Studies on molecular detection of *Leishmania* infection in stray dogs from Bangladesh

バングラデシュ人民共和国の野犬におけるリーシュマニア感染に関する研究

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CONTENTS

Chapter II: Potential of cell-free DNA as a novel diagnostic biomarker for parasite infections in dog

Abbreviations

BLAST	Basic Local Alignment Search Tool
DNA	Deoxyribonucleic acid
E-value	Expect value
ICT	Immunochromatographic test
ITS1	Internal transcribed spacer 1
M	DNA marker
NCBI	National Center for Biotechnology Information
Na_2EDTA	Disodium ethylenediaminetetraacetate
PCR	Polymerase chain reaction
TR	Tandem repeat
VL	Visceral leishmaniasis
WHO	World Health Organization
bp	Base pair
cfDNA	Cell-free DNA
kDNA	Kinetoplast DNA
rRNA	Ribosomal RNA

Unit measures abbreviations

min	Minute
ml	Milliliter
ng	Nanogram
pg	Picogram
s	Second
× *g*	Times gravity
°C	degree Celsius
κ	Kappa value
μM	Micromolar
μg	Microgram
μl	Microliter

General Introduction

Leishmaniasis is one of the most important vector-borne parasitic diseases caused by the haemoflagellate protozoan parasites of the genus *Leishmania*. This disease is reported to be endemic in 98 countries (Alvar et al., 2012) with 350 million people at risk of developing the disease annually (WHO, 2010). Based on main clinical symptoms, there are 3 different forms of leishmaniasis – visceral leishmaniasis (also known as kala-azar), cutaneous leishmaniasis (the most common), and mucocutaneous leishmaniasis.

Visceral leishmaniasis (VL), caused by the *Leishmania donovani* complex (*L. infantum* and *L. donovani*), is fatal and most serious form of the leishmaniasis. The annual incidence of VL ranges from 0.2 to 0.4 million and more than 90% of cases are reported from Bangladesh, India, Sudan, South Sudan, Ethiopia, and Brazil (Alvar et al., 2012). In Bangladesh, the current prevalence is estimated to be 40,000–45,000 cases with more than 40.6 million people at risk of developing the disease (Bern and Chowdhury, 2006; Salam et al., 2012).

There are two epidemiological patterns of VL. Zoonotic VL is mainly caused by *L. infantum*, with dogs as the main reservoir hosts, in the Mediterranean, the Middle East, Asia, and South America. On the other hand, in the Indian subcontinent and eastern Africa, the transmission of VL caused by *L. donovani* is thought to be anthroponotic. However, there are some reports of *L. donovani* infection in dogs in Sudan and northwest Ethiopia (Dereure et al., 2003; Kalayou et al., 2011; Shamboul et al., 2009). Other than dogs, red foxes in central Greece, cats in the western provinces of Turkey and Brazilian bats were reported to be the reservoirs of *Leishmania* parasites (Karayiannis et al., 2015; Oliveira et al., 2015; Pasa et al., 2015).

Few studies have investigated the role of animal reservoirs in maintaining *L.*

donovani in the Indian subcontinent. In recent studies, *Leishmania* DNA was detected in cows, buffaloes, and goats in Nepal (Bhattarai et al., 2010) and in goats in India (Singh et al., 2013). Recently, anti-*Leishmania* antibodies were detected in dogs (Sharma et al., 2009) in India. These findings strengthen a hypothesis of the presence of animal reservoirs for *L. donovani* in Bangladesh as well. In our recent study, *L. donovani* DNA was detected in one stray dog (1.2%) from VL-endemic areas of Bangladesh (Alam et al., 2013), which needs further verification to better understand the possible role of domestic and stray dogs as a reservoir host for VL transmission in this endemic area.

Although there is no official figure on the actual number of stray dogs in Bangladesh, officials estimate that there could be more than 2.5 million (Alam et al., 2013). Since the dog population is high and a close contact exists between dogs and humans especially in rural areas, disease transmission from these dogs is feasible. For example, Rabies, the fatal viral zoonotic disease, is endemic in Bangladesh with high public health significance and ranked the world's third highest death rate for human rabies (Hossain et al., 2011). These dogs can play an important role in the domestic transmission cycle of several pathogens including major zoonotic parasitic agents, but the information on this issue is extremely lacking in Bangladesh. Assessment of wide range of pathogens carried by stray dogs will be informative for the detection of new or unexpected parasitic agents which may have zoonotic importance and cause public health risks.

Traditionally, screening the parasites in dogs are based on microscopically observing the morphological characteristics of adult parasites through parasitological procedures and amplification of parasite DNA by PCR and real-time PCR. These traditional and conventional molecular approaches suffer from major limitations: (a) morphological identification of parasites requires highly specialized researchers and

sometimes needs host dissection and microscopic observation which is time consuming and labour-intensive. (b) molecular methods (PCR and real-time PCR) based on the use of specific primers can detect only the target species and lack the ability to detect unsuspected parasitic species. Therefore, the development of a rapid, easier and more sensitive method is much needed for screening parasites in dogs.

The concept of detecting cell-free DNA (cfDNA) is presently being applied in a range of clinical settings and in routine diagnostic practices as an efficient biomarker, particularly in the field of oncology and prenatal diagnosis (Wagner et al., 2012; Yu et al., 2014). cfDNA comprises fragments of DNA found extracellularly and mainly in the circulation. To date, the presence of cfDNA in the serum has been demonstrated for multiple parasites such as *Plasmodium*, *Trypanosoma*, *Schistosoma*, and *Wuchereria* using conventional molecular approaches (Ghayour et al., 2014; Russomando et al., 1992; Wichmann et al., 2009; Ximenes et al., 2014). Since the conventional molecular methods have some limitations as discussed above, cfDNA cannot be used as a target for parasite detection until recently.

Soon after high-throughput sequencing technologies become available, metagenomic studies have been utilized to reveal microbial diversity in the environmental samples. Most of the metagenomic studies performed to date have investigated the biodiversity of bacterial communities with great success (Amir et al., 2013; Sogin et al., 2006; Yatsunenko et al., 2012). The 18S rDNA-based amplicon analysis was employed to assess the biodiversity of parasites in rats (Hino et al., 2016; Tanaka et al., 2014). High-throughput techniques recently established for nematodes could be readily adapted to sequencing of mitochondrial genomes of *Taenia* species (Jex et al., 2008; Jex et al., 2009). Although diagnostic metagenomics has been little used for the detection of parasite

diversity, there is huge possibility that the field of parasitology research will soon catch up with other branches of microbiology.

For these reasons, In Chapter I, stray dogs were captured in VL-endemic areas of Bangladesh and screened for serological and molecular evidence of *Leishmania* infection. The presence of anti-*Leishmania* antibodies and *Leishmania* DNA was confirmed in several stray dogs which are supportive to the hypothesis that the dog is an animal reservoir and playing a potential role in VL transmission in this endemic focus.

In Chapter II, cfDNA was extracted from plasma of stray dogs previously captured in VL-endemic areas of Bangladesh and analyzed by the Illumina sequencing technology to identify parasite communities prevailing in those dogs. Several sequences associated with different parasites could be identified in this analysis. The results suggest that the new approach of analyzing cfDNA could be an efficient diagnostic method for screening the parasites in dogs.

Chapter I

Molecular and serological evidence of *Leishmania* infection in stray dogs from visceral leishmaniasis-endemic areas of Bangladesh

1. Introduction

Visceral leishmaniasis (VL), or kala-azar, is a fatal vector-borne parasitic disease caused by the *Leishmania donovani* complex (*L. infantum* and *L. donovani*) of intracellular protozoan parasites. VL is a serious public health problem in the Indian subcontinent; an estimated 200 million people are at risk, which represents approximately 67% of the global VL burden (Joshi et al., 2008; Sundor et al., 2008). In Bangladesh, the current prevalence is estimated to be 40,000–45,000 cases with more than 40.6 million people at risk of developing the disease (Bern and Chowdhury, 2006; Salam et al., 2012). The disease is prevalent in 45 districts of Bangladesh, and most reported cases are from the Mymensingh district (Bern and Chowdhury, 2006).

Two epidemiological patterns of VL are known. Anthroponotic VL is transmitted via infection from humans to humans and to a lesser extent from humans to animals. Zoonotic VL is transmitted from animals to humans and to a lesser extent from humans to humans. *L. infantum* is responsible for zoonotic VL, with dogs as the main reservoir hosts, in the Mediterranean, the Middle East, Asia, and South America. In areas where zoonotic VL is endemic, the prevalence of *L. infantum* infection in dogs is often high, although many infections are asymptomatic (Dantas-Torres et al., 2006). The transmission of VL caused by *L. donovani* is thought to be anthroponotic in the Indian subcontinent and eastern Africa (Desjeux, 2004). The importance of animal reservoirs in these regions is not well studied.

There are several reports of *L. donovani* infection in dogs showing typical symptoms of leishmaniasis in Sudan (Shamboul et al., 2009) and in apparently healthy ones in northwest Ethiopia (Kalayou et al., 2011). Infections in dogs with both *L. donovani* and *L. infantum* were reported in a village along the Albara River in eastern Sudan (Dereure et al., 2003). Few studies have investigated the role of animal reservoirs in maintaining *L.*

donovani in the Indian subcontinent. Recently, *Leishmania* amastigotes were detected in skin exudates of dogs in Sri Lanka (Nawaratna et al., 2009) and *Leishmania* DNA in cows, buffaloes, and goats in Nepal (Bhattarai et al., 2010). In Himachal Pradesh, India, anti-*Leishmania* antibodies were detected in 2 out of 31 dogs using the rK39 immunochromatographic test (ICT) (Sharma et al., 2009). Furthermore, *Phlebotomus argentipes*, the only known vector for *L. donovani* in the Indian subcontinent, is zoophilic, which supports the hypothesis of a zoonotic *L. donovani* transmission cycle.

In Bangladesh, the stray dog population is quite large, although the precise population size is unknown. These dogs typically live in or next to human houses, and thereby can contribute to the domestic transmission of major zoonotic diseases, including leishmaniasis. However, there is a lack of information about the importance of animals as a VL reservoir in Bangladesh. Recently, antibodies against the *Leishmania* parasite were detected in cattle from an endemic area of Bangladesh, but no parasitic DNA was detected by PCR (Alam et al., 2011). In our recent study, *Leishmania* DNA was detected in one stray dog from VL-endemic areas of Bangladesh (Alam et al., 2013). For further verification, we investigated additional stray dog samples from the same endemic areas and detected anti-*Leishmania* antibodies and *Leishmania* DNA, lending support to the hypothesis that dog is an animal reservoir for *Leishmania* parasites in the endemic area.

2. Materials and Methods

2.1. Sample collection and preparation

In May 2012, 50 stray dogs (IDs: BD1-39, 41-51; 30 males and 20 females) were captured in Trishal and Fulbaria Upazila (subdistricts) of the Mymensingh district in Bangladesh, which are two endemic areas for VL (Figure 1). Randomly captured dogs had no obvious clinical sings of leishmaniasis, but most were emaciated, with slight skin lesions.

From the saphenous/cephalic vein, 5 ml of venous blood was collected in tubes containing disodium ethylenediaminetetraacetate (Na_2EDTA). All tubes were immediately placed in a chilled ice box and stored until processing. The blood samples were centrifuged at 875× *g* for 10 min at 4°C. The plasma samples were stored at 4°C, and buffy coat samples were stored in lysis buffer for DNA extraction. Methods for stray dog capture and sample collection were approved by the Mymensingh Municipality Bureau and were described previously (Alam et al., 2013).

2.2. rK39 dipstick test

Of plasma samples, 20 µl was used for the rK39 ICT (Kalazar Detect™ Rapid Test, In Bios International, Inc., Seattle, WA, USA) according to the manufacturer's instructions. This test qualitatively detects anti-*Leishmania* circulating antibodies against a 39-amino-acid repeat that is conserved among viscerotropic *Leishmania* species (*L. donovani*, *L. infantum*, and *L. chagasi*) (Burn et al., 1993; Sharma et al., 2009). The presence of a red line in the test area indicated a positive result according to the manufacturer's instructions. Sera of uninfected dogs ($N = 3$) from a non-endemic region were tested as negative controls for the ICT test.

2.3. DNA extraction

DNA was extracted from 20 µl of blood buffy coat using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's instructions. Extracted DNA samples were kept at -20°C until further analysis.

2.4. ITS1-PCR assay

An internal transcribed spacer 1 (ITS1)-PCR assay was performed to amplify the ribosomal ITS1 region using the primers LITSR (5′-CTGGATCATTTTCCGATG-3′) and L5.8S (5′-TGATACCACTTATCGCACTT-3′) as previously described (Schönian et al.,

2003). The amplification conditions were as follows: initial heating at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 20 s, annealing at 53°C for 30 s, and extension at 72°C for 1 min, with a final extension step at 72°C for 6 min. PCR products were resolved by 2% agarose gel electrophoresis in 1×Tris-Borate-EDTA buffer at pH 8.0 and visualized under UV light after staining with RedSafe Nucleic Acid Staining Solution (iNtRON Biotechnology Inc., Sungnum, Korea). A positive control with *L. donovani* (strain MHOM/BD/2006/BD25) genomic DNA at 10 ng/µl and negative controls with DNA extracted from uninfected dogs (N = 3) from a non-endemic region and no-DNA (water) were included.

2.5. Real-time PCR

A quantitative real-time PCR assay based on the amplification of kinetoplast minicircle DNA (kDNA) was performed using the LightCycler® Nano system (Roche Diagnostics, Tokyo, Japan) with the primers RV1 (5′-CTTTTCTGGTCCTCCGGGTAGG-3′) and RV2 (5′-CCACCCGGCCCTATTTTACACCAA-3′) (Mary et al., 2004). The 20 µl reaction mixture contained 1×FastStart Essential DNA Green Master (Roche, Mannheim, Germany), 0.25 µM of each primer, and 2 µl of buffy coat DNA. The reaction conditions were as follows: initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The standard curve was established using *L. donovani* DNA extracted from 1.4×10^8 parasites from culture. Aliquots from serial dilutions (1 µl), ranging from 0.001 pg to 500 pg of parasite DNA, were added to the reaction tubes. The assay included negative controls with DNA of uninfected dogs from a non-endemic region (N = 3) and water.

2.6. Sequencing

The PCR products from the agarose gel were excised with a sterile gel cutter and purified using the NucleoSpin Extract II Kit (Clontech Laboratories Inc., MACHEREY-NAGEL, Düren, Germany). Sequencing reactions were performed with the BigDye v3.1 Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Direct cycle sequencing was performed using the ABI 310 Genetic Analyzer (Applied Biosystems). After generating a multiple alignment with a program BioEdit (Hall, 1999), the consensus sequences were compared with those in the NCBI database using BLASTn (Basic Local Alignment Search tool for Nucleotide). The obtained DDBJ/EMBL/GenBank accession number is LC123922.

2.7. *Statistical analyses*

A Fisher's exact test was used to determine statistical differences between the numbers of male and female dogs that were positive for *Leishmania* infection based on each of the three different diagnostic methods, i.e., rK39 ICT, ITS1-PCR, and real-time PCR (Table 1). Analyses were conducted using an online Fisher's exact test calculator (http://www.socscistatistics.com/tests/fisher/Default2.aspx). The level of agreement between the diagnostic techniques was evaluated by using kappa statistics with 95% confidence intervals (http://graphpad.com/quickcalcs/kappa1.cfm). Kappa values (*k*) of 0.20-0.60 indicate fair to moderate agreement and values of 0.60-0.80 indicate substantial agreement between observations (Altman, 2001).

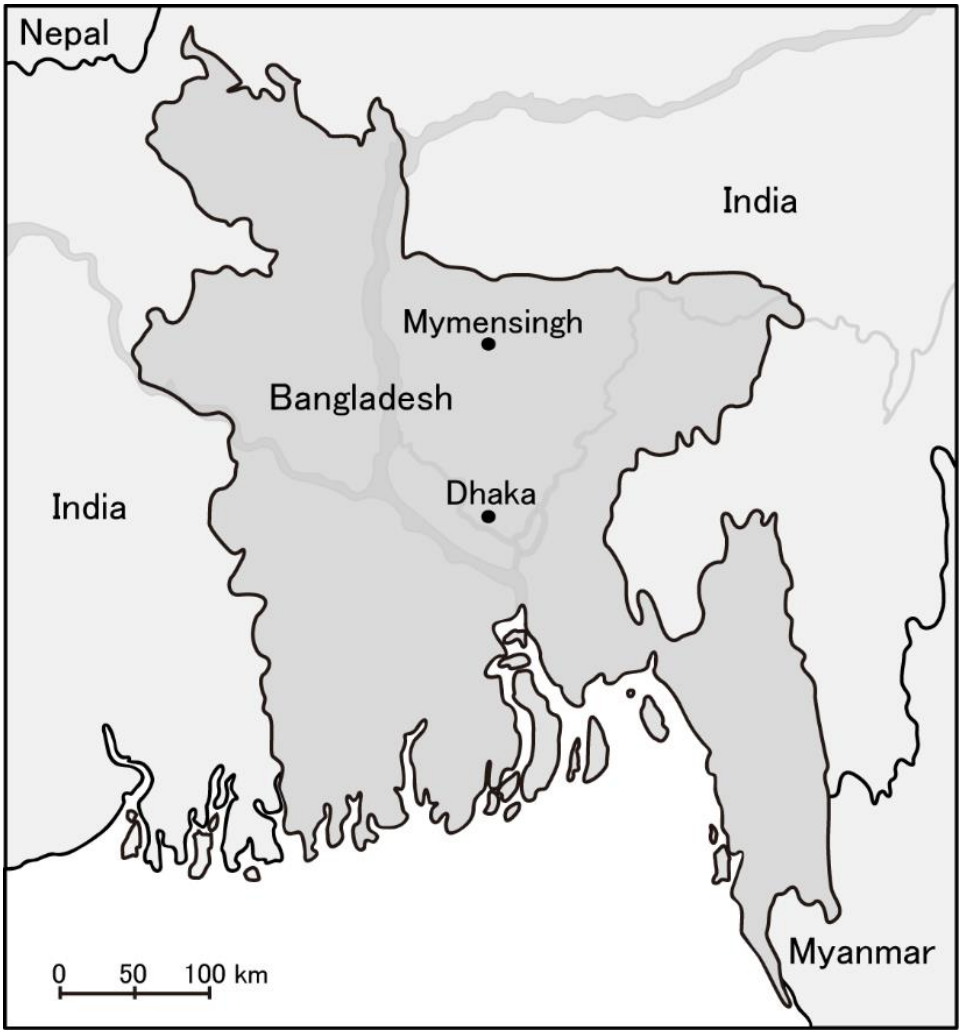

Figure 1. Map of Bangladesh.
Dog samples were collected in the Mymensingh district.

3. Results

3.1. rK39 dipstick test

Anti-*Leishmania* antibodies were detected in 6 out of 50 (12%) dog plasma samples. Moderately strong bands in the test line region were observed for four samples (for example, dog IDs; BD27 and BD28 in Figure 2, but faint bands in the rK39 dipstick test for two samples (data not shown). The ICT test showed negative results for the control dogs sera (N = 3) from a non-endemic region.

3.2. ITS1-PCR and sequencing results

Five (10%) out of 50 dog samples were positive for *Leishmania* DNA by ITS1-PCR (Figure 3). Sequencing analysis of the amplified products revealed 100% identity to *L. donovani* DNA sequences previously deposited in GenBank (accession nos. KT273408 and KR858307).

3.3. Real-time PCR

We obtained positive real-time PCR results for *Leishmania* kDNA amplification for 10 out of 50 (20%) dog samples. The concentrations of parasite DNA were 0.005–4.344 pg, equivalent to 0.02–21.72 parasites based on the typical amplification curves of control DNA (Figure 4).

3.4. Comparison of three diagnostic methods

Figure 5 shows a comparison of rK39 ICT, ITS1-PCR and real-time PCR results. Three dogs showed positive results by all the three diagnostic methods used in this study. Six dogs were serologically positive by rK39 ICT, in which *Leishmania* DNA could be detected by ITS1-PCR and/or real-time PCR in five dog samples. Of ten samples that were positive by real-time PCR, only five were positive based on ITS1-PCR. A moderate agreement was obtained between rK39 ICT and ITS1-PCR results (k = 0.50,

95% CI = 0.10-0.87) and between rK39 ICT and real-time PCR results (k = 0.56, 95% CI = 0.25-0.87). A substantial agreement was found between ITS1-PCR and real-time PCR results (k = 0.62, 95% CI = 0.32-0.91). There were no significant differences in infection rates between male and female dogs for any of the three diagnostic tests (Table 1).

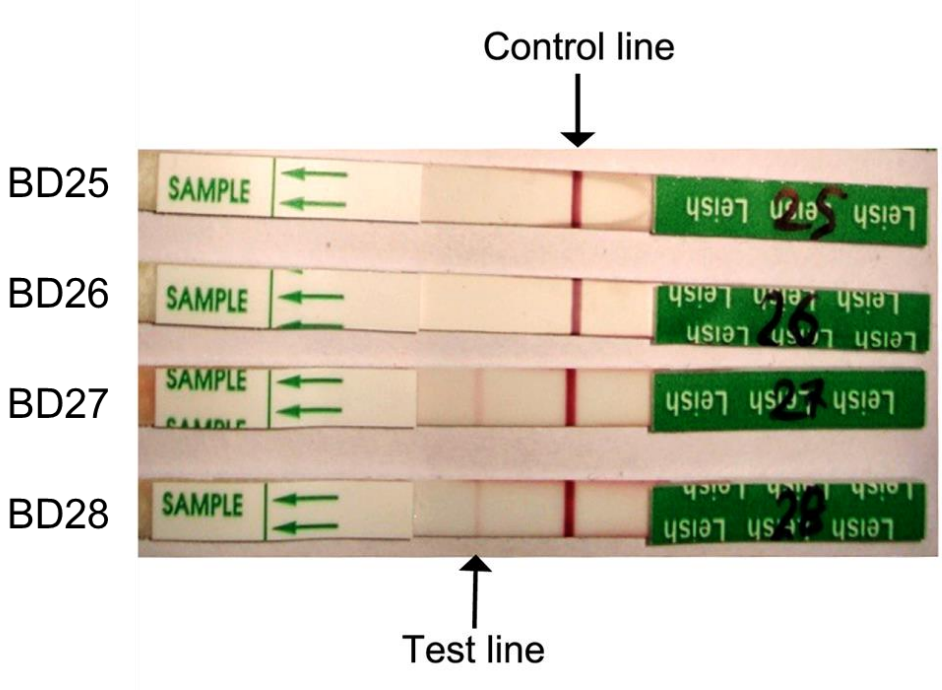

Figure 2. rK39 immunochromatographic strip test results.

Strips with only the control band (dog IDs; BD25 and BD26) represent negative results, while strips with both a control band and a positive test band (dog IDs; BD27 and BD28) reflect positive results.

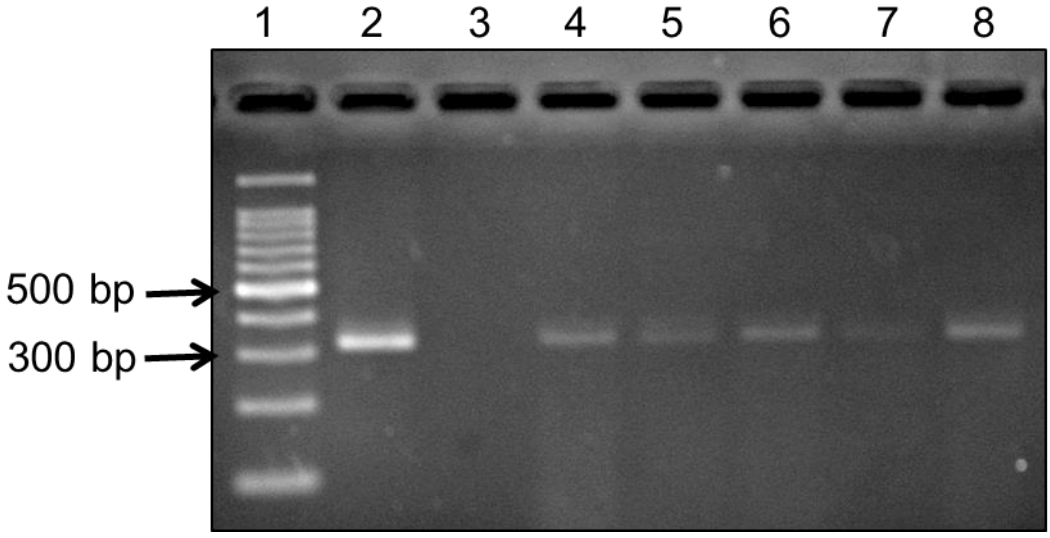

Figure 3. ITS1-PCR result.
Lane 1, 100 bp marker; lane 2, positive control; lane 3, negative control; lanes 4-8, positive samples.

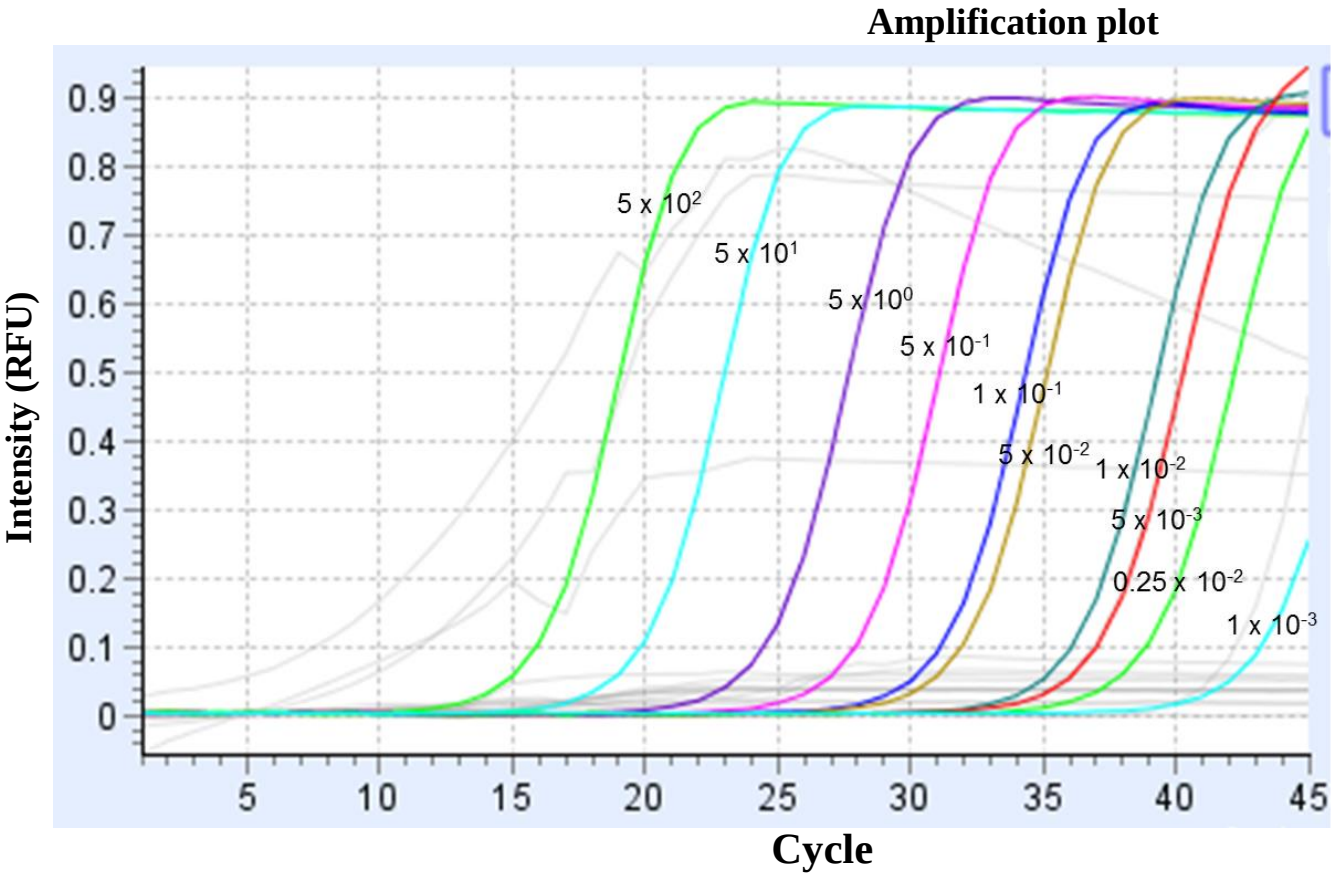

Figure 4. Typical amplification curves for standard *Leishmania* kDNA by real-time PCR using total *Leishmania* DNA ranging from 0.001 to 500 pg.

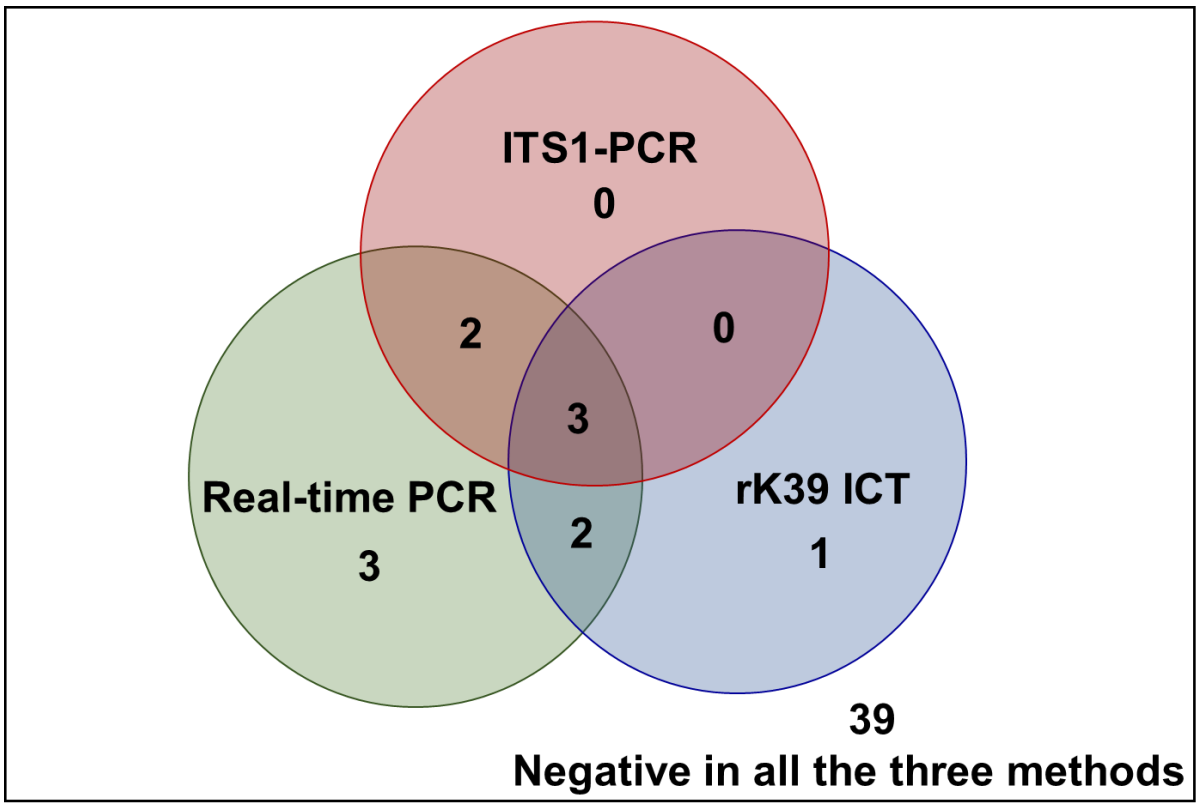

Figure 5. Comparison among the three diagnostic methods for detecting *Leishmania* infections in 50 stray dogs in Bangladesh.

Table 1. Infection rates for male and female dogs based on the three diagnostic methods.

Diagnostic methods	Positive dog samples (%)		
	Male	Female	*P*-value
rK39 ICT[a]	5 (16.7)	1 (5)	0.38
ITS1-PCR	4 (13.3)	1 (5)	0.63
Real-time PCR	8 (26.7)	2 (10)	0.28

[a]Immunochromatographic test.

4. Discussion

The presence of *Leishmania* infection in stray dogs were investigated and found evidence that dogs play a role in the maintenance of *Leishmania* parasites in the VL-endemic areas of Bangladesh. Knowledge of reservoir hosts and their potential role in disease transmission is a pre-requisite for understanding VL epidemiology and designing appropriate control strategies. Although VL in the Indian subcontinent is still thought to be anthroponotic, there is a good circumstantial evidence for a residual zoonotic reservoir. Disease emergence from stray dogs and other canids is of great concern, but the status of canine VL in Bangladesh is unclear.

In VL zoonotic foci, where dogs are the primary reservoir hosts, the disease is caused by *L. infantum* (Gavgani et al., 2002). However, there are also reports of canine infection with *L. donovani* in Sudan (Dereure et al., 2000; Shamboul et al., 2009), the causative agent of human VL in the Indian subcontinent and East Africa. It has been reported that the domestic dog may be an important reservoir host of *L. donovani* in eastern Sudan (Hassan et al., 2009). Some recent studies also reported reservoir hosts for *Leishmania* parasites other than dogs, such as red foxes in central Greece (Karayiannis et al., 2015), cats in the western provinces of Turkey (Pasa et al., 2015), and bats in Brazil (Oliveira et al., 2015). In India, *L. donovani* DNA was recently detected in goats (Singh et al., 2013).

The observations of anti-*Leishmania* antibodies and *Leishmania* DNA in blood samples obtained from stray dogs corroborate the findings of previous studies in Sri Lanka (Nawaratna et al., 2009), Sudan (Hassan et al., 2009), and India (Sharma et al., 2009). In Bangladesh, cattle that are seropositive for leishmaniasis have been found, but

there is no evidence of *Leishmania* DNA (Alam et al., 2011), suggesting that cattle do not play a role as reservoir hosts. In a recent study, *Leishmania* DNA was detected in a single (1.2%) dog among 85 stray dogs using DNA extracted from whole blood spotted on filter paper (Alam et al., 2013). In the present study, 20% and 10% of stray dogs were found to be positive based on real-time PCR and PCR using buffy coat DNA, respectively, despite sampling from the same VL-endemic foci. The higher positive rate in this study probably reflects a higher assay sensitivity using buffy coat DNA than whole-blood preparations, as demonstrated in previous studies (Lachaud et al., 2001; Sundor and Rai, 2002).

Some discrepancies were detected among the results of the three diagnostic methods used in this study. The highest positive rate (20%) was obtained using kDNA-based real-time PCR which is consistent with several previous studies showing that kDNA-based PCR is more sensitive than serological and ITS1-based PCR (Fallah et al., 2011; Mohammadiha et al., 2013). kDNA is considered the most sensitive target for leishmaniasis diagnosis, since it contains ~10,000 minicircles per parasite (Bensoussan et al., 2006). Samples that were positive based on PCR and/or real-time PCR, but negative based on rK39 ICT, might have a low infection burden and therefore lower levels of anti-*Leishmania* antibodies, consistent with previous studies (Hamarsheh et al., 2012; Wang et al., 2011), in which some seronegative dogs were PCR-positive. In this study, one serologically positive dog was negative for *Leishmania* DNA, this might be attributable to a past infection that was controlled via an immune response, as discussed elsewhere (Massunari et al., 2009). However, the possibility of false positive results of each diagnostic test should also be considered which might have led to the discrepancies among the diagnosing tests. For example, 3.6% (1/28) and 10.7% (3/28) of dogs from *Leishmania* non-endemic areas were positive by real-time PCR and ITS-based PCR,

respectively (Mohammadiha et al., 2013). The specificity of rK39 ICT with sera of dogs from non-endemic regions ranged from 94 to 100% according to some previous studies and a few false positive reactions were also reported in dogs infected with *Ehrlichia canis*, *Trypanosoma cruzi* or *Neospora caninum* (Lemos et al., 2008; Mettler et al., 2005; Paltrinieri et al., 2010).

It is important to isolate viable *Leishmania* from naturally exposed animals to clarify their role in the maintenance and transmission of VL. After a *Leishmania*-infected sand fly bites a mammalian host, promastigotes (flagellated forms) are phagocytized by dermal macrophages and transformed into round-shaped amastigotes, which replicate in macrophages, leading to cell destruction and the progressive infection of more phagocytes (Chance and Evans, 1999). Once an infection is established, *Leishmania* tends to localize in all tissues in which monocytic-macrophagic cells reside in high numbers, such as the liver, spleen, lymph nodes, bone marrow, gastrointestinal tract, and skin (Paltrinieri et al., 2010). Several strains of *L. donovani*, *L. infantum*, and *L. archibaldi* were isolated by culture lymph nodes of dog samples in eastern Sudan (Dereure et al., 2003). In the United States and Canada, *L. infantum* zymodeme MON1 was isolated from tissue specimen cultures of dogs (Duprey et al., 2006). As part of a preliminary study, it was attempted to detect *Leishmania* amastigotes in the spleen, liver, and lymph nodes of serologically positive dogs; however, the parasites were not observed in the hematoxylin/eosin-stained tissue sections (data not shown), probably owing to the low number of parasites in the reservoir host. Further studies with an increased sample size are required to demonstrate the existence of parasites in tissue specimens with more sensitive and specific tools and to isolate viable *Leishmania* from naturally exposed dogs.

In the present study, a higher infection rate in male dogs were observed than in

females, in agreement with the results of previous studies conducted in Iran (Haddadzade et al., 2013; Mohebali et al., 2005). Traditionally, canine leishmaniasis is transmitted directly from sand flies to dogs, but dog to dog transmission of *L. infantum* via direct contact with blood and secretions was recently detected in the United States and Canada (Duprey et al., 2006). The possible interaction between dogs and sand flies is an important issue with respect to the transmission of VL to humans. New and Old World sand fly species have varying degrees of host preferences and hence are opportunistic feeders (Lane et al., 1990; Quinnell et al., 1992). In eastern Sudan, *P. orientalis* and other sand flies are more attracted to dogs than to the mongoose, genet, and Nile rat (Hassan et al., 2009). Although there is a lack of information about the host preference of *P. argentipes*, the only known vector of *L. donovani* in Bangladesh, the feeding behavior of *P. argentipes* is mainly zoophilic (Palit et al., 2005) and animals act as the preferred blood meal source (Mukhopadhyay and Chakravarty, 1987). Hence, further studies were recommended to examine the host preferences of *P. argentipes* to dogs and other animals in the study area.

In conclusion, the present study confirmed the presence of anti-rK39 antibodies and *Leishmania* DNA in several stray dogs in the VL-endemic areas of Bangladesh. Although the number of animals examined was not adequate to incriminate dogs as a reservoir, the findings of this study imply that dogs are probable animal reservoirs for VL transmission in this endemic focus. However, detailed analyses of *Leishmania* infection in dogs and the ability of dogs to transmit the parasite to the vector sand fly in nature are needed to reveal the potential role of dogs in VL epidemiology in Bangladesh.

Summary

Visceral leishmaniasis (VL), or kala-azar, is mainly caused by two closely related *Leishmania* species, *Leishmania infantum* and *L. donovani*. *L. infantum* is responsible for zoonotic VL, with dogs as the main reservoir host in the Mediterranean, the Middle East, Asia, and South America. In the Indian subcontinent, VL is caused by *L. donovani* and is considered anthroponotic, although the only known vector, the sand fly, is zoophilic in nature. The role of domestic and stray dogs in VL transmission is still unclear in this area. In the present study, 50 stray dogs captured in VL-endemic areas of Bangladesh were screened for serological and molecular evidence of *Leishmania* infection. Anti-*Leishmania* antibodies were detected in six (12%) dog serum samples using rK39 immunochromatographic tests. Ten (20%) buffy coat DNA samples were found to be positive for *Leishmania* kinetoplast DNA by real-time PCR, five of which were positive based on internal transcribed spacer 1-PCR. Based on a sequencing analysis of the amplified products, the parasitic DNA was derived from *L. donovani*. The findings support the hypothesis that stray dogs are an animal reservoir for *L. donovani* in this endemic region. Further studies are required to determine the precise role of dogs in the epidemiology of VL in Bangladesh.

Chapter II

Potential of cell-free DNA as a novel diagnostic biomarker for parasite infections in dog

1. Introduction

Stray dogs maintain a close contact with humans and roam freely without or very low human care. In general, parasitic infestations in these dogs are very common and they have been sources of many zoonotic parasites of potential public health risk. For examples, stray dogs were suspected to contribute to the spread and increase of zoonotic visceral leishmaniasis caused by *Leishmania infantum* in urban areas of Portugal (Cortes et al., 2007). It is also documented that stray dogs maintain a permanent infection pressure of helminthic zoonotic agents including *Toxocara canis* and *Echinococcus* spp. towards the domestic dog population (Deplazes et al., 2011).

The information on the prevalence and diversity of parasites in dog is lacking especially in developing and low-income countries. Since stray dogs serve as sentinels of parasitic agents (Cabezón et al., 2010), assessment of wide range of parasites carried by dogs may lead to detection of new or unexpected parasitic agents which may have zoonotic importance and pose public health risks. However, such research has been limited due to the following technical reasons. Traditional approaches depending on microscopic observation are time consuming and labour-intensive and have low sensitivity. Conventional molecular-based methods such as PCR and real-time PCR require specific primers and thus are not suited to detect new or unexpected pathogens.

Cell-free DNA (cfDNA) comprises fragments of nucleic acids that have been liberated from cells and exist in the bloodstream, urine and other body fluids (Kato-Hayashi et al, 2015). The concept of detecting cfDNA is relatively new and is being explored for the non-invasive diagnosis of a variety of clinical settings. cfDNA has been studied as a routine and efficient biomarker, particularly in the field of oncology and prenatal diagnosis (Wagner, 2012; Yu et al., 2014). There are several studies analyzing

cfDNA as a diagnostic marker for human parasitic infections. Parasite-derived cfDNA has been detected in serum in cases of *Entamoeba histocytica* (Khairnar and Parija, 2008), *Plasmodium* spp. (Ghayour et al, 2014), *Schistosoma* spp. (Hussein et al., 2012; Kato-Hayashi et al., 2013; Kato-Hayashi et al., 2015), *Wuchereria bancrofti* (Ximenes et al., 2014), *Dirofilaria immitis* (Oi et al., 2015), and in urine in case of *Leishmania* (Veland et al., 2011) using conventional PCR-based methods.

Along with the recent progress of high-throughput sequencing techniques, it becomes feasible to detect cfDNA in a high sensitive manner (Chiu et al., 2008). In fact, identification of infectious microorganisms (viruses, bacteria and fungi) from cfDNA in plasma of human patients was successfully achieved by Illumina sequencing technology (Grumaz et al., 2016). The present study was therefore designed to evaluate the cfDNA as a potential diagnostic target for screening the parasite infections in dogs by a metagenomic approach. Plasma samples obtained from stray dogs in Bangladesh were employed, from which multiple pathogens were detected by PCR methods in the previous studies (Akter et al., 2016; Qiu et al., 2016; Terao et al., 2015). The results indicated that cfDNA could be a useful marker to detect parasite infections in dogs when employing high-throughput sequencing technologies followed by proper bioinformatics analysis and interpretation of the resulting data.

2. Materials and Methods

2.1. Samples

Fourteen plasma samples collected from stray dogs captured in visceral leishmaniasis (VL)-endemic areas of Bangladesh were employed. Previous studies conducted by our research group showed that some of the dogs were infected with several pathogens such as *Babesia gibsoni* (Terao et al., 2015), Anaplasmataceae (Qiu et al., 2016) and *Leishmania*

donovani (Akter et al., 2016) using conventional molecular methods. The details of the DNA extraction methods and PCR/real-time PCR results are shown in Table 2.

2.2. Extraction of cfDNA

Dog plasma samples stored at -80°C were thawed and immediately cfDNA was extracted from 0.6 to 1.6 ml of plasma using MagMAX™ Cell-Free DNA Isolation kit (Thermo Fisher scientific, Waltham, MA, USA) according to the manufacturer's protocol for the isolation of higher concentration of cfDNA. Briefly, the plasma samples were centrifuged at 6,000 × *g* for 30 minutes to remove any residual blood and cell debris. The resultant supernatants were mixed with MagMAX™ Cell-Free DNA Lysis/Binding solution and cfDNA was bounded to the MagMAX™ Cell-Free DNA Magnetic Beads. The beads were washed with MagMAX™ Cell-Free DNA Wash Solution and 80% ethanol. After a first elution with 400 µl of 0.1×TAE, the cfDNA was again rebounded with MagMAX™ Cell-Free DNA Magnetic Beads and washed with MagMAX™ Cell-Free DNA Wash Solution. Finally, cfDNA was eluted in 10 µl of the MagMAX™ Cell-Free DNA Elution Solution and stored at -20°C prior to further analysis.

2.3. Quantification of cfDNA

The quality and quantity of cfDNA were assessed with Agilent High Sensitivity DNA Analysis kit on Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). The total cfDNA yield was measured using the Qubit dsDNA HS Assay Kit with the Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.4. High-throughput sequencing

The quantified cfDNA was used to construct Illumina sequencing libraries using the TruSeq DNA LT Sample Prep Kit (Illumina, San Diego, CA, USA) following the manufacturer's instructions with some modifications. The fragmentation step was omitted

because the average size of cfDNA fragments was around 160 bp. For selective amplification of the libraries, the number of PCR cycles were increased to 15. Each sample was processed using different index sequences during adaptor ligation step. The final libraries were quantified using the KAPA Library Quantification Kit for Illumina platforms (KAPA Biosystmes, Wilmington, MA, USA) on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). To increase diversity of the libraries, nonindexed PhiX174 control library (Illumina) was used at a relative concentration of 5%. The final library pool was sequenced on Illumina MiSeq system using the Illumina MiSeq Reagent Kit v3 (150 cycles) with a single-end run. The reads that passed Illumina quality control filtering were used as raw data for further bioinformatics analysis.

2.5. Data analysis

The demultiplexed FASTQ files generated by MiSeq Reporter software (Illumina) were imported into CLC Genomics Workbench v 9.0 (Qiagen, Hilden, Germany). Low-quality sequences were removed prior to the analysis with default settings. The resultant reads were first mapped to the dog (*Canis lupus familiaris*) reference genome sequences (GenBank accession nos. NC_006583.3 to NC_006621.3 and NC_002008.4) to filter out the sequences possibly derived from the host. The unmapped reads were initially screened for homology to known parasites by local BLASTn searches against the NCBI NCBI nt (non-redundant nucleotide sequences) database (E-value < 0.0001) and the reads having a best hit with parasite sequences were extracted. These potentially parasite-derived sequences were further evaluated using the following criteria: 1) Read length is more than 50 bp; 2) second and third best BLAST hits, when available, were from the same taxonomic group of parasites. Only the reads fulfilling these two criteria were finally

retained as potential parasite-derived sequences. A schematic workflow for data processing is shown in Figure 6. Tandem Repeat Finder program version 4.09 (Benson, 1999) was used to screen for repetitive sequence motifs in parasite-derived reads.

Table 2. Dog plasma samples used for the present study.

	Previous PCR or real-time PCR results		
Dog ID	*Babesia gibsoni* [a]	Anaplasmataceae[a]	*Leishmania* [b]
BD27	-	-	+
BD28	-	-	+
BD30	+	-	-
BD32	-	+	+
BD34	-	-	-
BD36	-	+	-
BD37	+	-	-
BD39	+	+	+
BD43	-	-	-
BD45	-	+	+
BD46	-	-	-
BD47	-	-	-
BD49	+	-	-
BD51	-	+	-

[a]Screened by PCR using DNA extracted from whole blood spotted on FTA® Elute Micro cards (Qiu et al., 2016; Terao et al., 2015).

[b]Screened by real-time PCR using DNA extracted from blood buffy coat (Akter et al., 2016).

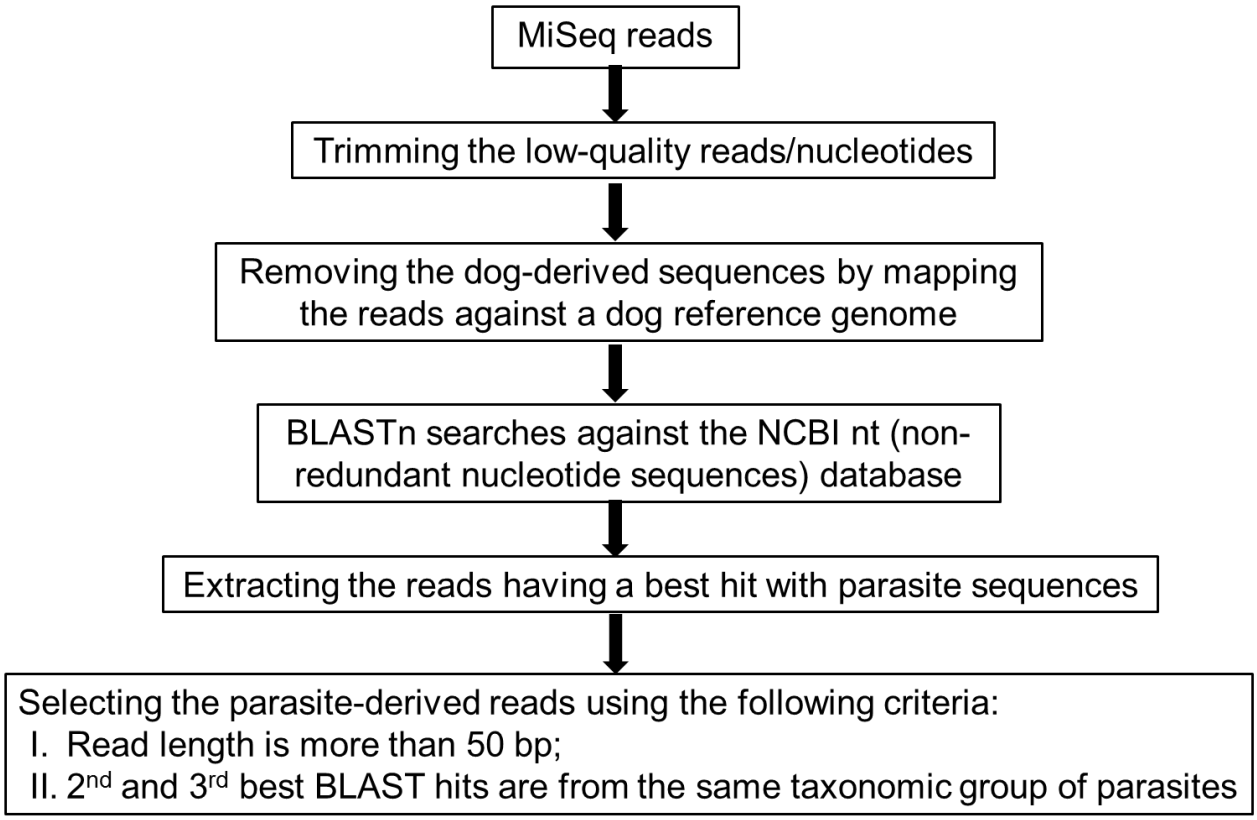

Figure 6. A schematic workflow of data analysis.

3. Results

3.1. Isolation of cfDNA

The concentration of cfDNA isolated from the plasma of 14 dogs ranged from 0.12 to 1.08 ng/µl. The presence of cfDNA approximately 160 bp in length was confirmed on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) (Figure 7).

3.2. Illumina sequencing and data trimming

A total of 32,726,663 reads with an average of 2.3 million reads per sample were generated from two independent MiSeq runs (Table 3). Trimming and removal of low-quality reads/nucleotides yielded 32,713,286 reads with an average length of 148.1 bp. The vast majority (97.8%) of these reads mapped to the dog reference genome. The remaining unmapped reads (*N* = 727,391) were used for the downstream analyses.

3.3. BLASTn analysis

Out of 727,391 reads used for BLASTn analysis, 467,936 (64.3%) had BLASTn hits with an E-value less than 0.0001 (Table 3). The plurality of the reads (90,006; 19.2%) showed the highest similarity with bacterial sequences (Figure 8), whereas 8.2% exhibited homology with canid sequences. Reads derived from nonindexed PhiX control were found in all samples (6,127-23,241) with an average of 10,329 (Table 4). In this initial screening, 11,639 and 4 reads had the highest similarity to parasites (39 genera) and Anaplasmataceae, respectively (Figure 8). After considering the read length and second and third best BLAST hits as described in Methods, 150 and 4 reads were ultimately assigned to parasite (19 genera) and Anaplasmataceae origins, respectively (Table 5). The top 5 parasite genera/species found in the present analysis were *Protopolystoma xenopodis* (*N* = 61), *Diphyllobothrium latum* (*N* = 14), *Babesia* species (*N* = 11), *W. bancrofti* (*N* = 9), and *Thelazia callipaeda* (*N* = 9). The number of reads

associated with *Leishmania* species was 4. Four reads determined as Anaplasmataceae origin were all associated with *Wolbachia* species. Among the total 150 parasite-derived reads, repetitive sequence motifs were found in 70 (Table 5).

3.4. Annotation of the sequences

Table 6 shows the details of the sequences with the highest similarity to*Leishmania*, *Babesia*, and *Wolbachia*. No reads from *Leishmania* and *Wolbachia* were assigned to any known genes, either because the sequences were non-coding or because the closest regions of the genome have not been annotated in the NCBI database. Out of 11 reads from *Babesia*, eight reads showed similarity with known genes, including six different protein coding genes and *B. gibsoni* 18S ribosomal RNA and *Babesia bigemina* 28S ribosomal RNA genes. Tandem repeat (TR) motifs were found in three reads associated with *Leishmania* (Table 6).

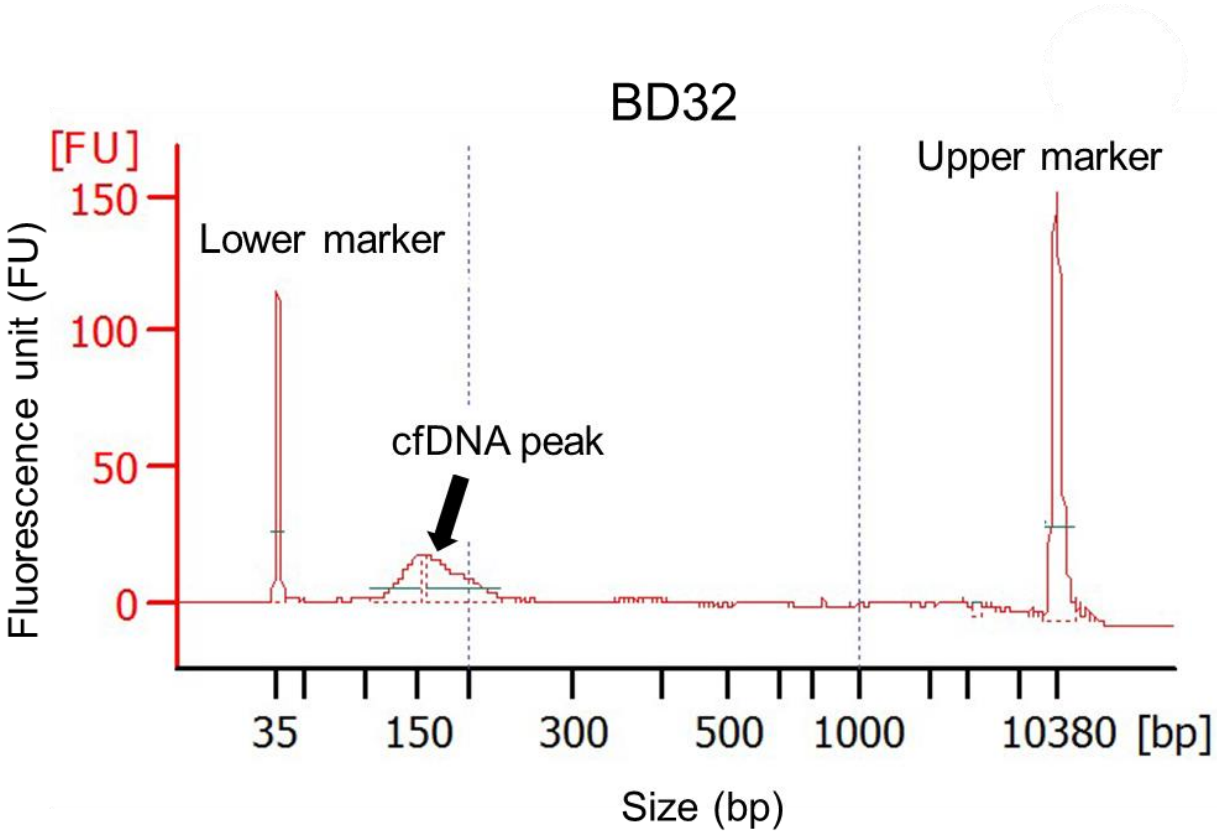

Figure 7. Representative example of cfDNA size distribution, as determined on an Agilent 2100 Bioanalyzer.

Table 3. Summary of Illumina sequencing and BLASTn searches.

Dog ID	Raw reads (Average length (bp))	Reads after trimming (Average length (bp))	Reads mapped to dog genome (%)	Reads not mapped to dog genome (%)	Reads with BLAST hits (%)
BD27	2,823,389 (150.1)	2,822,322 (149.0)	2,757,689 (97.7)	64,633 (2.2)	43,644 (67.5)
BD28	2,371,450 (147.7)	2,370,571 (146.8)	2,327,776 (98.1)	42,795 (1.8)	25,969 (60.6)
BD30	1,950,030 (150.4)	1,949,256 (149.4)	1,906,736 (97.8)	42,520 (2.1)	25,721 (60.4)
BD32	2,270,819 (146.3)	2,269,783 (145.3)	2,223,559 (97.9)	46,224 (2.0)	29,101 (62.9)
BD34	1,919,030 (150.3)	1,918,248 (149.4)	1,885,566 (98.2)	32,682 (1.7)	18,037 (55.1)
BD36	2,117,187 (146.8)	2,116,260 (146.0)	2,082,493 (98.4)	33,767 (1.5)	18,041 (53.4)
BD37	3,178,233 (150.8)	3,176,927 (149.9)	3,116,026 (98.0)	60,901 (1.9)	37,799 (62.0)
BD39	1,885,173 (149.6)	1,884,436 (147.0)	1,796,293 (95.3)	88,143 (4.6)	73,438 (83.3)
BD43	2,053,408 (142.8)	2,052,446 (141.8)	1,987,051 (96.8)	65,395 (3.1)	36,557 (55.9)
BD45	1,969,077 (148.3)	1,968,316 (147.3)	1,927,735 (97.9)	40,581 (2.0)	24,927 (61.4)
BD46	2,584,650 (154.1)	2,583,544 (153.2)	2,541,052 (98.3)	42,492 (1.6)	23,687 (55.7)
BD47	2,378,463 (150.6)	2,377,497 (149.8)	2,335,106 (98.2)	42,391 (1.7)	26,782 (63.1)
BD49	1,994,576 (152.1)	1,993,790 (150.9)	1,952,504 (97.9)	41,286 (2.0)	27,244 (65.9)
BD51	3,231,178 (147.7)	3,229,890 (146.7)	3,146,309 (97.4)	83,581 (2.5)	56,989 (68.1)
Total	32,726,663 (149.2)	32,713,286 (148.1)	31,985,895 (97.8)	727,391 (2.2)	467,936 (64.3)

Table 4. Initial classification based on the best BLAST hit.

	Dog ID													
	BD27	BD28	BD30	BD32	BD34	BD36	BD37	BD39	BD43	BD45	BD46	BD47	BD49	BD51
Parasites	74	210	51	35	43	26	67	68	697	9,897	147	80	137	107
Anaplasmataceae	0	0	0	0	0	0	0	0	1	0	0	0	3	0
Canids	3,601	3,050	2,746	3,790	2,626	2,917	2,828	2,292	1,675	2,521	2,202	2,004	1,575	4,397
Bacteria	8,102	6,208	5,712	4,378	3,958	3,725	8,696	5,009	10,322	3,866	5,402	6,320	4,462	13,846
PhiX174	14,129	11,112	6,685	7,727	6,205	6,360	15,536	7,421	9,837	6,127	9,137	13,751	7,336	23,241
Viruses	10	12	22	11	8	9	15	6	9	8	21	18	17	41
Undetermined	17,728	5,377	10,505	13,160	5,197	5,004	10,657	58,642	14,016	2,508	6,778	4,609	13,714	15,357
Total	43,644	25,969	25,721	29,101	18,037	18,041	37,799	73,438	36,557	24,927	23,687	26,782	27,244	56,989

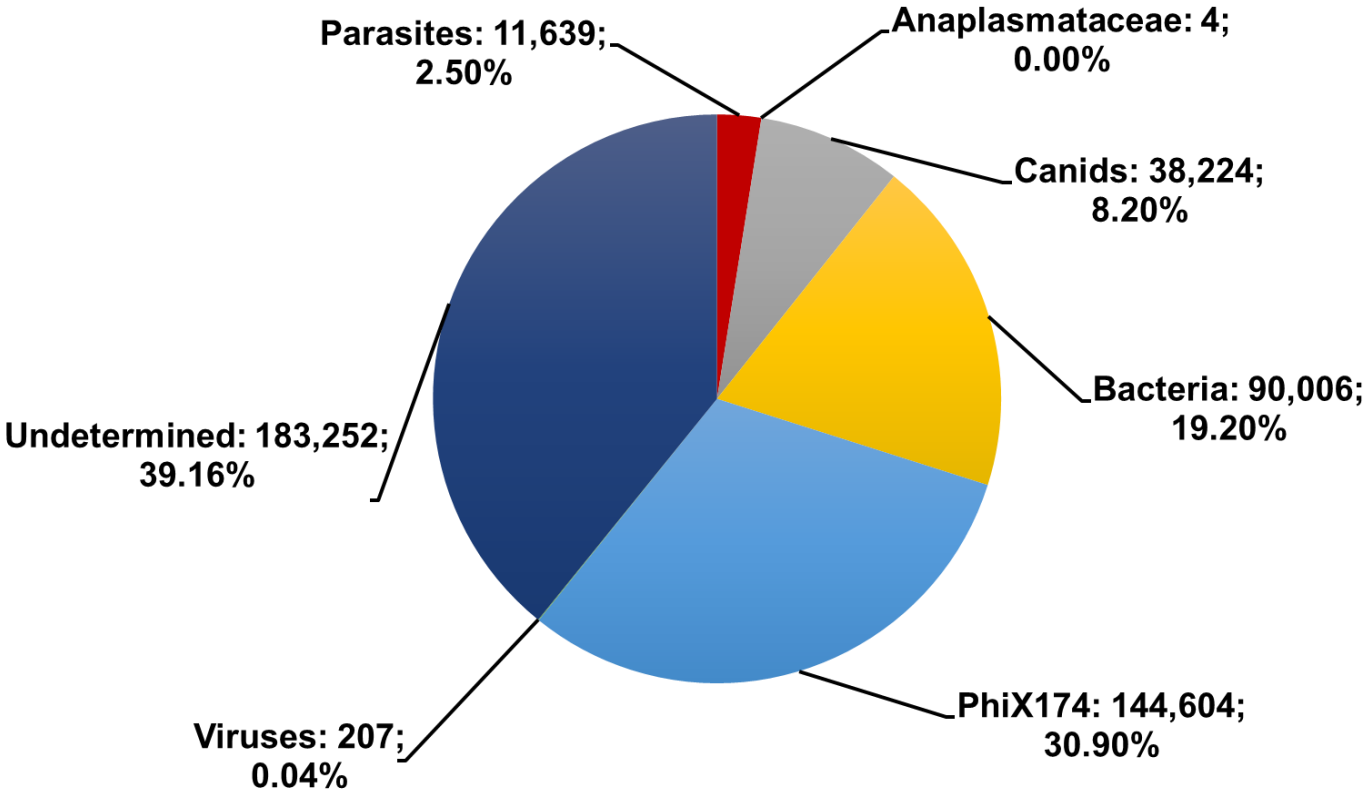

Figure 8. Distribution of top hit species of BLASTn searches.

Table 5. Detection of reads associated with parasites and Anaplasmataceae.

		Dog ID													
Phylum/Class/Order	Genus/Species	BD27	BD28	BD30	BD32	BD34	BD36	BD37	BD39	BD43	BD45	BD46	BD47	BD49	BD51	Total
Nematoda, Secernentea, Spirurida	*Wuchereria bancrofti*	2 (1)		2 (2)			1 (1)	3 (0)	1 (1)							9 (5)
Nematoda, Secernentea, Spirurida	*Thelazia callipaeda*		3 (1)		1 (0)		1 (1)	1 (0)	1 (0)	2 (1)						9 (3)
Nematoda, Secernentea, Spirurida	*Dirofilaria immitis*							2 (0)		1 (0)				2 (0)		5 (0)
Nematoda, Secernentea, Spirurida	*Brugia timori*							2 (0)						0		3 (0)
Nematoda, Secernentea, Spirurida	*Elaeophora elaphi*							1 (0)		2 (0)						3 (0)
Nematoda, Secernentea, Spirurida	*Loa loa*							1 (0)								1 (0)
Nematoda, Secernentea, Ascaridida	*Parascaris equorum*	1 (0)		1 (0)	1 (0)					1 (0)	1 (0)				3 (0)	8 (0)
Nematoda, Secernentea, Ascaridida	*Toxocara canis*				1 (1)											1 (1)
Nematoda, Secernentea, Rhabditia	*Parastrongyloides trichosuri*									2 (0)		1 (0)				3 (0)
Platyhelminthes, Cestoda, Pseudophyllidea	*Diphyllobothrium latum*	3 (1)	3 (3)					4 (3)	1 (1)			1 (0)			2 (1)	14 (9)
Platyhelminthes, Cestoda, Pseudophyllidea	*Spirometra erinaceieuropaei*						1 (0)	1 (0)		3 (1)				1 (1)	1 (0)	7 (2)
Platyhelminthes, Trematoda, Strigeidida	*Schistosoma* spp.					3 (0)			1 (0)				1 (0)	1 (0)		6 (0)
Platyhelminthes, Trematoda, Echinostomida	*Echinostoma caproni*							1 (0)								1 (0)
Platyhelminthes, Trematoda, Opisthorchiida	*Opisthorchis viverrini*									1 (0)						1 (0)
Platyhelminthes, Monogenea	*Protopolystoma xenopodis*	6 (5)	4 (4)	7 (5)	3 (3)	6 (3)	2 (2)	6 (4)	2 (2)	6 (3)	4 (3)	3 (3)	4 (4)	3 (3)	5 (2)	61 (46)
Apicomplexa, Aconoidasida, Piroplasmida	*Babesia* spp.			3 (0)				3 (0)	1 (0)					3 (0)	1 (0)	11 (0)
Apicomplexa, Aconoidasida, Piroplasmida	*Theileria* spp.							1 (0)								1 (0)
Euglenozoa, Kinetoplastida, Trypanosomatida	*Leishmania* spp.				1 (1)		1 (0)			2 (2)						4 (3)
Euglenozoa, Kinetoplastida, Trypanosomatida	*Trypanosoma* spp.			1 (0)					1 (1)							2 (1)
Proteobacteria, Alphaproteobacteria, Rickettsiales	*Wolbachia* spp.									1 (0)				3 (0)		4 (0)

The numbers in parentheses refer to the sequence number with repetitive sequences.

Table 6. Annotation of the reads with similarity with genera of *Leishmania*, *Babesia*, and *Wolbachia*.

Genus	Sequence ID	Length (bp)	Annotation	Tandem repeat
Leishmania	Leishmania1_32	149	Not available	6 bp x 9.7 repeats
	Leishmania2_36	77	Not available	Not detected
	Leishmania3_43	80	Not available	18 bp x 4.2 repeats
	Leishmania4_43	133	Not available	6 bp x 17.7 repeats
Babesia	Babesia1_30	79	Not available	Not detected
	Babesia2_30	147	*Babesia gibsoni* BgP32 gene	Not detected
	Babesia3_30	131	*Babesia gibsoni* small GTP binding protein rab11	Not detected
	Babesia4_37	169	Not available	Not detected
	Babesia5_37	92	Not available	Not detected
	Babesia6_37	151	*Babesia gibsoni* 18S ribosomal RNA gene	Not detected
	Babesia7_39	145	*Babesia bigemina* translation elongation factor gene	Not detected
	Babesia8_49	116	*Babesia bovis* histone 2B protein gene	Not detected
	Babesia9_49	72	*Babesia gibsoni* BgSA1 secretary antigen 1 gene	Not detected
	Babesia10_49	151	*Babesia gibsoni* thrombospondin-related anonymous protein (TRAP) gene	Not detected
	Babesia11_51	61	*Babesia bigemina* 28S ribosomal RNA gene	Not detected
Wolbachia	Wolbachia1_43	140	Not available	Not detected
	Wolbachia2_49	114	Not available	Not detected
	Wolbachia3_49	161	Not available	Not detected
	Wolbachia4_49	169	Not available	Not detected

4. Discussion

Parasitic diseases present a significant burden for public health, particularly in poor and marginalized communities. Currently, there is an extreme lack of information on the parasite diversity in stray dogs in Bangladesh. To the best of found knowledge, no previous study has focused on the potential of using cfDNA in conjunction with high-throughput sequencing techniques as a diagnostic marker for identifying parasite diversity in dogs.

Four sequences showing high similarity with *Leishmania* were detected in three samples (BD32, BD36 and BD43) (Table 5). As demonstrated in Chapter I, the dogs included in this analysis were screened for the infection with *L. donovani* using DNA extracted from blood buffy coat. Among the 14 dogs, five dogs (BD27, BD28, BD32, BD39, BD45) were positive by a quantitative real-time PCR assay based on kinetoplast minicircle DNA (kDNA). Only one dog (BD32) was scored as positive by both approaches. This fact may imply that the presence of cfDNA in the plasma does not represent the infection status of the parasites in blood circulating macrophages. Considering that the tested dogs, including ones positive by kDNA real-time PCR, had no obvious clinical sings of leishmaniasis, the infection was in the asymptomatic phase and the parasites resided in the tissues.

TR motifs were detected in three sequences associated with *Leishmania* (Table 6). The repeat sequences of the read IDs Leishmania1_32 and Leishmania4_43 were respectively found in 24 and 12 different chromosomes of the complete genome of *Leishmania mexicana* MHOM/GT/2001/U1103 strain (GenBank accession nos. NC_018305.1 to NC_018338.1), with which both reads showed the highest similarity in BLASTn analysis (data not shown). These two repetitive motifs were not found in the

reference genome sequence of *L. donovani*. (GenBank accession nos. NC_018228.1 to NC_018263.1).Like other protozoan parasites, *Leishmania* spp. contain a number of repetitive sequences in their genomes. This characteristic has been exploited by several diagnostic tools. For example, rK39, the most dependable antigen using for serodiagnosis of VL, is based on the protein encoded by TR sequences (Burns et al., 1993). Other candidate TR proteins have been proposed for serodiagnosis of leishmaniasis (Bhatia et al., 1999; Dillon et al., 1995; Goto et al., 2006). Theoretically, the chance of the detection of target sequence is related to the number of copies in the parasite genome. Therefore, cfDNA of repetitive sequences could be an especially suitable target for the molecular detection of *Leshmania* parasites.

Parasite sequences associated with *Babesia*, an intraerythrocytic protozoan parasite, were detected in five dogs (BD30, BD37, BD39, BD49, and BD51) (Table 5). This result agreed well with the result of previous study shown that four of the dogs (BD30, BD37, BD39, and BD49) were positive for *B. gibsoni* as determined by conventional PCR using DNA extracted from whole blood spotted on FTA Elute cards (Table 2). In contrast, four sequences associated with *Wolbachia* in the family Anaplasmataceae were found in two dogs (BD43 and BD49), whereas none of these were scored as positive by conventional PCR using DNA extracted from the FTA cards (Table 2). Three out of four *Wolbachia* sequences exibited the highest identities with the *Wolbachia* symbionts of *Onchocerca* parasites, indicating that the *Wolbachia* sequences were likely to originate from filarial nematodes. The difference between the results of two analytic methods might be partially explained by the difference of parasite localization within dogs. In addition, the differences in DNA extraction material (whole blood spotted on FTA Elute cards vs plasma), sample volume (several µl vs 0.6 to 1.6 ml), and the detection limit of each assay

should be taken into consideration.

Sequences related to a variety of filarial nematodes in order Spirurid, including *W. bancrofti*, *Dirofilaria immitis*, *Brugia timori*, *Elaeophora elaphi*, and *Loa loa* were obtained (Table 5). *D. immitis* is a common filarial parasite in dogs and the sample BD43 was infected with *D. immitis* by conventional PCR and sequencing analysis of the mitochondrial cytochrome oxidase I gene using DNA extracted from FTA Elute card (data not shown). On the other hands, three species, namely *W. bancrofti*, *B. timori*, and *L. loa*, are human filarial parasites (Metzger and Mordmüller, 2014; Small et al., 2014) and *E. elaphi* has only been reported as a parasite in red deer (*Cervus elaphus*) in Spain (Hernández et al., 1986). These facts collectively indicated that the sequences with the highest similarity with human and deer filarial parasites might have originated from *D. immitis*, but been misclassified. Since cfDNA is in general very short, it might not be suited for classification to lower taxonomic levels such as the genus and species.

One of the most significant technical problems of high-throughput sequencing technologies is the possible contamination between samples and from the environment. False positive results can be produced through even small level of cross-sample contamination (Cibulskis et al., 2011). There is also a chance of low-level carry-over contamination of samples from a previous sequencing run into the subsequent sequencing run (Illumina, 2013). In addition, it is challenging for researchers to work with small quantities of samples that may be swamped by the contaminating DNA, potentially resulting in false or misleading results (Salter et al., 2014; Strong et al., 2014). Another problem can occur in multiplexing different samples in the same sequencing run. In the present study, sequencing libraries of each sample were prepared using company-provided adaptors which include different index sequences. Based on the difference in the

index sequences, MiSeq Reporter software generated a set of data for each sample. However, the resultant data included the sequences originating from PhiX control library to certain extent despite that it does not have index sequence (Table 4). Therefore, it is necessary to acknowledge the limitations of current high-throughput sequencing technologies when interpreting the resultant data.

The present analysis detected sequences showing high similarity with 19 different parasitic genera (Table 5). The highest parasitic read counts were obtained for *P. xenopodis*, an oviparous monogenean that infects the urinary bladder of the pipid anuran *Xenopus laevis* (Jackson and Tinsley, 1998). Among the identified reads from *P. xenopodis*, 75% (46 out of 61) reads were the repetitive sequences (Table 5). Repetitive sequences are usually composed of transposable elements, which might make it difficult to correctly identify their species origins. Among the cestodes of the order Pseudophyllidea, the sequences associated with *D. latum* and *Spirometra erinaceieuropaei* were identified. *D. latum*, commonly called broad fish tapeworm, can infect humans, canids, felines, bears, pinnipeds, and mustelids, and is a rare zoonotic parasite of dogs (Schurer et al., 2012), which become infected by eating raw fish containing the infective pleurocercoids. Several reads were associated with *Schistosoma* spp., a trematode parasite living in the mammalian blood circulation. Although dogs have been suspected to play a role in transmission of *S. japonicum* to humans (Carabin et al., 2015), very little information is available regarding the presence of this parasite in dogs. Considering the life cycles of each parasite and analytical drawbacks discussed earlier, these results may not rigorously determine that the tested dogs were infected with those parasites. Further research including conventional molecular assays will be necessary to definitively establish the infection of those parasites in dogs.

Although the precise origin and distribution of cfDNA is not yet fully understood, it has been proposed that the cfDNA may be released passively into the circulation due to cellular apoptosis and/or active secretion from live cells (Weerakoon et al., 2016). There are many foreign sources of cfDNA reported such as bacteria (De Madaria et al., 2005), viruses (Ngan et al., 2002), and sometimes even foods (Sanderson et al., 1994). cfDNA is thought to be derived not only from human cells but from human microbiome as well. The passage of viable inhabiting bacteria or molecules and bacterial DNA from the gastrointestinal tract through the intestinal epithelial mucosa to the blood circulation is possible by a process called microbial/bacterial translocation (Giacona MB et al., 1998). In this analysis, we detected bacteria-derived cfDNA sequences in higher abundance (Figure 8), which could be attributable to the bacterial translocation from the gut of dogs. Additionally, few reads (0.04%) associated with viruses were detected which were mostly from bacteriophages and previously it was reported that phages could enter the blood stream via the gastrointestinal system (Zhang et al., 2006; Li et al., 2012). However, it is difficult to determine the actual source of bacterial and viral DNA in plasma without performing further researches.

There is still no standardization approach established for evaluating cfDNA in terms of extraction techniques, processing, standards and assay condition of cfDNA analysis. Efficacy of extraction procedures and quantification of cfDNA are the major technical issues which need to be consistent, robust, reproducible and accurate to improve the clinical utility of cfDNA. In addition, another key issue is the different platform assays used for cfDNA assessment. Recently, next generation sequencing-based approaches for the assessment of cfDNA are being developed, which are considered as more sensitive and specific than conventional PCR-based assays. However, as discussed earlier, this

technology still suffers from some technical drawbacks and further improvements are required to optimize laboratory procedures, control environmental contamination, and eliminate false-positive results. Thus, in order to realize the full potential of cfDNA as an efficient diagnostic biomarker, it will be necessary to standardize techniques for consensus evaluation and bioinformatics analysis of cfDNA sequencing data.

In conclusion, the present study demonstrated for the first time the detection of presumably parasite-derived cfDNA in dogs. The inventory of parasitic cfDNA content in dogs included both expected and unexpected parasites belonging to several genera. However, further elaborate studies are required to provide the precise data regarding the prevalence and impact of these parasites in stray dogs in Bangladesh.

SUMMARY

Stray dogs are native ownerless dogs and maintain a close contact with the human beings. Parasitic infections are very common in these dogs and the transmission of zoonotic parasites to human can cause significant public health risk. Accurate knowledge on the parasite communities in dogs will be useful to understand the epidemiology of parasitic diseases in an area. Here, the study was aimed to evaluate the cell-free DNA (cfDNA) as a potential diagnostic tool for screening the parasite infections in dog using Illumina sequencing technology. Fourteen plasma samples from stray dogs captured in Bangladesh were employed. A total of 32,726,663 reads with an average of 2.3 million reads per sample were generated from two independent MiSeq runs. After removal of the reads derived from the dog genome, the reads were screened for homology to known parasites by local BLASTn program. After quality filtering, a total of 150 reads were identified to show high similarity with the parasite sequences. Four sequences showing high similarity with *Leishmania* in three samples were detected. In addition, the reads related to *Babesia* spp. and filarial nematodes were detected. There was a good agreement with the result of the cfDNA analysis and that of conventional PCR conducted on DNA extracted from whole blood spotted on FTA cards. These findings implicate that cfDNA could be a useful marker to evaluate parasite diversity in dogs by employing high-throughput sequencing technologies.

General Conclusion

Visceral leishmaniasis (VL) is a fatal vector-borne parasitic disease of serious public health problem. This disease has two epidemiological forms; zoonotic form and anthroponotic form. In Mediterranean countries, the Middle East, Asia, and South America, *Leishmania infantum* is responsible for zoonotic VL, with dogs as the main reservoir host whereas in the Indian subcontinent, transmission of VL caused by *L. donovani* is thought to be anthroponotic with the transmission of infection from human to human by phlebotomine sand fly bites. However, the importance of dogs as reservoirs in *L. donovani* transmission is still not well investigated in the Indian subcontinent as well as in Bangladesh which is one of the most highly endemic countries for VL. In our previous study, *L. donovani* DNA was detected in one stray dog (1.2%) from VL-endemic areas of Bangladesh, which requires further verification to better understand the possible role of stray dog as a reservoir host for VL epidemiology in this endemic area. Considering that the stray dog's population in Bangladesh is high and that they are living very close to humans, there is a possibility of disease transmission from dogs to humans. Therefore, it is important to understand the parasite diversity in dogs to minimize the potential risk to humans. In this study, a total of 50 stray dogs were captured in VL-endemic areas of Bangladesh and tested for the presence of parasites by using serological and molecular methods.

In Chapter I, 50 stray dogs were screened for serological and molecular evidence of *Leishmania* infection. Anti-*Leishmania* antibodies were detected in 6 dog plasma samples using rK39 immunochromatographic tests. *Leishmania* kinetoplast DNA was observed in ten buffy coat DNA samples by real-time PCR, five of which were positive

based on internal transcribed spacer 1-PCR. Sequencing analysis of the amplified products revealed 100% identity to *L. donovani* DNA sequences reported from human VL patient in Bangladesh. These findings strengthen the assumption that stray dogs in this endemic focus could play a potential role as an animal reservoir for VL transmission.

In Chapter II, a metagenomic approach was employed to identify the parasite-derived cell-free DNA (cfDNA) sequences in the plasma of 14 stray dogs. An average of 2.3 million reads per sample were obtained from two independent Illumina MiSeq runs. After removal of the reads derived from the dog genome, the reads were screened for homology to known parasites by local BLASTn searches against the NCBI NCBI nt (non-redundant nucleotide sequences) database. After quality filtering, a total of 150 reads were identified to show high similarity with the sequences from parasites including *Leishmania* and *Babesia*, which were previously detected by molecular methods from the same dog group. The results suggest that the present approach of analyzing cfDNA would allow us to investigate the parasite diversity in a high throughput manner.

In conclusion, the present study demonstrated the presence of *L. donovani* in stray dogs. Further elaborate studies are required to analyze the *Leishmania* infection in dogs in a larger scale and to reveal the exact role of dogs in transmitting the parasite to vector sand fly in nature. The findings of this study also warranted that cfDNA in plasma could be a potential diagnostic marker to assess the parasite communities prevailing in dogs.

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Japanese Abstract

日本語要約

Studies on molecular detection of *Leishmania* infection in stray dogs from Bangladesh
（バングラデシュ人民共和国の野犬におけるリーシュマニア感染に関する研究）

内臓リーシュマニア症は、サシチョウバエによって媒介される公衆衛生上重要な寄生虫症である。地中海沿岸、中東、中央アジア、北アフリカなどの地域では、*Leishmania infantum*が人獣共通感染性の寄生虫種として、イヌが主要な自然宿主となっている。一方、インド、ネパール、バングラデシュを含むインド亜大陸では、*Leishmania donovani*がヒトとサシチョウバエでのみ生活環を完結する寄生虫種として知られている。しかしながら、*L. donovani*の自然感染環におけるイヌの役割は検討されてこなかった。わたしたちの研究グループは、予備調査においてバングラデシュ共和国の内臓リーシュマニア症流行地で捕獲した野犬の1頭の血液中から*L. donovani*のDNAを検出した。このことから、同地域の内臓リーシュマニア症流行における野犬の役割を詳しく調査する必要性が提起された。また、バングラデシュ共和国では、野犬がヒトの生活圏で生息しており、リーシュマニア原虫以外の寄生虫がイヌを介してヒトに伝播する可能性も考えられた。本研究では、バングラデシュのマイメンシン地域の内臓リーシュマニア症が高度に流行する地域で計50頭の野犬を捕獲し、リーシュマニア感染の診断ならびに保有する寄生虫種の網羅的検出を試みた。

第一章では、捕獲した野犬50頭について、リーシュマニア感染の血清診断とDNA診断を行った。リーシュマニア原虫のrK39抗原に対する抗体検査の結果、6頭（12%）のイヌで陽性反応が認められた。また、末梢血白血球層から抽出したDNAを材料にリーシュマニア原虫のミトコンドリア・ミニサークルDNAを標的としたリアルタイムPCRによる原虫DNAの検出を行ったところ、10頭（20%）のイヌで陽性反応が得られ、そのうち5頭からはInternal Transcribed Spacer（ITS）領域のPCR増幅が確認された。PCR産物のシーケンス解析により、得られた配列はバングラデシュ共和国の内臓リーシュマニア症患者から検出された*L. donovani*の配列と100%一致した。

第二章では、上記50頭のうち、採血した血液量の豊富であった14頭のイヌを対象に、血漿中に遊離するセルフリーDNA（cfDNA）を網羅的に解析し、寄生虫由来DNAの検出を試みた。抽出したcfDNAを材料にIllumina MiSeqによるメタゲノム解析を行い、各サンプルあたり約2,300万配列を得た。イヌ由来配列を参照ゲ

ノム配列へのマッピングにより除去した後、NCBI nt（non-redundant nucleotide sequences）データベースを用いて、BLASTnプログラムによる相同性検索を行った。その結果、寄生虫種の配列と高い相同性を示すリードが計150配列得られた。線虫類の配列は糸状虫の感染を示唆しており、それらの共生菌と考えられるヴォルバキアの配列も検出された。また、原虫類の検出結果はリーシュマニアとバベシアの感染を反映しており、赤血球内寄生性のバベシアではcfDNAの検出結果と血液を対象としたPCRによる原虫検出結果に一致が認められた。

以上の結果、バングラデシュ共和国における*L. donovani*の感染環の維持におけるイヌの役割が示唆された。今後、イヌ体内のリーシュマニア原虫のサシチョウバエへの伝播能を証明することにより、インド亜大陸に分布する*L. donovani*の自然界における存続様式が明らかになることが期待される。一方、イヌ血中のcfDNAは、寄生虫感染を診断するバイオマーカーとして有用であることが示された。今後、寄生虫のゲノム情報がさらに蓄積されることにより、cfDNA解析法がより精度の高い寄生虫感染診断法に発展し、また、野犬が保有する寄生虫のヒトへの感染リスクを評価するツールとして利用されることが期待される。