HAEMOPARASITES IN PET AND SHELTER DOGS OF KATHMANDU VALLEY, NEPAL

Umesh Acharya

T.U Redg: 52375382014

Roll no: Zoo 075/756

Batch: 2075

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

Submitted to

Central Department of Zoology

Institute of Science and Technology

Tribhuvan University

Kirtipur, Kathmandu

Nepal

September, 2022

DECLARATION

I hereby declare that the work presented in this thesis has been done by myself, and has not been submitted elsewhere for the award of any degree. All sources of information have been specifically acknowledged by reference to the author(s) or institution(s).

Umesh Acharya

Date: 23/08/2022

त्रिभुवन विश्वविद्यालय
TRIBHUVAN UNIVERSITY
प्राणी शास्त्र केन्द्रीय विभाग
CENTRAL DEPARTMENT OF ZOOLOGY
कीर्तिपुर, काठमाडौं, नेपाल ।
Kirtipur, Kathmandu, Nepal.
☎ ०१-४३३१८९६
01-4331896
Email: info@cdztu.edu.np
URL: www.cdztu.edu.np

पत्र संख्या :-
च.नं. Ref.No.:-

RECOMMENDATIONS

This is to recommend that the thesis entitled "Haemoparasites in Pet and Shelter dogs of Kathmandu Valley, Nepal" has been carried out by Umesh Acharya 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 thesis work has not been submitted for any other degree in any institutions.

.....

Prof. Dr. Mahendra Maharjan

Professor

Central Department of Zoology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

Date: 24/08/2022

त्रिभुवन विश्वविद्यालय
TRIBHUVAN UNIVERSITY
प्राणी शास्त्र केन्द्रीय विभाग
CENTRAL DEPARTMENT OF ZOOLOGY
कीर्तिपुर, काठमाडौं, नेपाल ।
Kirtipur, Kathmandu, Nepal.

०१-४३३१८९६
01-4331896
Email: info@cdztu.edu.np
URL: www.cdztu.edu.np

पत्र संख्या :-
च.नं. Ref.No.:-

Central Department of Zoology
TU Kirtipur

LETTER OF APPROVAL

On the recommendation of supervisor "Prof. Dr. Mahendra Maharjan" this thesis submitted by Umesh Acharya entitled "Haemoparasites in Pet and Shelter dogs of Kathmandu Valley, Nepal" is approved for the examination and submitted to the Tribhuvan University in partial fulfilment of the requirements for Master's Degree of Science in Zoology with special paper Parasitology.

.....

Prof. Dr. Tej Bahadur Thapa

Head of Department

Central Department of Zoology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

Date: 24/08/2022

त्रिभुवन विश्वविद्यालय
TRIBHUVAN UNIVERSITY
प्राणी शास्त्र केन्द्रीय विभाग
CENTRAL DEPARTMENT OF ZOOLOGY
कीर्तिपुर, काठमाडौं, नेपाल।
Kirtipur, Kathmandu, Nepal.
☎ ०१-४३३१८९६
01-4331896
Email: info@cdztu.edu.np
URL: www.cdztu.edu.np

पत्र संख्या :-
च.नं. Ref.No.:-

Central Department of Zoology T.U., Kirtipur

CERTIFICATE OF ACCEPTANCE

This thesis work submitted by Umesh Acharya entitled "Haemoparasites in Pet and Shelter dogs of Kathmandu Valley, Nepal" has been accepted as a partial fulfilment for the requirements of Master's Degree of Science in Zoology with special paper Parasitology.

EVALUATION COMMITTEE

.....

Supervisor
Prof. Dr. Mahendra Maharjan
Central Department of Zoology,
Tribhuvan University
Kirtipur, Kathmandu

.....

Head of Department
Prof. Dr. Tej Bahadur Thapa
Central Department of Zoology,
Tribhuvan University
Kirtipur, Kathmandu

.....

External examiner
Prof. Dr. Ranjana Gupta
Central Department of Zoology,
Tribuvan University
Kirtipur, Kathmandu

.....

Internal Examiner
Assoc. Prof. Dr. Kishor Pandey
Central Department of Zoology,
Tribuvan University
Kirtipur, Kathmandu

Date of Examination: 13 / 09 / 2022

ACKNOWLEDGEMENTS

I would like to convey my heartfelt gratitude to my supervisor, Prof. Dr. Mahendra Maharjan, Central Department of Zoology, Tribhuvan University, Kirtipur Kathmandu, for supervising my thesis work and providing continuous encouragement and valuable advice.

Likewise, I am also grateful to Dr. Pragya Koirala, Senior Veterinary officer, Molecular biology section, Central Veterinary Laboratory, Tripureshwor, Kathmandu, Nepal for her kind support and co-operation.

I am very thankful to Mr. Prakash Devkota, Veterinary officer, Haematology and Biochemistry unit for continuous support and help in completion of this study.

Similarly, I would like to express my deepest gratitude to Prof. Dr. Tej Bahadur Thapa, Head of Department, Central Department of Zoology for his prolonged inspiration and continuous support.

My sincere thanks to all teaching and non-teaching staffs of Central Department of Zoology, Tribhuvan University.

Finally, I would like thank my parents, family members and my dear friends for their cooperation that have been collectively helpful in thesis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PHOTOGRAPHS

LIST OF ABBREVIATIONS

Abbreviated form	Details of abbreviations
EDTA	Ethylene diamine tetraacetic acid
CRVH	Central referral veterinary hospital
CBC	Complete blood counts
SPSS	Statistical Package for the Social Sciences
RBC	Red blood cell
WBC	White blood cell
PCV	Packed cell volume
Hb	Hemoglobin
CVBDs	Canine vector borne diseases
TU	Tribhuvan University
TLC	Total leucocyte count
TEC	Total erythrocyte count
CVL	Central veterinary laboratory
DLC	Differential leucocyte count
PCR	Polymerase chain reaction

ABSTRACT

Haemoparasitic infections in dog are a significant economic burden worldwide but have received less attention than that of rabies. Present study was carried out in shelters and pet dogs from March to August 2021 in Kathmandu valley. Purposive sampling was carried out to take a blood samples from shelter and pet dogs irrespective of age sex and breed. About 3 ml of blood was collected in a clean ethylene diamine tetraacetic acid (EDTA) tube from a saphenous, cephalic, or jugular vein with the help of veterinary technicians. Altogether 200 blood samples from dogs were collected. Among them, 100 blood samples were collected from shelter home Lalitpur (Sneha's care) and 100 blood samples were collected from domestic dogs, which were brought to Central referral animal hospital Tripureshwor. All the information like owners' name, address, sex of dogs, age of dogs, breed of dogs were taken. Similarly, presence and absence of tick in dogs was also noted. Hematological analysis was performed to complete blood counts using haematology analyzer. A thin blood smear was prepared for the examination of haemoparasites. Data were analyzed in SPSS software to test significance. A total of three species of haemoparasites, *Babesia*, *Anaplasma*, and *Ehrlichia* sp. were recorded in this study. Pet dog's parasitic prevalence was recorded at 18%. Among them, male dogs were found to be the higher prevalence (11%) followed by females (7%). Similarly, shelter dogs showed a 31% of prevalence. Among them, 21% of males and 10% of females were affected by haemoparasites. The age, sex, and breed of the dogs found to have no statistically significant effect on the prevalence of haemoparasites. However, significant difference was noted between ticks-infected dogs with haemoparasitic prevalence. Hematological analysis revealed a significant decrease in red blood cell count ($P<0.05$) in Parasite-positive dogs. However, other blood parameters like platelets, packed cell volume, haemoglobin, lymphocytes, monocytes, and eosinophil and basophils were not found to be correlated in haemoparasites in both shelter and pet dogs. Hence, dogs of Kathmandu valley need to treat haemoparasites regularly in order to break the transmission chain.

Keywords: Prevalence, Haemoparasites Breed, dog, Haematological parameter

1. INTRODUCTION

The dogs are domesticated descendent of the wolf and belong to the CANIDAE family of the order CARNIVORE. They are multipurpose highly demanding companion animals worldwide (Morey, 2006) and the first animal to be domesticated 1500 years back (Diaz Reganon *et al.,* 2020). They are intimately connected to human beings and can act as reservoirs of many parasites and can transmit disease to humans, livestock, and wildlife. Haemoparasites infestation in a dog is a major burden worldwide (Manandhar, 2008) and has a significant economic impact from the veterinary standpoint. The common haemoparasite known to infect dogs are *Babesia* spp, *Trypanosoma* spp, *Leishmania* spp, *Hepatozoon* spp, *Ehrlichia* spp, *Anaplasma* spp, *Mycoplasma* spp and *Dirofilaria* spp. which are transmitted through different arthropod vectors like Ticks, Lice, Triatomines, Mosquitoes, Tabanids, and Phlebotomine Sandflies. These parasites affect mainly erythrocytes and intra-leukocytes or those that of living freely (Urquhart, 1987). The vector-borne disease has a seasonal character primarily because the population density of vectors varies throughout the year. Arachnids like ticks tend to be more active during warmer months (Bowman, 2008; Pavlovic *et al.,* 2012).

Babesia is tick-transmitted haemoprotozoans that infect vertebrate hosts and birds causing a major impact on farm and pet animal health with additional economic costs worldwide (Andersson *et al.,* 2017). The geographical distribution of *Babesia* differs according to the species. The larger species, *Babesia canis*, usually transmitted by tick *Dermacentor reticularis* is considered endemic in most European countries whereas smaller *B. gibsoni* transmitted by *Haemaphysalis bispinosa* and *H. longicornis* is considered endemic in Asian countries (Schoeman, 2009). In Giemsa-stained blood smear of red blood cells (RBC), *B. canis* is recognized as paired structure in a typical pear-shaped manner of size 4–5 μm long, and *B. gibsoni* as merozoites of size 1.5–2.5 μm long (Laha *et al.,* 2015). Similarly, *B. rossi* transmitted by *H. elliptica* is considered endemic in South Africa and *B. vogeli,* transmitted by *Rhipicephalus sanguineus* is considered endemic in Australia, the Southern part of USA and Brazil (Schoeman, 2009). Apart from a tick bite, the blood transfusion from an infected donor can transmit the disease and the infected bitch can infect new borne puppies via the transplacental route (Adaszek *et al.,* 2016).

Anaplasma is an Intracellular rickettsial organism, which causes canine Anaplasmosis. There are two species of *Anaplasma*, which cause pathogenesis in dogs. It infects granulocytes, predominantly neutrophils but also eosinophils, where it exists and reproduces in membrane-bound vesicles, forming micro colonies called morulae (Latin for mulberry). *A. phagocytophilum* is transmitted by ticks, which include *Ixodes pacificus* in the western United States, *Ixodes scapularis* in the upper Midwestern and the northeastern United States, *Ixodes ricinus* in Europe, and *Ixodes persulcatus* and *Dermacentor silvarum* in Asia and Russia (Cao *et al.,* 2000; Ritcher *et al.,* 1996; Telford *et al.,* 1996). Other *Ixodes* spp. ticks also have been implicated in transmission, including *Ixodes trianguliceps, Ixodes hexagonus*, and *Ixodes ventalloi* in Europe (Bown *et al.,* 2008; Santos *et al.,* 2008; Nijhof *et al.,* 2007). Most dogs naturally infected with *A. phagocytophilum* probably remain healthy, as indicated by widespread serological evidence of exposure in endemic areas in the absence of a history of clinical illness (Bella *et al.,* 2008; Folley *et al.,* 2001). To date, there are no case reports documenting fatalities in dogs. The disease Ehrlichiosis is caused by *Ehrlichia* species which affects dogs, humans, and other domestic and wild animal species. Global warming, expansion of tick habits, etc. has increased the spread of disease in non-endemic areas. *Ehrlichia canis* has a worldwide distribution; high infection rates and disease in dogs are primarily observed in tropical and subtropical areas (Lanza– Perea *et al.,* 2009). Canine ehrlichiosis is also known as Tropical Canine Pancytopenia. It is a tick-borne disease of canines caused by intracellular, gram-negative bacteria. *Ehrlichia* species that have been detected in the blood and tissues of clinically ill dogs are granulocytic or monocytic ehrlichiosis. Commonly found species of *Ehrlichia* in dogs are *E. canis* and *E. ewingi*. In humans, *E. chafeensis* is known as human monocytic ehrlichiosis (Rani *et al.,* 2010).

1.2 Objectives of the study

1.2.1. General objective

- To study prevalence of haemoparasites and association of hematological alteration in dogs of Kathmandu Valley.

1.2.2 Specific Objectives

- To determine prevalence of haemoparasities in dogs of Kathmandu valley.
- To find out association between haemoparasite and haematological parameters.

1.3 Rational of the study

In the context of Nepal, there is an increasing trend of rearing dogs as pet animals, and are several organizations, shelter houses, veterinary hospitals, and private clinics, which provide treatments, and food, and actively involved to advocate animal welfare. However, there is a huge population of dogs roaming as stray dogs in Kathmandu valley. Free roaming dogs in Kathmandu valley are quite high, however, minimal information is available about their demographics (Massei *et al.*, 2016). The stray dog's density was reported 2,930 stray dogs per kilometer, and the ratio of stray dogs to a human was 1: 4.7 (Kato *et al.*, 2003). These dogs are associated with different haemoparasites.

There is an increasing number of sick dogs reported in both pet and shelter houses. The people are much more concerned about their pet's health and take them to clinics. However, the dog taken to hospitals is treated symptomatically without a diagnosis. In the Nepalese context diagnosis of disease as per blood examination is not common practice. Several studies suggested that there is an increased prevalence of haemoparasites in hyperthermic dogs (Manandhar and Rajawar, 2008). However, many dogs can remain healthy without showing symptoms but can act as reservoirs. Thus, the present study is undertaken to determine haemoparasite infections and their association with blood parameters, of dogs both pet and shelter dogs of Kathmandu valley.

2. LITERATURE REVIEW

2.1 Background

Haemoparasites cause disease in dogs that adversely influence their health globally (Greay *et al.,* 2018; Ikejiofor *et al.,* 2021; Meyers *et al.,* 2020; Rucksaken *et al.,* 2019; Vichova *et al.,* 2018). The common haemoparasite is known to infect dogs are *Babesia* spp, *Trypanosoma* spp, *Leishmania* spp, *Hepatozoon* spp, *Ehrlichia* spp, *Anaplasma* spp, *Mycoplasma* spp (*Haemobartonella*) and *Dirofilaria* spp, which are transmitted through different arthropod vectors like Ticks, Lice, Triatomines, Mosquitoes, Tabanids and Phlebotomine Sandflies (Urquart *et al.,* 1987). The prevalence of blood parasites is not similar globally. In general, Asian countries have a high prevalence (Sarmah *et al.,* 2019; Reganon *et al.,* 2020; Singh *et al.,* 2016; Mittal *et al.,* 2019) of haemoparasites in comparison to Europe (Tabar *et al.,* 2009; Trotta *et al.,* 2009) and Africa (Obeta *et al.,* 2020; Ombugadu *et al.,* 2021). Canine vector-borne disease comprises a group of globally distributed and spreading illnesses that are caused by a wide range of pathogens transmitted by arthropods (Otranto *et al.,* 2009; Beneth *et al.,* 2012; Cardoso *et al.,* 2012; Miro *et al.,* 2013). Some of these organisms can cause life-threatening diseases in dogs and zoonotic diseases in many countries (Menn *et al.,* 2010; Chungpivat and Taweethavonsawat, 2008; Dontas, 2008; Rani *et al.,* 2011).

2.2 Global Context

Haemoparasites infection in dogs is a major burden worldwide (Manandhar, 2008). In European countries, the study suggested that there was a low prevalence of haemoparasites in the country (Maia *et al.,* 2015; Tabar *et al.,* 2009; Trotta *et al.,* 2009). In contrast, studies reported from Serbia and Romania suggested a high prevalence in this region (Pavlovic *et al.,* 2017; Anderson *et al.,* 2017). In comparison to European countries, Asian countries have a high prevalence of haemoparasites (Rajamanickam *et al.,* 1985; Kelly *et al.,* 2013; Diaz-Reganon *et al.,* 2020).

Canine babesiosis is commonly known as ‘malignant jaundice’ and is a clinically significant disease caused by the bite of ticks primarily (Schnittger *et al.,* 2012). About 100 *Babesia* species infect vertebrate hosts (El-bahnasawy *et al.,* 2011). There are 12 species of Piroplasms recorded that causes canine babesiosis (Irwin, 2010). Out of 12 species, eight species, *B. gibsoni, B. conradae, B. microti, B. vogeli, B. canis, B. rossi,*

and Babesia. Sp., can be visualized microscopically. The other four species, *T. anulata, T. equi, T. sp,* and *B. caballi* are only detected by molecular analysis (Terao *et al.,* 2015). In addition to this several unclassified *Babesia* spp. have also been detected (Kubo *et al.,* 2015 Lethinan *et al.,* 2008). The most pathogenic species of canine babesiosis are *B. rossi* and *B. gibsoni* having poor prognoses (Irwin, 2009).

Babesia rossi is endemic in southern Africa but is also recorded in other parts of the country (Oyamada *et al.,* 2005). It is mainly transmitted by the tick *Haemophysalis elliptica,* which is a more virulent species and causes hemolytic anemia and other complications (Apanaskevich *et al.,* 2007). *Babesia. canis* is endemic in Europe (Solano-Gallego *et al.,* 2011) and sporadically reported in all parts of the world. It is transmitted by *Dermacentor* spp. and causes mild clinical signs which might be anorexia, depression, fever, jaundice, anemia, and thrombocytopenia (Boozer and Macintire, 2003). *Babesia gibsoni* is considered a 'small' Babesia and is endemic in Asia and is transmitted by *Haemophysalis cornis*. *Babesia vogeli* is found worldwide and transmitted by *Rhipicephalus sanguineus* (Inokuma *et al.,* 2004). It never causes clinical signs and is the least pathogenic. *Babesia conradae* is considered more pathogenic and causes higher parasitemia, more pronounced anemia, and higher mortality. Its possible vector is *R. sanguineus* and its method of transmission is unknown (Irwin, 2009 Kjemtrup *et al.,* 2006). *Babesia microti* is endemic in Northwest Spain (Garcia, 2006) and causes severe anemia and thrombocytopenia with azotemia (Garcia, 2006). The suspected vector is *Ixodes hexagonous* (Chamacho *et al.,* 2003). *Babesia* sp. (coco) has only been found in the immune-suppressed dog where it has been associated with anemia and thrombocytopenia.

The clinical signs of canine babesiosis in dogs depend on many factors like which species of *Babesia* are infecting, co-infection with other parasitic diseases, and immunity of the host. The incubation period is about 10-28 days (Schoeman, 2009) and clinical signs include fever, lethargy, hemolytic anemia, thrombocytopenia, hemoglobinuria, marked splenomegaly, and hepatomegaly (worjiack *et al.,* 1997; Goo *et al.,* 2008). Sometimes dogs become chronically infected with no or only poorly characterized signs (Conrad *et al.,* 1991). Different virulence has been described among *Babesia* species infecting dogs. The most virulent species of canine *Babesia* species are *B.rossi* and *B.gibsoni* (Irwin 2009). Both species were reported from Asian countries, however, *B. rossi* was only reported from Iraq and its prevalence was less than 1%.

These *Babesia* species have moderate to severe pathogenicity (Solano-Gallego *et al.,* 2011; Irwin, 2009; Irwin and Hutchinson, 1991). *Babesia gibsoni* positive dogs were found to have various clinical features like fever, anorexia, depression, pale mucous membrane, and weakness, reddish to dark urine, icterus, and splenomegaly. However, 31% of dogs were asymptomatic during the study (Lee *et al.,* 2009). Thrombocytopenia is a prominent feature of *B.gibsoni* infections (Meinkoth *et al.,* 2002). Mean platelet counts were found to be low in *B. gibsoni* infected dogs (Matsuu *et al.,* 2004).

Another important haemoparasite is *Anaplasma*. A case of canine anaplasmosis in North America has been reported in California, Washington, Illinois, Minnesota, Wisconsin, Missouri, and British Columbia (Madewell *et al.,* 2004; Greig *et al.,* 1996). In Europe, infected dogs have been reported in Austria, Italy, Sweden, Switzerland, Germany, Poland, and the United Kingdom (Egenvall *et al.,* 1997; Gravino *et al.,* 1997; Pusterla *et al.,* 1997; Skotarczak *et al.,* 2004). In Asia, it is reported in India, Pakistan, Nepal, and Thailand (Bhattacharjee and sarmah 2013; Gadahi *et al.,* 2008; Diaz Reganon *et al.,* 2020; Rajamanickam *et al.,* 1985). Most dogs naturally infected with *A. phagocytophilum* probably remain healthy, as indicated by widespread serological evidence of exposure in endemic areas in the absence of a history of clinical illness (Beall *et al.,* 2008; Foley *et al.,* 2001).

The family EHRLICHIACEAE includes the alpha-proteobacterium *Ehrlichia*. Dogs, people, and other domestic and wild animal species are all effected by ehrlichiosis. The spread of illness to formerly non endemic places is a major issue due to global warming, expanding tick habitats, and rising international travel. *Ehrlichia canis* is present across the world, however tropical and subtropical regions tend to have higher rates of infection and sickness in dogs (Lanza– Perea *et al.,* 2009). Tropical canine Pancytopenia is another name for canine ehrlichiosis. It is a gram negative, intracellular bacterial disease that affects dogs and is spread by ticks. Blood and tissues from dogs with clinical illnesses have been found to include granulocytic or monocytic ehrlichiosis (Rani *et al.,* 2010).

Donatein and Lestoquard (1935) first characterized the illness in Algeria at the Pasteur Institute. Blood smears from infected dogs stained with the giemsa technique revealed a small rickettsia-like organism in monocytes that was identified as *Rickettsia canis*. They also noticed that the experimental dogs infested with ticks, *Rhipicephalus*

sanguineus, developed a severe illness characterized by anemia. For the first time in India, Mudaliar (1944) reported Ehrlichiosis in Chennai. Later, *E. canis* infections in Hyderabad were documented by Ragavachari and Reddy in 1958. A highly lethal and hemorrhagic disease was identified by Wilkins *et al*. (1967) in military dogs in Singapore and South-East Asia. Nyindo *et al*., (1971) noted tropical canine pancytopenia during the Vietnam War. Keefe *et al*., (1982) found that the disease was more prevalent in temperate and tropical regions compared to cold regions. Rikihisa (1991) reported rickettsial illness in humans and animals. *Ehrlichia* species are prevalent in tropical and subtropical areas, according to Stiles (2000). Australia is the only continent where the disease is not endemic (Sykes, 2014). Numerous vector-borne parasite diseases, such as canine ehrlichiosis, are endemic to India as a result of its diverse agro-climatic zones.

2.3 National Context

Only a few studies on canine hemoparasites have been done in Nepal. Most studies conducted up to this date have employed microscopy methods. Although molecular methods are state-of-the-art, there is not a lot of documentation regarding the outcomes for the detection of blood parasites in canine samples. Studies by Maharjan *et al.*, (2014), Subedi (2009), Manandhar and Rajawar (2006), Phuyal *et al.*, (2017), and Bhatta *et al*., (2017) microscopic prevalence between 10% and 17.14% and in the molecular study in the Kathmandu valley indicated molecular prevalence of 81.43% (Diaz Reganon *et al*., 2020).

Dog hemoparasite infections have traditionally been a significant financial burden in Nepal. These hemoparasites can harm in both red and white blood cells. Recent investigations revealed that hyperthermic dogs have a higher prevalence of hemoparasites (Manandhar *et al.*, 2008). Many dogs, however, continue to exhibit no symptoms and serve as reservoirs. Clinical symptoms and hemoparasites may not always correlate. Numerous investigations have revealed that canine haemoparasitic infections were associated with alterations in blood parameters. Due to the particular nature of these alterations, vets can suspect blood hemoparasites and confirm the diagnosis by analyzing blood parameters.

In context of Nepal, Haemoparasites were considered as potential cause of illness in dogs. There are very few information is there in the prevalence of blood parasitic

disease (Bhatta *et al.*, 2017). The present study was undertaken to determine the status of haemoparasites infection and their association with the change in hematological profile, which will be helpful on the timely diagnosis and proper treatment of febrile cases of canines.

3. MATERIALS AND METHODS

3.1. Materials Required

The materials used during study were:

i. Gloves

ii. Forceps

iii. Microscope

iv. Slides, cover slips, slide box

v. 10 ml Syringes

vi. Needle (21 Gauze)

vii. Hematology Analyzer

viii. Micropipette

ix. Micropipette tips

x. Coupling Jar

xi. Diamond pencil

Chemicals required

i. Methanol

ii. Giemsa staining reagents

iii. Ethyl alcohol 70%

iv. Distilled water

v. Immersion oil

3.2 Study Area

A study on hemoparasites in shelters and pets was carried out from March to August 2021 in Kathmandu valley of Bagmati Province, Nepal. The valley is located from 85.3240ºE to 27.7172ºN in an altitude of 1400m above sea level. Three districts, namely Kathmandu, Bhaktapur, and Lalitpur, were taken as study areas. The Kathmandu valley

is highly populous and harbors a huge number of companion and community dogs. The pet dog's blood samples were taken from the central referral veterinary hospital (CRVH) at Tripureshwor, Kathmandu and the shelter dog's blood samples were collected from Sneha's care shelter for dogs, Bhainsepati, Lalitpur.

Central referral veterinary hospital is Nepal's leading provider of veterinary services. It was founded in 1996 BS (1940 AD). This hospital was founded in response to the perceived need for veterinary services for domesticated animals such as cattle, buffalo, sheep, goats, and horses in the Kathmandu valley and surrounding areas.

Sneha's Care is a non-profit organization dedicated to protecting street dogs and animals from any form of torture, cruelty, or ill-treatment. It was founded in Bhaisepati, Lalitpur in 2014. It has launched several missions to improve the lives of street dogs and other animals. The organization was established to provide human care and long-term solutions or managing the street dog/animal population and meeting their medical needs.

Figure 1: Map of Study Area.

3.3. Sampling method

Purposive sampling carried out to take blood sample from Shelter and pet dogs irrespective of age sex and breed. Altogether 200 blood samples of dog had collected.

Among them 100 blood samples were collected from shelter home Lalitpur (Sneha's care) which were rescued previously by shelter owners from different localities of Kathmandu valley. All the information like owners name, address of dogs, sex, age, breed, were taken from representative of shelter home. Similarly, ticks presence or absence in dogs was noted. Other 100 samples of blood sample were collected from domestic dogs, which were brought in Central referral animal hospital Tripureshwor. All the information like owners name, address of dogs, sex, age, breed, were taken from veterinary hospital. Similarly, ticks presence or absence in dogs was noted. The obtained samples were brought to Lab of Central referral animal hospital Tripureshwor. Hematological analysis was performed to complete blood counts using haematology analyzer. Thin blood smear was prepared for examination of haemoparasites using standard protocols.

3.4. Sample preparation and staining

With the assistance of veterinary professionals, approximately 3 ml of blood was drawn from the saphenous, cephalic, or jugular vein using a 22G * 1" needle and placed in a clean ethylene diamine tetraacetic acid (EDTA) tube. If the lab work had to be postponed, it was kept at 4ºC. Hematology analyzer was used to analyze for complete blood count (CBC). Then, thin blood smear was prepared. For this, a drop of blood was placed on a clean glass slide, and a thin smear was made and promptly dried by air using a slide that was tilted at a 45-degree angle. The smear was then left to fix in methanol for around five minutes. The fixed slide was stained for 35–40 minutes with a working solution (10%) of Giemsa stain. The slide was cleaned and dried before being examined under an oil immersion compound microscope.

3.5. Hematological examination

The samples collected in EDTA tube had subjected for determination of haemoglobin (Hb), packed cell volume (PCV), total erythrocyte count (TEC), and total leukocyte count (TLC) and Differential counts like Neutrophils, Lymphocytes, Eosinophils, Monocytes, Basophil and Platelets count. The blood parameters had recorded with auto haematology analyzer (TEK 5000P Auto Haematology Analyzer) present at CRVH, Tripureshwor.

3.6. Microscopic Examination

Giemsa stained blood films examination under light microscopy is consider the gold standard of diagnosis (Njunda *et al.,* 2013). The stained slides were examined under high power magnification (10x by 100x) with the help of immersion oil. Starting from tail end of the slides to the whole field parasites encountered and focused for photograph. Following morphological characters to identify the parasites as well as literature review (Merk Vet Manual, 2016). *Babesia* has a pear-shaped, located in pairs, round, oval or irregular depending on the stage of development of the parasite in erythrocytes. *Ehrlichia* in Microscopic examination of Giemsa stained blood smears were identified by the presence of morulae in mononuclear cells and neutrophils. *Anaplasma* occurs intracellularly as solid dots on the margin or on center

3.7. Data Analysis

Data entered in MS-excell, and coded for SPSS. Effects of sex, age, location, breed, tick infestation, complete blood count etc. and statistical association of parasite calculated by chi-square test. Values of $P<0.05$ was considered significant at 95% level of confidence.

3.8. Ethical Approval

The study was approved by the Ethical Committee of Nepal Veterinary Council. Blood samples of dogs was collected after obtaining written informed consent from Sneha's care shelter for stray dogs, Bhaisepati Lalitpur.

4. RESULTS

4.1. Prevalence of Haemoparasites

Altogether 200 dogs blood samples, both shelter (n = 100) and pet (n = 100) from Kathmandu valley were examined. The overall prevalence of haemoparasites in Kathmandu valley was found to be 24.5%. The Parasitic prevalence of haemoparasites in shelter dogs found to be higher than that of pet dogs (Figure 2).

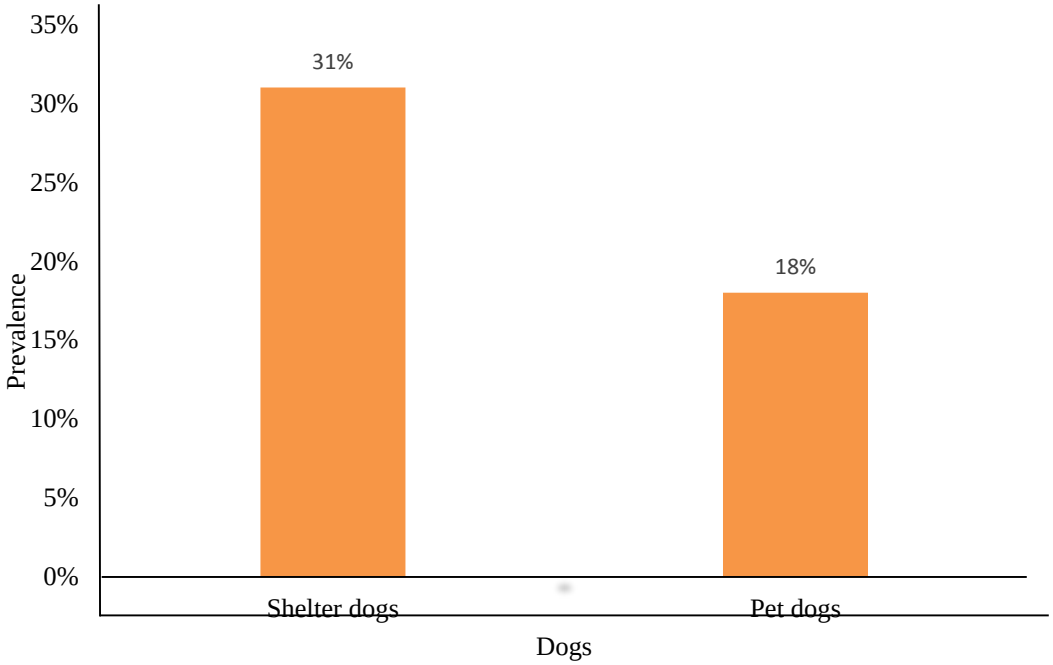


Figure 2: Prevalence of Haemoparasites in dogs of Kathmandu valley

In both shelter and pet dogs three species of Haemoparasites *Babesia* sp., *Anaplasma* sp. and *Ehrlichia* sp. were recorded in Kathmandu valley. The most prevalent haemoparasite was *Babesia* sp. in both shelter and pet dogs followed by *Ehrlichia* sp. and *Anaplasma* sp. (Figure 3).

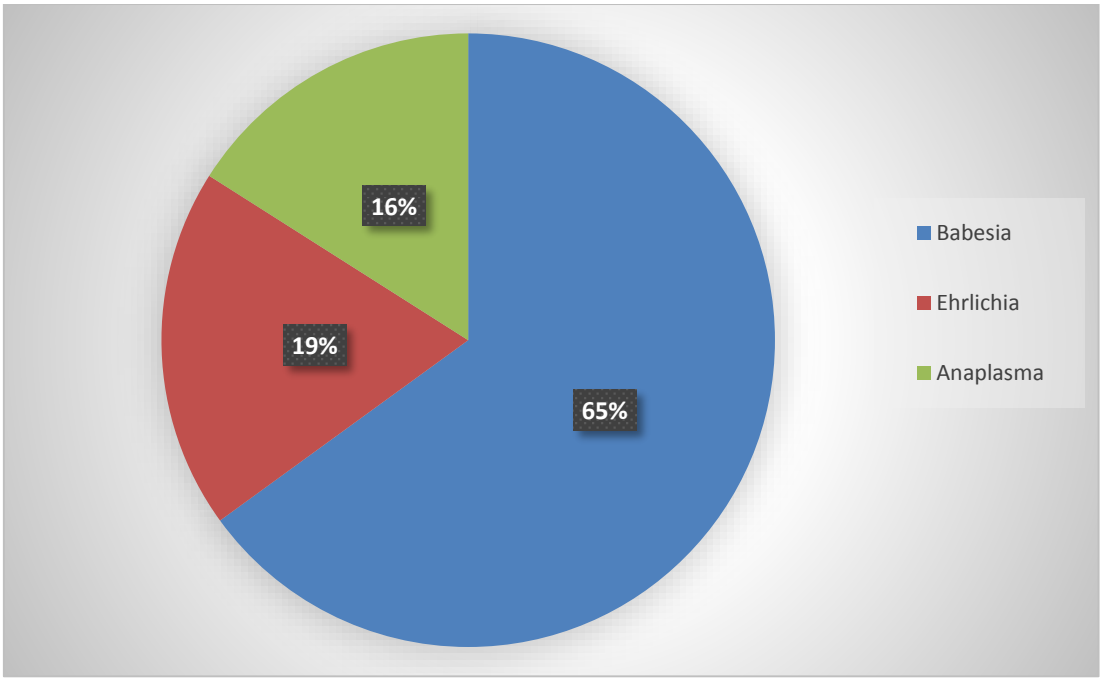


Figure 3: Species wise prevalence of Haemoparasites in dogs of Kathmandu valley

Shelter and pet wise parasitic analysis also showed that shelter dogs have high prevalence than that of pet dogs. All three parasitic species *Babesia* sp., *Anaplasma* sp. and *Ehrlichia* sp. were recorded more in shelter dogs than that of pet dogs. However, no significant difference seen in prevalence of parasites between shelter and pet dogs of Kathmandu valley (Table 1).

Table 1: Species Wise prevalence of Haemoparasites

Species	**Parasite prevalence in shelter dogs (n = 100)**	**Parasite prevalence in pet dogs (n = 100)**	**Chi square value**	**P value**
Babesia	21 (21%)	11 (11%)		
Ehrlichia	6 (6%)	3 (3%)	16.879	0.393
Anaplasma.	4 (4%)	4 (4%)		

During the study period a total of 66 blood samples from 0-2 years, 52 blood samples from 2-5 years and 82 from 5 years above dogs were collected. The overall prevalence of different age group of dogs showed that old dogs (5 years above) had high prevalence of haemoparasites followed by puppies (0-2 years) and adult dogs (2-5 years) (Figure 4).

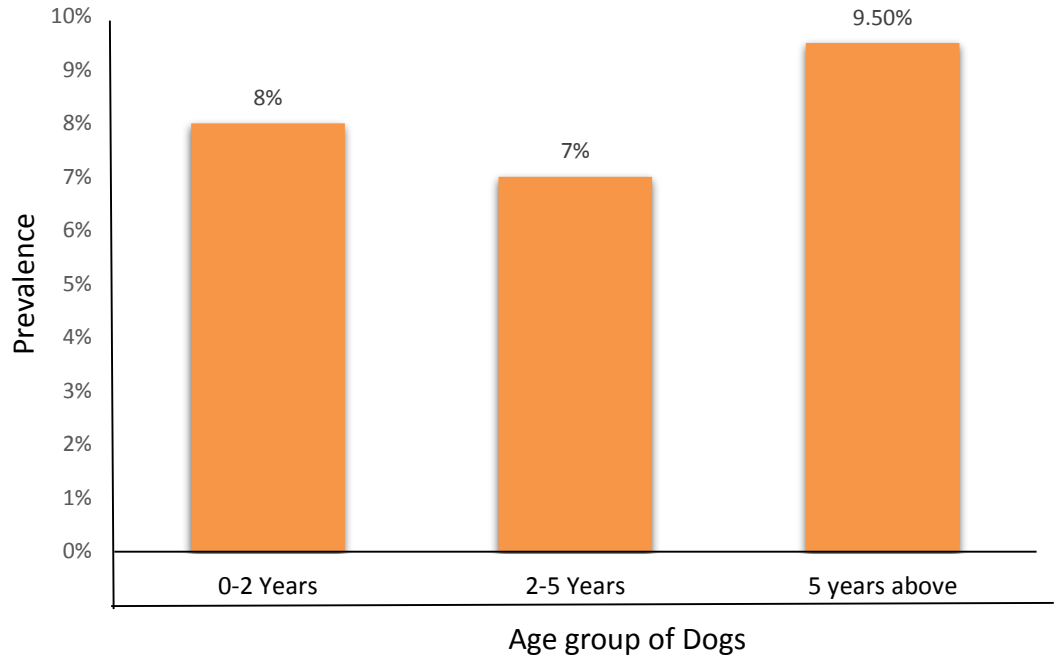


Figure 4: Age wise prevalence of Haemoparasites in Dogs of Kathmandu valley

In the study total puppies, adult and senior dogs in shelter dogs were 31, 27 and 42 respectively. Similarly, in pet dogs, they were 35, 25 and 40. The parasite positive among them were 8, 4 and 16 respectively in shelter dogs and that of pet dogs were 8, 4 and 16. The age wise blood prevalence analysis showed that there is high prevalence in 2 to 5 years old age (Adult) in shelter dogs. However, in pet dogs prevalence was high in 0 to 2 years old age (puppies) dogs (Figure 5). There was no significant difference ($p= 0.123$) seen in age groups and haemoparasites in both shelter and pet dogs of Kathmandu valley.

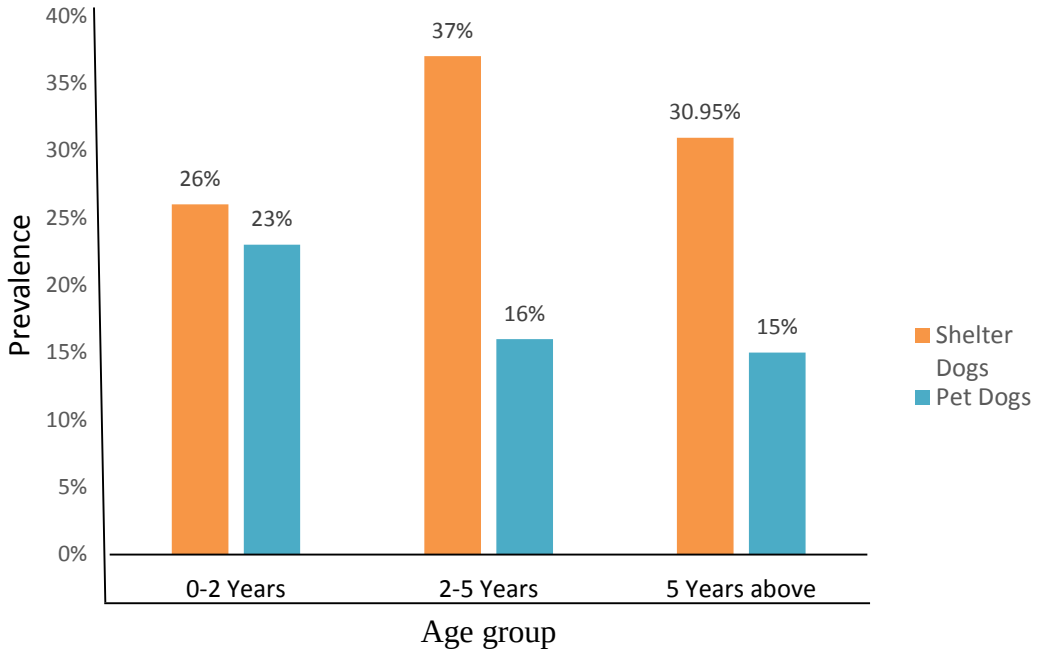


Figure 5: Age wise Prevalence of Haemoparasites in shelter and pet dogs

Total of 113 male and 87 female were included in the study. Among them 52 were male in pet dogs and 61 in shelter dogs. Sex wise prevalence analysis showed that male are more infected than that of female dogs in both shelter and pet dogs. However, no significant difference (p=0.399) seen between sex and parasites of Kathmandu valley (Figure 6).

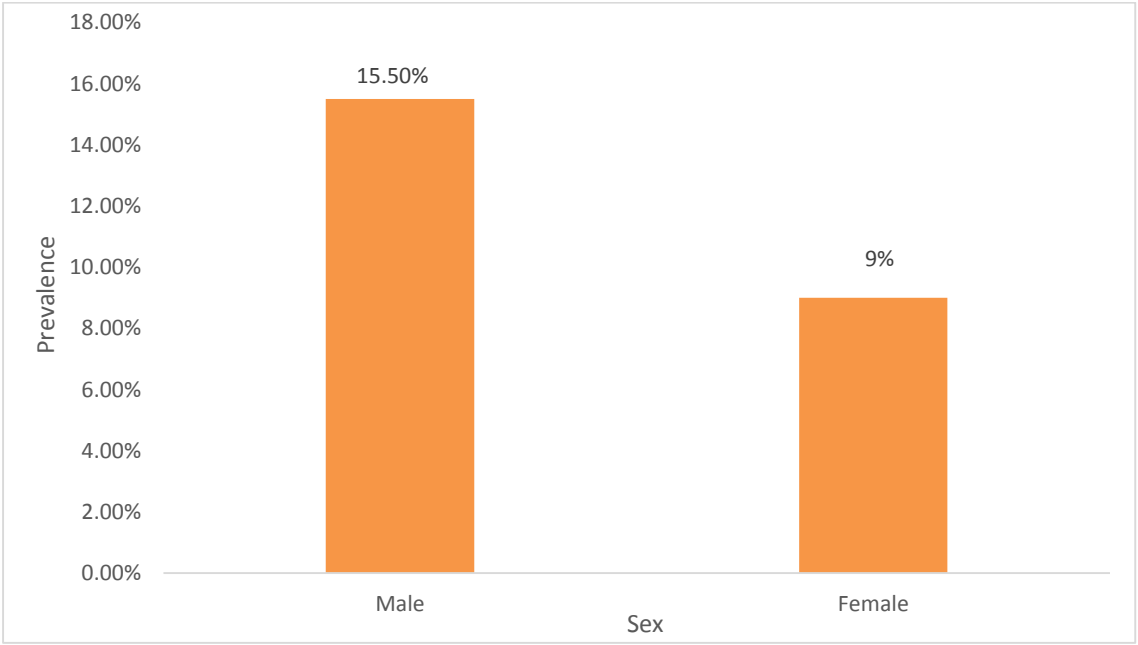


Figure 6: Sex wise Prevalence of Haemoparasites in Kathmandu Valley

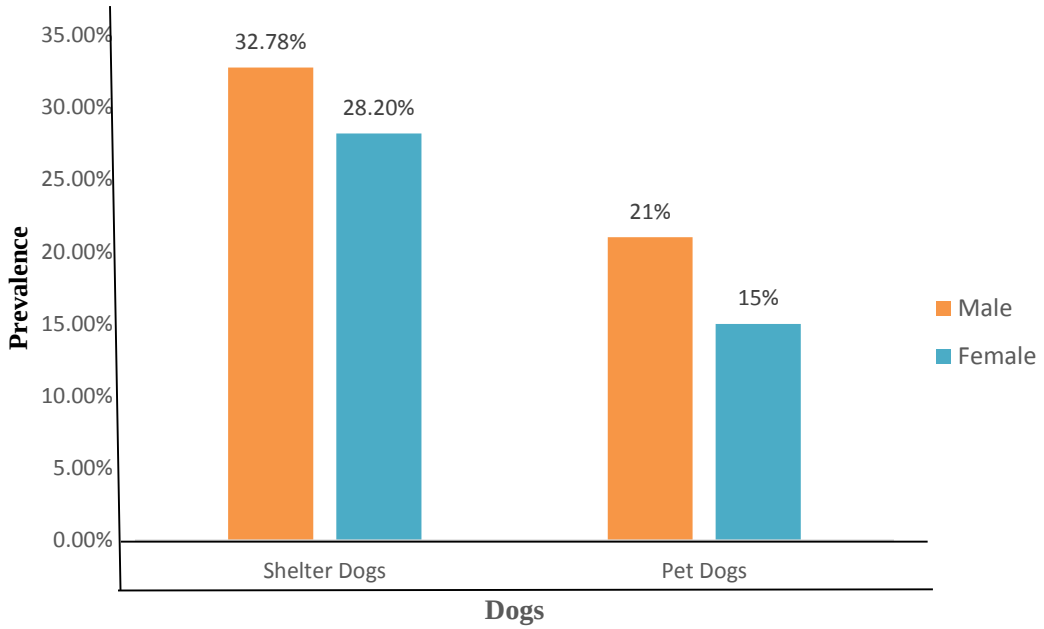


Figure 7: Sex wise Prevalence of Haemoparasites in shelter and pet dogs

Total 136 samples were from Kathmandu, 42 from Lalitpur and 22 from bhaktapur was taken. Among them 33 from Kathmandu, 9 from Lalitpur and 10 from Bhaktapur were found to be parasite positive. Overall location wise prevalence was highest in Kathmandu districts followed by Lalitpur and Bhaktapur (Figure 8). Similarly, in shelter dogs prevalence was high in Kathmandu followed by Lalitpur and Bhaktapur. In contrast to shelter dogs, in pet dogs parasitic prevalence was high in Bhaktapur followed by Kathmandu and least prevalence was seen in Lalitpur districts (Table 2).

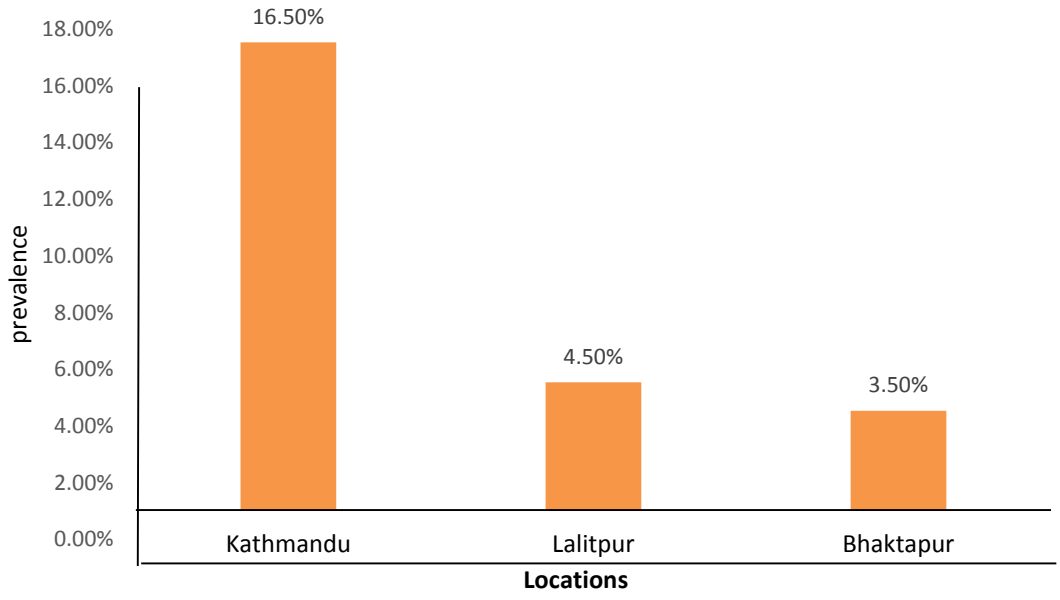


Figure 8: Prevalence of Haemoparasites by Location in Kathmandu valley

Table 2: Location wise prevalence of Haemoparasites in Shelter and pet dogs of Kathmandu valley

Location	**Shelter dogs**	**X^2**	**P value**	**Pet dogs**	**X^2**	**P value**
Kathmandu	21 (30.88%)			12 (17.64%)		
Lalitpur	7 (33.33%)			2 (10%)		
Bhaktapur	3 (27.27%)	0.125	0.939	7 (36%)	3.541	0.170

In shelter dogs very few breeds of dogs were observed. However, in pet dog's altogether nine breeds were observed. Breed wise prevalence of parasite showed that Doberman breed was mostly affected by Haemoparasites followed by local dogs and pug. Boxer breed dog was only breed in which no parasites were observed. (Table3).

Table 3: Breed Wise prevalence of Haemoparasites in Kathmandu valley

Breeds	**Shelter Dogs (n=100)**	**Pet dogs (n=100)**	**Total (200)**
Bhote (n=10)	1 (1%)	1(1%)	2 (1%)
Pit-bull (n=6)	1 (1%)	1 (1%)	2 (1%)
German shepherd (n=28)	-	4 (4%)	4 (2%)
Japanese spitz (n=25)	0 (0%)	2 (2%)	2 (1%)
Pug (n=8)	0 (0%)	2 (2%)	2 (1%)
Doberman (n=8)	-	3 (3%)	3 (1.5%)
Mongrel (n=13)	-	4 (4%)	4 (2%)
Local (n=97)	29 (29%)	1 (1%)	30 (15%)
Boxer (n=5)	-	0 (0%)	0 (0%)

Tick infested and non-tick infested both dogs were found to be positive for Haemoparasites. However, prevalence is high in non-tick infested dogs than that of tick infested dogs in both shelter and pet dogs of Kathmandu valley. Prevalence of Haemoparasites of both shelter and pet dogs are significantly difference in tick infestation dogs.

Table 4: Prevalence of Haemoparasites in tick infestation and non-tick infestation dogs

	Parasitic prevalence in Tick positive Dogs (n=26+16)	**Parasitic prevalence in Tick Negative Dogs** (n=74+84)	**X^2**	**P value**
Pet Dogs	6 (37.5%)	12 (14.28%)	4.90	0.027
Shelter Dogs	14 (53%)	17 (22.97%)	8.57	0.003

4.2 Prevalence of Haemoparasites with blood parameters

During the study, complete blood count was performed. Each parameters were separated as normal range, below normal range and above normal range. Prevalence of each range was evaluated. Similarly, significant test was performed using chi square test between each parameters range and haemoparasites in both shelter and pet dogs. The prevalence of haemoparasites when blood parameters were altered were shown in table (Table 5 and Table 6). Prevalence of haemoparasites and blood parameters analysis showed that there was increased level of WBC seen in both shelter and pet dogs which were parasite positive. In contrast, there is decrease level of RBC, Hemoglobin, Pack cell volume, platelets, lymphocytes, monocytes and Eosinophil's in parasite positive dogs in both shelter and pet dogs of Kathmandu valley. There was significant difference in RBC with prevalence of haemoparasites in both shelter and pet dogs of Kathmandu valley (Table 5 and 6).

Table 5: Prevalence of Haemoparasites and Blood parameters abnormalities in Pet dogs

Blood Parameters	Below normal range	Above normal range	Chi sq. value	P value
WBC	7.14%	19.60%	1.302	0.521
RBC	35%	10%	9.680	0.008
Hb	23%	15%	0.827	0.661
PCV	20%	0%	0.271	0.398
Platelets	15%	0%	0.407	0.524
Neutrophils	29%	19%	3.353	0.187
Lymphocytes	24%	22%	1.920	0.383
Monocytes	5%	0%	2.59	0.273
Eosinophils	12%	0%	1.586	0.208
Basophils	20%	0%	2.171	0.38

However, there were no significant differences between the parasitized and non-parasitized dogs in Kathmandu valley in terms of Hemoglobin, PCV, Platelets, Neutrophils, Lymphocytes, monocytes and eosinophil in both shelter and pet dogs of Kathmandu valley.

Table 6: Prevalence of Haemoparasites and Blood parameters abnormalities in Shelter dogs

Blood Parameters	**Below normal range**	**Above normal range**	**Chi sq. value**	**P value**
WBC	5.26%	26.08%	5.13	0.077
RBC	41%	0%	6.722	0.035
Hb	40.47%	0%	3.902	0.142
PCV	29.62%	0%	0.611	0.737
Platelets	51.06%	0%	16.89	0.00
Neutrophils	26.66%	42.85%	1.91	0.385
Lymphocytes	41.66%	27.86%	1.688	0.201
Monocytes	57.14%	0%	3.21	0.201
Eosinophils	36.36%	0%	0.379	0.538
Basophils	0%	0%	0.917	0.338

5. Discussion

5.1 Overall Prevalence

Dogs are closely related to humans and serve as a reservoir for numerous parasites. To humans, livestock, and wildlife they can spread disease. Numerous arthropod vectors, such as ticks, lice, triatomines, mosquitoes, tabanids, and phlebotomine sandflies, transmit hemoparasites, including *Babesia* species, *Trypanosoma* species, *Leishmania* species, *Hepatozoon* species, *Ehrlichia* species, *Anaplasma* species, *Mycoplasma* species (*Haemobartonella*), and *Dirofilaria* species. These parasites mostly affect erythrocytes and intra leukocytes, as well as those who are living freely (Urquhart 1987).

In this investigation, a high prevalence of hemoparasites was found. Similar investigations carried out in the Kathmandu Valley had discovered an overall prevalence of haemoparasites in dogs between 10 and 17.14 percent (Maharjan *et al.,* 2014; Subedi, 2009; Manadhar and Rajwar, 2008). The current study, however, found a higher frequency of 24.5%. This might be accounted for by the fact that prior research only included dogs that were owned. Unlike the present study, which involved both owned and rescue dogs. The prevalence discovered in this study was lower than that discovered in the Kathmandu Valley by Diaz-Reganon et al. (2020), where the prevalence was reported to be 81.43%. This might be because only shelter dogs were used for the sample selection, and because different pathogen detection methods—such as real-time PCR—were used to identify hemoparasites. Real-time PCR has been shown to be significantly more sensitive than detecting hemoparasites in blood smears (Salano Gallego *et al.,* 2011; Otranto *et al.,* 2011 and Sainz *et al.,* 2015). Similar research done in Serbia on strays and household pets dogs (Pavlovic *et al.,* 2017) revealed a slightly higher prevalence (31.32%) than the current study's (24.5%). In contrast to a study conducted in Serbia, shelter dogs were more prevalent in this study than pet dogs (Pavlovic *et al.,* 2017). This can be because the sample sizes and regions are different. The current findings also differed from those of a study conducted in India by Bhattacharjee and Sarmah (2013), which revealed prevalence rates of 57.31% in hospital population dogs, 58.03% in working dogs, and 63.64% in stray dogs. This can be as a result of sample size, length of study, and sampling from the community of stray dogs.

In this investigation, three different types of hemoparasites were found in dogs from shelters and private homes. This study's findings on the pattern of parasite species differed slightly from those of Manandhar and Rajwar (2008), who found just two species in the Kathmandu Valley: *Ehrlichia* sp. and *Babesia* species. Similar to Subedi (2012) who recorded three species (*Babesia* sp. 10%, *Ehrlichia* sp. 3.4%, and *Anaplasma* sp. 2%), Bogicevic et al. discovered *Ehrlichia canis* (11.06%) and Phuyal et al. (2014) reported two species *Babesia canis* 4% and *Ehrlichia canis* 8%.

The current findings differ from those of Pavlovic et al. (2017) as well, who found that *Ehrlichia sp*. was present in 15.93% of pet dogs and 28.35% of shelter dogs whereas *Babesia* sp. was recorded in 39.75% of dogs kept as pets and 71.64% of dogs in shelters. *Anaplasma* sp. was discovered in 19.40% of shelter dogs and 6.04% of dogs kept as pets. In a study conducted by Nwoha *et al.,* three kinds of hemoparasites were discovered, including *Babesia* sp. (94.4%), *Trypanosoma* sp. (5.6%), and *Anaplasma* sp. (45%). In accordance with previous investigations, Bhattacharjee and Sarmah (2013) discovered *Babesia* sp. (47.72%), *Ehrlichia platy* (4.54%), *Ehrlichia canis* (2.27%), *Ehrlichia ewingi* (2.27%), and *H. canis* (2.27%) in the blood of stray dogs in Khanapara, Guwahati, India. Depending on the geographic area and the accessibility of vectors, there may be variations in the prevalence of different types of parasites.

The current study found that dogs older than 5 years were more likely than not to have hemoparasites (9.50%). This result is comparable to that of Bhatta *et al*., (2017), who found that canines 5 years and older were most prevalent (23.07%). Similar findings from other studies by Manandhar and Rajwar (2008), Subedi (2012), Gadhi *et al*., (2008), Jalali *et al*., (2013), and Akhtardanesh *et al*., complement these findings (2010). The higher parasite prevalence in older dogs may be brought on by weakened immune systems. In contrast to Maia et al. 2015, when 61% of dogs in the age category with 1-7 years old were more infected, the current study found a lower infection rate.

Similar to this, a study by Phuyal *et al*., found that the prevalence of hemoparasites was higher in the population of dogs aged 1 to 5 years. Blood parasites are prevalent in shelter dogs, according to the current study (37%). Another recent research of stray dogs in the Kathmandu Valley supported this finding, showing a high incidence (60%) in adult dogs (Diaz Reganon *et al.*, 2020). According to a study conducted in Pakistan,

adult dogs have a higher frequency of parasites than puppies (Gadahi *et al.,* 2008). This may be due to mature dogs' poor immunity to blood parasite infections.

The results of the current investigation indicated that males had a higher prevalence of hemoparasites than females. This discovery was consistent with discoveries made in the Kathmandu Valley and reported by Diaz Reganon *et al*., (2020) According to Maia *et al*., (2015), Subedi (2009), and Bhatta *et al*., (2017), male dogs are substantially more common than female dogs. This might be explained by male dogs having a higher inclination than female dogs (Papa, 2016). However, the results of this study differed from those of Phuyal *et al*., (2014), Shitta (2009), and Gadahi *et al*., (2008). Different immunological states in the two sexes of dogs may account for the differential in haemoparasite prevalence. Highest overall prevalence of haemoparasites recorded in Kathmandu districts. However, prevalence was found similar to dogs of Lalitpur and Bhaktapur. Contrary to general predominance, Lalitpur districts had more infected shelter dogs than Kathmandu, then Bhaktapur. The incidence of pet dogs was also higher in Bhaktapur districts than in Kathmandu, then Lalitpur districts (6.66%). Within these three Kathmandu valley districts, the climate and ecology are similar. It's possible that different methods of sample collection account for the heterogeneity in the prevalence of hemoparasites in dogs by region. In the findings of breed wise prevalence, the overall breed prevalence was found to be high in local dogs (15%). In contrast with the findings of Subedi (2012) Manandhar and rajwar (2006) and Phuyal (2014) in which German shepherd had high prevalence compared to other breeds. In present study tick infected dogs (both shelter p= 0.027 and pet p= 0.003) and prevalence of parasite showed statistically significant differences among non-tick infected dogs. But findings of our study were contrast to Diaz Reganon *et al.,* (2020) and Maia *et al.,* (2015) in which parasitic prevalence in tick infected dogs and non-tick infected dogs, were not significantly difference.

The hematological parameter under study revealed statistically significant low RBC, high WBC in infected cases than in non-infected cases whereas there was no significant difference in the Haemoglobin and PCV estimation between infected and non-infected group. However, lower mean PCV, platelets was recorded in the study. This finding agrees with the findings of (Maharjan*et al.,* 2014; Shitta *et al.,* 2012) who also have observed a lower mean PCV in the infected dogs than the non-infected dogs. This might be because hemoparasites induced immune-mediated harm to the bone marrow stem

cells. There was no statistically significant difference in neutrophil and eosinophil, lymphocyte, and monocyte counts between infected and non-infected subjects in differential leucocyte count (DLC). However, Weiser *et al*., (1991) noted that neutropenia was seen in infected individuals and that the infection was caused by a hemoprotozoan (Manandhar and Rajawar, 2008).

7. Conclusion and Recommendation

7.1. Conclusion

Overall prevalence of canine haemoparasite was 24.5% examined by blood smear examination. Maximum prevalence of haemoparasite was found in shelter dogs compared to pet dogs. Dogs were found to be infected by three Haemoparasites *Babesia* sp., *Anaplasma* sp. and *Ehrlichia* sp. Among them *Babesia* sp. was found to be most prevalent. Males were more infected with parasites than that of female dogs. Similarly, old age (5 years above) dogs have high prevalence than other age group dogs in Kathmandu valley. Similarly, location wise result indicated that Kathmandu districts had high parasitic prevalence than that of Lalitpur and Bhaktapur districts. High occurrence of parasite was encountered in local dogs followed by pit bull and mongrel dogs. In this study tick infected dogs and parasitic prevalence was found to be significantly different.

Haematological parameter between the infected and no infected dogs revealed statistically significantly difference ($p < 0.05$) in RBC, whereas, other parameters does not have any significance difference. However, lower mean PCV, haemoglobin and Platelets recorded in parasite positive dogs. It can be conclude that ticks borne parasitic disease is major problem in Kathmandu valley. Blood parameters play significant role in parasitic prevalence.

7.2. Recommendations

1. Tick controlled method should be employ in shelter dogs
2. The recorded parasite can transmit from dogs to humans and other animals. So, owners or handlers of dogs, Veterinarians, laboratory workers should be aware about the disease and care must be taken during handling of affected of dogs and its sample.
3. Boxer, German shepherd, Japanese spitz dogs seems resistance to blood parasite and very few number of dogs were infect with the parasites compared to other breeds. These breed dogs were recommended to rear in home.

REFERENCES

Anderson, M. O., Tolf, C., Tamba, P., Stefanache, M., Waldenström, J. and Dobler, G. et al., 2017. Canine tick-borne diseases in pet dogs from Romania, Parasites and Vectors **10:** 1–6.

Baneth, G., Bourdeau, P., Bourdoiseau, G., Bowman, D. Breitschwerdt E, Capelli G, *et al*. 2012. Vector-borne diseases constant challenge for practicing veterinarians: recommendations from the CVBD World Forum, Parasite and Vectors **5:** 55-60.

Baneth, G., Samish, M. and Shkap, V. 2007. Life cycle of *Hepatozoon canis* (Apicomplexa: Adeleorina: Hepatozoidae) in the tick *Rhipicephalus sanguineus* and domestic dog (*Canis familiaris*), Journal of Parasitology **93:** 283–99.

Beall, M.J., Chandrashekar, R. and Eberts, M.D. 2008. Serological and molecular prevalence of *Borrelia burgdorferi*, *Anaplasma* phagocytophilum, and *Ehrlichia* species in dogs from Minnesota, Vector Borne Zoonotic Disease **8:** 455–464.

Bowman, D., Little, S. and Lorentzen, L. 2009. Prevalence and geographic distribution of *Dirofilaria immitis*, *Borrelia burgdorferi*, *Ehrlichia canis*, and *Anaplasma phagocytophilum* in dogs in the United States: Results of a national clinic-based serologic survey, Veterinary Parasitology **160:** 138–148.

Bown, K.J., Lambin, X. and Telford, G.R. 2008. Relative importance of Ixodes ricinus and Ixodes trianguliceps as vectors for *Anaplasma* phagocytophilum and *Babesia* microti in field vole (Microtus agrestis) populations, Applied Environmental Microbiology 74:7118–7125.

Bhattarcharjee, K. and Sarmah, P. 2013. Prevalence of haemoparasites in pet, working and stray dogs of Assam and North–East India: A hospital based study, Veterinary World **6:** 874- 878.

Bhatta, T., Acharya, N., Acharya, K.P. and Thapa, B.R. 2018. Prevalence of Haemoparasites in hyperthermic dogs of Kathmandu valley, Nepal, Asian Journal of Animal Vetereniary **13:** 67–72.

Cao, W.C., Zhao, Q.M. And Zhang. P.H. 2000. Granulocytic *Ehrlichia*e in *Ixodes persulcatus* ticks from an area in China where Lyme disease is endemic, Journal of Clinical Microbiology **38:** 4208–4210.

Cardoso, L., Mendao, C. and Madeira, L. 2012. Prevalence of Dirofilaria immitis, *Ehrlichia canis*, *Borrelia burgdorferi* sensu lato, *Anaplasma* spp. and *Leishmania infantum* in apparently healthy and CVBD-suspect dogs in Portugal – a national serological study, Parasite and Vectors **5:** 62-65.

Diaz-Reganon, D., Agulla, B., Piya, B., Fernandez-Ruiz, N., Villaescusa, A., García-Sancho, M., Rodríguez-Franco, F. and Sainz, A. 2020. Stray dogs in Nepal have high prevalence of vector-borne pathogens: A molecular survey, Parasites and Vectors **13:**1–8.

Egenvall, A.E., Hedhammar, A.A. and Ersdorff. A.I. 1997. Clinical features and serology of 14 dogs affected by granulocytic ehrlichiosis in Sweden, Veterinary Record **140:** 222–226.

Foley, J.E., Kramer, V.L. and Weber, D. 2002. Experimental ehrlichiosis in dusky footed wood rats (*Neotoma fuscipes*), Journal of Wildlife Disease **38:** 194–198.

Gadahi, J., Arijo, A., Abubakar, M., Javaid, S.B. and Arshed, M.J. 2008. Prevalence of Haemoparasites in stray and pet Dogs in Hyderabad Area: Comparative sensitivity of different Diagnostic techniqes for the detection of microfilaria, Veterinary World **1:** 229-232.

Garcia, A.T. 2006. Piroplasma infection in dogs in Northern Spain, Veterinary Parasitology **138:** 97–102.

Gravino, A.E., Caprariis, S. and Manna, L. 1997. Preliminary report of infection in dogs related to *Ehrlichia equi*: Description of three cases, New Microbiology journal **20:** 361–363.

Irwin, P.J. 2009. Canine babesiosis: from molecular taxonomy to control, Parasites and Vectors **2:** 4-7.

Irwin, P. J. 2010. Canine Babesiosis. Veterinary Clinics of North America - Small Animal Practice, **40:**1141–1156.

Irwin, P.J. and Hutchinson, G.W. 1991. Clinical and pathological findings of *Babesia* infection in dogs, Australia Veterinary Journal **68:** 204–9.

Ivan, P., Radoje, K., Doder, B., Rangelov, N. and Ivana T. 2017. Haemoparasites in dogs from the Belgrade area in the period 2014–2015, Zbornik Matice srpske za prirodne nauke **41:** 100-106.

Jalali, M., Mosallanejad, B., Avizeh, R., Alborzi, A.R., Nejat, H.H. and Taghipour, R. 2013. *Babesia* infection in urban and rural dogs in Ahvaz district, Southwest of Iran. Archives of Razi Institute **68:** 37-42.

Kato, M., Yamamoto, H., Inukai, Y. and Kira, S. 2003. Survey of the stray dog population and the health education program on the prevention of dog bites and dog-acquired infections: a comparative study in Nepal and Okayama Prefecture, Japan, Acta Medica Okayama **57:** 261–266.

Keefe, T., Holland, C.J., Salyer, P.E. and Ristic, M. 1982. Distribution of *Ehrlichia canis* among military working dogs in the world and selected civilian dogs in the United State, Journal of American Veterinary Medical Association **181:** 236-238.

Lanza-Perea, M., Kumthekar, SM., Sabarinath, A., Karpathy, S., Sharma, R.N. and Stone, D.M. 2009. Doxycycline treatment of asymptomatic dogs seropositive for *Ehrlichia canis*, West Indian Veterinary Journal **9:** 11-13.

Maharjan, D., Jha, A., Singh, D.K. and Poudel, S.K. 2014. Prevalence of Haemoparasites in Feral Dogs of Lalitpur District. Proceedings of Scientific presentation and Conference information 11th National conference of Nepal veterinary association **8:** 54-60.

Manandhar, S. and Rajawar, N.B. 2008. Incidence of Haemoparasites as the causative agent of hyperthermia in dogs of Kathmandu valley, Proceedings on 8th national veterinary conference of Nepal Veterinary Association **13:** 145-150.

Matsuu, A., Koshida, Y., Kawahara, M., Inoue, K., Ikadai, H., Hikasa, *et al*., 2004. Efficacy of atovaquone against *Babesia gibsoni* in vivo and in vitro, Veterinary Parasitology **124:** 9-18

Massei, G., Fooks, A., Horton, D., Callaby, R., Sharma, K., Dhakal. and Dahal, U. 2017. Free roaming dogs in Nepal: demographics, health and public knowledge, attitudes and practices, Zoonoses Public Health **64:** 29–40.

Menn, B., Lorentz, S. and Naucke, T.J. 2010. Imported and travelling dogs as carriers of canine vector-borne pathogens in Germany, Parasite and Vectors **3:** 34-44.

Morey, D. 2006. Burying key evidence: The social bond between dogs and people, Archeology of Science **33:** 158-175.

Miro, G., Montoya, A., Roura, X., Galvez, R. and Sainz, A. 2013. Seropositivity rates for agents of canine vector-borne diseases in Spain: a multicentre study, Parasite and Vectors **6:** 117-126.

Mudaliar, VS.1994. Canine rickettsiosis in south India, Indian Veterinary Journal **20:** 163-165.

Nijhof, A.M., Bodaan, C. and Postigo, M. 2007. Ticks and associated pathogens collected from domestic animals in the Netherlands, Vector Borne Zoonotic Disease **7:** 585–595.

Njunda, A. L., Assob, N. J. C., Nsagha, S. D., Kamga, F. H. L., Mokenyu, M. D., and Kwenti, T. E. 2013. Comparison of capillary and venous blood using blood film microscopy in the detection of malaria parasites: A hospital based study, Scientific Journal of Microbiology, **2:** 89–94.

Nwoha, R., Daniel-lgwe, G., Onuekwasi, A., Onyeabar, K. and Okah, U. 2013. Incidence of Haemoparasites in dogs in Ikwunao local government area of Abia state, Journal of Veterinary Science and Technology **4:** 41-72.

Otranto, D., Dantas-Torres, F., Breitschwerdt, E.B. 2009. Managing canine vector borne diseases of zoonotic concern: part one. Trends Parasitology **25:** 157–63.

Otranto, D., Dantas-Torres, F., Weigl, S., Latrofa, MS., Stanneck, D., Decaprariis, D., *et al*. 2011. Diagnosis of Hepatozoon canis in young dogs by cytology and PCR. Parasite and Vectors **4:** 55-64.

Oyamada, M., Davoust, B. and Boni, M. 2005. Detection of *Babesia canis rossi*, *B. canis vogeli*, and *Hepatozoon canis* in dogs in a village of eastern Sudan by

using a screening PCR and sequencing methodologies, Clinical Diagnostic Laboratory Immunology **12:** 1343–1346.

Papa, K., Isaac, M., Lucy, M A. and Samuel, O. 2016. Prevalence of Helminths in Dogs and Owners' Awareness of Zoonotic Diseases in Mampong, Ashanti, Ghana Journal of Parasitoloy research **16:** 171-183.

Phuyal, S., Jha, V. and Subedi M. 2017. Prevalence of Haemoparasites in dogs of Kathmandu Valley, Nepalese Veterinary Journal **34:** 107–112.

Pusterla, N., Huder, J., Wolfensburger, C. 1997. Granulocytic ehrlichiosis in two dogs in Switzerland, Journal of Clinical Microbiology **35:** 2307– 2309.

Raghavachari, K., Reddy, A.M.K. 1958. Rickettsia canis in Hyderabad, Indian Veterinary Journal **35:** 63-68.

Rani, PAM., Irwin, P.J., Coleman, G.T., Gatne, M. and Traub, R.J.2011. A survey of canine tick borne diseases in India, Parasites and Vectors **4:** 141-148.

Richter, P.J., Kimsey, R.B. and Madigan, J.E. 1996. Ixodes pacificus (*Acari*: *Ixodidae*) as a vector of *Ehrlichia equi* (Rickettsiales: Ehrlichieae), Journal of Medical Entomology **33:** 1–5

Rikihisa, Y. 1991. The tribe *Ehrlichia*ceae and Ehrlichiosis diseases, Clinical Microbiology **4:** 286-308.

Santos, A.S., Santos-Silva, M.M., Sousa, RD. 2008. PCR-based serosurvey of *Anaplasma phagocytophilum* in Portuguese ticks (*Acari*: *Ixodidae*), Vector Borne Zoonotic Disease, **33:** 1–5.

Solano-Gallego, L., Miro, G., Koutinas, A., Cardoso, L., Pennisi, M.G., Ferrer, L., *et al*. 2011. *Leishmania* Veterinary guidelines for the practical management of canine leishmaniosis, Parasite and Vectors **4:** 86-94.

Sainz, A., Roura, X., Miro, G., Estrada-Pena A., Kohn, B., Harrus, S., *et al*. 2015. Guideline for veterinary practitioners on canine ehrlichiosis and anaplasmosis in Europe, Parasite and Vectors **8:** 75-84.

Sarma, K., Nachum-Biala, Y., Kumar, M and Baneth, G. 2019. Molecular investigation of vector-borne parasitic infections in dogs in Northeast India, Parasite and Vectors **12:** 122-126.

Schoeman, J.P. 2009. Canine babesiosis, Journal of Veterinary Research **76:** 59–66.

Singh, M.N., Raina, O.K., Sankar, M., Rialch, A., Tigga, M.N., Kumar, G.R. and Banerjee, P.S. 2016. Molecular detection and genetic diversity of *Babesia* gibsoni in dogs in India, Infectious Genetics Evolution **41:** 100-106.

Shitta, K.B., James-Rugu, N. and Azonci, A.2012. *Babesia* canis infection in dogs in Jos North and Jos South LGAs of Plateau State, Nigeria, International Journal Science Technology Development Study **7:** 42-47.

Schnittger, L., Rodriguez, A. E., Florin-Christensen, M. and Morrison, D. A. 2012. *Babesia*: A world emerging Infection, Genetics and Evolution **12:** 1788–1809.

Skotarczak, B., Adamska, M., Rymaszewska, A. 2004. *Anaplasma phagocytophilum* and protozoans of *Babesia* genus in dogs from endemic areas of Lyme disease in north-western Poland, Wiad Parazytol **50:** 555–561.

Sykes, J.E. 2014. Bacterial diseases In: Canine and feline infectious disease, Saunders Elsevier **4:** 278-289.

Stiles, J. 2000. Canine rickettsial infections, Veterinary Clinics of North America, Small Animal Practice Journal **30:** 1135-1149.

Tabar, M.D., Francino, O., Altet, L., Sanchez, A., Ferrer, L. and Roura, X. 2009. PCR survey of vector borne pathogens in dogs living in and around Barcelona, an area endemic for leishmaniasis, Vetereniray Record, **164:** 112–116.

Telford, S.R., Dawson, J.E. and Katavolos P. 1996. Perpetuation of the agent of human granulocytic ehrlichiosis in a deer tick-rodent cycle, Proceeding of National Academy of Science **93:** 6209–6214.

Trotta, M., Fogliazza, A., Furlanello, T. and Solano-Gallego, L. 2009. A molecular and serological study of exposure to tick-borne pathogens in sick dogs from Italy, Clinical Microbiology Infection **15:** 62–63.

Urquhart, G.M., Armour, J., Duncan, J.L., Dunn, A.M. and Jenning, F.W. 1987. Veterinary Parasitology 2nd ed. Black well publishing, The University of Glasgow, Scotland 243-246.

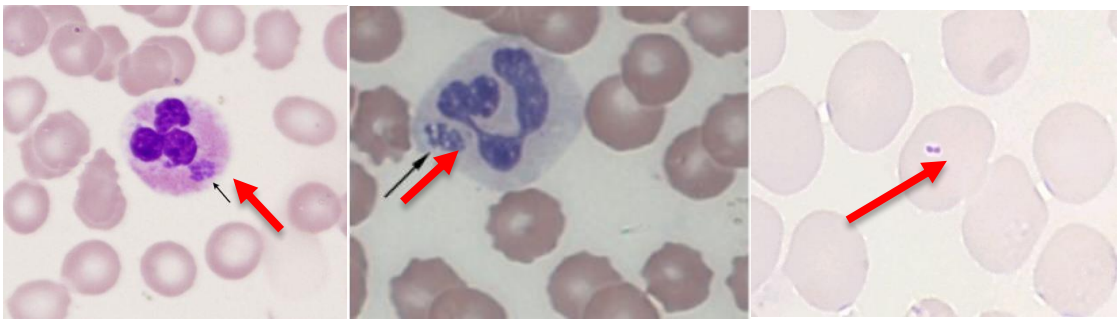
Wilkins, R.J., Hurwitz, A.I. and Dodds-Laffin, W.J. 1967. Immunologically mediated thrombocytopenia in the dog, Journal of American Veterinary Medical Association **163:** 882-89.

Weiser, M.G., Thrall, M.A., Fulton, R., Beck, E.R., Wise, L.A. and Van, J.L. 1991. Granular lymphocytosis and hyperproteinemia in dogs with chronic ehrlichiosis, The Journal of the American Animal Hospital Association **27:** 84-88.

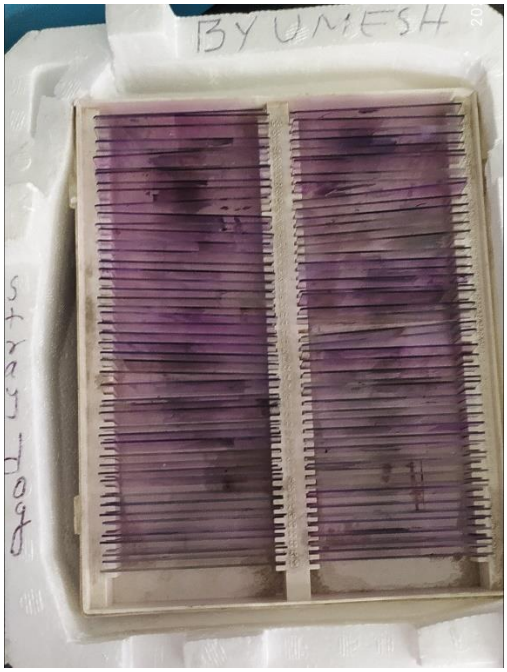
Zamokas, G., Grigonis, A., Daunoras, G. and Academy, V. 2014. Importance of haematological changes in diagnosing canine, Parasite and vectors **67:** 89-94.

APPENDICES

PHOTOGRPHS

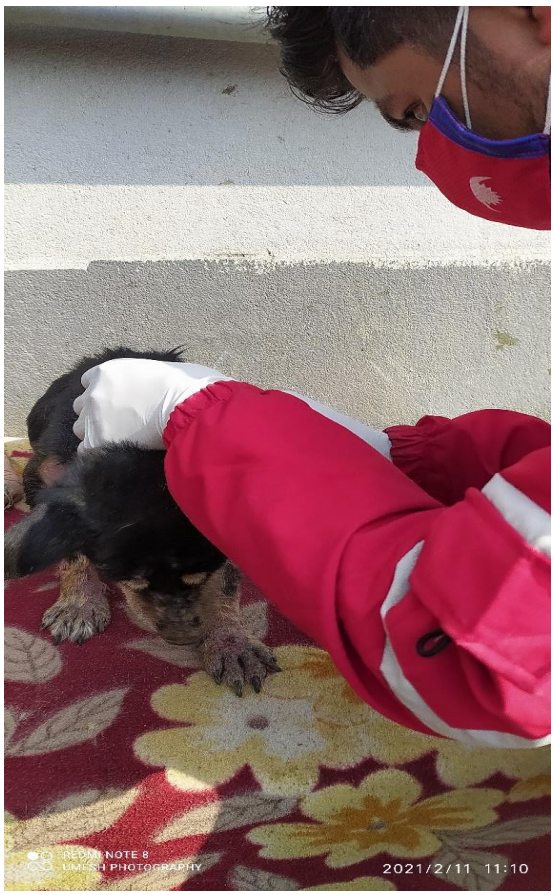


Ehrlichia species *Anaplasma* species *Babesia* species



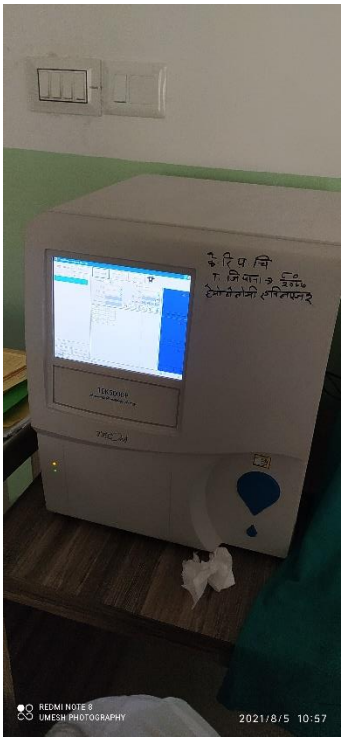
Slides prepared during study

Sneha's care shelter for dogs



Blood collection in shelter dogs

Microscopic observation during study



Haematology Analyzer in CRVH

ETHICAL APPROVAL

Government of Nepal

Nepal Veterinary Council

Ref. no. Ethical. 200/2077.78

Date: March 26, 2021

Subject: **Ethical clearance for a research entitled "Serological and Molecular Study on Leishmaniasis in Stray Dogs from Kathmandu Valley. "**

To

Mr. Umesh Acharya

M.Sc. Student

Central Department of Zoology, Tribhuvan University

Dear Mr. Acharya,

With reference to your application dated 2077.12.02 regarding the ethical approval for a research study described below, I have the pleasure to inform you that an ethical clearance has been approved for the specified research study with the following terms and conditions.

9. Study detail :
   a. Title: **" Serological and Molecular Study on Leishmaniasis in Stray Dogs from Kathmandu Valley "**
   b. Nature of study: **Requirement of M.Sc. course in Tribhuvan University.**
   c. Principal Investigator: **Mr. Umesh Acharya**
   d. Veterinarian : **Dr. Gyanendra Thakur, NVC Regd no. 859**
   e. Resesearch methodology:
      i. Animal used : **Stray dogs from Kathmandu**
      ii. Sample : **Blood sample collection under supervision of veterinarian**
      iii. Lab. test : **Hematological study in laboratory.**
   f. Research laboratory:
      i. **Central Department of Zoology, TU, Kirtipur**
      ii. **Central Veterinary Laboratory, Tripureswor, Kathmandu.**

रजिष्ट्रार
नेपाल पशु चिकित्सा परिषद्

Government of Nepal

Nepal Veterinary Council

10. The researcher shall satisfy the requirement prescribed in the prevailing law for export (if necessary) of biological material outside of the country.

11. The study described above shall be carried out according to standard protocol under the supervision of the registered veterinarian mentioned above.

12. The welfare aspect of animal will be well taken care during the research. Any activity posing threat to animal welfare shall be dealt in accordance with the prevailing law.

13. NVC shall retain the right to withdraw or amend this Ethical Approval, if

    a. any unethical principal or practices are revealed or suspected

    b. relevant information has been withheld or misrepresented and

    c. regulatory changes of whatsoever nature so require

    d. in case of violation of animal welfare or detection of activities intended cruelty to animal.

14. 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 the completion report at the end of the project.

    b. However, NVC must be informed immediately of

        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

15. 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.

16. The validity of this ethical clearance is one year effective from the **March 26, 2021 to March 25, 2022 AD.** You will be required to apply for renewal of ethical clearance on an annual basis till the study is not completed.

Wishing you well in your research

26.03.2021

Dr. Narayan Prasad Ghimire

Copy to: Chairperson, Nepal Veterinary Council

रजिस्टार
नेपाल पशु चिकित्सा परिषद्

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