, *Babesia* species infecting other domestic animals were identified, including *B. canis* in dogs and *B. caballi* in horses, described by Piana and Galli-Valerio (1895) and Koch (1904), respectively (Schnittger et al., 2012) . Yang and Wang were the first to identify and document *Babesia bovis* and *Babesia bigemina* in China's Guizhou province in 1964. *Babesia* species, widely recognized as animal pathogens over the past three decades have also been identified as occasional causes of human infection (Dhakal et al., 2023). Since the late 1950s, *Babesia divergens* in cattle (Europe) and *Babesia microti* in rodents (North America) have been identified as responsible for a significant number of human infections (Häselbarth et al., 2007).

2.2 Etiology and Pathogenesis of Babesiosis

Babesiosis is a parasitic disease caused by hemotropic protozoa of the *Babesia* genus. These protozoa are classified under the family Babesidae, order Ixoplasmida, class Piroplasmea, phylum Apicomplexa, subkingdom Alveolata, and kingdom Protozoa (Dhakal et al., 2023) . Worldwide, bovine babesiosis, also known as tick fever, cattle fever, Texas fever, red water disease, or piroplasmosis, is caused by six different species of *Babesia* parasites. These include *B. bigemina*, *B. bovis*, *B. divergens*, *B. major*, *B. occultans*, and *B. argentina*. Among them, *B. bigemina* (commonly referred to as African red water) is the most widespread, whereas *B. bovis* (known as Asiatic red water) is considered the most virulent species (Jacob et al., 2020) . In the host, intraerythrocytic *Babesia* species appear oval, round, or pear-shaped, with sizes varying from 1 to 5 μm in length (Boustani & Gelfand, 1995). The primary causative agents of bovine babesiosis in tropical and subtropical regions are *Babesia*

bigemina and *Babesia bovis*, while additional species such as *B. ovata*, *B. majori*, *B. occulata*, *B. divergens*, *B. jakiwiri*, and *B. veneturum* can also infect cattle(Dhakal et al., 2023; He et al., 2021) .

Babesia parasites possess most of the apicomplexan invasion organelles, except for conoids (Suarez et al., 2019). The pathogenesis of parasites in red blood cells begins with the invasion of sporozoites from the tick's salivary glands, leading to their development, replication, multiplication, and eventual egress from RBCs through asexual reproduction (Dhakal et al., 2023). The asexual reproduction of these parasite species results in the extensive destruction of the host's red blood cells (RBCs), causing severe intravascular hemolytic anemia in affected animals (Bock et al., 2004) . *Babesia*, along with those from the closely related genus *Theileria*, rank among the most widespread and commonly found blood parasites globally having a substantial impact on the economy, human and animal health worldwide (Homer et al., 2000).

2.3 Transmission and life cycle

Babesia is primarily transmitted through the bite of an infected tick, with blood transfusion being a less frequent mode of transmission (Ord & Lobo, 2015) . Four genera of the Ixodidae family known to transmit *Babesia* include *Rhipicephalus*, *Ixodes*, *Haemaphysalis*, and *Hyalomma* while the genus *Ixodes* serve as the primary vectors for *Babesia* species (Antunes et al., 2017; Leiby, 2006) . *Babesia* spp. are transmitted when ticks feed on the blood of a vertebrate host (Ord & Lobo, 2015). Unlike *Theileria*, *Babesia* species are transmitted to the next generation of vector ticks through eggs (transovarial transmission), aiding their global spread (Mehlhorn & Schein, 1985) . *B. bigemina* is transmitted by ticks such as *Boophilus microplus*, *B. decoloratus*, *B. annulatus*, *B. geigyi*, and *Rhipicephalus evertsi*. Similarly, *B. bovis* is transmitted by *Boophilus* species like *microplus*, *annulatus*, and *geigyi*. *B. divergens* is carried by *Ixodes ricinus* and *Ixodes persulcatus*, while *Haemaphysalis punctata* and *Haemaphysalis longicornis* are responsible for transmitting *B. major* and *B. ovata*, respectively (Bock et al., 2004).

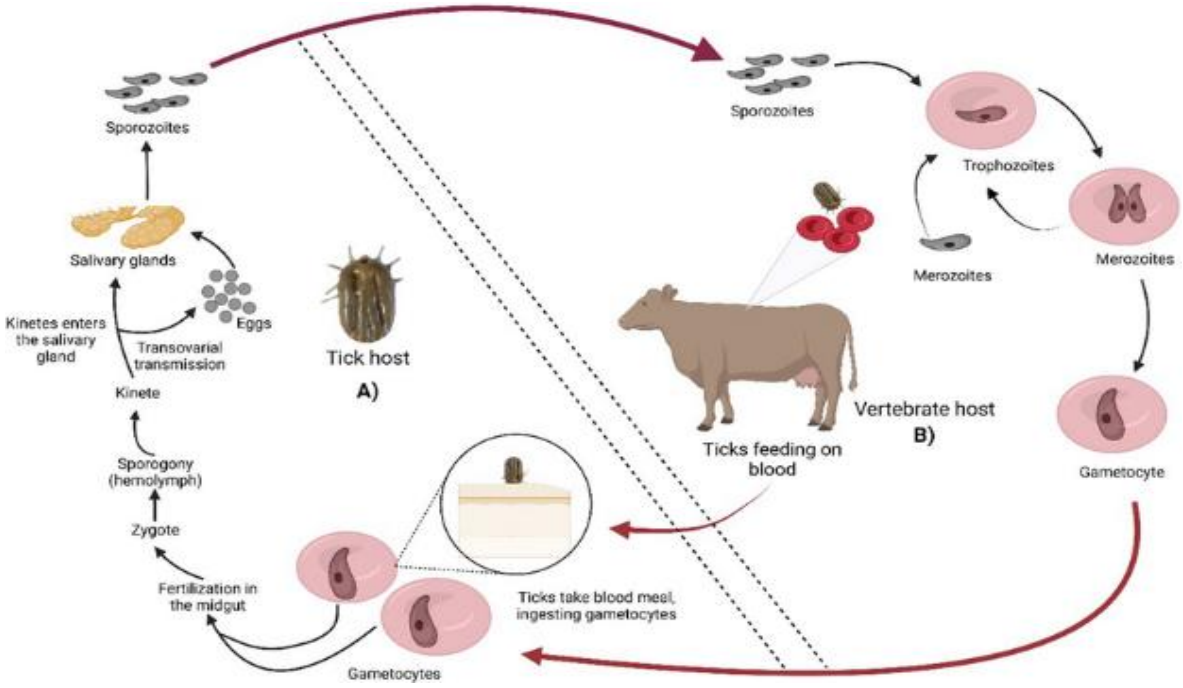


Figure 1: life cycle of *Babesia*

(source:https://www.researchgate.net/figure/Life-cycle-of-Babesia-bovis-Babesia-bovis-has-two-main-phases-A-The-sexual-cycle-takes_fig1_373019133)

These parasites undergo a complex life cycle, with hard ticks serving as their definitive hosts and vertebrates acting as intermediate hosts(Suarez et al., 2019). *Babesia* species infect vertebrate hosts by invading red blood cells and multiplying asexually through binary fission, while in their definitive hosts, ixodid ticks, they develop sexual forms and undergo sexual multiplication in the midgut(Mehlhorn & Schein, 1985) . After entering erythrocytes, merozoites reproduce rapidly. Within RBCs, *Babesia* spp. continuously replicate, exit, and reinvade, leading to the destruction of host red blood cells (Elsworth & Duraisingh, 2021) . In most species, they divide by binary fission, resulting in the characteristic paired appearance inside the erythrocyte, while in some species, four parasites form simultaneously (Mehlhorn & Schein, 1985). inside the red blood cells, some merozoites develop into male and female gametocytes, which persist inside the RBCs of the bovine host. When ticks feed on the host, they ingest these gametocytes (Santos et al., 2023). After being ingested with the tick's blood meal, gametocytes transform into gametes, which are then released from the RBCs into the tick's midgut. These gametes fuse to form a diploid zygote, which subsequently penetrates the tick's gut epithelial cells and undergoes meiosis, producing haploid kinetes (Jalovecka et al.,

2018). *Babesia* spp. kinetes invade tick tissues, including the ovaries (except *B. microti*, which targets nephrocytes), enabling transmission to larvae. They then migrate to the salivary glands (Howell et al., 2007). Upon reaching the salivary glands, *Babesia* kinetes multiply to form sporozoites, the stage that infects vertebrate hosts. SGs serve as the last obstacle, much like the midgut, that parasites must overcome to complete their life cycle in the vector (Chauvin et al., 2009) .

2.4 Geographical distribution

2.4.1 Babesia in Global context

Tick borne apicomplexan parasites are a major global threat, leading to serious illness in many animal species such as dogs, cattle, wildlifes and occasionally humans(Andersson et al., 2017). Tick borne diseases are highly recorded in tropical and sub tropical regions of continents such as Africa, Asia, Australia and Latin America (Jongejan & Uilenberg, 2004) where *Babesia bovis* and *B. bigemina* are found as the major cause of Babesiosis in cattle (Prado et al., 2022). The occurance of *Babesia* and *Theileria* depends on the availavility of their vector which are ixodid ticks . Central Asia is considered as an endemic region for *Babesia* and *Theileria* infection (Kuibagarov et al., 2023). *Babesia bovis* infection is often more severe in susceptible cattle, leading to their high mortality (Terkawi et al., 2011) .

2.4.2 Babesia in Nepal

Research conducted in various regions of Nepal has identified ticks as vectors responsible for transmitting diseases such as babesiosis and anaplasmosis in cattle, which severely affect their health and productivity (Lamichhane & Basnet, 2020). A research showed higher numbers of ticks in Chitwan where cattle were found grazed in jungle (Dhital et al., 2018). A molecular study on piroplasm in cattle of Kathmandu valley by (Dhakal et al., 2023) recorded 52.8% prevalence via nPCR where *B. bovis* and *B. bigemina* were seen via DNA sequencing . Similarly,(Shrestha, 2017) recorded a 0.64% prevalence of *Babesia* in Banke and Surkhet districts using PCR. Despite some research on erythrocytic parasites, including *Babesia*, through microscopy in various animals in Nepal, there has been very less molecular investigation of bovine babesiosis using PCR. Since PCR is a highly specific technique for detecting *Babesia*, the lack of such studies highlights a significant research gap in this field.

2.5 Clinical findings

The time between initial exposure to a pathogen and the appearance of the first symptoms, known as the incubation period, varies based on the parasite load and typically ranges from 7 to 21 days (Etiology, 2022). The main symptoms of babesiosis includes pale mucous membranes, jaundice, rapid breathing, hemoglobinuria and fever. These symptoms were observed in infected animals, consistent with previously published findings (Bal et al., 2016). Severe anemia and hemoglobinuria in cattle occur due to the destruction of a large number of red blood cells by *Babesia* inside them. Leading to the release of hemoglobin into the bloodstream and its excretion in urine (hemoglobiuria). However, Zlotnik (1953) reported a cerebral form of babesiosis in cattle, showing neurological signs instead of typical symptoms. Affected cattle had sudden high fever (106-107°F), teeth grinding, loss of appetite, head twisting and wall pressing behaviours. While *B. bigemina* was rarely found in blood and spleen smears, it was abundant in brain capillaries, heavily parasitizing red blood cells (Infections, n.d.).

2.6 Diagnosis

Babesiosis is usually identified through clinical signs and assessment of epidemiological risk factors, including travel history to endemic regions or activities that increase exposure to tick-infested areas while the confirmation is achieved through microscopic examination of stained blood films taken from affected cases or molecular testing (Krause et al., 2021; Ravindran et al., 2007). This process is mainly useful for detecting infected erythrocytes during the acute phase(Bock et al., 2004) . The location, size, and shape of intraerythrocytic stages are distinctive for *Babesia* species and are used for diagnosis (Mehlhorn & Schein, 1985). Microscopy generally has low sensitivity in detecting pathogens in animals with a low parasite load, such as infected carrier animals (Villanueva-saz et al., 2022). Alternatively, serological diagnostic methods, including the indirect immunofluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA), are used to detect parasite-specific antibodies (J. Mosqueda et al., 2012). A modified ELISA method, SELISA (Slide enzyme-linked immunosorbent assay) is also used in detection of antibodies against some *Babesia* spp (Ravindran et al., 2007).

Recently, enhanced nPCR assays with high sensitivity have been developed for direct detection of *B. bovis* and *B. bigemina*, demonstrating detection limits 100 times lower for *B. bovis* and 1,000 times lower for *B. bigemina* compared to previous nPCR assays (Romero-Salas et al., 2016). While acute infections are usually detected through microscopic examination, subclinical infections are best identified using serological and PCR methods however due to cost and practicality, PCR-based methods are not always accessible for diagnosis (Liu et al., 2012).

DNA sequencing is a powerful tool used in many fields like archaeology, genetics, forensics and molecular diagnosis. Some well-known DNA sequencing methods include the Sanger method, Maxam-Gilbert method, Pyrosequencing™, and single-molecule sequencing using exonuclease (França et al., 2002). Recent improvements in sequencing technology are allowing researchers to explore questions at the level of species, whole organisms, individual cells and the processes happening inside cells in which next-generation DNA sequencing is used at each of these levels (Shendure & Aiden, 2012). Many researchers have used DNA sequencing to identify the blood parasites including piroplasms where they sequence the DNA, align and edit them using various softwares such as MEGA, followed by a BLAST analysis to comparing with the reference samples in National Center for Biotechnology Information (NCBI) to identify the species (Dhakal et al., 2023; Köseoğlu et al., 2021).

2.7 Importance of Hematological study

Study of hematological and biochemical values help in understanding disease progression, prognosis, and guiding supportive therapy in animals (Ganguly et al., 2015). Hematologic analysis is important not only for diagnosing blood-related disorders but also for identifying various organ and systemic diseases (Roland et al., 2014). Complete blood counts (CBC) help diagnose diseases by revealing abnormalities in blood components (Lindholm-Perry et al., 2018). While a complete blood count (CBC) alone rarely confirms a diagnosis, the hemogram provides valuable insights for diagnosing, monitoring, and predicting disease progression (Roland et al., 2014). Cattle infected with *Babesia* exhibit changes in hematological parameters compared to non-infected animals, including a statistically significant decrease in total RBC count and mean hemoglobin concentration, along with an increase in total WBC count (Dhakal et al., 2023). The decrease in total RBC count and mean hemoglobin concentration, along with the increase in total WBC count, may be caused

by the breakdown of RBCs and the activation of phagocytic cells to remove the toxic remnants (Sharma et al., 2013) .

3. Materials and methods

3.1 Study area

Nepal is a landlocked country bordered by India to the east, west, and south and China to the north. It spans diverse geographical landscapes, ranging from the high Himalayas in the north to the flat plains of the Terai in the south. Covering an area of 147,516 square kilometers, Nepal extends approximately 850 km from west to east and varies between 145 - 241 km in width from north to south. The country is divided into three main ecological regions: Mountain, Hill, and Terai, each with distinct climatic conditions. Agriculture is a major livelihood for the majority of the population, with around 50.4 % of people engaged in farming and livestock rearing. This study was conducted in two regions of Nepal: Jiri Municipality of Dolakha district and Bardaghat Municipality of Nawalparasi district .

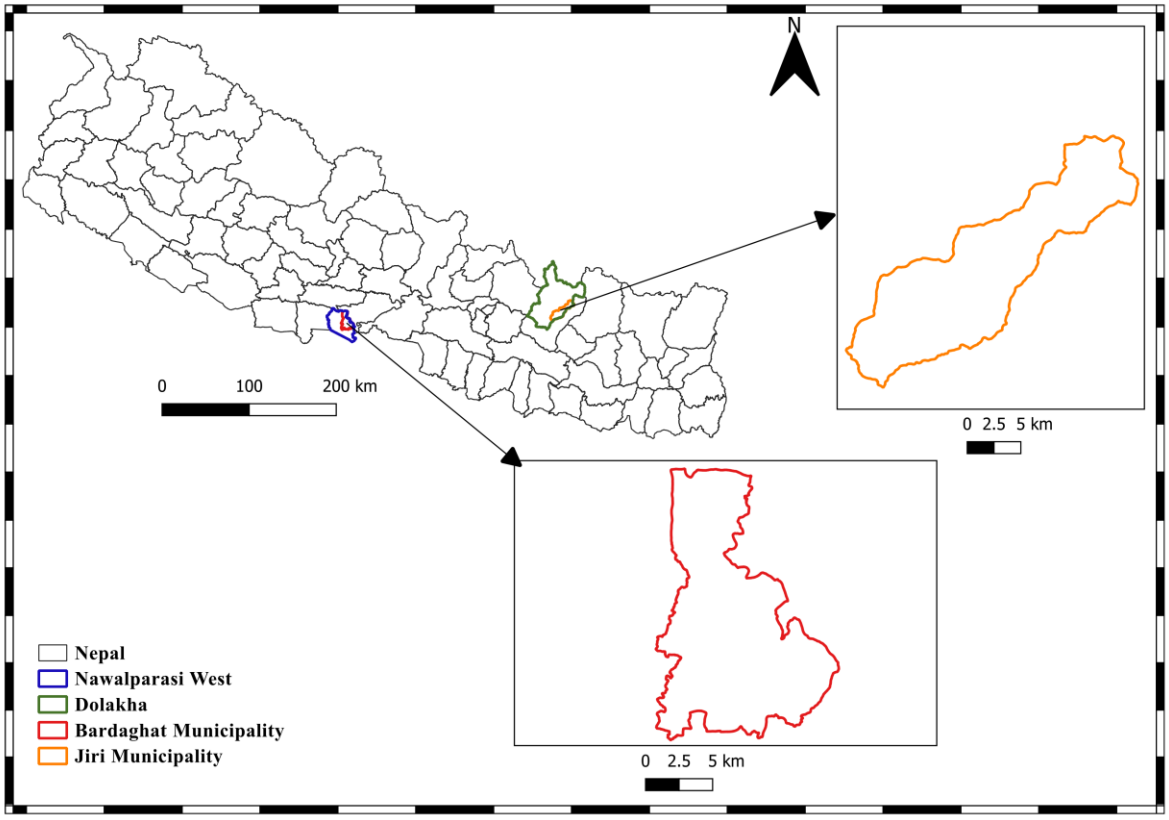


Figure 2: Map of Nepal showing the study areas.

Samples from Dolakha were taken from Cattle Genetic Resource Centre (CGRC) which is situated at the heart of Jiri Valley spanning approximately 209 hectares and positioning at an

altitude of 1935 meters above sea level . Dolakha lies in Bagmati Province of Nepal, positioned between approximately 27 ◌ 28' to 28°00' north latitude and 85°50' to 86°32' east longitude. The district covers an area of 2,191 square kilometers and has an altitude ranging from 732 meters to 7,181 meters above sea level . Being located in the himalayan region, Dolakha has a cooler climate and diverse topography, which influences tick infestation and disease prevalence. CGRC was established as the Livestock Development Farm (LDF) in 1957 AD. Initially been managed and financially supported by the Swiss government, the center was officially handed over to the Government of Nepal on 1963. In the fiscal year 2023/24, 125.3 cattle on average of various breeds are being reared. These cattle graze on open pasture land near the center.

Figure 3: Cattle Genetic Resource Center, Jiri Dolakha

In contrast, Nawalparasi West, also known as Parasi, is a district in the Lumbini Province of Nepal, situated between 27°21' to 27°47' north latitude and 83°36' to 84°15' east longitude. It covers an area of 634.88 square kilometers and lies at an altitude ranging from 100 meters to 1,491 meters above sea level. Being located in the terai belt, Nawalparasi West experiences a warmer and more humid climate, providing a suitable environment for tick proliferation. According to the data from MOAL 2022/23, the population of cattle in Nawalparasi West is 12,602. Sample was collected from multiple farms from several wards of Bardaghat Municipality . The cattle selected from this study area are confined and are not allowed to graze outside.

3.2 Ethical Clearance

This research received ethical approval from the Ethical Clearance Committee of the Nepal Veterinary Council (Ref No: Ethical/152) .

Informed consent was taken from the cattle owners.

3.3 Sample collection

A total of 190 blood samples from female cattle were collected from the two study areas, Jiri Municipality (n=90) from the Cattle Genetic Research Center and Bardaghat Municipality (n=100) from the selected 10 farms from different wards with higher cattle popultion. The samples in Jiri and Bardaghat municipality were collected in september and october of 2024 respectively. By puncturing the jugular vein, 3 ml blood was taken from each cattle as shown in Figure 3, using sterile syringes that was immediately transferred into EDTA vials to prevent coagulation. To preserve the integrity of the samples for microscopic examination, thin blood smears were prepared soon after the blood collection before preserving the sample.

3.4 Microscopy process

3.4.1 Thin smear preparation

For thin smear preparation, a glass slide was cleaned properly and a small drop of blood was placed at one end of the dry and clean slide. Using a spreader slide held at a 45° angle, the blood was smoothly spread across the slide in a forward motion to form a tongue-shaped smear. The prepared smear was then allowed to air-dry immediately. This immediate smear preparation helped minimize potential cell damage and ensured better staining quality. The prepared smears were allowed to air dry and packed into a slide rack before being carefully transported to the microscopic unit of Central Department of Zoloogy, Kirtipur, Kathmandu along with the remaining blood samples in EDTA vials for further analysis.

3.4.2 Methanol fixing

After air-drying, the thin blood smear was fixed using absolute methanol to preserve cell morphology and improve staining quality. The dried smears were placed on a coupling jar filled with methanol carefully, ensuring complete coverage. It was left undisturbed for 3-5

minutes to allow proper fixation. After that, the excess methanol was washed and the slide was air-dried before proceeding with Giemsa staining.

3.4.3 Giemsa Staining

Firstly, Giemsa's stain was prepared by mixing one part of the Giemsa standard solution with nine parts of phosphate-buffered water at pH 7.2. The solution was then filtered and stored in an amber-colored bottle to protect it from light. Then the methanol fixed blood smears were placed on a flat surface and and carefully flooded with Giemsa's stain using a dropper, ensuring complete coverage. The smear was left in the prepared Giemsa stain for 20-30 minutes. After staining, the slide was gently rinsed with phosphate-buffered water to remove excess stain and then air-dried complitely.

3.4.4 Microscopic analysis

To identify the presence of *Babesia* parasites, the stained blood smears were examined under a microscope using an oil immersion objective lens (100x) . Focusing on the parasites, the slide was viewed from its end, scanning the entire field for proper identification of the intracellular parasites within red blood cells. Observations were recorded, focusing on the morphology, size, and distribution of the parasites . In microscopy, *B. bovis* is generally seen forming paired structured merozoites at obtuse angles measuring 1.5 to 2 µm in the center of erythrocytes while *B. bigemina* appears as round merozoites with size varying 2-3 µm to 5 µm and are pear-shaped at acute angles (Laha et al., 2015).

Figure 4: Sample collection

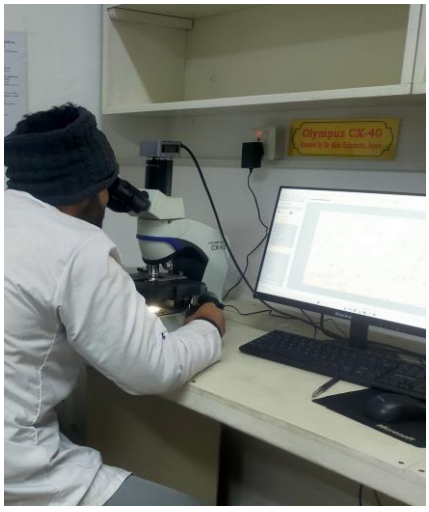


Figure 5 : Microscopic Analysis

3.5 Molecular analysis

Out of the total sample, 100 samples were taken forward for the molecular processes including 50 samples from each study area. Priority was given to the microscopic positive samples as well as the samples of symptomatic cattle . For the molecular study, DNA was extracted from the blood samples which was followed by nPCR assays. Blood sample was preserved in -20°C.

3.5.1 DNA Extraction

DNA extraction was done by using QIAamp@ DNA Blood Mini Kit (QUIGEN) by following the manufacurer's protocol. Before starting the extraction process, buffers AW1 and AW2 were made ready by adding indicated volume of ethanol . A volume of 200 µl blood sample was taken in a microcentrifuge tube where 20 µl Proteinase K and 200 µl buffer AL were added before vortexing and incubating for 10 minutes at 56°**C** . 200 µl ethanol was added followed by a quick vortex. All the contents were then pipetted into QIAGEN columns and centrifuged for 1 minute at 8000 rpm. The collection tube was discared and fresh new 2 ml collection tube was used to place the column . 500 µl buffer AW1 was added and centrifuged for 1 minutes at 8,000 rpm. The collection tube was again discarded and replaced with new one and 500 µl of buffer AW2 was added and the mixture was centrifuged for 3 minutes at 14,000 rpm. The collection tube was discarded and the column was placed into a new microcentrifuge tube. 50µl buffer AE was added to the column carefully in the centre and incubated at room temperature for 1 minute. It was then centrifuged for 1 minute at 8000 rpm . Finally, the column was discarded and thus collected DNA was stored in -20°C.

3.5.2 PCR assays

The selected 100 samples were carried forward for PCR and nPCR process. The 18S ribosomal RNA gene were amplified by using different primers for the primary and secondary amplification. Primers BTH 18S – 1F and BTH 18S – 1R were used for primary amplification while BTH 18S- 2F and BTH 18S- 2R were used for secondary amplification. These primers are designed for the amplification of DNA fragement of 1508 base pairs (bp). The details of the primers used is presented in Table 1.

Table 1: Details of the primers used in the PCR

Primers	Name	Sequencing	Band size (bp)	Tm
PCR Primers	BTH 18S – 1 F	GTGAAACTGCGAATGGCTCATTAC	1577	55
	BTH 18S – 1 R	AAGTGATAAGGTTCACACAAAACTTCCC		
nPCR Primers	BTH 18S – 2 F	GGCTCATTACAACAGTTATAGTTTATTTG	1508	
	BTH 18S – 2 R	CGGTCCGAATAATTCACCGGAT		

3.5.3 Preparation of master mix

A PCR mastermix is a premixed solution containing essential components that simplifies the reaction setup and ensures consistency. For the single PCR volume of 10 µl, mastermix was made by adding various components as shown in Table 2 in appropriate amount for the first PCR process.

Table 2: Composition of the Mastermix

S.N.	Component	Volume	Concentration
1.	Premix Taq™ (Ex Taq™ Version 2.0)	5 µl	
2.	Forward Primer (BTH 18S-1F)	0.3 µl	0.2 µM
3.	Reverse Primer (BTH 18S-1R)	0.3 µl	0.2 µM
4.	DNA Sample	1 µl	<500 ng
5.	Nuclease free water	3.4 µl	

Each of the volume mentioned above was for single sample which was multiplied with the total sample and one extra volume was added to ensure the sufficient amount of components.

3.5.4 First PCR process

PCR was performed to get the first PCR products that were later used as DNA sample for the second PCR (nPCR). The PCR was performed in "MyGeneTM L Series Peltier Thermal Cycler". A thermal cycle shown in Table 3 was set in the PCR machine. The thermal conditions were previously used for the similar researches (Masatani et al., 2017).

Table 3: Thermal conditions used in PCR and nPCR

Process	Temperature	Time	No. of cycles
Initiation	94°C	3 minutes	1
Denaturation	95°C	10 seconds	30
Annealing	55°C	15 seconds	
Extention	68°C	45 seconds	
Final extention	68°C	5 minutes	1

3.5.5 nPCR assay

The First PCR products were used as DNA samples for the nested PCR (nPCR) using different Primers (BTH 18S- 2F and BTH 18S- 2R) . The rest of components of the mastermix was similar to the previous one. Initially, the nPCR assay was performed using the undiluted first PCR products . Samples that showed negative results in this process were then subjected to a second round of nPCR with different dilutions of the first PCR product. For this process, the first PCR products were diluted to the concentration of 10x and 50x with nuclease free water. These dilutions were then used as the DNA sample for the nPCR assay. The PCR condition for nPCR was similar to the first PCR.

3.5.6 Gel electrophoresis

To separate the amplified DNA product, gel electrophoresis was performed using a 1.5 % agarose gel. First, 0.6 g of agarose powder was dissolved in 40 ml of diluted TBE buffer. The mixture was heated in an oven until the agarose completely dissolved. The solution was left to cool in room temperature. Once the solution cooled to room temperature, 3 µl of Ethidium Bromide was added, and the mixture was shaken thoroughly. The gel was then poured into a casting tray, a comb was inserted, and the gel was allowed to set for about 30 minutes. After solidification, the comb was removed, and the casting tray was carefully placed into a buffer tank filled with TBE buffer.

About 5 µl of the nPCR product was mixed with 1 µl loading dye and loaded into the wells carefully using the micropipette. A negative control and positive control was also used for the proper evaluation. Gel was run in 120 volts for 30 minutes which is shown in Figure 7. The gel was then placed on a UV transilluminator, and the bands of amplified DNA were visualized.

Figure 6: Peforming PCR

Figure 7: Running Gel Electrophoresis

3.6. DNA Sequencing

DNA sequencing was done in Obihiro University, Japan following the previously described method (Masatani et al., 2017). DNA fragments from the second PCR were cut from the gel

and purified using the NucleoSpin Gel and PCR Clean-up Kit (MACHEREY-NAGEL, Germany). Their sequences were determined using the dideoxy chain termination method (BigDye v3.1 Cycle Sequencing Kit, Applied Biosystems, USA) with BTH 18S 2nd F, BTH 18S 2nd R, and Inner Seq primers. A DNA fragment containing mixed parasite infections was inserted into a pGEM-T easy vector (Promega) after adding adenine residues to its ends using A-attachment Mix (Toyobo, Japan). The obtained sequences were analyzed and compared with those in the NCBI nucleotide database.

3.7 Sequence editing and Alignment

The Seqman software was used for editing, trimming the ends and checking error of the sequenced data from sequencing platform. The file was saved in FASTA format. FASTA file was used for alignment using software program (MEGA 11). The pairwise sequence alignment was generated by running the alignment program. The alignment was visualized using the software.

3.8 NCBI BLAST

The NCBI BLAST webpage was searched and opened in google chrome. The FASTA file of the desired sequence was selected and the parameters of search were set. The “BLAST” button was clicked. High similarity between the obtained sequence and the hits in the database were analyzed. The identity with high percentage was analyzed and compared. The homology between the two sequences were compared to differentiate species.

3.9 Phylogenetic analysis

The obtained sequences were aligned using bioinformatics tools, and a phylogenetic tree was constructed to determine the evolutionary relationships between the detected species and reference sequences from GenBank. The analysis was performed using MEGA software with the Neighbor-Joining method, and bootstrap values were calculated to assess the reliability of the tree branches.

3.10 Hematological analysis

The blood samples collected in EDTA vials were tested for hemoglobin concentration (Hb), total red blood cell (RBC) count, and total white blood cell (WBC) count. This analysis was done using Sahli's hemoglobinometer and a hemocytometer at the Hematology laboratory of

CVL, Tripureshwor for Jiri samples, while PE7010VET Veterinary hematology analyzer was used in Province veterinary laboratory, Parasi for the hematological analysis of Baradaghat samples.

3.11 Statistical analysis

The collected data were initially documented in a notebook, then organized in a Microsoft Excel spreadsheet. Prevalence was determined by calculating the ratio of infected animals to the total number of animals examined. R-studio was used to measure the various statistical values such as the Chi-square test to assess statistical significance, with a confidence level (CL) of 95% and a significance threshold of $P \leq 0.05$. The results were presented in tables and graphs.

4. Results

4.1 Microscopic Examination

Out of 190 blood samples from two study areas Jiri and Bardaghat, 17 samples (8.95%) were seen positive for *Babesia* species. Higher prevalence of *Babesia* was seen in Jiri (11.11%) while the microscopic result of Bardaghat municipality showed only 7 % positive.

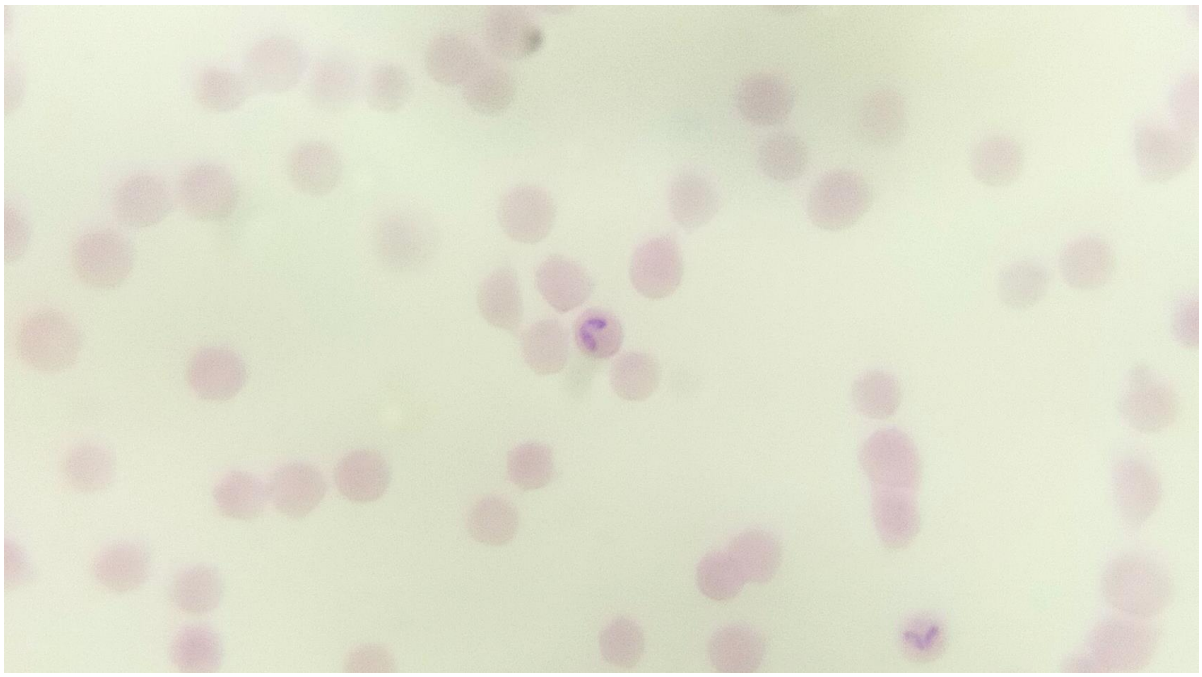
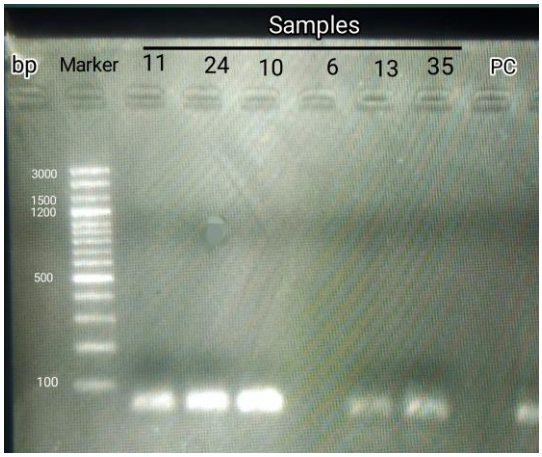


Figure 8 : Microscopic image of *Babesia*

4.2. PCR and nPCR result

A total of 100 samples were selected for PCR including 50 samples each from both study areas. Two sets of PCR were done for single sample including the first PCR and nested PCR. The DNA samples amplified by first PCR didn't show any bands while nPCR showed high prevlance of piroplasms exhibiting bright fluorescence under UV light for the positive samples as seen in Figure 9 a and b. The band size of these samples was compared to a molecular marker, revealing that positive samples had an approximate length of 1508bp.



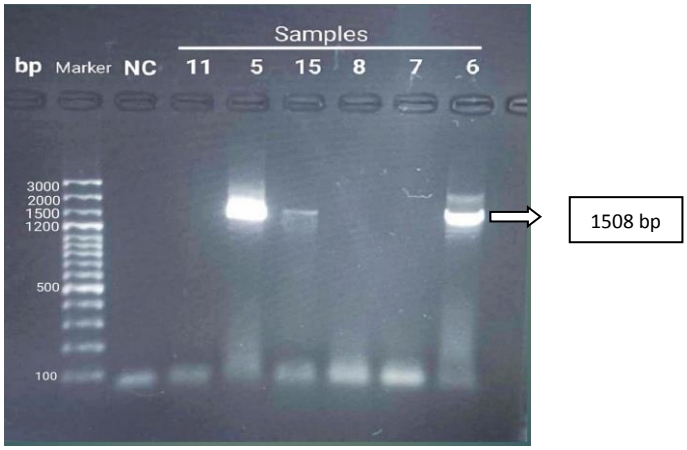


Figure 9 a **Figure 9 b**

Figure 9 a shows the gel electrophoresis image of negative PCR products. Left to Right: bp, Basepair; 11 to 35, Samples; PC, Positive control. Similarly Figure 9. b shows nPCR products with some positive samples. Left to Right: bp, Basepair; NC, Negative Control; 11 to 6, Samples;

The overall prevalence rate via microscopy was only 8.95% (17/190) but nPCR showed higher prevalence rate which was 53 % (53/100). In Jiri Municipality 28 out of 50 samples were seen positive via nPCR while 25 out of 50 samlples were identified positive from Bardaghat Municipality. Figure 10 shows the overall results obtained via both processes.

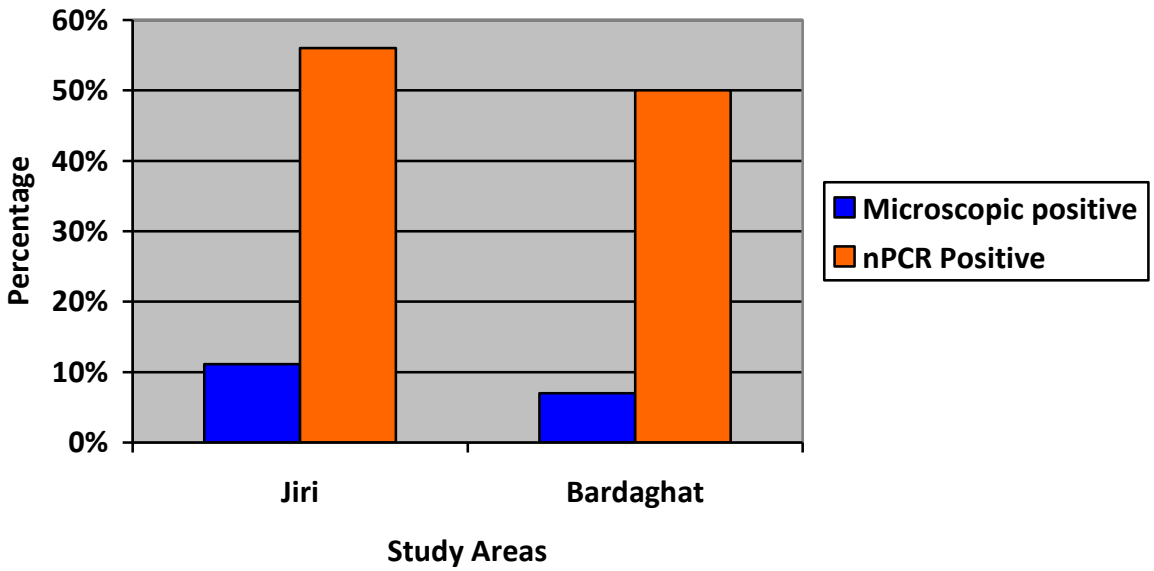


Figure 10: Compararive Microscopic and nPCR results in a bar diagram

All the samples that were positive in microscopy didn't appear positive in nPCR. This mismatch may be due to the relatively low sensitivity of stained smears in microscopy. Several studies have suggested that microscopic methods are more prone to producing false-negative results, especially when parasitemia is low (Antonio Alvarez et al., 2019) On the other hand, while PCR is a highly sensitive and reliable method, it can also face challenges in detecting low parasitemia. Various researches highlighted that PCR sometimes shows reduced sensitivity when parasitemia levels are very low, which can affect the accuracy of detection in such cases (Kim et al., 2007).

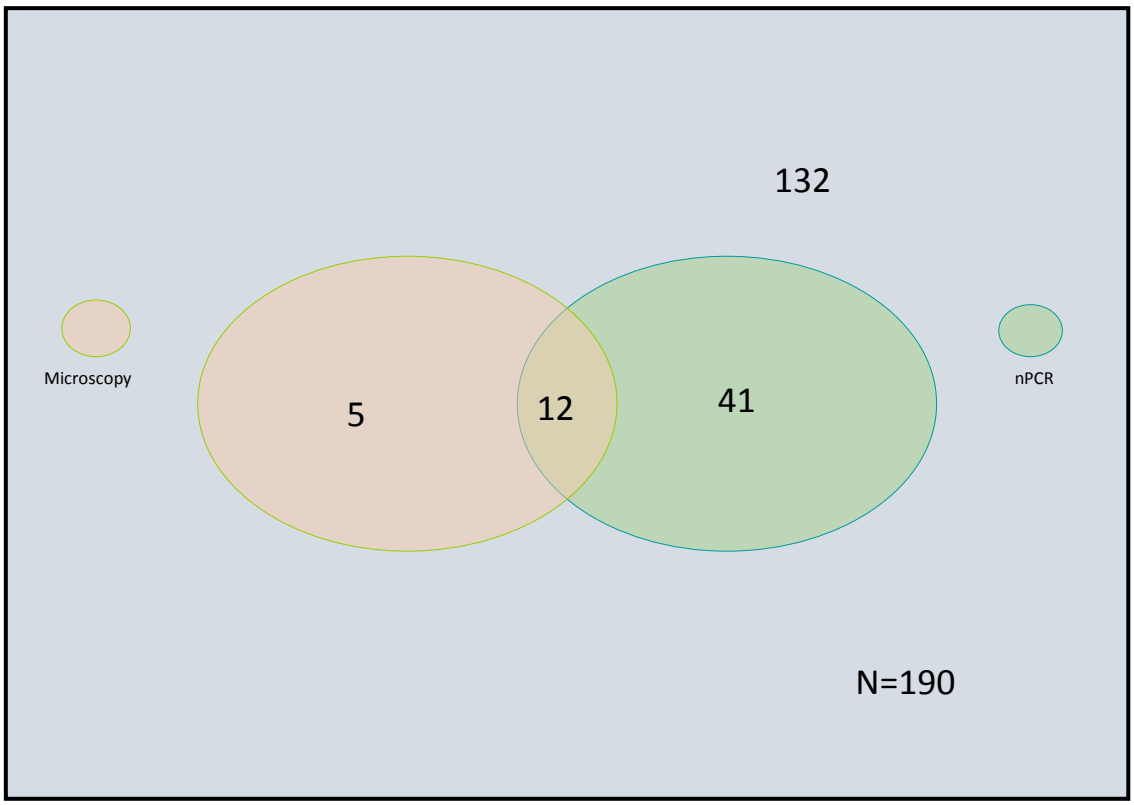


Figure 11: Venn-diagram showing microscopic and nPCR results

4.3 Comparative result

The comparision of diagnostic techniques highlights the superior sensitivity of nPCR over the microscopy. Microscopy of blood smears was found to be less sensitive and specific for detecting *Babesia*. A statistically significant difference in prevalence rates was observed between the two methods, microscopy and nPCR which is shown in Table 5. (χ^2=67.049,

p=2.648x10^{-16} & df =1) . In the other hand, all microscopic positive samples were not positive in nPCR.

Table 4: Comparative analysis of Microscopy and nPCR results.

Samples	Diagnosis Methods		p-value
	nPCR (n=100)	Microscopy (n=190)	
Positive	53	17	2.648x10^{-16} ($p < 0.05$)
Negative	47	173	

4.4 Sequencing Results

Out of 53 nPCR positive samples, 14 samples were selected randomly for DNA sequencing . The sequencing revealed distinct nucleotide sequences of *Babesia* in eight samples. Specifically, *Babesia bovis* was seen in 5 samples and *Babesia bigemina* was seen in 3 samples while 2 samples showed both species. Similarly 7 *Theileria* positive samples infected by 4 *Theileria* species were seen. *Theileria orientalis* and *Theileria annulata* were seen in 5 and 3 samples respectively while *Theileria buffeli* and *Theileria sergenti* were seen in one same sample as shown in Table .

Table 5: Piroplasm species recorded in DNA sequencing

Piroplasm Species	**No. of Infected cattle**
Babesia bovis	n = 5
Theileria orientalis	n = 5
Theileria annulata	n = 3
Babesia bigemina	n = 3
Theileria buffeli	n = 1
Theileria sergenti	n = 1

High homology was seen in the sequence obtained for the *Babesia* and *Theilera* species. The sequence obtained for *Babesia bovis* (83bp) and *Babesia bigemina* (882bp) showed homology of 99.19% and 99.75% respectively . Similarly *Theileria orientalis* (943) showed 99.76% homology with the various isolates from the Gene Bank as shown in Figure 12 and 13.

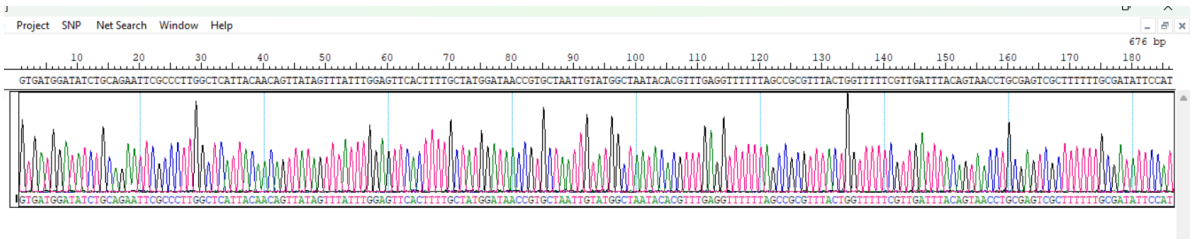


Figure 12: Raw Sequence of Chromatography

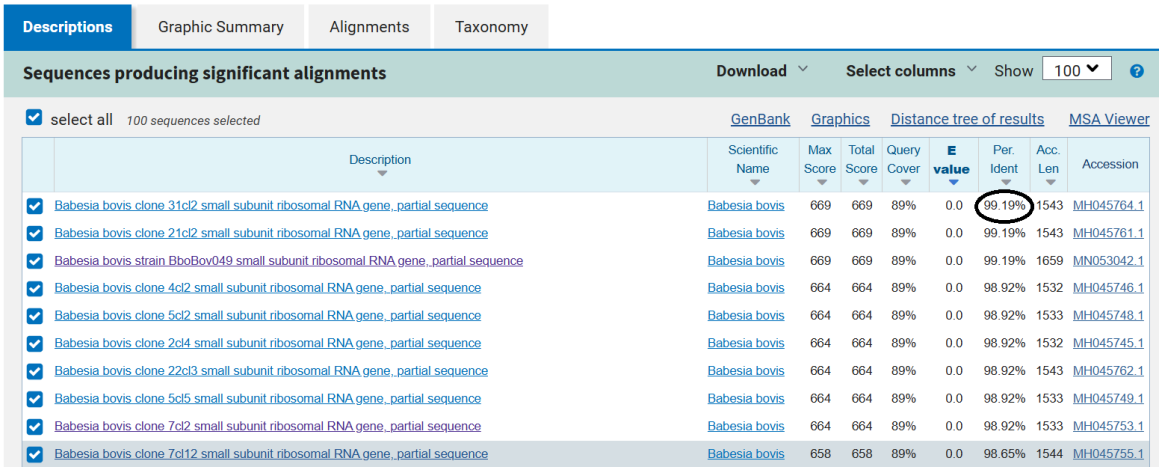
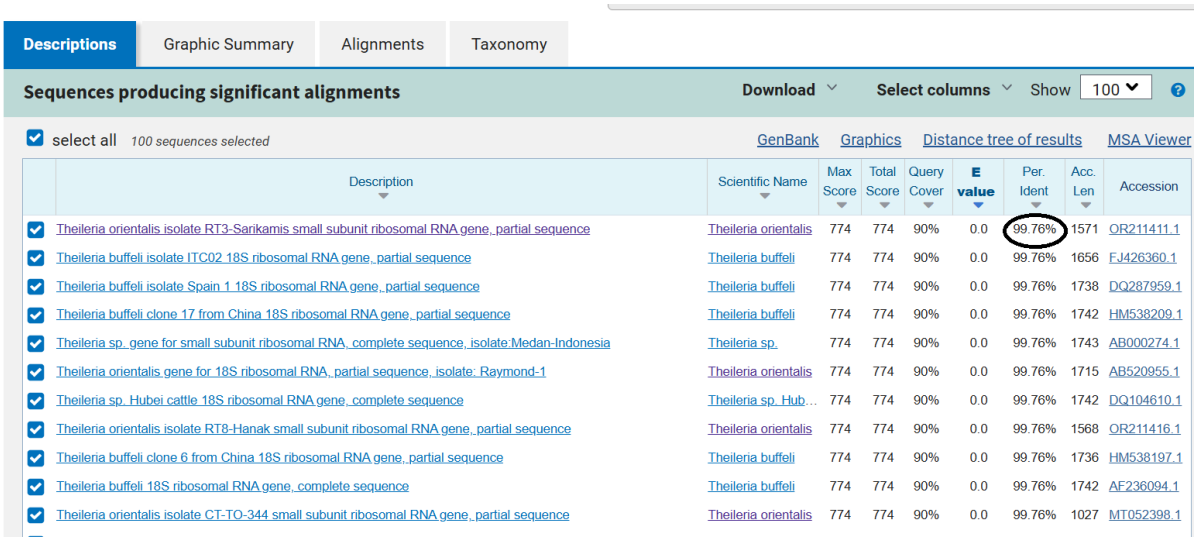


Figure 13 a: BLAST search result of *Babesia bovis*



Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments — Download — Select columns — Show 100

select all 100 sequences selected — GenBank | Graphics | Distance tree of results | MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Theileria orientalis isolate RT3-Sarikamis small subunit ribosomal RNA gene, partial sequence	Theileria orientalis	774	774	90%	0.0	99.76%	1571	OR211411.1
Theileria buffeli isolate ITC02 18S ribosomal RNA gene, partial sequence	Theileria buffeli	774	774	90%	0.0	99.76%	1656	FJ426360.1
Theileria buffeli isolate Spain 1 18S ribosomal RNA gene, partial sequence	Theileria buffeli	774	774	90%	0.0	99.76%	1738	DQ287959.1
Theileria buffeli clone 17 from China 18S ribosomal RNA gene, partial sequence	Theileria buffeli	774	774	90%	0.0	99.76%	1742	HM538209.1
Theileria sp. gene for small subunit ribosomal RNA, complete sequence, isolate:Medan-Indonesia	Theileria sp.	774	774	90%	0.0	99.76%	1743	AB000274.1
Theileria orientalis gene for 18S ribosomal RNA, partial sequence, isolate: Raymond-1	Theileria orientalis	774	774	90%	0.0	99.76%	1715	AB520955.1
Theileria sp. Hubei cattle 18S ribosomal RNA gene, complete sequence	Theileria sp. Hub…	774	774	90%	0.0	99.76%	1742	DQ104610.1
Theileria orientalis isolate RT8-Hanak small subunit ribosomal RNA gene, partial sequence	Theileria orientalis	774	774	90%	0.0	99.76%	1568	OR211416.1
Theileria buffeli clone 6 from China 18S ribosomal RNA gene, partial sequence	Theileria buffeli	774	774	90%	0.0	99.76%	1736	HM538197.1
Theileria buffeli 18S ribosomal RNA gene, complete sequence	Theileria buffeli	774	774	90%	0.0	99.76%	1742	AF236094.1
Theileria orientalis isolate CT-TO-344 small subunit ribosomal RNA gene, partial sequence	Theileria orientalis	774	774	90%	0.0	99.76%	1027	MT052398.1

Figure 13 b: BLAST search result of *Theileria orientalis*

4.5 Phylogenetic analysis

A phylogenetic tree was constructed to determine the evolutionary relationships between the detected species and reference sequences from GenBank. R3 2F, R4 1F, R5 1F, R7 1F , R22 1F, R21 1F and R10 2F samples were used to construct the phylogenetic relationship of piroplasm sequences of cattle. The sequences were aligned with the help of Mega 11 software. Maximum likelihood analysis was used for construction of phylogeny. The bootstrap value was 1000. R21 1F and R10 2F formed clade with *Babesia bovis* with a bootstrap value 54. R22 1F formed clade with *Babesia bovis* with highest bootstrap value i.e. 100. R3 2F, R7 1F, R4 1F and R5 1F are on one clade with bootstrap value 39 forming clade with *Theileria orintalis, Theileria buffeli, Theileria sergenti, Theileria annulata, Babesia bovis* and *Babesia bigemina.*

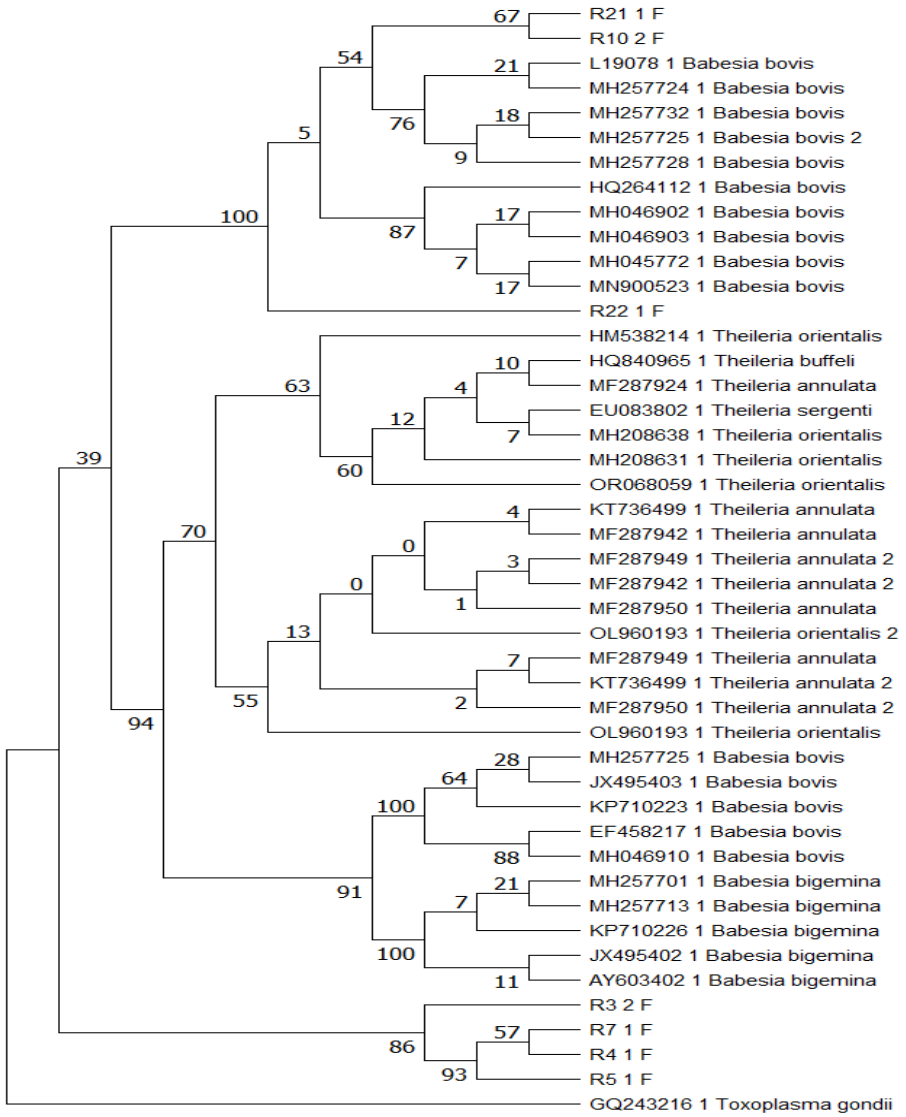


Figure 14: Phylogenetic relationship of piroplasm sequences of cattle

4.6 Hematological result

For the hematological analysis the parameters of healthy and infected cattle was compared . Parameters of healthy cattle was taken from (Dhakal et al., 2023). Complete Blood Count of 25 infected cattle was compared to that of the controlled ones. A significant alteration was seen in the hematological parameters such as Hb level, RBC and WBC count of the infected and controlled cattle as shown in Table 6.

Table 6: Hematological analysis

Hematological parameters	Control (n=10) (Dhakal et al., 2023)	Infected (n=25)	p-value	Reference value
Hb (g/dL)	11.32 ± 3.40	8.45 ± 0.46	0.017*	8-15
RBC (×10^6/µl)	7.16 ± 2.40	5.39 ± 0.68	0.038*	5-10
WBC (×10^3/µl)	12.32 ± 4.16	13.46 ± 5.44	0.606	4-12

Mean ± SD

Note: *Significant

The p-value for haemoglobin concentration and RBC count is less than 0.05 in the infected cattle that shows significant decrease in Hb concentration and RBC count. However, p value for WBC count is greater than 0.05, so there is increase in WBC count of the infected cattle as per statistical analysis.

4.7 Clinical Findings:

Out of the examined cattle, 32 % exhibited clinical signs suggestive of *Babesia* infection. As per the questionnaire done with the cattle owners, the most commonly observed signs included fever and lethargy. Additionally, some demonstrated jaundice, and weight loss. However, not all symptomatic cattle were tested positive for *Babesia* .Only 19 % of symptomatic cattle showed positive for *Babesia* infection having the recorded symptoms. Upon hematological analysis, infected cattle showed lower Hb and RBC count.

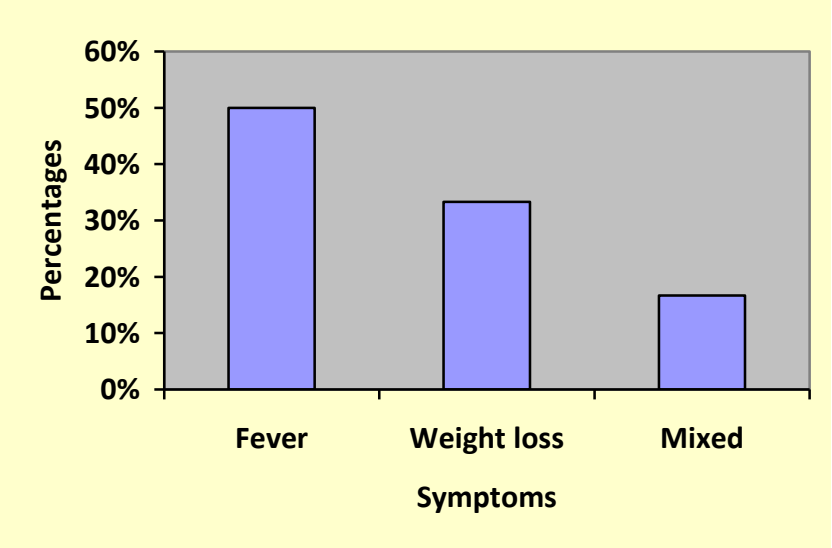


Figure 15: Analysis of Clinical Findings

5. Discussion

5.1. Prevalence of *Babesia* infection in cattle

The study aimed to determine the prevalence of *Babesia* infection in cattle from Jiri Municipality (Dolakha) and Bardaghat Municipality (Nawalparasi) using both microscopy and PCR techniques. The results revealed a notable difference in detection rates between these methods. Microscopy detected *Babesia* in 17 out of 190 cattle (8.95%), whereas PCR demonstrated a significantly higher prevalence of 53 out of 100 samples (53%). These findings suggest that PCR is a more sensitive diagnostic method than microscopy, as also reported in previous study by (Dhakal et al., 2023) where microscopy and nPCR result showed prevalence of 39.6% and 71.1% respectively. Similarly (Kaur et al., 2021) also reported similar findings in North India having the prevalence based on microscopy 12.9% where PCR recorded 30.22% animals positive for haemoparasitic infections including *Babesia* and *Theileria*. The significant difference in detection rates between microscopy and PCR highlights the importance of molecular diagnostic techniques. PCR showed higher sensitivity, detecting infections that were missed by microscopy. This Variation aligns with previous research suggesting that PCR is more effective in identifying low-parasitemia cases .A research in another South Asian Country Bangladesh also revealed similar findings where the detection rates of hamoparastites including *Babesia* were higher in PCR methods (18.7%) compared to microscopy (14.3%) (Hossain et al., 2023) . All the microscopic positive sample were not seen positive in nPCR analysis. It might be due to the low sensitivity of stained smears in microscopy as suggested by various researches there is a high likelihood finding false negative results during diagnosis by microscopic method (Antonio Alvarez et al., 2019). Similary lack of skilled personal is another problem in microscopic diagnosis. Therefore, detection by PCR is suggested due to its higher sensitivity however this has some issues of requiring multiple process, less sensitive in low parasitemia and high cost (Kim et al., 2007). Hence, nPCR should be considered as a standard diagnostic tool for *Babesia* surveillance in Nepal's cattle populations.

5.2. Findings of DNA sequencing

The easiest way to understand the relationships between *Theileria* and *Babesia* species is by studying genes, especially the 18S rRNA gene (Allsopp & Allsopp, 2006). Same gene was targeted by the current study. The DNA sequencing of nPCR positive samples confirmed the

presence of various six piroplasm species in cattle with various species of *Babesia* and *Theileria.* Among all, *Babesia bovis* and *Theileria orintalis* became the most prevalent. All the piroplasm showed high sequence similarity to *Theileria* and *Babesia* species available in GenBank.Furthurmore, phylogenetic analysis revealed that the detected sequences clustered closely with strains reported from various study areas. other regions of Nepal and neighboring countries (Dhakal et al., 2023; Oliveira-Sequeira et al., 2005). This suggests potential regional transmission patterns and common tick vectors and also the findings of this study highlight the importance of molecular diagnostics not only for species level identification but also for understanding the genetic diversity and epidemiology of piroplasms affecting cattle.

5.3. Piroplasm infecton in different farming styles

Ticks responsible for the transmission of *Babesia* are widely distributed across Nepal. According to the findings of (Dhital et al., 2018), cattle ticks are found abundantly from the mid-hills to the plains of Nepal**,** indicating their extensive presence across diverse ecological regions . This widespread distribution suggests that *Babesia* infection in cattle could be a significant concern in various geographic areas. A study in Bhutan reported *Babesia* infections in cattle across various ecological zones, including Cool Temperate, Warm Temperate, Dry Subtropical, Humid, Subtropical, and wet Subtropical regions while the higher infection was seen in cattle from lower Agro-Ecological Zones (Pem et al., 2021) . This might be due to the higher tick burden seen in lower altitudes as comparision to the higher altitudes (RLDC 2019). Many Studies have shown that tick species distribution is influenced by temperature and humidity (Namgyal et al., 2021), which could explain the differing prevalence rates between the two study areas of current research. Several studies in Europe has shown a decline in *Babesia* infection likely due to the changes in grazing practices and improved pastures (Springer et al., 2020).

In this study, Jiri, Dolakha showed higher prevalence of piroplasm despite of being a high-altitude, cooler region, compared to the lowland of Bardaghat. This might be due to the difference in the farming style and sampling methodology. The samples from Jiri, Dolakha were collected from a single farm, while samples from Bardaghat, Nawalparasi were gathered from multiple farms. This could explain some of the variations in findings. In Jiri Municipality, the high infection rate within a single farm suggests localized transmission,

potentially due to shared housing conditions and limited movement of cattle. Since the cattle from Jiri graze outside which likely increased their exposure to the tick vectors showing the significant role of grazing behavior and role of environment in the transmission of the disease. On the other hand, Bardaghat Municipality's lower prevalence may result from diversified farm management practices, leading to reduced infection risk. Ticks are spread by their hosts, the movement of these host populations across different areas is important for understanding tick density, especially in regions where the climate supports their habitat (Estrada-Pena, 2009). Condition of the sheds also affects the tick distribution. Sheds that are not cemented and having cracks and crevices provide ideal hiding and breeding spaces for ticks, significantly contributing to their population growth (Buteikienė, 2008) . Tick-borne pathogens have been widely associated with poor tick management and environmental factors that favor vector survival (Dhital et al., 2018).

5.4. Hematological findings

This study found that Piroplasm-infected cattle had significantly lower RBC count and hemoglobin concentration ($p < 0.05$) compared to non-infected cattle. Additionally, there was a notable increase in total WBC count, indicating hematological alterations due to infection . Similar alterations in hematological parameters were also reported by (Dhakal et al., 2023) . The decrease in total RBC count and mean hemoglobin concentration, along with the increase in total WBC count, may be caused by the breakdown of RBCs and the activation of phagocytic cells to remove the toxic remnants (Sharma et al., 2013) .

6. Conclusions and recommendations

6.1. Conclusions

The findings of this study underscore a significant prevalence of *Babesia* infection in cattle from two distinct regions of Nepal, showing an overall prevlene of 29.47 % by both microscopic and nPCR methods. nPCR showed more prevalence than microscopic examination. These findings suggest that geographical and environmental factors, alongside farm management practices, may influence the level of infection, particularly since cattle in Dolakha originated from the same farm and open grazing showed higher prevalence than those of Bardaghat that came from indoor of multiple farms. Hematological analysis further highlighted the pathological impact of *Babesia* infection, with affected cattle exhibiting a marked increase in white blood cells (WBC) and a decrease in red blood cells (RBC) and hemoglobin (Hb). These alterations are indicative of the systemic nature of the infection, which compromises the overall health of the animals and may lead to long-term consequences if left unaddressed. This study emphasizes the urgent need for comprehensive control strategies and continuous monitoring to mitigate the spread and impact of *Babesia* in cattle populations.

6.2. Recommendations

- Regular screening for Babesia infection in cattle should be implimented, especially in areas with higher prevalence.
- Farmers should be educated on the early signs of *Babesia* infection to facilitate prompt treatment and reduce the spread.
- Appropriate preventive measures, including vaccination and tick control programs should be promoted to mitigate the impact of *Babesia* on cattle health.

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Appendices

Photographs

Fig. 1 Sample Collection

Fig 2. Transferring blood in EDTA Vials

Fig. 3 Analyzing Hematological Parameters

Fig 4. Running Gel Electrophoresis

Annex 1

Government of Nepal

Nepal Veterinary Council

Date : 2024-12-11

Letter. no.: 2081-82

Ref. no.: Ethical / 152

Subject: **Ethical Clearance for study on "Prevalence of Babesia spp. in cattle of Bardaghat Municipality"**

To

Madhav Pandey

Dear Mr. Pandey,

With reference to your letter dated November 29th, 2024 regarding the ethical approval of a research study on the area mentioned below, I have the pleasure to inform you that the Council has approved ethical clearance for the specified research study with the following terms and conditions.

1. Study detail:
   a. Title: " **Prevalence of Babesia spp. in cattle of Bardaghat Municipality** "
   b. Nature of study: Master's Thesis
   c. Principal researcher: Madhav Pandey, N.V.C Regd No.
2. Supervisor / Co-supervisor: Dr. Kishor Pandey, N.V.C Regd No.
3. Research laboratory: Central Department of Zoology
4. The study described above shall be carried out according to standard protocol in compliance with the Code of Conduct, 2058 prescribed by "Nepal Veterinary Council (NVC)"
5. Care must be taken to ensure animal welfare. Any activity posing threat or cruelty to animal shall be punishable in accordance with the prevailing law.
6. The NVC shall retains the right to
   a. Withdraw or amend this Ethical Approval, if
      i. Any unethical principal or practices are revealed or suspected
      ii. Relevant information has been withheld or misrepresented
      iii. Regulatory changes of whatsoever nature so require
      iv. The conditions contained in the directive have not been adhered to
   b. Access to any information or data or experimentation site at any time during the course or after completion of the study.
7. NVC shall have the right to stop the study any time in case of :
   a. Violation of animal welfare or
   b. Detection of activities intended cruelty to animal.
   c. Any other matter related to animal welfare or cruelty shall be dealt in accordance with the decision of NVC.
8. The Principal Researcher shall report to NVC in the prescribed format, where applicable,
   a. Six-monthly progress report regarding the ethical compliance, status of animal and
   b. The completion report at the end of the project
   c. However, NVC must be informed immediately if
      i. Any material change in the conditions or undertakings mentioned in the document,
      ii. Any material breaches of ethical undertakings or events that impact upon the ethical conduct of the research.
      iii. Any change or revision in protocol during the course of the study, and
9. NVC may carry out monitoring of the study as and when required. However, it is the responsibility of researcher to organize periodic monitoring of study by NVC.
10. The validity of this ethical clearance is one year effective from the December 11th, 2024. You will be required to apply for renewal of ethical clearance on an annual basis till the study is not completed.
11. Permission from NVC is mandatory before publication of the finding of study.
12. Wishing you well in your research

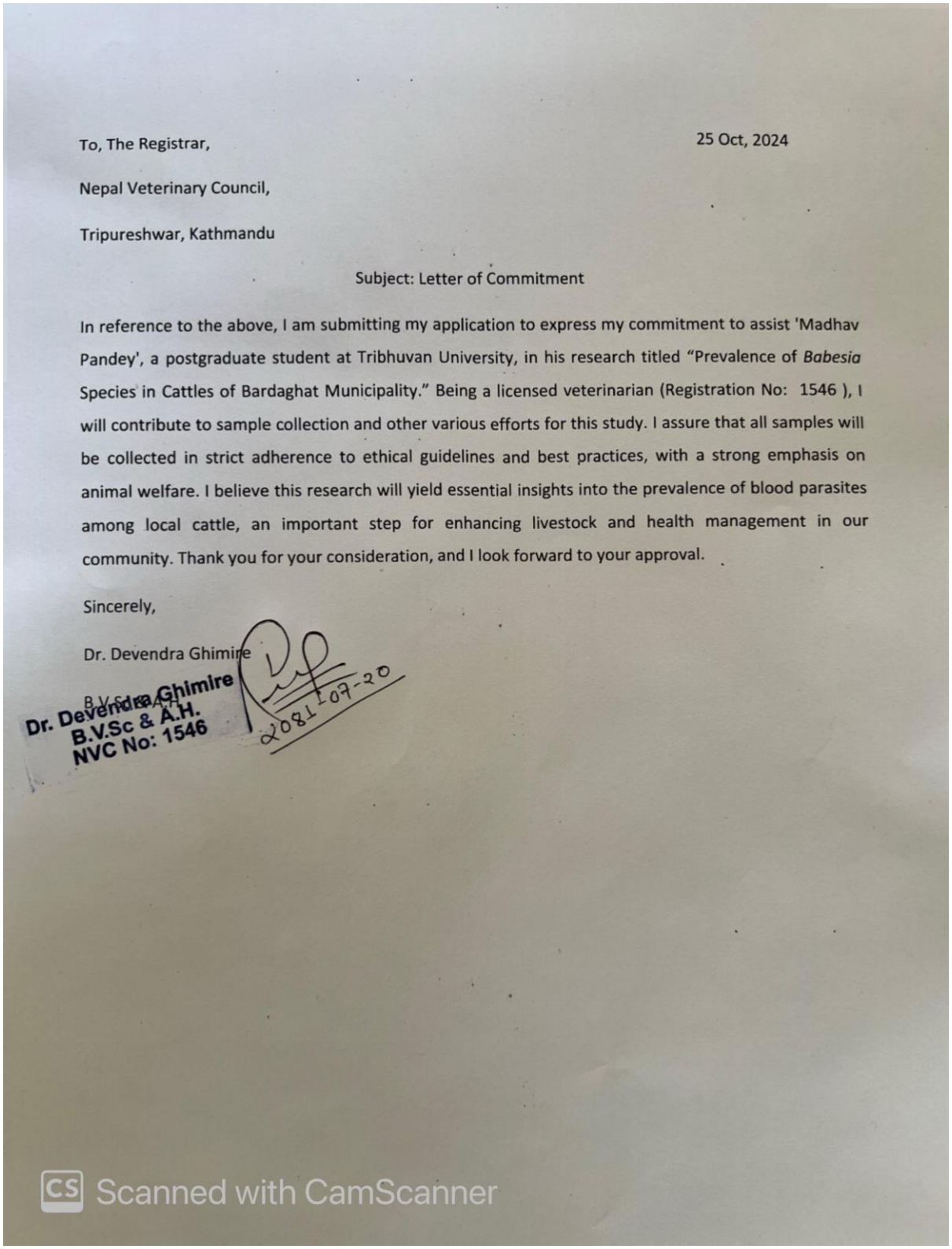
Dr. Ram Kumar Mandal
Registrar

Copy to: Chairperson, Nepal Veterinary Council

Copy to: Vice Chairperson, Professional Standard and Complaint Assessment Committee, Nepal Veterinary Council

Veterinary Complex, Tripureswor, P.O. Box No.: 21655, Kathmandu, Nepal
Tel.: +977 1 5359144, 5361210; Fax: +977 1 5359144, Email: info@vcn.gov.np, Wesite: www.vcn.gov.np

Annex 2



To, The Registrar,

25 Oct, 2024

Nepal Veterinary Council,

Tripureshwar, Kathmandu

Subject: Letter of Commitment

In reference to the above, I am submitting my application to express my commitment to assist 'Madhav Pandey', a postgraduate student at Tribhuvan University, in his research titled "Prevalence of *Babesia* Species in Cattles of Bardaghat Municipality." Being a licensed veterinarian (Registration No: 1546), I will contribute to sample collection and other various efforts for this study. I assure that all samples will be collected in strict adherence to ethical guidelines and best practices, with a strong emphasis on animal welfare. I believe this research will yield essential insights into the prevalence of blood parasites among local cattle, an important step for enhancing livestock and health management in our community. Thank you for your consideration, and I look forward to your approval.

Sincerely,

Dr. Devendra Ghimire

B.V.Sc & A.H.

Dr. Devendra Ghimire
B.V.Sc & A.H.
NVC No: 1546

2081-07-20