ORIGINAL ARTICLE

Duplex real-time PCR methods for molecular detection and characterization of canine tick-borne haemoparasites from Punjab state, India

Aparna M. Thomas[1] · Harkirat Singh[1] · Harsh Panwar[2] · Ram S. Sethi[3] · Nirbhay K. Singh[1]

Abstract

Background Microscopy is a routinely used technique for the diagnosis of canine tick-borne haemoparasitic diseases in various clinical laboratories worldwide. In an attempt to provide better diagnostic assay to the clients for effective management of these diseases duplex real-time PCR assays were applied.

Methods and results Blood samples (n = 338) aseptically collected from suspected dogs of Central Plain Zone of Punjab state, India were subjected to SYBR Green based real-time duplex PCR assays for simultaneous detection of *B. vogeli* & *E. canis* and *B. gibsoni* & *H. canis*. Results revealed an overall prevalence rate of canine tick-borne haemoparasites as 54.1%, amongst which *H. canis* was the predominant (25.4%), followed by *B. gibsoni* (16.3%), *E. canis* (10.7%) and *B. vogeli* (1.8%). Sensitivity and specificity of the duplex assays ranged from 59.04 to 100.0% and 58.12 to 92.52%, respectively and their strength of agreement was "fair" with kappa value statistics. A significant ($p < 0.05$) association between prevalence of *B. gibsoni*, *H. canis* and *E. canis* infection with risk factors like sex, breed, season and location was recorded. The ancestral background of the field isolates of haemoparasites was also studied by phylogenetic analysis of their nucleotide sequences.

Conclusions SYBR Green dye based duplex real-time PCR assays proved to be highly sensitive, specific, rapid and affordable diagnostic tests for use by clinicians to save the life of pets.

Keywords Canine haemoparasites · Duplex real-time PCR assays · Molecular characterization · Risk factors

Introduction

Tick-borne diseases are amongst the most important emerging problems in dogs worldwide particularly in the tropical and semi-tropical regions. The geo-climatic conditions of the Indian sub-continent, including Punjab state, characterized by high humidity and ambient temperature for most parts of the year is highly conducive for development and propagation of ticks [1]. The brown dog-tick *Rhipicephalus sanguineus* sensu lato acting as a common vector for several pathogens in dogs often leads to multiple infections upon exposure. The important canine tick-borne pathogens include *Babesia* spp., *Ehrlichia* spp. and *Hepatozoon canis* and co-infections of these parasites have been reported especially in the endemic areas [2].

Conventional microscopic detection of parasites is considered as the "gold standard" test but has limited utility in sub-clinical or chronic cases owing to its lower sensitivity [3]. Serological assays like enzyme linked immunosorbent assay (ELISA), indirect fluorescent antibody test (IFAT), and immunoblot assays are sensitive, but suffer from the problem of cost incurred, cross-reactivity and inability to distinguish between present or past infection [4]. However, molecular diagnostic techniques, like conventional polymerase chain reaction (PCR) and real-time PCR assays offer sensitive and specific diagnostic tests for detection of the pathogens.

✉ Harkirat Singh
drharkiratsingh@hotmail.com

1 Department of Veterinary Parasitology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab 141004, India

2 Department of Dairy Microbiology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab 141004, India

3 Department of Animal Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab 141004, India

Recently, conventional singleplex PCR assays have been extensively used for the detection of *E. canis*, *Babesia* spp. and *H. canis* in dogs from India [5] particularly Punjab state [6–10]. Although, multiplex PCR assays for simultaneous detection of these pathogens would save time, labour and cost but there are limited reports on development and application of these assays from the region [11–13]. In contrast to conventional PCR approach, the real-time PCR assay has an advantage of monitoring the formation of target specific amplicon throughout the reaction and can hence replace these diagnostic protocols [14]. As scanty literature is available regarding the use of multiplex real-time PCR assays [15–17], the present study was aimed to utilize the duplex real-time PCR assays for the simultaneous detection of canine haemoparasites of Punjab state, India and their molecular characterization to reveal the genetic diversity, if any.

Materials and methods

Study area

The Punjab state is located in north-western India which extends from the latitudes 29.30°N to 32.32°N and longitudes 73.55°E to 76.50°E covering a geographical area of 50,362 km^2 and lies between altitudes of 180 and 300 m above sea level. The current study was conducted in 11 districts of Central Plain Zone of Punjab viz., Amritsar (31.63°N, 74.87°E), Barnala (30.38°N, 75.54°E), Fatehgarh Sahib (30.68°N, 76.41°E), Jalandhar (31.32°N, 75.57°E), Kapurthala (31.37°N, 75.37°E), Gurdaspur (32.04°N, 75.40°E), Ludhiana (30.9°N, 75.85°E), Moga (30.81°N, 75.17°E), Patiala (30.34°N, 76.37°E), Sangrur (30.25°N, 75.84°E) and Tarn Taran (31.45°N, 74.93°E). The climate of the study area falls under warm semi-arid (BSh) and humid subtropical climate (Cfa) type as per the Koppen-Geiger climate classification [18].

Collection of blood samples

Blood samples (n = 338) were collected aseptically following the guidelines of animal care after approval from the Institute Animal Ethics Committee (IAEC/2018/1090-1125 dated 19.06.2018 and IAEC/2019/63-97 dated 29.04.2019) from suspected dogs. Animals presented at Multi-speciality Veterinary Hospital, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana and government/private veterinary clinics of selected districts with tick infestation, pyrexia, anaemia, cachexia, haemoglobinuria, posterior paralysis etc. were selected for the study. Approximately 2–5 mL of blood samples were collected in EDTA coated vacutainers, utilized for the preparation of thin blood smears and subsequently kept at −20 °C till further use for DNA extraction.

Microscopy

Thin blood smears were prepared and stained with diluted Giemsa stain as per the protocol outlined by Juyal et al. [19] and examined microscopically. The intra-cellular piroplasms of *Babesia* spp. in RBCs, morulae of *E. canis* in monocytes or lymphocytes and gamonts of *H. canis* in the neutrophils or monocytes were identified morphologically. At least 100 oil immersion microscopic fields were tested before declaring the sample negative [10].

Genomic DNA extraction

For conducting the PCR assays, whole genomic DNA was extracted from the blood samples using QIAamp® DNA blood mini kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations with minor modifications like increasing the centrifugation time by 30 s and eluting the DNA in a final volume of 100 μL as per Singh et al. [20]. The eluted DNA was stored at −20 °C till further use.

Babesia vogeli and *Ehrlichia canis* duplex real-time PCR assay

The 18S ribosomal gene sequence of *B. vogeli* and virB9 gene sequence of *E. canis* were selected for synthesis of self-designed primers in a previous study carried out in our laboratory [17]. The criteria used for selection was compatibility of the primers, similar annealing temperature, variable amplicon size as well as T$_m$ values that can be interpreted with ease by melt-curve analysis. The laboratory standardized assay was set up in 20 μL final volume using KAPA SYBR® FAST qPCR Kit Master Mix (2X) Universal (Kapa Biosystems, MA, USA) and consisted of 1X molar concentration of Master Mix, 0.5 pmol each of the respective primers for *B. vogeli* and *E. canis* and 0.75 pmol for primer of RPS5 as internal control [17]. The field samples were added @ 4 μL per reaction as a template and final volume was made up to by nuclease free water (Thermo Scientific, MA, USA). The details of the primers used in the study areas under:

Forward primer (Bv-18S-337-F): 5′CTTAAAGGAAGG AGAAGTCGTAACA 3′

Reverse primer (Bv-18S-337-R): 5′CAGTCAAGCGGA GTTGCAAAT 3′

Forward primer (EC-virB9-234-F): 5′TGACCTGATATG CCGTAGCG 3′

Reverse primer (EC- virB9-234-R): 5′ACAAGGTAG TTGTCGCTTGTA 3′

Forward primer (RPS5-141-F):5′TCACTGGTGAG/AAC CCCCT 3′

Reverse primer (RPS5-141-R):5′CCTGATTCACACGGC GTAG 3′

A 3-step PCR standardized protocol was selected with melt curve analysis and cycling conditions comprising of hot start at 95 °C for 3 min, 30 cycles of amplification comprising of denaturation at 95 °C for 20 s, annealing at 63.5 °C for 20 s and extension and data acquisition at 72 °C for 40 s followed by melt or dissociation at 95 °C for 30 s, 65 °C for 30 s and 95 °C for 30 s. The analytical sensitivity of the assay and cross-reactivity studies were also carried out previously using the DNAs of *B. gibsoni* and *H. canis* in our laboratory [17].

Babesia gibsoni and *Hepatozoon canis* duplex real-time PCR assay

The 18S ribosomal gene sequence of *B. gibsoni* and *H. canis* were selected for synthesis of self-designed primers in a previous study carried out in our laboratory [17] with the same selection criteria as discussed above. The PCR assay was set up as above with 1.25 pmol of the respective primers for *B. gibsoni*; 2.5 pmol for *H. canis* and 2.0 pmol for β-actin as internal control [17]. The details of the primers used are as under:

Forward primer (Bg-18S-126-F): 5′CCGTCGTAGTCC TAACCATAAAC 3′

Reverse primer (Bg-18S-126-R): 5′ TTCAGCCTTGCG ACCATAC 3′

Forward primer (Hc-18S-106-F):5′ TCAACTTTATTA GAAGAGGCGCATT 3′

Reverse primer (Hc-18S-106-R):5′TTTTCACTTTGC GATTTGCTAAGTT 3′

Forward primer (β-actin-218-F):5′CTGTCCCTGTAT GCCTCTG 3′

Reverse primer (β-actin-218-R):5′ATGTCACGCACG ATTTCC 3′

A 3-step standardized PCR protocol was selected as above except for the annealing that was carried at 63.0 °C for 20 s and extension at 72 °C for 20 s. The analytical sensitivity of the assay and cross-reactivity studies were also carried out previously using the DNAs of *B. vogeli* and *E. canis* in our laboratory [17].

The results obtained from the duplex real-time PCR assays were compared with microscopy and the sensitivity and specificity of the developed assays were calculated. The association of related risk factors like age (< 6 m; 6–12 m; > 12 m); sex (male and female); breed (defined and non-defined); season (summer, monsoon and winter) and locations (districts) with the prevalence of these haemoparasitic infections was determined.

Molecular characterization, sequencing and analysis

The positive representative samples from various districts by duplex real-time PCR assays were amplified by parasite specific conventional PCR assays using the same set of primes as described above but at final concentrations of 10 pmol/reaction. The cycling conditions for performing the PCR assay for *B. gibsoni* and *H. canis* were: initial denaturation at 95 °C for 3 min, 40 cycles of denaturation (95 °C for 30 s), annealing (62 °C for 30 s) and extension (72 °C for 30 s) and thereafter final extension (72 °C for 7 min). For *B. vogeli* and *E. canis* the assay conditions were same as above with the exception of extension which was carried at 72 °C for 45 s.

The PCR products for *B. gibsoni*, *B. vogeli*, *E. canis* and *H. canis* were purified using PCR Purification Kit (Real-Gene, CA, USA) as per manufacturer's protocol. The purified PCR products were subsequently cloned using CloneJET PCR cloning kit containing pJET1.2/blunt cloning vector (Thermo Scientific, USA) and the recombinant plasmids were transformed into *E. coli* DH5α cells (Invitrogen, MA, USA). The positive clones (containing the inserts) were confirmed by colony PCR assays and plasmid DNA from the positive colonies were isolated by QIAprep® Spin Miniprep Kit (Qiagen) as per manufacturer's instruction and used as a template source for plasmid PCR assays. The recombinant plasmids were outsourced to DNA sequencing facility at Bioserve Biotechnologies Pvt. Ltd. (India) for sequencing by Sanger's sequencing method. The sequence data were aligned and analyzed by multiple sequence alignment using Clustal W method in Lasergene software (DNASTAR Inc., Madison, USA) and compared with homologues in the GenBank using nucleotide BLAST (NCBI).

Statistical analysis

The data obtained from microscopy and duplex real-time PCR assays were analysed by GraphPad for 2-tailed Fisher's exact test (https://www.graphpad.com/quickcalcs/ contingency1.cfm) and kappa value (https://www.graphpad.com/quickcalcs/kappa1.cfm). The strength of agreement between microscopy and duplex real-time PCR assays was interpreted as per Landis and Koch [21]. Furthermore, diagnostic sensitivity and specificity of these PCR assays in comparison to microscopy were analysed by MedCalc Software Ltd. Diagnostic test evaluation calculator version 20.008 (https://www.medcalc.org/calc/diagnostic_test.php). The association of various risk factors with prevalence of tick-borne haemoparasitic infections were determined by the Chi-square and Fischer's exact test by SPSS software (Version 20) and probability of error was accepted up to 5% ($p < 0.05$).

Results

Prevalence of canine tick-borne haemoparasitic infections

The SYBR Green based duplex real-time PCR assays previously developed in our laboratory revealed the T_m values for *B. vogeli*, *E. canis* and RPS5 as 89.5 °C, 78.0 °C and 85.0 °C, respectively while T_m values for *B. gibsoni*, *H. canis* and β-actin were 81.5 °C, 75.5 °C and 86.5 °C, respectively. A C_T value of 25 was set as the cut-off for positivity for both the assays as per our previous study. Similarly, the analytical sensitivity of PCR assays was 0.3125 pg/μL, 2.5 pg/μL, 0.15625 pg/μL and 0.039 pg/μL for detection of *B. vogeli*, *E. canis*, *B. gibsoni* and *H. canis*, respectively.

An overall prevalence rate of 54.1% was recorded for these haemoparasitic infections by duplex real-time PCR assays, amongst which *H. canis* was the predominant parasite (25.4%), followed by *B. gibsoni* (16.3%), *E. canis* (10.7%) and *B. vogeli* (1.8%). Mixed infections of two (*B. gibsoni* and *H. canis*; *E. canis* and *B. gibsoni*; *E. canis* and *H. canis*; *B. gibsoni* and *B. vogeli*) and three (*B.*

gibsoni, *B. vogeli* and *H. canis*; *B. gibsoni*, *B. vogeli* and *E. canis*; *B. gibsoni*, *E. canis* and *H. canis*) parasites were also recorded (Figs. 1, 2, details presented in Table 1). Microscopy revealed an overall prevalence rate of 10.7% which included *B. gibsoni* (7.1%), *E. canis* (1.8%), *H. canis* (1.5%) and *B. vogeli* (0.3%) with no mixed infections (Table 1).

The sensitivity and specificity of duplex real-time PCR assays showed significant variation (p-value < 0.0001) by Fisher's exact test over microscopy, the ″gold standard″ test. The sensitivity (95% CI) and specificity (95% CI) of the *B. vogeli* and *E. canis* duplex real-time PCR assay was 100.00% (59.04 to 100.00%) and 89.43% (85.60 to 92.52%), whereas that of *B. gibsoni* and *H. canis* assay was 100.0% (88.06 to 100.00%) and 63.75% (58.12 to 69.12%). The kappa (value ± SE) for the *B. vogeli* & *E. canis* and *B. gibsoni* & *H. canis* PCR assays were estimated at $0.259 ± 0.08$ and $0.232 ± 0.038$, respectively with ″fair″ strength of agreement for both the assays.

Molecular characterization, sequencing and analysis

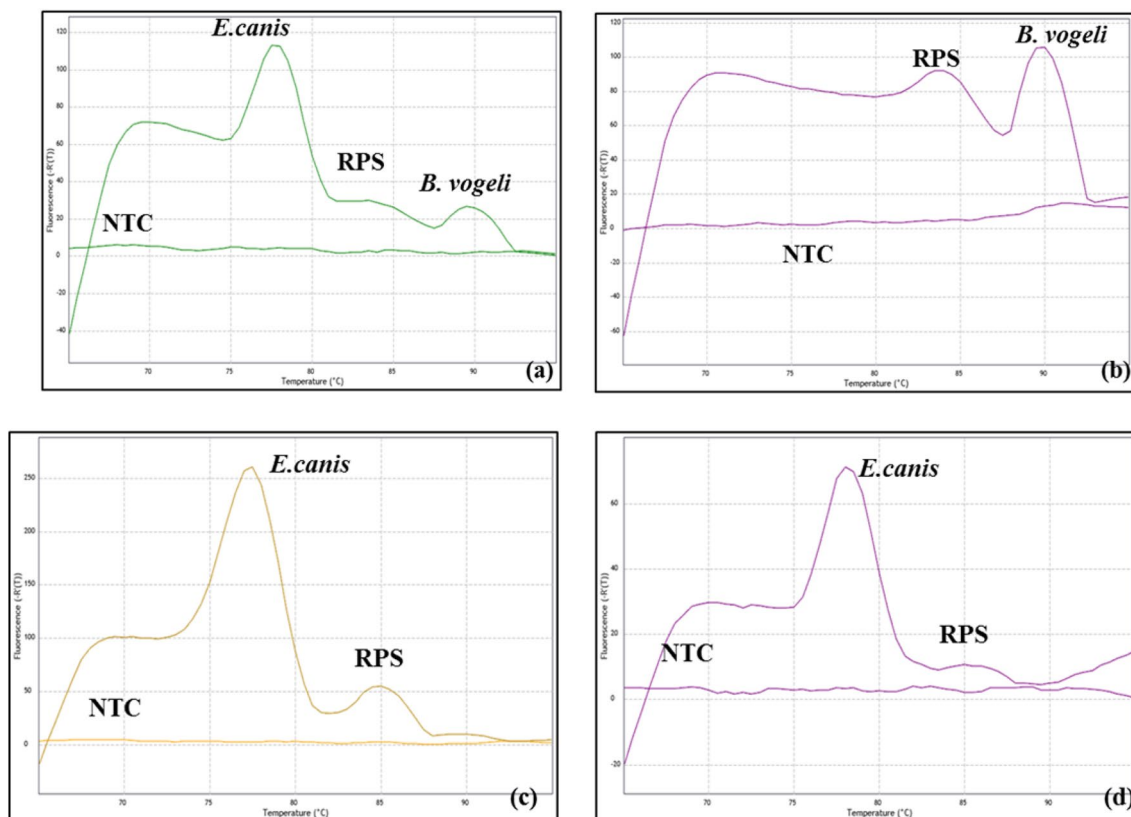Amplicons of 126 bp, 337 bp, 234 bp and 106 bp corresponding to 18S rRNA gene for *B. gibsoni*, *B. vogeli* and

Fig. 1 **a–d** Field application of *B. vogeli*, *E. canis* and RPS 5 duplex real-time PCR assay
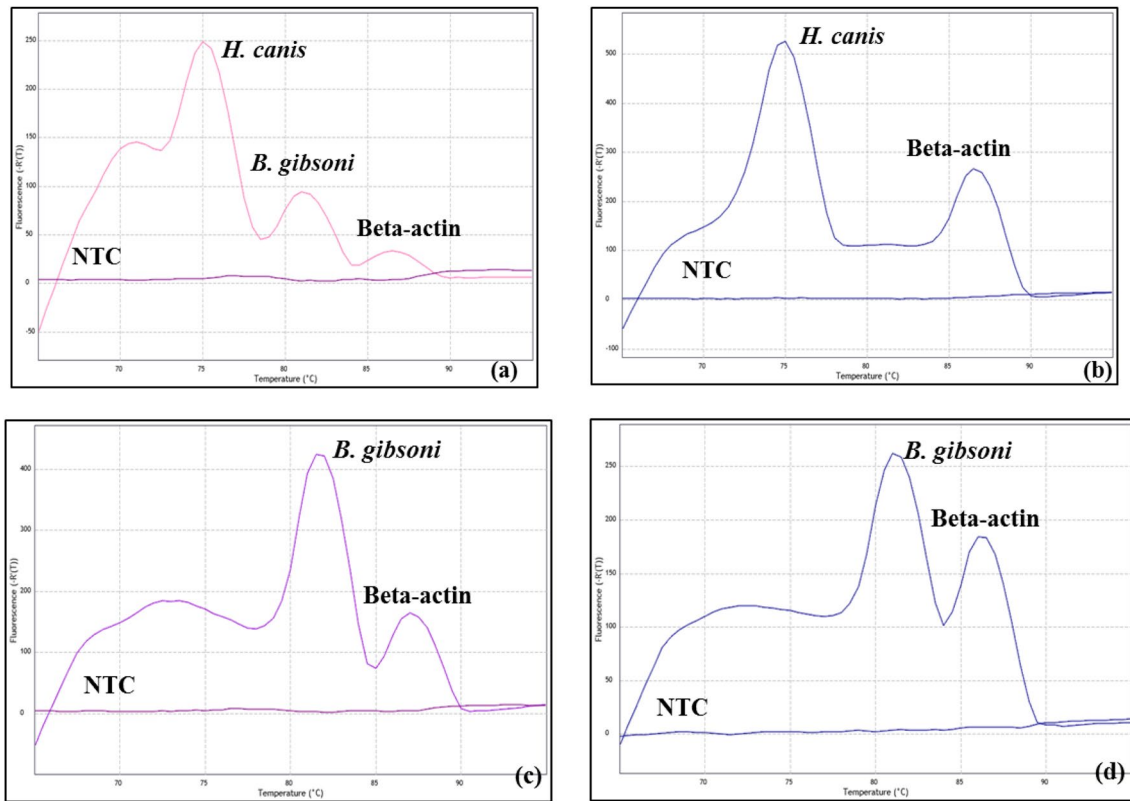
Fig. 2 **a–d** Field application of *B. gibsoni*, *H. canis* and β-actin duplex real-time PCR assay

Table 1 Prevalence of haemoparasites by microscopy and duplex real-time PCR assays

Test	N	BV	BG	EC	HC	M₁	M₂	M₃	M₄	M₅	M₆	M₇
Microscopy	338	1 (0.3)	24 (7.1)	6 (1.8)	5 (1.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Duplex real-time PCR assays	338	6 (1.8)	55 (16.3)	36 (10.7)	86 (25.4)	16 (4.7)	8 (2.4)	7 (2.1)	2 (0.6)	2 (0.6)	2 (0.6)	2 (0.6)

Values in brackets indicate %; BV, *Babesia vogeli*; BG, *Babesia gibsoni*; EC, *Ehrlichia canis*; HC, *Hepatozoon canis*

M_1 Mixed infection of BG & HC, M_2 Mixed infection of EC & BG, M_3 Mixed infection of EC & HC, M_4 Mixed infection of BV, HC & BG, M_5 Mixed infection of EC, BV & BG, M_6 Mixed infection of BV & BG, M_7 Mixed infection of EC, HC & BG

H. canis and virB9 gene for *E. canis* were obtained, without any non-specific amplification by colony and plasmid PCR assays (Supplementary Fig. 1, 2). The sequences of six isolates of *B. gibsoni* (MZ321033, MZ321032, MZ413880, MZ467324, MZ457927 and MZ467328), three of *B. vogeli* (MZ320524, MZ458122 and MZ467325), one of *E. canis* (MZ326696) and nine of *H. canis* (MZ318674, MZ323362, MZ323363, MZ323361, MZ411573, MZ411572, MZ467327, MZ467326 and MZ458102) were published in the repository of GenBank, NCBI. The nucleotide sequences of local isolates of *B. gibsoni*, *B. vogeli* and *H. canis* revealed homology ranging from 7.6 to 100.0%; 4.0% to 100.0% and 86.8% to 100.0% for the partial 18S rRNA gene sequence while for *E. canis* 100% homology was observed for partial

virB9 gene when compared with other isolates (Supplementary Fig. 3). Based on this information a phylogenetic tree using bootstrap method was constructed and the phylogenetic linkage of various isolates for the respective parasites is presented (Fig. 3).

Assessment of various risk factors

Microscopic examination revealed significant ($p < 0.05$) association between sex of host and *B. gibsoni* infection with higher prevalence in males. Duplex real-time PCR assays revealed significantly ($p < 0.05$) higher prevalence of *H. canis* in German Shepherd dogs and significant ($p < 0.05$)
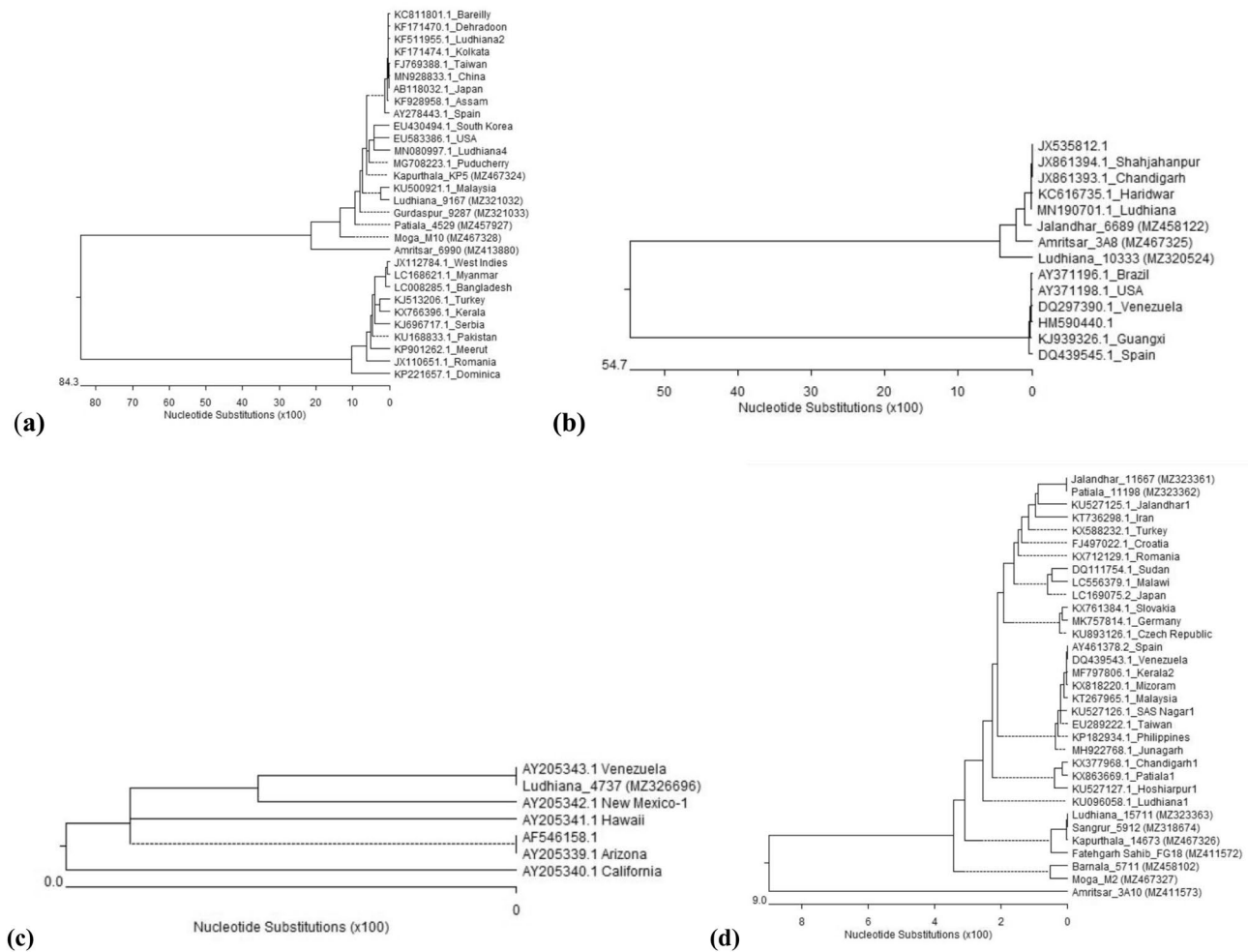
Fig. 3 Phylogenetic tree showing genetic relationships of **a** various isolates of *B. gibsoni*. **b** various isolates of *B. vogeli*. **c** various isolates of *E. canis*. **d** various isolates of *H. canis*

variation in *E. canis* infection among the seasons and locations (districts) of sample collection (details in Table 2).

Discussion

Although, demonstration of the parasite or its stages in samples of blood, cerebrospinal fluid, lymph node aspirates, etc. collected from suspected animal has been considered as the "gold standard" technique but microscopy suffers from the inherited disadvantage of low sensitivity and individual interpretation of results. Therefore, despite being a rapid and cost-effective technique, microscopy is not very useful for detection of latent/chronic cases with low parasite levels [7]. However, amplification of the parasitic DNA by nucleic acid-based assays in such cases gains importance because of their inherited property of high threshold detection limits with higher sensitivity and specificity over the conventional tests.

Published reports of molecular detection of canine haemoparasitic infections by various PCR assays from Punjab, India reveals the percent prevalence of *B. gibsoni*, *B. vogeli*, *E. canis* and *H. canis* infections in range of 15.4–15.42, 0.26–0.93, 0.39–41.59 and 0.26–30.0, respectively [6–10, 13]. The wide variation regarding the prevalence of *E. canis* and *H. canis* might be attributed to the fact that Milanjeet et al. [6] utilized a nested PCR assay-based protocol while Singh et al. [10] used LAMP based assay, respectively in their studies. In the present study, the prevalence rates recorded for these haemoparasites were in the range as reported earlier.

The higher sensitivity (threshold detection limits) and specificity of duplex PCR based assays with an added advantage of simultaneous detection of more than one parasitic DNAs in the same tube renders them to be more cost effective with high field applicability [14]. Likewise, reports are available worldwide regarding simultaneous detection of multiple infections in dogs by different nucleic acid-based

Table 2 Distribution of tick borne haemoparasitic infections of dogs in accordance with various risk factors

Risk factor	Parameter	N	Microscopy (%)				Duplex real-time PCR assays (%)			
			BG	BV	EC	HC	BG	BV	EC	HC
Age	< 6 m	39	1 (2.6)	0 (0.0)	0 (0.0)	0 (0.0)	6 (15.4)	1 (2.6)	6 (15.4)	9 (23.1)
	6– 12 m	35	2 (5.7)	0 (0.0)	1 (2.9)	0 (0.0)	4 (11.4)	0 (0.0)	3 (8.6)	10 (28.6)
	> 12 m	264	21 (8.0)	1 (0.4)	5 (1.9)	5 (1.9)	45 (17.0)	5 (1.9)	27(10.2)	67 (25.4)
	Pearson's χ^2 value	-	1.611	0.281	0.961	1.423	0.741	0.793	1.127	0.296
Sex	Female	116	3 (2.6)	0 (0.0)	1 (0.9)	1 (0.9)	14 (12.1)	4 (3.4)	14(12.1)	26 (22.4)
	Male	222	21 (9.5)	1 (0.5)	5 (2.3)	4 (1.8)	41 (18.5)	2 (0.9)	22 (9.9)	60 (27.0)
	Pearson's χ^2 value	-	5.456*	0.524	0.844	0.462	2.290	2.835	0.373	0.855
Breed	German Shepherd	49	3 (6.1)	0 (0.0)	1 (2.0)	0 (0.0)	7 (14.3)	0 (0.0)	8 (16.3)	20 (40.8)
	Labrador	59	3 (5.1)	0 (0.0)	0 (0.0)	2 (3.4)	9 (15.3)	1 (1.7)	4 (6.8)	12 (20.3)
	Non-descript	58	4 (4.8)	0 (0.0)	2 (2.4)	2 (2.4)	11 (13.3)	2 (2.4)	10(12.0)	27 (33.7)
	Pit bull	23	1 (4.3)	1 (4.3)	0 (0.0)	0 (0.0)	4 (17.4)	1 (4.3)	1 (4.3)	6 (26.1)
	Pomeranian	15	2 (13.3)	0 (0.0)	1 (6.7)	0 (0.0)	4 (26.7)	0 (0.0)	3 (20.0	2 (13.3)
	Pug	38	7 (18.4)	0 (0.0)	1 (2.6)	0 (0.0)	10 (26.3)	2 (5.3)	5 (13.2)	4 (10.5)
	Rottweiler	12	1(8.3)	0 (0.0)	1(8.3)	0 (0.0)	2 (16.7)	0 (0.0)	3 (25.0)	3 (25.0)
	Others#	59	3 (5.1)	0 (0.0)	0 (0.0)	1 (1.7)	8 (13.6)	0 (0.0)	2 (3.4)	11 (18.6)
	Pearson's χ^2 value	-	10.011	13.736	7.938	4.047	5.086	6.158	11.212	16.984*
Season	Summer	128	11 (8.6)	0 (0.0)	3 (2.3)	0 (0.0)	25 (19.5)	5 (3.9)	17(13.3)	33 (25.8)
	Monsoon	113	9 (8.0)	1 (0.9)	2 (18)	2 (1.8)	20 (17.7)	1 (0.9)	15 (13.3)	28 (24.8)
	Winter	97	4 (4.1)	0 (0.0)	1 (1.0)	3 (3.1)	10 (10.3)	0 (0.0)	4 (4.1)	25 (25.8)
	Pearson's χ^2 value	-	1.864	1.997	0.545	3.720	3.698	5.601	6.090*	0.040
Location	Amritsar	27	2 (7.4)	0 (0.0)	1 (3.7)	0 (0.0)	5 (18.5)	1 (3.7)	5 (18.5)	8 (29.6)
	Barnala	25	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (8.0)	1 (4.0)	4 (16.0)	5 (20.0)
	Fathegarh Sahib	30	1 (3.3)	0 (0.0)	1 (3.3)	0 (0.0)	4 (13.3)	0 (0.0)	2 (6.7)	9 (30.0)
	Gurdaspur	28	2 (7.1)	0 (0.0)	0 (0.0)	0 (0.0)	4 (14.3)	0 (0.0)	0 (0.0)	9 (32.1)
	Jalandhar	30	3 (10.0)	0 (0.0)	1 (3.3)	1 (3.3)	5 (16.7)	1 (3.3)	3 (10.0)	9 (30.0)
	Kapurthala	28	1 (3.6)	0 (0.0)	0 (0.0)	1 (3.6)	3(10.7)	0 (0.0)	1 (3.6)	4 (14.3)
	Ludhiana	64	7 (10.9)	1 (1.6)	3 (4.7)	1 (3.3)	18 (28.1)	3 (4.7)	16(25.0)	11 (17.2)
	Moga	28	3(10.7)	0 (0.0)	0 (0.0)	0 (0.0)	5 (17.9)	0 (0.0)	1 (3.6)	8 (28.6)
	Patiala	31	4(12.9)	0 (0.0)	0 (0.0)	2(6.5)	6 (19.4)	0 (0.0)	1 (3.2)	8 (25.8)
	Sangrur	26	1(3.8)	0 (0.0)	0 (0.0)	0 (0.0)	2 (7.7)	0 (0.0)	3 (11.5)	9 (34.6)
	Tarn Taran	31	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (4.8)	0 (0.0)	0 (0.0)	6 (28.6)
	Pearson's χ^2 value	-	9.059	4.294	7.904	9.588	12.579	8.287	27.476*	7.504
	Total	338	24 (7.1)	1 (0.3)	6 (1.8)	5 (1.5)	55 (16.3)	6 (1.8)	36(10.7)	86 (25.4)

N number of examined samples; #[Saint Bernard (5), Spitz (5), Tibetan Mastiff (1), American Bully (5), Cocker Spaniel (1), Dogo Argentino (1), Bull Dog (2), Golden Retriever (4), Husky (3), Bull Terrier (2), Dachshund (6), Beagle (5), Greyhound (9), French Bully (2), Gaddi (3), French Mastiff (1), Pakistan Bully (1), Dalmatian (1), Doberman (1), Bully Pointer (3)]; *P < 0.05

assays with higher threshold detection limits [22–24]. However, in Indian scenario, reports of multiplex PCR assay-based detection of canine diseases are available only from selected regions including states of Tamil Nadu [11], Kerala [12] and Punjab [13].

Among the two variants of real-time PCR assay i.e., TaqMan probe and SYBR green based protocols the former has the inherited disadvantage of higher cost due to individual probe requirement for each sequence [15, 16, 25]. The SYBR-Green methodology being cheaper, easy to use and having acceptable level of sensitivity in detection of

DNA of infectious agents make it an affordable diagnostic test. Hence, this protocol was applied in the present study for the simultaneous detection of the commonly prevalent canine haemoparasitic infection to be utilized by clinicians to save the life of pets.

Assessment of associated risk factors with the prevalence of these parasitic infections in recent past revealed non-significant variations with respect to age, sex and breed [6, 9, 13]. However, the present study reports significant variations in prevalence of *B. gibsoni* among sexes, *H. canis* among breeds and *E. canis* infection among the seasons and

locations that can be attributed to the seasonal activity of vector tick. Similar to our findings, location wise significant variation in prevalence of *E. canis* infection has been recently reported by Kaur et al. [13].

Declarations

Conflict of interest The authors declare that they have no competing interests.

Ethical approval Animal protocols were approved by the Institute Animal Ethics Committee at College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana vide reference IAEC/2018/1090-1125 dated 19.06.2018 (GADVASU/2018/IAEC/45/11) and IAEC/2019/63-97 dated 29.04.2019 (GADVASU/2019/IAEC/49/20).

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