PREVALENCE OF *BABESIA* SPP IN SUSPECTED CATTLE OF KATHMANDU VALLEY, NEPAL

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A thesis submitted in partial fulfillment of the requirements for the award of the degree of Masters of Science in Zoology with special paper Parasitology

Submitted to

Central Department of Zoology

Institute of Science and Technology

Tribhuvan University

Kirtipur, Kathmandu

Nepal

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DECLARATION

I here declare that the work presented in this thesis has been done by myself, and has not been submitted anywhere for the ward of any degree. All the source of information has been specifically acknowledged by reference to the authors or institutions.

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RECOMMENDATION

This is to recommend that the thesis entitled "**PREVALENCE OF *BABESIA* SPP IN SUSPECTED CATTLE OF KATHMANDU VALLEY, NEPAL**" has been carried out by Medhavi Dhakal for the partial fulfillment 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 thesis work has not been submitted for any other degree in any institution.

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On the recommendation of the supervisor Associate Professor **Dr. Kishor Pandey**, this thesis submitted by Miss. Medhavi Dhakal entitled "**PREVALENCE OF *BABESIA* SPP IN SUSPECTED CATTLE OF KATHMANDU VALLEY, NEPAL**" is approved for the examination and submitted to the Tribhuwan University in partial fulfillment of the requirement for the Degree of Master of Science in Zoology with special paper Parasitology.

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This thesis work submitted by Miss. Medhavi Dhakal entitled **"PREVALENCE OF *BABESIA* SPP IN SUSPECTED CATTLE OF KATHMANDU VALLEY, NEPAL"** has been accepted as a partial fulfillment for the requirement of Master's Degree of Science in Zoology with special paper Parasitology.

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LIST OF ABBREVATIONS

Abbrevative form	Details of Abbrevations
µl	Micro litre
µm	Micro meter
CBC	Complete Blood Count
DNA	Deriboneucleic Acid
ELISA	Enzyme-linked Immunosorbent Assay
GDP	Gross Domestic Product
i.e.	id est
ICTs	Immunochromatography Tests
IFAT	Indirect Fluroscent Antibody Technique
MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Corpuscular Volume
nPCR	Nested Polymerase Chain Reaction
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
RBCs	Red blood Cell
rRNA	ribosomal Ribonucleic Acid
TBBPs	Tick Borne Blood Protozoan
TEC	Total Erythrocyte Count
TLC	Total Leucocytes Count

ABSTRACT

Babesia is an intraerythrocytic apicomplexan hemoprotozoan which causes a fatal disease babesiosis and is transmitted by ticks. *Babesia* is one of the major causes of low productivity and poor health condition in wide variety of animals. *Babesia* is diagnosed based on labor-intensive microscopy of blood smears. Therefor, there is an urgent need of rapid and more sensitive method to detect *Babesia* in Nepal. The aim of this study is to identify *Babesia* among livestock of Kathmandu Valley, Nepal. For this purpose, 106 blood samples of cattle was collected in EDTA tubes from different areas of Kathmandu valley. Thin blood smears were prepared for microscopic examination. Parasites DNA was extracted from blood sample. PCR test was performed to identify *Babesia* present in cattle. The prevalence of *Babesia* was found to be 39.62% through microscopy while the rate of prevalence of piroplasma were 71.1% through nPCR. Sequencing of randomly selected nine nPCR products revealed unique sequences of *Babesia* spp. from two samples. The existence of two distinct populations of *Babesia* spp. that is *B. Bovis* and *B. bigemina* circulating in the blood of infected cattle was verified. The hematological observation revealed significant ($P \leq 0.05$) reduction in Hb concentration, RBCs in infected cattle. Thus, this study shows higher prevalence of *Babesia* in cattle of Kathmandu Valley. This study was the first research at molecular level provides new data about *Babesia* infections among cattle in Kathmandu valley which could be useful for the control of disease in this area.

1. INTRODUCTION

1.1 Background

Nepal is a landlocked country which is divided into three ecological belt. Around 60.4% population of Nepal is involved in agriculture during 2018. The agriculture sector of Nepal contributes about 23.95% of nation's gross Domestic Product (GDP) with substantial to the nation's economy (MOF, 2022). The agriculture sector of Nepal provides jobs and employments for Nepalese people. The mixed farming system contributes the agriculture sector where crops and animal husbandary are mutually dependent. Livestock is an important element of Nepal's agricultural production system. The contribution of livestock to the national economy in 2020/2021 was 27 % of agricultural value added and 13% of national GDP. In the livestock sector, cattle and buffaloes are one of the major livestock of Nepal which contribute approximately 80% of the total livestock of Nepal (MoALD, 2021). Livestock raising is widespread in all ecological regions of the country, including the Mountain, Hill and Terai belts, with variations due to climate, topography and socio-economic factors. The livestock sector in Nepal is represented specifically by small farm holders to fulfill the requirements of vitamins and proteins, meals security, and income.

1.2 Livestock farming scenario in Nepal

The livestock industry is economically important in Nepal. The group of animals that forms the livestock includes buffaloes, cattle, pigs, sheep, goats, fowl and ducks. The livestock are not only employed solely for agricultural purpose, they also have worth and uses of their own. Farmers of Nepal are keeping livestock for milk wool, manure, meat, drought, transportation and also for various farming purposes. Farm families rely on livestock as a significant source of animal protein and for draught power in both agriculture and transportation. On the basis of animal mass unit, cattle and buffaloes are the major livestock of Nepal. Cattle are large domestic four footed herbivores that includes cow, calves, bulls and oxen. Amritmahal, Siri,Sahival, Red Sindhi, Holstein, Friesian, Haryana, Ongola and Kankrej are some important indegeous and exotic cattle breeds that are reared in Nepal (Adhikari, 2018). It is believed that

about 1.5 billion of cattle are present globally (FAO, 2021). According to the Livestock census (2078), 7.46 million cattle are present in Nepal which is the main source of traction and manure. In Nepal, cattle are raised only for the milk as they are worshipped as goddess Lachhmi; their slaughter for meat production is prohibited (Bhatta et al., 2018). Moreover, the annual growth of cattle is 0.1% only but the production of milk increased by 15.22% during 2020/2021. Buffaloes are the major producers of milk and meat as well as dung and draft force for improving soil fertility. Nepal has 5.15 million buffaloes where 1,630,642 are milikng buffaloes which produces 1,419,412 metric tons of milk during 2020/2021 (MoALD, 2021). Due to the expansion in cows and buffaloes of better breeds and higher production capacity than the native livestock, milk output has significantly increased in recent years. The dairy industry has been ranked as the second-most significant value chain commodity (MOF, 2022). Other livestock such as pigs, sheeps, goats, fowl and ducks are kept for the purpose of meat production. Chicken is highly popular for meat production as they have high nutritive and economic values. During 2020/2021, there is about 226,959 metric tons of chicken had produced for consumption. Primarily, goat was the second most major producer of meat that was about 534,956 metric tons during 2011/2012. The total meat production during 2020/2021 is 1,493,550 metric tons with the annual growth rate of -6.8%. The pig population is 1.58 million which produce about 31,450 metric tons of pork with the annual growth rate of 6.6% in the pork production during 2020/2021. The population of Sheep is 793,725. Sheep is also reared for production of milk but they are highly popular for the production of wool which produces about 584,000 kg of wools with annual growth rate of -0.95%/ during 2020/2021. The population of Duck is 0.43 million which produces about 17,930 eggs and 442 metric tons of meat during 2020/2021. Bagmati province has around 41.94 million livestock during 2020/2021 which is heighest as compared to other province (MoALD, 2021).

1.3 Livestock Disease

Livestock disease threatens local and national food security and lowers the income and quality of life of livestock keepers. Human health is also significantly impacted by animal disease, which accounts for 60% of infectious disease in human (Gizaw et al., 2020). Numerous diseases brought on by bacteria, viruses, fungi, and parasites to the livestock industry. Among parasitological issues, the harm brought on by TBBPs is regarded as particularly high (Ghosh

et al., 2007). International Organization of Animal health (IOE), has listed the fatal diseases of cattle which includes foot and mouth disease, vesicular stomatitis, rinderprest, contagious bovine pleuropneumonia, lumpy skin disease, rift valley fever, heartwater, hydatidosis, anthrax, bovine anaplasmosis, bovine babesiosis, bovine brucelliosis, cysticercosis, bovine tuberculosis, ezoonotic bovine leukosis, haemmorrhagic sepiticemia, theileriosis, trichomonas, trypanosomosis.

Endoparasitic illness has long been significant threat to the health of animal species. The blood vascular system is negatively impacted by blood parasites, which might include intraerythrocytic parasites and those that dwell freely (Phuyal et al., 2017). The prevalence and spread of their vectors are generally corelated with the presence of disease brought on by hemoparasites (Hamsho et al., 2015).

1.4 *Babesia*

Babesia is a tick borne hemoprotozoosis which affect wide varieties of domestic and wild mammalian vertebrates throughout the world (Jaimes-Duenez et al., 2018). The round, intraerythrocytic parasite of genus *Babesia* which was present in the blood of infected cattle was first described by Russian Researcher Victor Babes in 1888. There was huge threat of Babesiosis on economy because of reduced meat and beef production as well as death of animal at the time of Babes, Smith and Kilborne (Mosqueda et al., 2012).

Babesia mostly affects millions of cattle population in tropical, subtropical, and temperate regions of the world following the rainy and winter seasons, the summer season is when these diseases are most frequently recorded (Farooqi et al., 2017). Babesiosis has a significant economic impact on the cattle sector, but it can also infect other domestic animals like horses, sheep, goats, pigs, and dogs. About 1.2×10^6 cattle in the globe, 500 million of them are thought to be at danger of developing bovine babesiosis, which is one of the most significant tick-borne illness of cattle in the world (Chaudhry et al., 2010). The main tick vector of *Babesia* is various Ixodid tick *Boophilus spp., Rhipicephalus spp, Ixodes spp., Haemaphysalis spp., Dermacentor spp, Hyalomma spp* (Bock et al., 2004). Ticks are the most significant group of arthopods for the transmission of disease from one animal to another, and they are second only to mosquitoes globally as vectors of infectious diseases to humans (Ghosh

et al., 2007). Ticks affect both directly and indirectly to livestock. Direct infection of livestock includes blood and weight loss whereas indirectly it serve as vector for various infectious diseases (Sahara et al., 2019).

Till date about hundred plus species of *Babesia* have been identified and reported in animals and humans throughout the world. *B. bovis, B.orientalias, B.ovata, B. majori, B. occulatans, B. divergens, B. Jakiwiri,* and *B.veneturum* are the species which infect cattle. *B.bovis and B.bigemina* are the two most commonly found species in water buffalo and cattle. *B.orientalisis* is the only one species of *Babesia* know infecting Buffalo only whereas *B.bovis* and *B.bigemina* are known infecting the variety of bovid (He et al., 2021). According to reports, babesiosis brought on by *B. bovis* is more severe than babesiosis brought on by *B. bigemina*. If animals recover from infection, *Babesia* piroplasms are left in low numbers of erythrocytes for an extended period of time, creating a long-lasting carrier status. These animals play a critical part in the spread of the tick-borne illness (Rajabi et al., 2017).Through the established clinical findings of the disease, established finding include fever above 41^0C, anemia, depression, anorexia, dehydration, hemoglobinuria and loss of life of some affected animals. Nervous sign are generally discovered at result of sequestration of *B. bovis* infected erythrocytes in cerebral capillaries. Thrombocytopenia and leukopenia following babesial infection emphasize hematological abnormalities (Fonseca et al., 2017).All infectious illnesses spread by ticks have the potential to directly or indirectly alter hematological parameters (Thongsahuan et al., 2020). The constant infestation by ticks deranges the blood and worsens the vital blood parameters, such as the amount of hemoglobin, PCV, MCHC, MCV, TLC, and TEC. Hematological parameter is important for determining the causes of hematological illness as well as for determining the cause of several organ and systematic diseases. A CBC count's results are frequently useful for illness diagnosis, monitoring, and prognosis (Roland et al., 2014). Many methods for both indirect and direct diagnosis of the BBA complex have been established globally. Giemsa-stained thin blood smears can be used to directly look for parasites inside red blood cells, or parasite DNA can be amplified using the polymerase chain reaction for diagnosis (Souza et al., 2009).

Antibodies against the BBA complex agents are indirectly detected as part of the diagnosis (Amorim et al., 2009). Polymerase Chain Reaction is molecular diagnostic tool that has been

used in the detection and diagnosis of bovine babesiosis. PCR was used to amplify and sequence the 18S rRNA gene of *B.bovis*. The result of the study showed that the 18S rRNA gene is a useful target for the molecular characterization of *B.bovis* (Hira et al., 2005). Although apicomplexan parasite infect many hosts and cell types, members of the Apicomplexan have comparable host invasion methods (Gaffar et al., 2004). The secretary organelles; Microneme and Rhoptries are the part of the complicated apical complex of the invasive stages of the Apicomplexa. The invasion of the host cell and the formation of the PV are the both crucially dependent on these secretary organelles (Solidati et al., 2001).

The apical complex of the parasites releases variety of chemicals during the invasion process, including Associated-membrane-antigen-1 (AMA-1), Rhoptry-Associated-Protein-1 (RAP-1), Thrombospondin-Related-Anonymous-Protein (TRAP), and Spherical Body Protein (SBPs) during cell entry, intracellular development and escape from the cell. It has been demonstrated that use of released chemicals from the parasite's apical complex as a medication target and tool for the development of diagnostic procedures which is highly effective (Guswanto et al., 2017; Niu et al., 2015).

1.5 Statement of Problem

Babesiosis is responsible for severe economic losses in dairy cattle industry due to incurring cost for parasite control. Little is known regarding the transmission of dangerous pathogens and parasites carried by ticks, such as *Babesia*, despite the broad distribution of several tick species across various areas of Nepal. Despite their crucial role in disease transmission, sufficient study on these vector has not been done, and none has been able to examine the relationship between the presence of particular tick species and disease causing agents. In absence of inappropriate control strategies these tick-borne blood parasitic diseases have serious economic impact due to high mortality rate, and reduction in production of milk. The research regarding babesiosis is not well documented from Nepal. In order to address the above problems, a study is required to fill the gaps in knowledge about bovine babesiosis and its vectors, as well as to create baseline information that can be used to develop efficient disease control and prevention methods.

1.6 Rationale of study

Many researchers concluded that *Babesia* parasites cause great economic loss to the dairy industry. The prevalence studies are of great aid to boost productivity by timely identification and treatment of potential risk of babesiosis. Moreover, little information is available about piroplasmosis and their effects in affected animals in Nepal as a whole. Furthermore, the present study will provide baseline data regarding risk factor associated with *Babesia* parasite infection and the potential effect of the parasite on the selected hematological parameters of livestock of Nepal.

1.7 Objective of the study

1.7.1 General objectives

- To determine the prevalence of *Babesia* spp. in suspected cattle of Kathmandu Valley, Nepal.

1.7.2 Specific Objectives

- To determine the prevalence of *Babesia* by microscopy and nPCR.
- To differentiate the various species of *Babesia* in suspected cattle through sequencing.
- To study the alteration on hematological parameters in *Babesia* infected cattle.

1.8 Significance of the study

Animal health is important factor in the economic aspects of livestock production in Nepal. Many animals perish or recover after lengthy period of time, resulting in a loss of meat and milk output. The mortality rate can be decreased with early diagnosis and good treatment. The detection of *Babesia* by molecular technique is very useful which can detect the invading species in the blood of cattle even before the appearance of clinical sign in cattle. Therefore, this study might be helpful for the early diagnosis and treatment of the disease as well as understanding the prevalence of *Babesia* and the harm caused by it among Cattle of Kathmandu Valley, Nepal. It will also guide future Researcher when babesiosis management initiatives are implemented in the research area.

2. LITERATURE REVIEW

2.1 Etiology of Babesiosis

Babesiosis is a parasitic infection caused by hemotropic protozoa of the genus *Babesia*, belonging to the family Babesidae, order Ixoplasmida, class Piroplasmea, phylum Apicomplexa, subkingdom Alveolata, and kingdom Protozoa. Due to their pear-shaped appearance while replicating in erythrocytes, these parasites are also known as Piroplasms (Azagi et al., 2021). Redwater Fever, Cattle Tick Fever, Spanish Fever, or Texas Cow Fever are some of the common names for this illness (Florin-Christensen et al., 2014). Intraerythrocytic *Babesia* species are oval, round, or pear-shaped in the host and range in size from 1 to 5 μm in length (Boustani & Gelfand, 1995). *Babesia* parasites (as well as those of the closely related genus *Theileria*) are among the most pervasive and prevalent blood parasites of the world, second only to *Trypanosomes*, and as a result, they have a significant global economic, medical, and veterinary effect (Homer et al., 2000). Mainly two etiological agents of *Babesia* i.e. *B. bigemina* and *B. bovis* are primarily responsible for bovine babesiosis in the tropics and subtropics (Rajabi et al., 2017). *B.ovata, B. majori, B. occulatans, B. divergens, B. Jakiwiri,* and *B.veneturum* are the additional species which infect cattle (He et al., 2021). These most pathogenic species, *B. bovis* and *B. bigemina*, are spread by the globally dispersed tick species *Rhipicephalus microplus* (Hassan et al., 2018). According to reports, babesiosis brought on by *B. bovis* is more severe than babesiosis brought on by *B. bigemina* (Rajabi et al., 2017). Babesiosis primarily affects millions of Cattle population in tropical, subtropical and temperate part of the world (Farooqui et al., 2017).

2.2 History of *Babesia*

Babesia species are well-known animal infections, and during the past thirty years, they have also come to be understood as sporadic human pathogens. The first known outbreak of babesiosis, often known as the Plague or Divine Murrain that struck the livestock of the Pharaoh Ramses II, most likely took place in biblical times (Boustani & Gelfand, 1995). Babes claimed that intraerythrocytic "Bacteria" was reason for the fever hemoglobinuria-related deaths of 30,000–50,000 head of Romanian cattle in 1888 (Babes, 1888). The protozoan known

as *Babesia* was found responsible for Texas Cattle Fever in 1893 by Smith and Kilborne which was spread by blood-sucking ticks (Smith and Kilbourne, 1893). Yang and Wang originally discovered and cataloged *B.bovis* and *B.bigemina* in Guizhou province, China in 1964 (He et al., 2021). Bovine babesiosis has been coined to characterize *Babesia* infections in Cattle since the genus *Babesia* was established (Florin-Christensen et al., 2014).

2.3 Transmission and life cycle:

Babesia is primarily transmitted through the bite of an infected tick as well as transovarially and, less frequently, through blood transfusions (Suarez et al., 2019; He et al., 2017). The tick vector *Rhipicephalus microplus* was identified as the vector of *Babesia. R. microplus* larvae, nymphs, and adults can all transmit *B. bigemina*, but only the tick larval stage can transmit *B. bovis* (He et al., 2021).

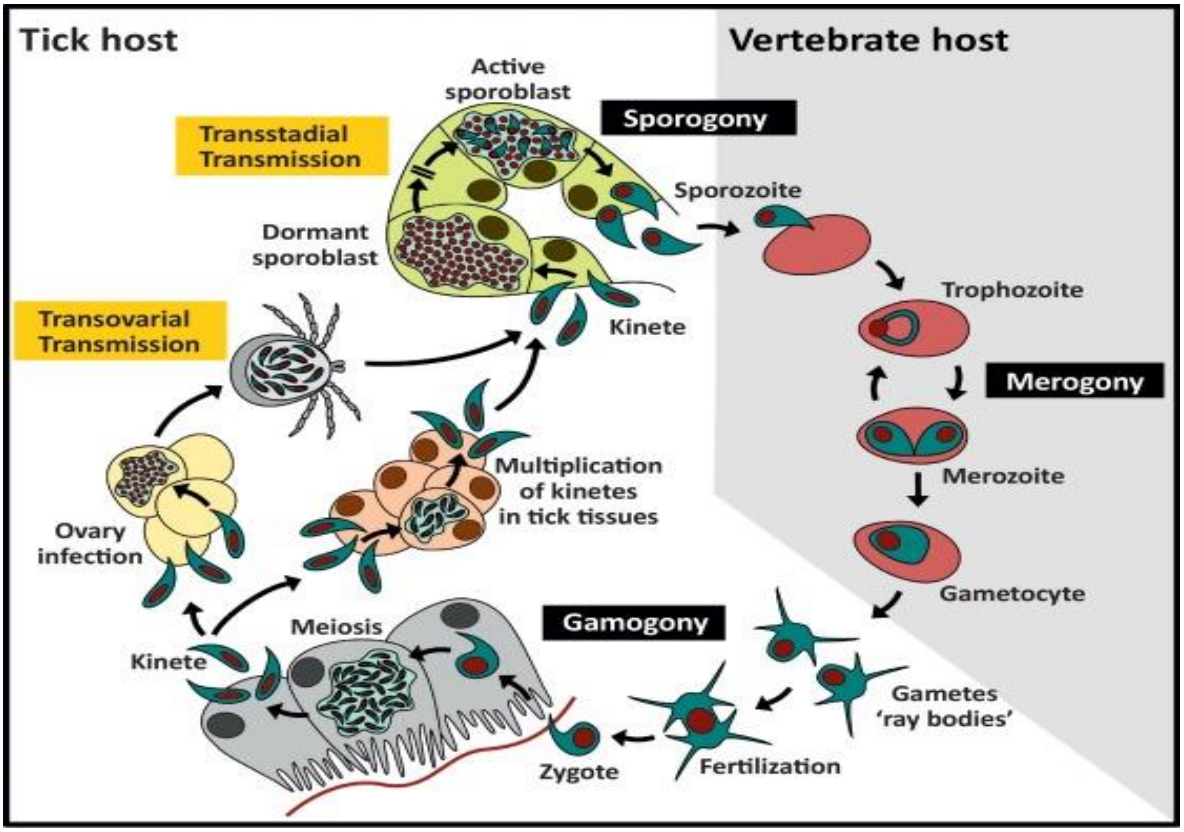


Figure-1 Life cycle of *Babesia*

(Source:https://www.google.com/search?q=babesia+life+cycle&sxsrf=APwXEdfXhZfbdCdChw_mXhqwRecFxV9u3A:1685020959476&source=lnms&tbm=isch&sa=X&ved=2ahUKEwit4emdyJD_AhVu8zgGHdCuBIUQ_AUoAXoECAEQAw&biw=1034&bih=620&dpr=1#imgrc=gjkgysChaegKuM)

B. bigemina is transmited by *Boophilus microplus*, *B. decoloratus*, *B. annulatus*, *B.geigyi*, *Rhipicephalus evertsi. Boophilus microplus*, *Boophilus annulatus*, *Boophilus geigyi* transmit *B. bovis. Ixodes ricinus*, *Ixodes persulcatus* transmits *B. divergens* while *Haemaphysalis punctata Haemaphysalis longicornis* transmits *B.major* and *B. ovata* respectively (Bock, 2004).

Babesia has two stages in its life cycle: one occurs inside the host RBCs, when the Sporozoites transform into piroplasms, and the other takes place inside the tick vector (Salem and Farag, 2014). The *Babesia* life cycle includes the three sequential stages of Merogony, Gamogony, and Sporogony, which correspond to the archetypal developmental pattern of Apicomplexa members. During tick bite, the infectious sporozoite infects the vertebrate host. Sporozoites enter the host's red blood cells (RBCs), where they transform into trophozoites and split into merozoites through Merogony (Yokoyama, 2006). Later egress, reinfecting more host erythrocytes. Merozoites, sexually commit to intraerythrocytic gametocytes to begin gamogony (sexual reproduction) in ticks. The ookinete enters the tick gut cells through the peritrophic matrix. The zygote proceeds through meiotic division once it is inside the epithelium, producing kinetes. Kinetes spread to peripheral tick tissues, such as ovarian cells, by the bodily fluid of the tick (haemolymph). *Babesia* transovarial transmission arises from the invasion of tick ovaries, which infects the eggs. Parallel invasions by primary kinetes result in secondary kinetes that infiltrate tick salivary glands via schizogony (multiple fission) and other tick organs. Invading kinetes transform into a multinucleated syncytium (sporoblast) inside salivary gland cells, where it remains latent during the tick ecdysis to ensure transracial transmission of the parasite. The sporoblast is triggered as soon as the next infected tick instar begins blood-feeding on a susceptible host, and many infectious sporozoites are continually generated and disseminated into the host circulation through the process of sporogony (Jaloveka et al., 2019)

2.4 Pathogenesis

Babesia parasite contains RBC invading organs known as apical complex. Within the RBCs of their infected vertebrate hosts, *Babesia* spp. invade and multiply asexually by binary fission (Suarez et al., 2015). Pathogenesis of parasites in red blood cells is caused by asexual

reproduction. The invasion of parasite sporozoites from the salivary glands of the tick sets off the infection, which is then followed by parasite development, DNA replication, parasite multiplication, and eventually parasite egress from RBC. This cycle keeps on because the egressed merozoites infect fresh RBCs. These alterations are probably caused by stage-specific gene expression in the parasite (Hakimi et al., 2021).

The two species that affect Cattle the most frequently, *B. bovis* and *B. bigemina*, are typically found as co-infections in field animals. Contrary to *B. bovis* infection, which causes significant parasitaemia and a more severe illness than *B. bigemina*, *B. bigemina* infection is often characterized by a low degree of parasitaemia (Niu et al., 2015). *B. bovis* infections are frequently acute or subacute and have a shorter time course, with more severe neurologic symptoms quickly leading to death or a slow pace of recovery in situations when it is not deadly (Githaka et al., 2022).

2.5 Epidemiology and Geographical Distribution

In tropical and sub-tropical areas, such as Asia, Africa, Australia, and the America, *Babesia bovis* and *B. bigemina* are mostly the main reason for the disease in cattle (Prado et al., 2022) as they affect millions of cattle population causing decline in economic sector (Terkawi et al., 2011). The distribution of *Babesia* parasite depends on the presence of Ixodid tick species, which are known to spread these parasites amongst the Bovine populations (Farooqui et al., 2017). The *B. bovis* infection is frequently more severe and causes higher mortality in susceptible cattle. This is most likely because of the ability of parasitized erythrocytes to sequester in the kidney, lungs, and brain's micro-capillaries causing organ failure and systemic shock, which ultimately cause death (Terkawi et al., 2011). The important *Babesia* species *B. bovis, B. bigemina, B. orientalis,* and *B. ovata* are known to infect cattle throughout Asia (Galon et al., 2019).

2.5.1. Babesia in Global context

Babesia has been reported from different country of the world. Large number of study has been done in different parts of Asia such as Bangladesh (Roy & Majumder, 2019), Thailand (Terkawi et al., 2011), Philippines (Herrera et al., 2017), China (He et al., 2021) and so on. The research conducted in Northwestern region of Thailand revels the high prevalence of

Babesia parasites in the livestock of these areas. The prevalence of two species of *Babesia* i.e *B.bovis* and, *B. bigemina* were 11.2% and 3.6% by nPCR, 14.7% and 5.9% by ELISA, and 16.8% and 5.6% by IFAT, respectively (Terkawi et al., 2011). The cross sectional study that was performed in different areas of Bangladesh has shown 1.04% prevalence of *B. bigemina* and 0.52% of *B. bovis* (Roy & Majumder, 2019).

The south America also highlights the high prevalence of *Babesia* and indicate that *B.bovis* and *B. bigemina* as the major hindrance of milk and beef production in livestock of Columbia through the molecular study (Jaimes-Duenez et al., 2018). The molecular study in South Africa shows the highest prevalence of *Babesia* where *B. begemina* and *B. bovis* where infection rate was 64.7% and 35.1% respectively by nested PCR (Mtshali & Mtshali, 2014). In Portugal, a very first molecular study regarding babesiosis has shown the higher incidence of two species of *Babesia* i.e. *B.bovis* and *B.bigemina* with infection rate of 71% and 34% respectively by nPCR (Silva et al., 2009).

2.5.2. Babesia in context of Nepal:

According to the research conducted in the Terai belt of Nepal concluded the tick as a vector transmits diseases like babesiosis and anaplasmosis which is causing detrimental impact on cattle health and production (Lamichhane & Basnet, 2020). The molecular study on piroplasmosis and anaplamosis by (Shrestha, 2017) reported the prevalence rate of *Babesia* 0.64% by PCR in Banke and Surkhet of Mid-western Nepal. Only a few number of the research in erythrocytic parasites including *Babesia* through microscopy has been conducted in different animals in Nepal. However, No any study has conducted on bovine babesiosis through molecular method like PCR which is known as highly specific method for the detection of *Babesia* parasite. This shows the knowledge gap in research.

2.6 Clinical findings:

The incubation period, which is the duration of time between exposure to a pathogenic organism and the onset of the first symptoms, is dependent on the parasite load and can last anywhere between 7 and 21 days (Karasova et al., 2022). The indications and symptoms of bovine babesiosis vary based on the afflicted species, age, local climate, tick population, and host immune response. Bovine babesiosis is a highly pathogenic disease (Neamat-allah &

Hashem, 2018). The age and cleanliness of the host have an impact on the severity of the damage as well (Salem and Farag, 2014). Depending on how the host's immune system responds, the disease can manifest in a variety of ways, from a preclinical, asymptomatic infection to a life-threatening one (Akel & Mobarakai, 2019).

Babesia species can induce a variety of clinical symptoms since each specie strain's varies in their virulence and pathogenicity (Canto et al., 2006). Pale mucosa and jaundice, hemoglobinuria, fever, hemolytic anemia, depression, decreased appetite, ruminal stasis, prostration, lachrymation, increased salivation, and in extreme instances, mortality are all symptoms of this infection. Fever, significant anemia, and hemoglobinuria lasting up to 3 weeks are among the symptoms of infected animal's clinical presentations, which also include modified hematological and serum profiles. In extreme circumstances, death might happen 24 hours after the infection (Siddique et al., 2020). *B. bovis* infections always result in an acute syndrome, albeit the degree of the illness might vary. *B. bovis* infection causes a strong immune response, but it does not get rid of the parasite, leaving a chronic infection that persists (Canto et al., 2006).

2.7 Diagnosis:

The observation of clinical indicators in combination with laboratory testing is typically used to make the diagnosis of illnesses transmitted by ticks. Bovine babesiosis may be diagnosed using a variety of methods, but often a thin or thick blood Giemsa-stained smear is used to microscopically show the presence of parasites as the cause of the clinical symptoms (Alvarez et al., 2019). The classical microscopic examination of *Babesia* piroplasms in Giemsa stained thin blood smear is a gold standard test that is relatively cheap and quick method; however, in chronic infection, it has low sensitivity and usually fails to detect carrier animals (Bal et al., 2016). However, it is difficult to discriminate between pathogenic and non-pathogenic species that coexist often when piroplasms are seen under the microscope (Farooqui et al., 2017)

Serological assays are used to detect circulating antibodies of *Babesia* parasites (Farooqui et al., 2017). *Babesia*-specific antibodies can be found in serum samples using serological assays i.e. enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence assay (IFAT). The IFAT is now recognized as the "gold standard" test for serological diagnosis of

bovine babesiosis and is the test that sero-epidemiological investigations employ the most frequently. Recent studies have shown that fast immunochromatography tests (ICTs) are useful for identifying specific antibodies against *B. bovis or B. bigemina* in cattle (Lira-amaya et al., 2021). These techniques are sometimes referred to be time-consuming, difficult-to-perform diagnostic tests that lack specificity. Cross-reactions and a lack of distinction between earlier exposure and present illness are further drawbacks. PCR identifies the presence or absence of short DNA sequence and amplify them to make millions of copies. The PCR-DNA technique, when combined with non-radioactive DNA probe, was sensitive enough to detect Cattle that had been identified with *B. bigemina* for a long time. Analytical sensitivity experiments revealed that as little as 100 fg of parasite genomic DNA, equivalent to 0.0000001% of infected erythrocytes, could be detected (Alvarez et al., 2019).

Current infections can be discovered using PCR, which is specific. Another benefit of PCR is that it has been shown to be sensitive to between one and three infected erythrocytes. When nested PCR is used, the sensitivity of a PCR experiment can be boosted by several fold (Abdel-Shafy et al., 2022).

2.7.1 PCR

It is an indirect method of finding parasites is by identifying nucleic acid. With the development of molecular genetics and genomics, particularly PCR-based technologies, it is now possible to determine genetic diversity along the genome of numerous pathogens (Schnittger et al., 2012). However, these molecular techniques have very high sensitivity and specificity, and during the past year, a variety of molecular methods have been developed to identify *Babesia* spp. in their host and vectors (Mosqueda et al., 2012). The majority of diagnostic assays used in hematozooan research are based on final time PCR and other comparable techniques. The small ribosomal subunit is clearly one of the most widely used genes in PCR experiments, however other genes (membrane proteins, cytochrome b) have also been used. The number of copies of the genes used in amplification methods has a significant impact on molecular diagnostic sensitivity. It is commonly known that PCR performance can be improved by selecting highly repetitive components, but this is often limited because the number of gene sequences available in database is rather small (Criado-Fornelio et al., 2007).

2.7.2 Sequencing

Babesia species and strains have been studied using whole genome sequence. Comparing the genomes of two *B. micorti* strains from the United States and Europe using whole genome sequencing the researchers discovered genetic differences across the isolates, such as variances in virulence factors and metabolic pathways, which could be utilized to create new diagnostic tools and treatments in the United States and Europe (Cornillot et al., 2012).

Babesia has also been studied using transcriptome sequencing. The researchers used transcriptome sequencing to discover differently expressed genes in *B. bovis* during its life cycle. The study discovered many genes that could be used to generate novel therapies or vaccinations (Suarez et al., 2017).

Babesia has been identified in complicated samples, such as blood samples from sick people, using metagenomics sequencing. The metagenomics sequencing to identify *Babesia* species in blood samples from feverish patients in the United Kingdom. The study discovered several *Babesia* species, emphasizing the need for improved diagnostic methods and surveillance in locations where *Babesia* is not typically considered endemic (Chiodini et al., 2019). While sequencing has provided important insights into *Babesia* genetics and pathogenesis, there are still several gaps in our knowledge.

2.8 Importance of Babesiosis:

Babesiosis is a blood-borne illness which is economically important that affects free-living animals and is seen as a developing zoonosis in humans. Sometimes it presents without any traditional clinical symptoms. The subclinical infection persists for several months or years in the recovered animals, who continue to act as carriers (Barman et al., 2018). The economic security of the nation is significantly impacted by these illnesses, which also have a detrimental negative effect on the communities that depend on livestock production for food (meat and milk), revenue, and labor for fieldwork and transportation (Mtshali & Mtshali, 2014). As a

consequence, this disease results in reduced production, increased mortality, and the expense of treating ill animals leading to economic losses (Prado et al., 2014).

3. MATERIALS AND METHODS

3.1. Study Area

Nepal is landlocked country bordered by India to the east, west and south and China to the north. Nepal is located between 26 and 30 degrees north latitude and 80 and 88 degrees east longitude has a total area of 147,181 square km and stretches roughly 145-241 km north to south and 850 km west to east. Nepal covers 0.1% of earth's land surface and is topographically divided into three regions: Mountain, Hill and Terai. The total human population of Nepal is 30.44 million where about 66% people of the country are engaged in agriculture.

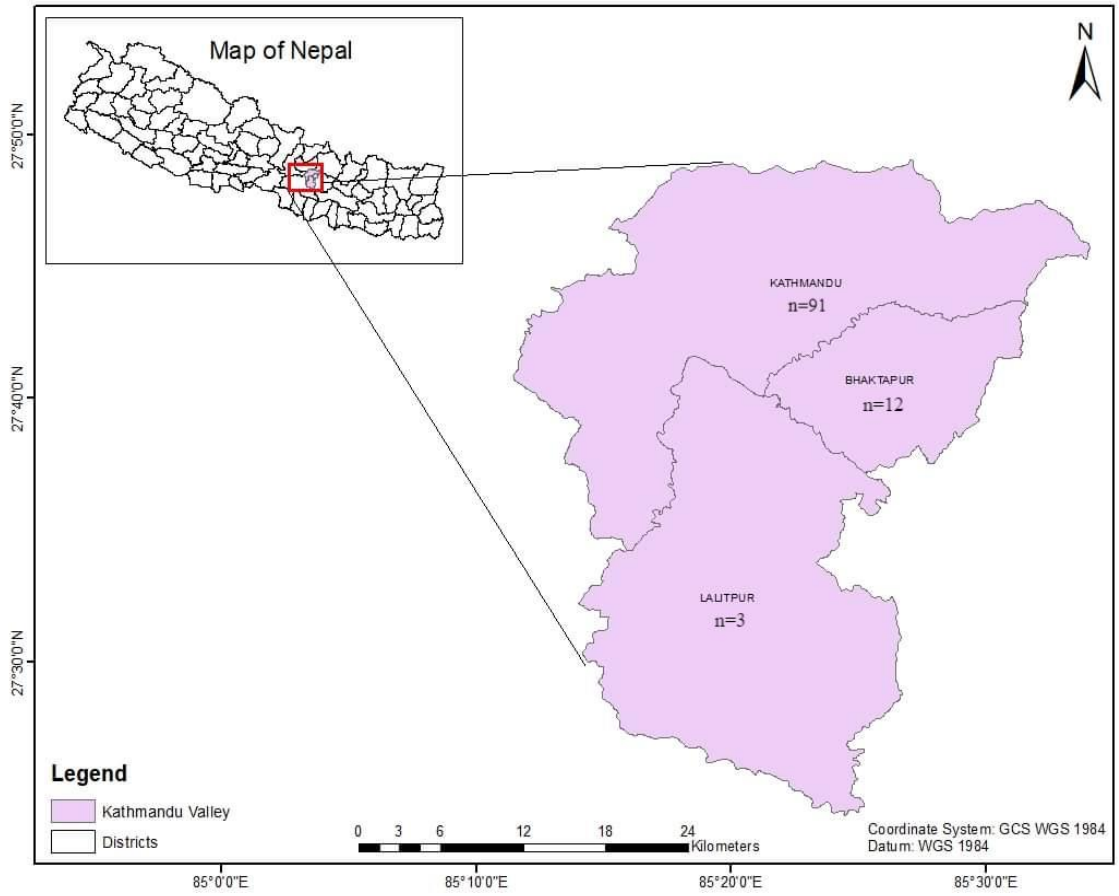


Figure 2: Map of Study Area

The Kathmandu Valley is located in Bagmati province of Nepal which is surrounded by four mountain ranges: Shivapuri hills, Phulchoki, Nagarjun and Chandragiri. The valley is made up of three districts: Kathmandu district, Lalitpur district and Bhaktapur district. It is situated between 27°32'13" and 27°49'10" N latitude and 85°11'31" and 85°31'38" E longitude. The valley is 665 square kilometers in size and is located on average 1350 meters above mean sea level.

The Kathmandu Valley is located in Nepal's central hill where temperature varies from 35^0C to 3^0C. Due to the favorable condition for tick, the prevalence of tick borne disease including *Babesia* has been expected in Kathmandu valley. In this study, blood samples from 100 cattle were collected from different areas of three districts of Kathmandu Valley, whereas the samples were collected from different age group and breeds of cattle.

According to MoALD, (2022), The population of livestock in Kathmandu Valley is 7,932,035. Among them Cattle 104,159, Buffalo 105,993,Goat165,129, Sheep 4,545, Pig 64,957, Fowl 7,438,028 and Duck 49,224. The district profile report of three districts of Kathmandu revealed that the production of milk 69,609 metric tons.

3.2 Research Design

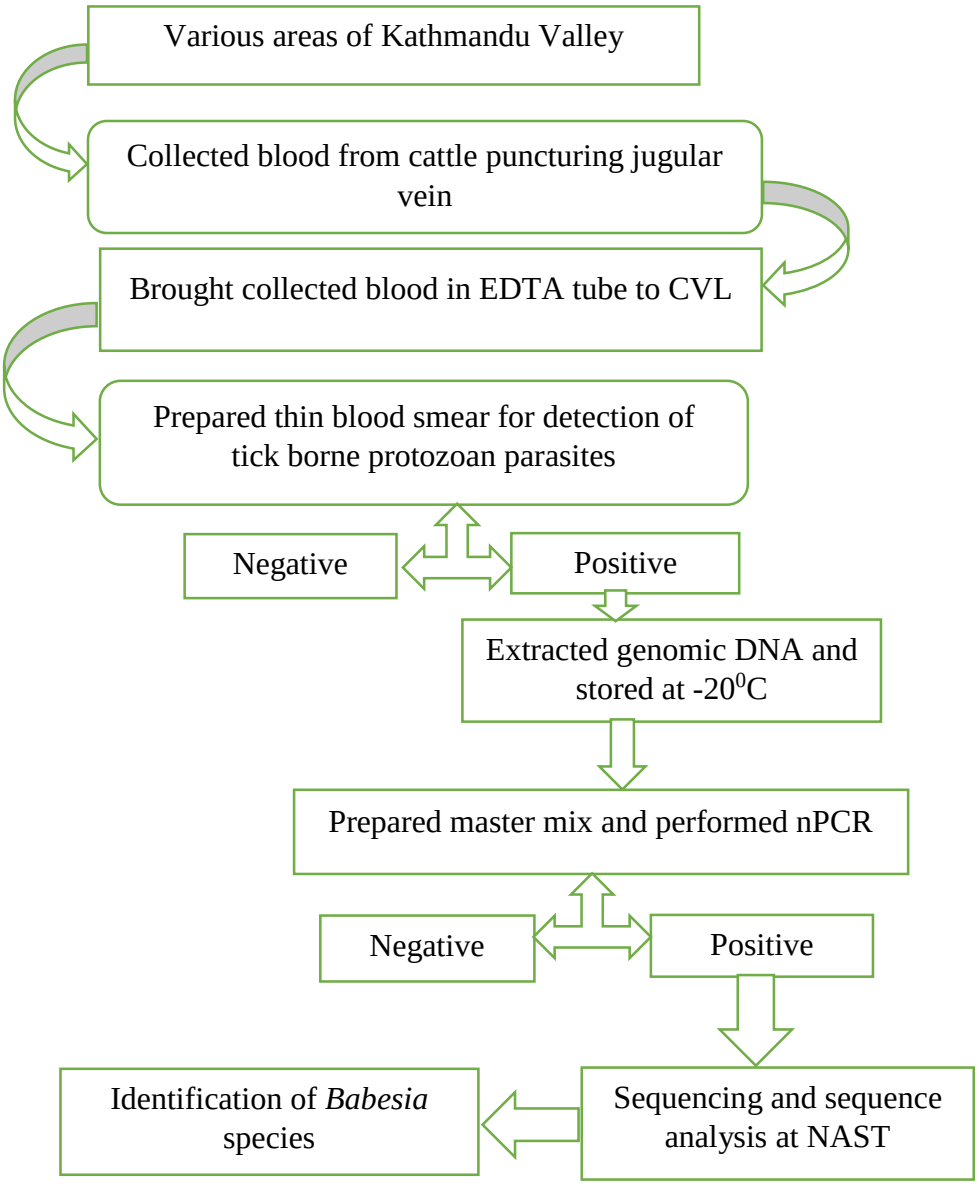


Figure 3: Research design of the study

3.3 Blood Sampling

This was a Hospital based study and all the blood samples used for the thesis work had been received from **Central Veterinary Laboratory, Tripureshwor, Kathmandu** between March to November, 2022. A total 106 blood samples from suspected female Cattle were collected from three districts of Kathmandu Valley Kathmandu (n=91); Lalitpur (n=3); Bhaktapur (n=12). The samples were collected during summer and rainy season followed by March (n=9), April (n=16), May (n=28), August (n=17), September (n=12), October (n=11), November (n=13). Blood samples were collected by puncturing Jugular veins of Livestock and brought to **Central Veterinary Laboratory, Tripureshwor, Kathmandu** in EDTA tubes for smear preparation, staining and microscopic examination. The Molecular method (PCR) for confirmation of *Babesia* was conducted at the **Molecular Laboratory of Central Department of Zoology, Kirtipur, Kathmandu.**

3.4 Laboratory Analysis

3.4.1 Preparation of thin smear

The glass slide was cleaned prior for the preparation of the thin smear. At one end of the dry, spotless slide, a little drop of blood was inserted. To create a smear with a tongue shape, a spreader slide inclined at a 45^0 was pushed along the horizontal from one end to the other. The prepared smear was quickly air-dried and then fixed in methanol for 3-5 minutes**.**

3.4.2 Giemsa's stain solution preparation

Giemsa's stain preparation was created by dissolving one volume of Giemsa's standard solution in nine volumes of phosphate-buffer water at P^H 7.2. Filtered, stored, and diluted with distilled water, the solution was kept in an amber-colored container with a stopper. Buffering water with 3.0 gm. 1-1 Na_2HPO_4 and 0.6 gm. 1-1 KH_2PO_4 controls the p^H of the solution.

3.4.3 Microscopic Examination

With the use of immersion oil, the stained slides were viewed at high magnification (10x by 100x). Looking from the end of the slides to the entire field, concentrating on the parasites for the photo. Depending on the stage of growth of the parasite in erythrocytes, *Babesia* has a pear-shaped, paired, round, oval, or irregular shape and located in the center of erythrocytes.

Theileria forms n red blood cells are primarily rod-shaped, measuring 1.5-2µm by 0.5-1µm; however, round, oval, comma, and ring-shaped forms may also be seen in erythrocytes (Soulsby, 2012).

3.5 PCR

3.5.1 DNA Extraction

The collected blood samples were stored at -20^{0}C until DNA extraction. The blood samples were extracted using QIAamp$^@$ DNA Blood Mini Kit (QUIGEN, Germany).

About 200µl of collected blood was mixed with 20 µl Proteinase K adjusting volume to 220 µl. In that mixture 200µl Buffer AL was mixed thoroughly vortexing and incubated at 56^{0}C for 10 min. Then 200µl ethanol was added to the sample and mixed thoroughly by vortexing. The prepared mixture were then pipette to the DNeasy Mini spin column placing in 2ml collection tube and then centrifuged at 8000 rpm for 1 min.

500µl of Buffer AW1 was added and the DNeasy Mini spin column to a fresh 2 ml collecting tube before centrifuging at 8000 rpm for 1 minute. The collecting tube and flow through was discarded. Similarly, 500µl of Buffer AW2 was added and the DNeasy Mini spin column to a fresh 2 ml collection tube before centrifuging at 14000 rpm for three minutes. The collecting tube and flow through was discarded.

After that the DNase Mini spin column was placed in clean 2ml microcentrifuge tube and 200µl Buffer AE was added and the mixture was then incubated at room temp for 5 minutes and then centrifuged for 1 minute at 8000rpm. The isolated genomic DNA was then stored in -20^{0}C for further use.

3.5.2 nPCR assays

Among the total collected blood samples, only those blood samples which were TBBPs positive through microscopy were further used for PCR. The TBBPs positive samples were analyzed by nPCR with primers provided by Masahito Asada from Japan. The 18S ribosomal RNA gene of the parasite was amplified by PCR to detect *Babesia*, *Theileria*, and *Hepatozoon* parasites utilizing BTH 18S-1 as the primary amplification and it was followed by nPCR using

BTH 18S-2 primer for secondary amplification. The details of primer mentioned above are given below in (Table1):

Table 1: Primers used for PCR amplification

	Name	Sequencing	Tm	Size
Primary	BTH 18S-1F	GTGAAACTGCGAATGGCTCATTAC	55	1500
	BTH 18S-1R	AAGTGATAAGGTTCACACAAAACTTCCC		
Secondary	BTH 18S-2F	GGCTCATTACAACAGTTATAGTTTATTTG		
	BTH 18S-2R	CGGTCCGAATAATTCACCGGAT		

3.5.3 Preparation of master mix for PCR

A 25µl mixture containing 12.5 µl Taq (Emerald AMP max X PCR master mix), 0.2µl of each primer (BTH 18S-1F and BTH 18S-1R), 1µl of template DNA, and the rest nuclease free water was used to prepare master mix for first PCR. The first PCR products were then diluted 5 times (1 µl PCR product + 4 µl nuclease free water) and then used for nPCR.

For preparation of master mix for nPCR, same volume of constitute as first round PCR were used but as DNA template dilute first PCR product were used.

Table 2: Constitute and volume of master mix for PCR

Constitute	**Volume**	**Final concentration**
Taq (Emerald AMP max X PCR master mix)	12.5 µl	
Primer(F)	0.2 µl	0.2 µM
Primer (R)	0.2 µl	0.2 µM
DNA template	1µl	<500 ng
Nuclease free water	11.1 µl	Up to 25 µl

Each of the above volume was for a single sample and these values should be multiplied from the sample number and additional one to minimize the possible pipetting error.

3.5.4 Thermal Cycler Condition of first and nPCR

Thermo cyclic Conditions applied during the course of PCR is given below in the (Table 3). The PCR was performed in "MyGene^{TM} L Series Peltier Thermal Cycler".

Table 3: Thermal cycler condition of first and nPCR

Primers	BTH 18S-1F, 1R/BTH 18-2F &2R		No of cycles
	Temperature (^{0}C)	Time (min)	
Initiation	95	3	1
Denaturation	95	0.5	40
Annealing	55	0.5	
Extension	68	1.5	
Final extension	68	5	1

3.5.5 Gel Electrophoresis:

The amplified DNA product was separated by gel electrophoresis on 1% agarose gel. For this, 0.25 gm agarose powder was mixed with 25ml of diluted TBE buffer and heated in oven until the powder dissolved completely in the buffer. The gel was left to cool down at room temperature and 4µl of Ethidium Bromide was added shaking the mixture vigorously. The gel was then transferred into the casting tray, inserted the comb and left to set the gel completely into the casting tray for about 30 minutes. At the last the comb was removed after solidification and then the casting tray was placed properly into the buffer tank filled with TBE buffer.

3.5.6 Sample loading and running the gel

About 5µl of PCR product was loaded carefully with the pipette into the wells allowing the gel to run for 45 minutes at 80 Volts. To check the result the Clear View UV Transilluminator was used for the detection of DNA bands.

3.6 Sequencing

To further confirm and validate the nPCR results, randomly selected positive 9 samples were sequenced. The sequencing was performed in **National Academy of Science and Technology**

(NAST), Lalitpur by Dr. Deegendra Khadka and Dr. Ram Chandra Poudel. For sequencing, the second PCR products' contaminating primers were eliminated using 2 µl of Sap-Exo (Jena Bioscience, Jena, Germany) for 5 µl of PCR product, which was then incubated in a thermocycler at 37^0C for 10 min and 80^0C for 10 min. Following the manufacturer's instructions, the purified products were used in a sequencing reaction with a BigDyeTM Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA) utilizing BTH 18S primers. These sequencing reactions were purified using an Applied Biosystems Big Dye XterminatorTM Kit before being analyzed by an automatic 3500XL Genetic Analyzer (Applied Biosystems). Using Sequencer 5.0 (Thompson et al., 1997), raw DNA sequence data were assembled, aligned, and DNA contigs were constructed utilizing forward and reverse sequences.

3.7 Sequence editing and Alignment

The raw sequencing data obtained from sequencing platform trimmed their end using a software program (MEGA V7) checking ambiguities or error. The accuracy of the edited sequence was verified comparing it to known reference sequence. The aligned sequence was obtained from database. The FASTA was chosen selecting the appropriate parameters for alignment. The alignment program was run to generate the pairwise sequence alignment. The alignment was visualize using software.

3.8 NCBI BLAST

The NCBI BLAST webpage were accessed and the sequence was entered against the desired sequence. The desired database were choosen by selecting the option "Choose Search Set" section. Then the appropriate BLAST programme for piroplasma sequence were search and selected. The parameters of search were setted. And after submission of all the necessary information "BLAST" button were clicked. The similarity between the obtained sequence and the hits in database were analyzed. The homology between two sequence were analyzed to differentiat species.

3.9 Hematological analysis

The sample collected in EDTA tubes were subjected for the estimation of hemoglobin concentration (Hb), total Red blood cell (RBCs) counts and total White blood Cells (WBCs) count in this study using Sahli's haemoglobinometer at Hematology laboratory of CVL, Tripureshwor.

3.10 Statistical Analysis

The obtained data were first recorded in notebook and then managed in Microsoft Excel spreadsheet and then later transferred to SPSS (Statistical Package for the Social Sciences) software. The prevalence was calculated as a proportion of infected animals out of the total animal examined. Chi-square test was used for testing of significance, the confidence level (CL) for all analyses was set at 95%, while the significance level was set at $P \leq 0.05$. The analyzed data was represented in tabulated form with graph.

3.11 Ethical Clearance

The ethical approval for this research was obtained from Ethical Clearance Committee of Nepal Veterinary Council (Ref No: 236/2078.79).

4. RESULTS

4.1 Microscopic examination of tick borne protozoan parasites

Out of 106 blood samples of suspected female domestic Cattle from different areas of Kathmandu Valley examined using blood smear examination revealed 42.45% (45) prevalence of tick-borne protozoan parasites. During the microscopic examination of blood smear two genus of tick-borne protozoan parasites were detected which was *Babeisia* and *Theileria*. Few samples were also found positive for *Anaplasma* which is a Bacteria and it does not lie under the category of Blood borne protozoan parasites, so that it didn't include under this study. During the microscopic examination prevalence of *Babesia* parasite was found maximum with the prevalence rate of 39.6% (42/106) whereas the prevalence rate of *Theileria* was found to be 2.8% (3/106) which is lower than *Babesia*.

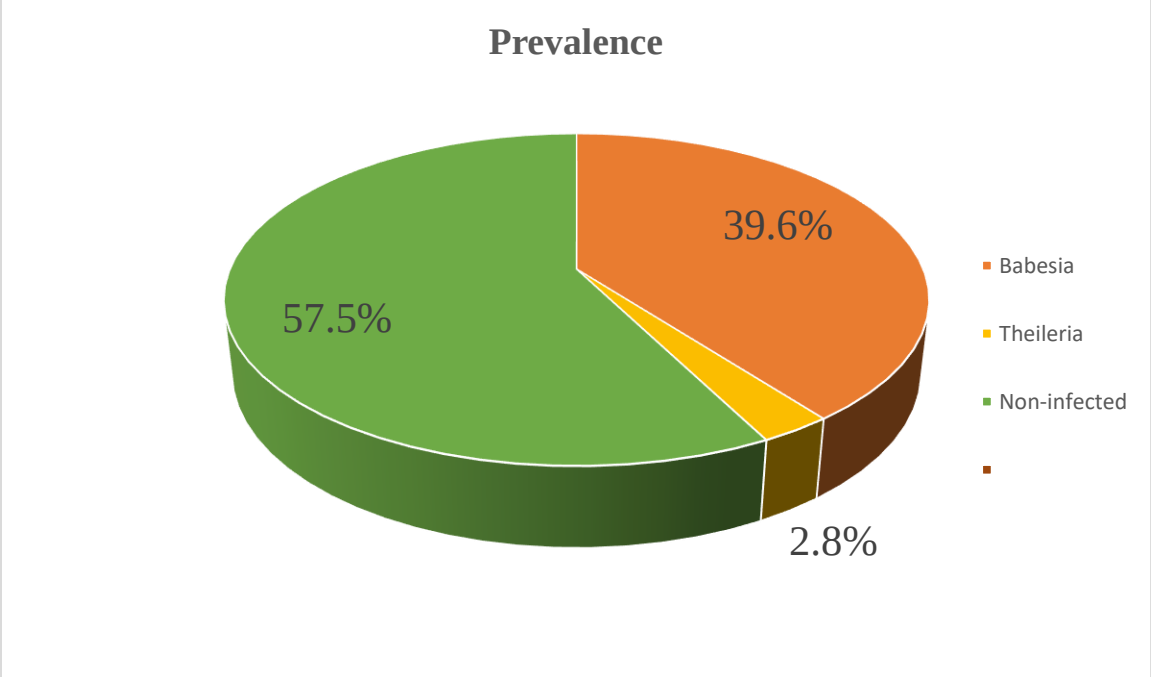


Figure 4: Prevalence of tick borne protozoan parasites in suspected cattle

According to microscopy, the species of *Theileria* was detected only from Kathmandu district with the prevalence rate of 2.8% while remaining two districts of Kathmandu valley did not report the presence of *Theileria*. The Kathmandu districts alone reports 35.8% (38/106) of TBPPs where prevalence rate of *Babesia* and *Theileria* were 30% (35/106) and 2.8% (3/106) respectively.

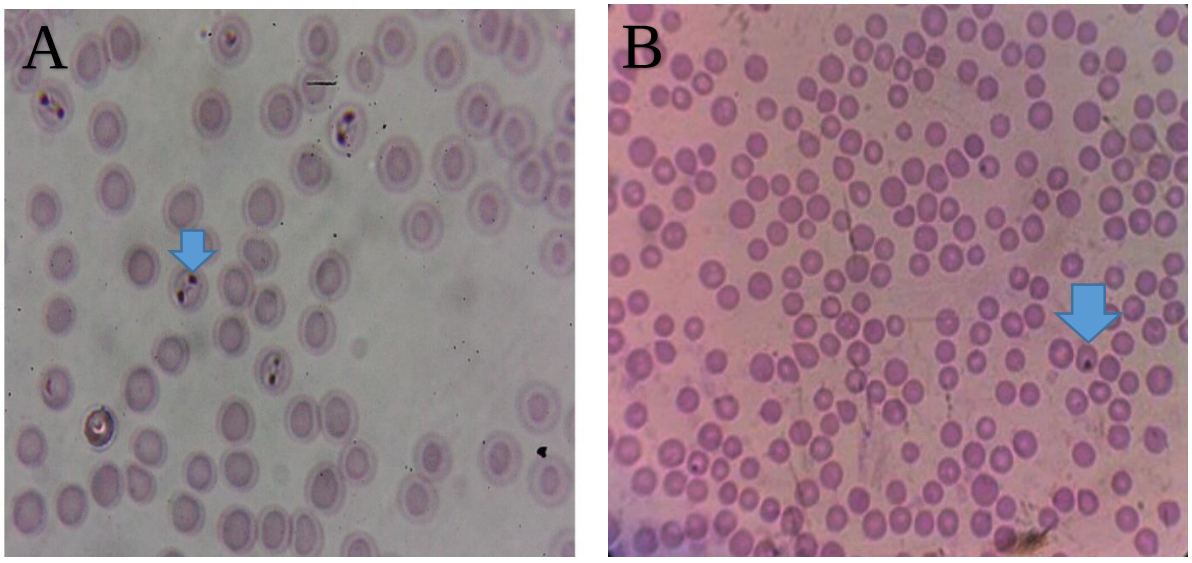


Figure 5: Microscopic figure of A. *Babeisa* spp. B. *Theilerial* spp.

4.2 District wise Prevalence of *Babesia*

The result of this study has revealed the prevalence of *Babesia* among the livestock of Kathmandu Valley, Nepal. Among total 106 samples collected and examined through thin smear, the prevalence rate of *Babesia* were 32.9% from different areas of Kathmandu Valley through microscopy. Among overall 39.6% (42/106) *Babesia* positive cases of Kathmandu valley 38.4% (35/91), 33.3% (4/12), and 100% (3/3) reported from three districts of Kathmandu Valley i.e. Kathmandu, Bhaktapur and Lalitpur respectively. The study reports higher rate of prevalence of *Babesia* from the Lalitpur district 100% among other districts of Kathmandu Valley. Similarly, Kathmandu reports higher rate of prevalence of 38.4% of *Babesia* than Bhaktapur district while Bhaktapur district reports lowest rate of prevalence among other two districts of Kathmandu Valley. Although, prevalence rate of *Babesia* parasites in cattle of three districts was statically significant ($p=0.023$, $X^2=7.506$ at df =2).

Table 4: Overall prevalence of *Babesia* spp. in suspected cattle of Kathmandu Valley, Nepal through microscopy

Kathmandu valley	Total no. of sample examined	Prevalence (%)	P-value
Kathmandu	91	38.4% (35)	0.023*
Bhaktapur	12	33.3% (04)	
Lalitpur	03	100% (03)	
Total	106	39.6% (42)	

4.3 Detection of *Babesia* through nPCR

The samples which were positive for TBBPs through microscopic examination of Blood smear were selected for nPCR. Microscopic examination of Blood smear revealed 42.4% (45/106) prevalence rate for piroplasma.

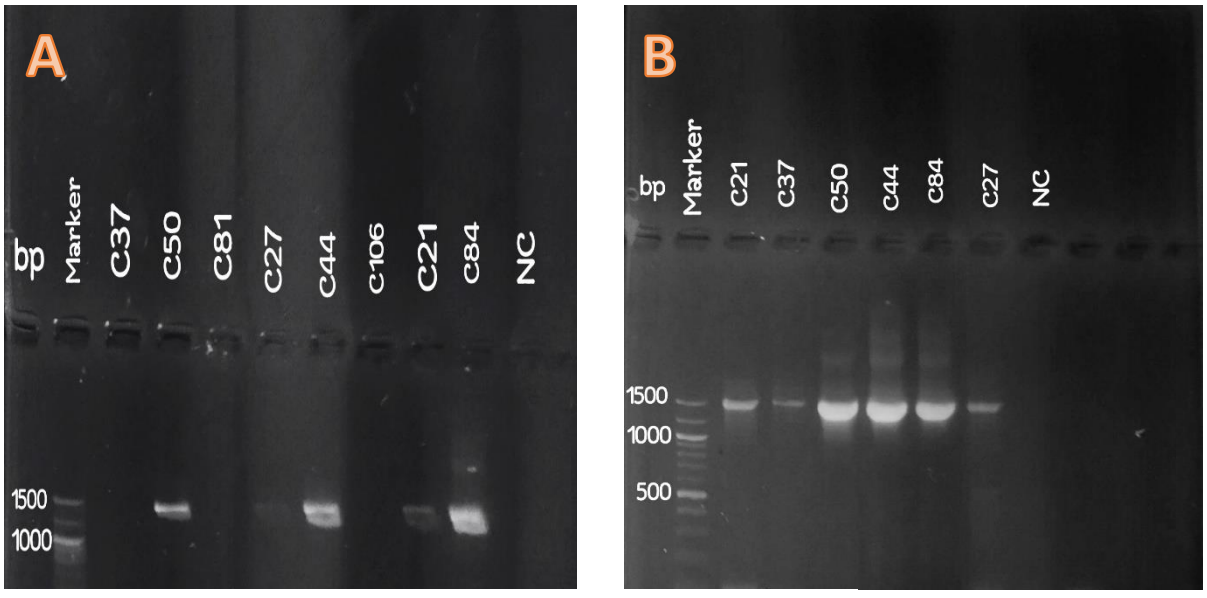


Figure 6: A. First PCR B. nPCR product under UV-illuminator

For nPCR, those 45 TBBPs positive samples used where two set of primer: BTH 18S-1F, 1R and BTH 18S-2F, 2R used for amplification of target gene of TBBPs. The nPCR examination confirmed 71.1% (32/45) as positive for piroplasma. In this study the microscopic examination of Blood smear revealed 42.45% prevalence of piroplasma while nPCR reported 71.1 % of prevalence. The samples which were positive for PCR appeared bright under UV-light. The band size of sample were compared to the marker which shows the positive sample had the band size of approximately 1500bp in length. The PCR product which were negative under UV-light appeared positive after the nPCR amplification. While analyzing the first and nPCR product, it was found that the nPCR product highly sensitive than PCR products. The prevalence rate was found maximum through nPCR

The comparative analysis of diagnostic test made in this study reveals the higher sensitivity of nPCR. Whereas microscopic examination of Blood smear examination was found less sensitive and specific for the diagnosis of *Babesia*. There is significant difference in the prevalence rate analyzed by the two diagnostic method i.e. microscopy and nPCR (p= 0.048, X^2=3.895 & df =1).

Table 5: Comparisons of data obtained through microscopy and nPCR

Samples	Diagnostic technique used		P-value
	Microscopy	nPCR	
No. of samples examined	106	45	0.048*
No. of positive samples	42.4% (45)	71.1% (32)	

4.4 Overall Seasonal prevalence of *Babesia* through nPCR

Out of 106 samples n=53 were collected during summer season and n=53 during rainy season. The rate of prevalence of *Babesia* was 33.96% (18/53) and 26.4% (14/53) recorded during summer and rainy season respectively through nPCR. The prevalence rate of *Babesia* were found to be higher in summer through nPCR.

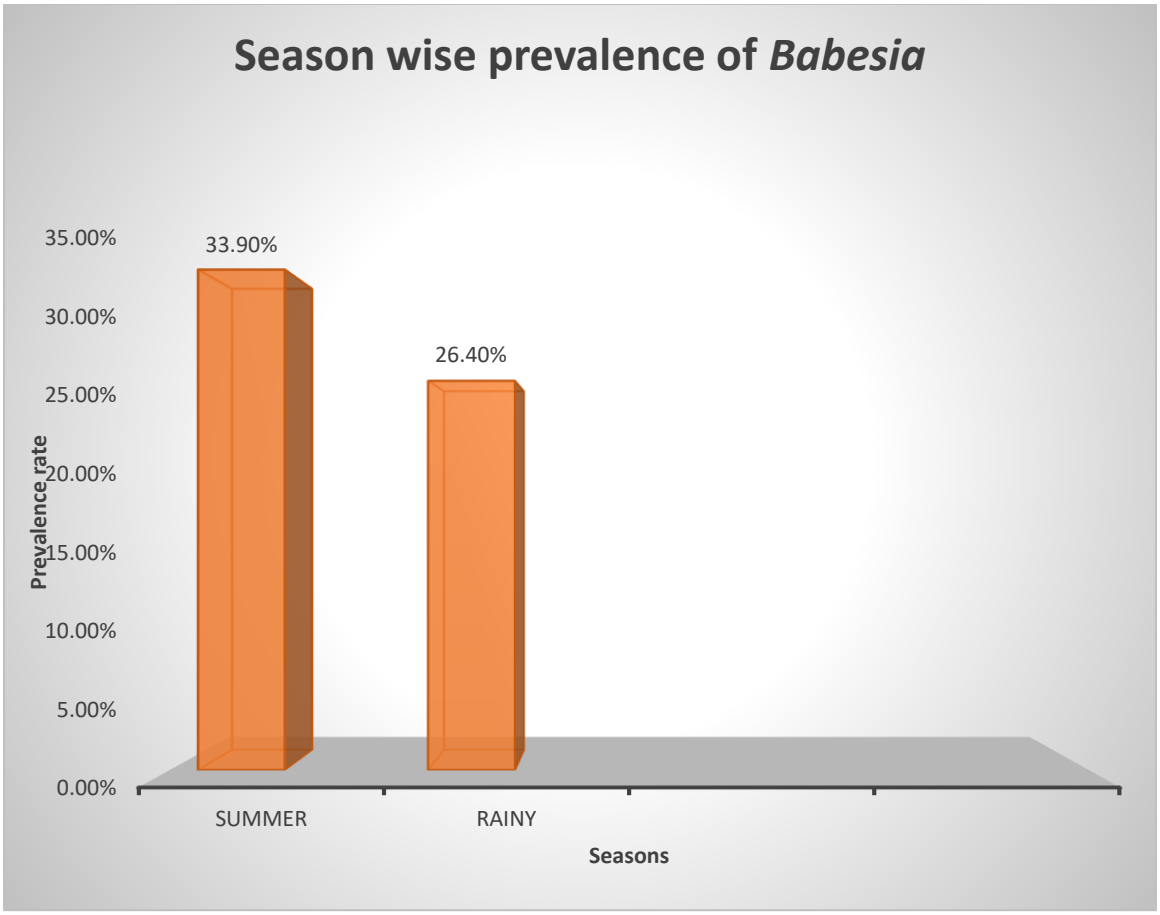


Figure-5: Season-wise prevalence of *Babesia* through nPCR

The high rate of prevalence of *Babesia* have been recorded from both seasons. The prevalence rate of *Babesia* parasites among cattle of Kathmandu Valley in rainy and summer seasons were statically not significant ($p=0.713$, $X^2=0.135$ at df =1).

4.5 Sequence Analysis

Among total 32 PCR positive samples 9 samples were randomly selected for the sequencing and sequence analysis. The study used forward primers in PCR to sequence nine samples that were PCR-positive for *Babesia* and *Theileria* species. The obtained sequences were then compared to reference accession from GenBank using multiple sequence alignment.

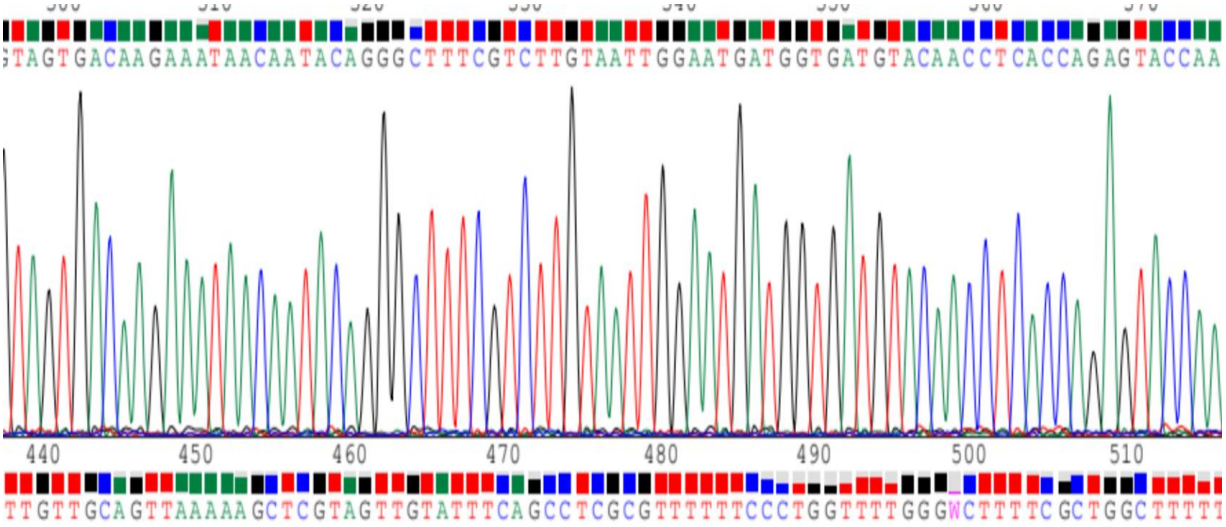
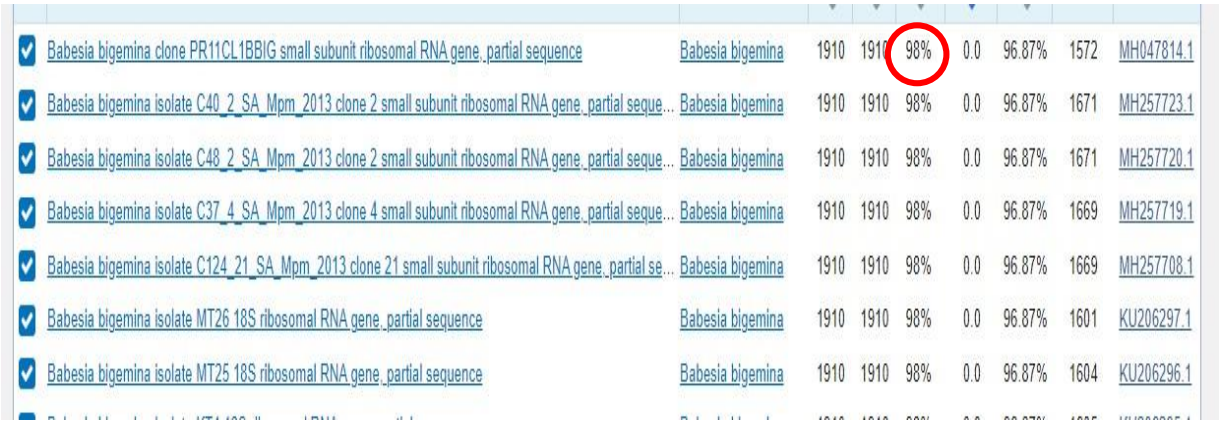


Figure 7: Raw sequence of Chromatography

Distinct nucleotides sequences were found for *Babesia* species in two samples and for *Theileria* species in seven samples in the second PCR products that were sequenced. Specifically, *B. bovis* was identified in one sample and *B. bigemina* in another sample while rest of 5 samples were identified for *Theileria spp*. For *Theileria* species, four samples contained *T. annulata* and three samples contained *T. orientalis*.

Figure 8: BLAST search result of *B. bovis*

The nucleotide sequences for the identified *Babesia* and Theileria species were further examined. The sequence obtained for *B. bigemina* (1020 bp) showed high homology of 96.87% with various *B. bigemina* isolates from Gene bank. The sequence obtained for *B. bovis* (890bp) showed strong homology (100%) with different *B.bovis* isolates. This study highlights mainly the presence of two prevalent species of *Babesia* i.e. *B.bovis* and *B. bigemina* among the cattle of Kathmandu Valley. Overall, the findings from this study support the presence of *B. bigemina* and *B. bovis* infections in cattle at molecular level.



Babesia bigemina clone PR11CL1BBIG small subunit ribosomal RNA gene, partial sequence	Babesia bigemina	1910	1910	98%	0.0	96.87%	1572	MH047814.1
Babesia bigemina isolate C40_2_SA_Mpm_2013 clone 2 small subunit ribosomal RNA gene, partial seque...	Babesia bigemina	1910	1910	98%	0.0	96.87%	1671	MH257723.1
Babesia bigemina isolate C48_2_SA_Mpm_2013 clone 2 small subunit ribosomal RNA gene, partial seque...	Babesia bigemina	1910	1910	98%	0.0	96.87%	1671	MH257720.1
Babesia bigemina isolate C37_4_SA_Mpm_2013 clone 4 small subunit ribosomal RNA gene, partial seque...	Babesia bigemina	1910	1910	98%	0.0	96.87%	1669	MH257719.1
Babesia bigemina isolate C124_21_SA_Mpm_2013 clone 21 small subunit ribosomal RNA gene, partial se...	Babesia bigemina	1910	1910	98%	0.0	96.87%	1669	MH257708.1
Babesia bigemina isolate MT26 18S ribosomal RNA gene, partial sequence	Babesia bigemina	1910	1910	98%	0.0	96.87%	1601	KU206297.1
Babesia bigemina isolate MT25 18S ribosomal RNA gene, partial sequence	Babesia bigemina	1910	1910	98%	0.0	96.87%	1604	KU206296.1

Figure 8: BLAST search result of *B. bigemina*

4.6 Analysis of Hematological parameters

The finding of present study show that presence of tick borne protozoan parasites also effects and alters the hematological parameters as compared to the non-infected healthy cattle. On the basis of comparison made on Control (Healthy cattle) of n=10 and infected cattle of n=43, there were some alteration on hematological parameters has been detected.

The statistical analysis has shown that there is significant ($p<0.05$) decrease in total RBC count and Hemoglobin concentration. Moreover, significant increase in WBC count has been recorded. Hematological parameters significantly varied in case of cattle infected with *Babesia.*

Table-6 Comparison of hematological parameters of control and infected group of cattle

Hematological parameters	Control (n=10)	Infected (n=43)	p-value	Reference value
RBC×10^6/µl	4.7±3.15	4.26±1.24	0.0021*	5-10
Hb (g/dl)	8.11±1.75	8.26±2.4	0.0013*	8-12
WBC×10^3/µl	9.74±4.35	9.23±2.83	0.25	4-12

Mean±SD

Note: *Significant

5. DISCUSSION

Bovine babesiosis is a tick-borne illness that affects cattle and is often known for having a high morbidity and death rate on a global scale. *Babesia* infection in cattle is prevalent worldwide. Designing workable preventative and control strategies requires knowledge of the condition's prevalence and factors associated with it. Prevalence studies are of utmost relevance for illness mapping and examining the epidemiological triad. According to the researcher, this is the first study to determine prevalence of bovine *Babesia* among livestock at molecular level in Nepal.

The overall prevalence of tick-borne haemoprotozoan in the present study on the basis of microscopic examination is found to be 42.45% in cattle. A higher prevalence similar to our finding was found in many earlier studies conducted in many areas of the world. The prevalence reveled by present study is comparatively higher than the study conducted by (Bohara & Shresth, 2016) in western regions of Nepal with the overall prevalence rate of 0.64% only. Similarly, the study conducted by (Yadav, 2015) in Terai region of Nepal; the prevalence of Tick-borne haemoprotozoan was reported 11.28 % which is lesser than our study. No any finding similar to this study has been reported from any part of Nepal. However, the result similar to this finding has been reported by (Zahid, 2005) from Pakistan with the prevalence rate of 44% through microscopy. The higher rate of prevalence of Tick-borne haemoprotozoans i.e. 73.1% has been reported from Shrilanka by (Sivkumar et al., 2012) which is higher than this study.

Infection with the blood parasite like *Babesia* is distributed throughout the world. The overall prevalence of infection with *Babesia* in the present study on the basis of microscopic examination is 39.6%, which is higher than those of (Khankhawash, 2018) and (Velusamy et al., 2014), who recorded 16.25% in Gorkha, Nepal and 16.64% in Tamil Nadu, India respectively. Babesiosis also has been reported from the various areas of Nepal such as western Nepal i.e. Banke and Surkhet (Bohara & Shrestha, 2016), Terai i.e. Chitwan and Nawalparasi (Pandey et al., 2013), Kathmandu (Díaz-Reganon et al., 2020; Phuyal et al., 2017), Sirah, Saptari, Udayapur, Sunsari, Morang, Jhapa, Jumla, Kavrepalanchok and Banke (Shrestha, 2017), Gorkha (Khankhawash, 2018). The prevalence rate of *Babesia* parasite of present study is higher as compared to (Shrestha, 2017; Bohora & Shrestha, 2016; Khankhawash, 2018)

which documented 6.02%, 0.64%, 6.25% prevalence respectively. This is a hospital-based study and the samples used for this study was from sick unhealthy cattle as well as suspected for TBBPs were brought to the laboratory seeking diagnosis and treatment. The cases chosen for this research was from suspected and poor health condition cattle population which were suffering from fever, weight loss, anemia and many other symptomatic conditions. Consequently, this could be one of the significant reasons of higher prevalence rate of *Babesia* in this research work. Moreover, higher the prevalence of *Babesia* infection might be due to sampling bias, variation in geo-climatic condition, cross breed with poor immunity and exposure of vectors and age of the animals might contribute to variable prevalence of babesiosis in the study areas.

The nPCR examination of TBBPs positive samples confirmed 71.1% infection of piroplasma in cattle. The recent study for the detection of prevalence of *Babesia* was related to the experiment performed by (Terkawi et al., 2011; Galon et al., 2019) who reported two species of *Babesia* i.e. *B. bovis* and B. bigemina from Thailand and Philippines with the prevalence rate of 11.2% and 3.6%, 21% and 3% respectively . The molecular test (nPCR) is considered as the most sensitive and specific test to detect these parasites even though in the case of presence of low parasitemia (Mosqueda et al., 2012). However, the microscopic test is labor intensive and requires skill to detect and identify parasites in RBC and less sensitive for the detection of parasites (Kolte et al., 2017). Many findings has reported lower rate of prevalence than this study from Indonesia, Pakistan, Brazil, and India with prevalence rate of 50.7%, 29%, 20.4%, and 7.35% respectively by rate of prevalence by (Guswanto et al., 2017; Chaudhry et al., 2010; Souza et al., 2014; Bhat et al., 2015). The finding which is similar to this study was reported from Sri-Lanka with prevalence rate of 73.1% by (Sivakumar et al., 2012).

While analyzing the seasonal prevalence of *Babesia* the rate of infection was found higher 33.9% through nPCR during summer season. According to (Alam-Tufani et al., 2017), the high prevalence rate during the summer may be caused by the hot and humid climate as the tick infestation is influenced by temperature, rainfall, and relative humidity, which in turn directly determines the prevalence of blood protozoan diseases.

Sequence analysis performed in present study presents the first molecular evidence of presence two *Babesia* species i.e., *B. bovis* and *B. bigemina* circulating among the population of cattle

in Nepal. These two species are most prevalent species among cattle and has been reported from many countries of Asia. The molecular study conducted in the Kenya of India revealed the presence of mainly these two species of *Babesia* i.e. *B. bigemina* and *B.bovis* with the prevalence rate of 13.8% and 17.2% respectively (Githaka et al., 2022). Similarly, (Kolte et al., 2017) also reports the presence of *B. bigemina* and *B. bovis* among the cattle of Maharastra, India. PCR amplification of Spherical Body Protein (SBP) reveals 10% prevalence of *B. bovis* from various areas of Pakistan (Farooqui et al., 2017). Furthermore, a number of cases of *B. bigemina* has been reported from various part of India by (Singh et al., 2013; Mushahary et al., 2020). The presence of *B. bigemina* and *B. bovis* among the cattle of Nepal may due to the close boundary Nepal shares with India. The presence of these two species has also been reported by many county of the world including Thiland by (Terakawi et al., 2011), China by (Tian et al., 2015; Niu et al., 2015), Egypt by (Abdel-Shafy et al., 2022), Philiphines by (Galon et al., 2019; Ybanez et al., 2013), United states of America by (Calder et al., 1996), Sri-lanka by (Sivakumar et al., 2012), Bangladesh by (Roy et al., 2019), Indonesia by (Guswanto et al., 2017), South Africa by (Mtshali & Mtshali, 2014), Portugal by (Silva et al., 2009), Iran by (Rajabi et al., 2017).

Hematological parameters are the indicators of diseases and are considered to be good tools for diagnosis, prognosis and effective therapy (Ganguly et al., 2015). In this study, *Babesia* infected Cattle had lower RBCs, Hb concentration. The result of this study shows alteration in hematological parameters in infected Cattle as compared to non-infected cattle as there is statistically significant ($p<0.05$) decrease in total RBC count, mean hemoglobin concentration and increase in total WBCs count could be attributed due to the breakdown of RBCs and stimulation of phagocytic cells to clean up the toxic remnants (Sharma et.al., 2013).

6. CONCLUSION AND RECOMMENDATION

This is the first molecular study conducted in *Babesia* among Nepalese cattle. The present study provides a basic information on the prevalence of *Babesia* and it's circulating species among livestock of Nepal at molecular level. The study revealed prevalence of *Babesia* 39.6% through microscopic examination of Giemsa's-stained blood smears whereas nPCR revealed presence of 71.1% piroplasma among the cattle of Kathmandu Valley. Similarly, sequence analysis revealed two most prevalent species of *Babesia* around the world i.e., *B. bovis* and *B. bigemina* circulating among the cattle of Kathmandu Valley. This study also reveals the presence of *Theileria* spp. i.e., 2.83% among the livestock of Kathmandu Valley. Moreover, analysis of hematological parameters shown the alternation of hematological parameters in infected cattle which shows significant ($P<0.05$) decrease in the total RBC count and Hemoglobin concentration in infected Cattle.

Therefore, based on the current findings and the above conclusions the following recommendations are given as follow:

- To prevent the spread of bovine babesiosis, Nepal should create and implement surveillance systems and action strategies.
- The disease causing vector eradication programs should be conducted on regular basis making people aware about the vector and harm caused by it.
- Farmers should be made aware of the disease's characteristics and how to prevent their animals from this disease.

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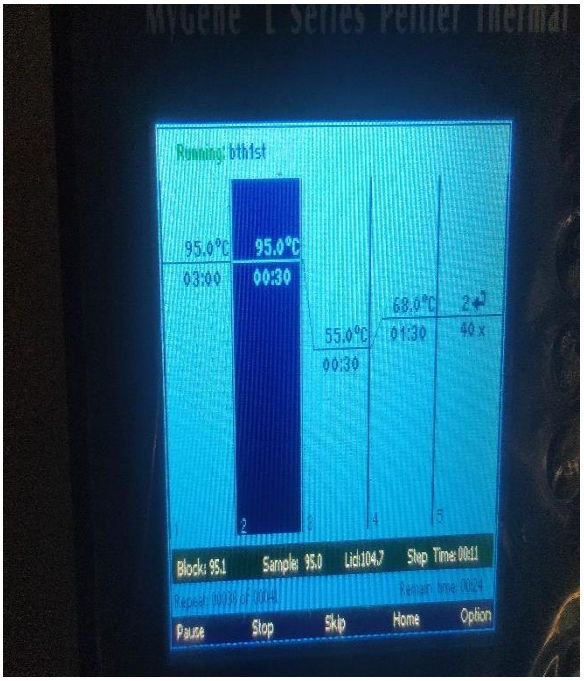
PHOTOGRAPHS

Photograph 1: Blood smear preparation

Photograph 2: Microscopic examination

Photograph 3: Preparation of master-mix



Photograph 4: Processing in thermal cycler

ANNEX-1

नेपाल सरकार

नेपाल पशु चिकित्सा परिषद्

Ref. no. 236 /2078.79

Date: May 08, 2022

Subject: **Ethical Clearance for study on "Prevalence of Babesia Parasites among livestock at Kathmandu Valley, Nepal"**

To

Ms. Medhavi Dhakal

medhavidhakal514@gmail.com

Dear Ms Dhakal,

With reference to your letter dated April 27, 2022 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 Parasites among livestock at Kathmandu Valley, Nepal"**
   b. Nature of study: A cross sectional study using questionnaire, Interview and analysis of biological samples from Cattle.
   c. Principal researcher: Ms. Medhavi Dhakal
2. **Supervisor / Co-supervisor: Dr.** . Kishor Pandey, PhD

   Dr Tulsi Ram Gompo, NVC Reg. No. 482
3. **Research laboratory: (1) Central Department of Zoology, Institute of Science and Technology, Tribhuvan University**
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

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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 9nd May 2022 to 15th Jan 2023. 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.

Wishing you well in your research

Dr. Manoj Kumar Shahi

Copy to: Chairperson, Nepal Veterinary Council

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

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