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RESEARCH ARTICLE

Bm86 Genetic Diversity of Indigenous Tick Population from Punjab Province Pakistan

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Abstract

Ticks transmit a number of bacterial, protozoal and viral pathogens that cause many diseases like ehrlichiosis, hemorrhagic fever, theileriosis, babesiosis and anaplasmosis in livestock. This study was designed for molecular characterization of BM86 gene of *Rhipicephalus (Boophilus) microplus* tick. In this study, the BM86 gene was amplified, using primers flanked by restriction enzyme sites. The molecular detection of *R. microplus* was studied in three districts belonging to different ecological zones in the province of the Punjab, Pakistan. Tick samples were collected and initially screened through microscopy and further analyzed by PCR and sequencing. The phylogenetic tree was generated by using the MEGA 7 through Neighbor Joining method employing best model through the phylogenetic analysis of *R. microplus*. Pairwise comparisons of nucleotide sequences showed nucleotide differences ranging between 0.007 and 0.01%. Haplotype and nucleotide diversity in Bm86 gene was found among different districts. Six single nucleotide polymorphisms were seen in sequences of BM 86 from indigenous tick populations collected from the Punjab province. More interestingly, out of these 6 polymorphisms, we got 2 from district Okara, 3 from district Sahiwal, and 1 in Mandi Bahauddin. Conserved regions were observed among the local strains for BM86 gene. A common convergence in similar clade was with the local Pakistan *R. microplus*. Local mean diversity was 0.005 and overall mean diversity was 0.038. Field strain has been isolated as candidate specie for local tick vaccine, which in turn will increase the efficacy of future tick vaccine including reduction of economic burden on the farmer.

Keywords: *Rhipicephalus (Boophilus) microplus, BM86 gene, Molecular characterization*

Pakistan'ın Punjab Eyaletine Özgü Kene Popülasyonunun Bm86 Genetik Çeşitliliği

Öz

Keneler, çiftlik hayvanlarında erlişiyozis, hemorajik ateş, theileriozis, babeziozis ve anaplazmozis gibi birçok hastalığa neden olan bir dizi bakteriyel, protozoal ve viral patojeni bulaştırırlar. Bu çalışma, *Rhipicephalus (Boophilus) microplus* kenesinin BM86 geninin moleküler karakterizasyonu için tasarlanmıştır. Bu çalışmada, BM86 geni restriksiyon enzim bölgeleri ile çevrili primerler kullanılarak amplifiye edildi. *R. microplus*'un moleküler tespiti, Pakistan'ın Punjab eyaletindeki farklı ekolojik bölgelere ait üç yörede gerçekleştirildi. Kene örnekleri toplanıp ilk mikroskopik tarama yapıldıktan sonra, PCR ve sekans analizleri gerçekleştirildi. Filogenetik ağaç, *R. microplus*'un filogenetik analizi için en iyi model olan komşu birleştirme yöntemi kullanılarak MEGA 7 ile oluşturuldu. Nükleotid dizilerinin ikili karşılaştırmaları %0.007 ile %0.01 arasında değişen nükleotid farklılıkları olduğunu saptadı. Çalışılan farklı bölgelerde Bm86 geninde haplotip ve nükleotid çeşitliliği saptandı. Punjab eyaletinden toplanan lokal kene popülasyonunda BM86 geninde altı adet tek nükleotid polimorfizmi belirlendi. Daha da ilginci, bu 6 polimorfizmden 2'si Okara, 3'ü Sahiwal ve 1'i Mandi Bahauddin bölgesine aitti. Yerel suşlara ait BM86 geninde korunaklı bölgeler saptandı. Pakistan'ın lokal *R. microplus* suşlarında benzer sınıfta ortak bir kümelenme gözlendi. Lokal ortalama çeşitlilik 0.005 ve genel ortalama çeşitlilik 0.038 olarak saptandı. Lokal kene aşı aday türü olarak saha suşunun izolasyonu, çiftçiler üzerindeki ekonomik yükün azaltılması da dahil olmak üzere gelecekteki kene aşısının etkinliğini artıracaktır.

Anahtar sözcükler: *Rhipicephalus (Boophilus) microplus, BM86 geni, Moleküler karakterizasyon*

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Introduction

Tick infestation not only causes pathogen transmission but also restlessness, stress induction and damage to hide quality [1,2]. Ticks transmit bacterial, protozoal and viral pathogens that cause many diseases like ehrlichiosis, hemorrhagic fever, theileriosis, babesiosis and anaplasmosis in livestock [3] likewise mediterranean spotted fever, lyme disease (borreliosis), Q fever, rocky mountain spotted fever, relapsing fever, babesiosis, congo fever and tick-borne encephalitis in humans [4]. Two major families of ticks are prevalent around the globe; first is Argasidae (soft ticks) having about 193 species, second is Ixodidae (hard ticks) with approximately 702 species [5]. Infestation with hard tick *R. microplus* economically impacts the livestock and causes huge losses [6]. Tick infestation significantly reduces milk and meat production [7] which is major contributor in overall cost associated with tick borne diseases [8-10]. Kivaria [11] reported an annual loss of $364M with approximate 1.3 M mortality in cattle due to tick borne diseases.

During 2019-20, Pakistan exported Rs. 13644 M leather and leather products, 18,139M hides [12].

The cattle and buffalo population in Pakistan are at risk of tick infestation throughout the year, although very little is known about the biology, diversity and distribution of tick species across different agro-ecological zones (AEZ) of the country [13]. The occurrence and prevalence of tick-borne pathogens (TBPs) in bovines have been reported from different parts of Pakistan [14-16]. In north east Khyber Pakhtunkhwa (KPK) and Gilgit Baltistan, the overall prevalence of tick infestation in livestock was 75.03% [17] while in northwest of KPK it was 77.9% [18]. The overall prevalence in Punjab (farm animals) was 36.52% [19], point prevalence in south Punjab was 75.1% [20] while in Sargodha, Khushab and Rawalpindi districts it was 54.76% [21].

In Pakistan, ticks are found frequently during mid-April to September, principally the summer season. Ticks prefer to bind on animals at neck, around and inside ear, udder region and inner side of thighs [22]. Traditionally many practices have been observed in Pakistan to control the ticks including burning of grass and temporary sheds but mostly it is through use of injectable ivermectins and use of acaricides [23]. Acaricides are being used in the form of spray and dip but this activity is not feasible due to presence of residual effects in food and water, and of high cost [24]. In recent studies, resistance to acaricides has also been reported [25-30]. Therefore vaccination may be considered as best alternate to overcome this problem. In this regard, BM 86 is considered as candidate gene for vaccine production against *Rhipicephalus (Boophilus) microplus.* BM 86 derived vaccines cause reduction in weight of blood engorged female ticks, reduction in egg mass weight, reduction in tick population in the field over one generation, a significant declined reproductive efficacy of *R. microplus* females, reduced rate of treatments with acaricides and a helpful addition for integrated control programs [31]. In silico analysis of BM86 gut glycoprotein showed that it has antigenic epitopes, one of which has showed more than 80% efficacy in vaccinated cattle against *R. microplus* [32]. Advanced and well planned strategies will be beneficial to achieve the goals for controlling ticks in meat and dairy animals [33].

Keeping in view the economic importance of tick born infestations, present study was designed for molecular characterization, phylogenetic association, haplotype diversity and nucleotide diversity of BM86 gene of *R. microplus* tick, a local strain for future vaccine production to reduce the cost of tick control measures in livestock.

Material and Methods

Study Plan

The three different ecological regions from where ticks were sampled are from district Okara (representative district of Northern irrigated agro-ecological zone), district Sahiwal (representative district of Northern irrigated agro-ecological zone) and district Mandi Bahauddin (representative district at junction of barani lands and Northern irrigated agro-ecological zone) in the Punjab province, Pakistan. These areas were selected due to presence of favorable climate condition for ticks and high population of dairy animals. Total livestock population in these three districts is estimated to be 1.09 million cattle (local, cross bred and exotic breeds), 0.36 million buffaloes, 0.36 million sheep and 1.4 million goats.

Collection of Ticks

Tick collection was performed systematically according to the recommended procedures [20]. Ticks collected were placed in falcon tubes. The tick samples were dispatched to Parasitology laboratory in clean and properly labeled plastic containers. The outer covering of these containers was covered by cheese cloth. The collected ticks were characterized microscopically on the basis of morphology with the help of key described by [34] and [35].

RNA Extraction, cDNA Synthesis & Polymerase Chain Reaction

Total RNA was extracted from the mid gut of female ticks, by using a Trizol reagent (Sigma) according to the given instructions. The extracted RNA was amplified by reverse transcription polymerase chain reaction (RTPCR). The cDNA strand synthesis reaction was performed using a cDNA Synthesis Kit (Amersham, UK), following the manufacturer's instructions. All RNA samples were quantified by nano drop method, 2 µL distilled water was used to calibrate blank then 2 µL sample loaded and reading was noted.

Reverse Transcriptase PCR was performed for synthesis of cDNA with the following method.

Total extracted RNA (5 µg) was taken and cDNA was prepared by adding 1 µL oligo dT and 1 µL 10 mMdNTPs to RNA. Total volume of 10 µL was obtained by adding distilled water. After proper mixing, it was heated at 65°C for five minutes. The sample was chilled hurriedly in ice for 2 min. Then it was micro centrifuged to get the solution to the bottom. RNA mixture was placed on ice while preparing the reaction mixture. In reaction mixture 2 µL 10X RT, 4 µL 25 mM $MgCl_2$, 2 µL 0.1M DTT and 1 µL RNase were added to the reaction mixture. It was softly mixed; a quick spin was applied to collect the mixture in the tube. Sample was incubated at 42°C for 2 min. 1 µL SS II Reverse Transcriptase (was kept on ice for the entire time) was added. Sample was incubated at 42°C for 50 min. Heat inactivation of the enzyme at 65°C to 70°C for 15 min was performed. The sample was kept on ice for 5 min and mixed gently. After that 1 µL RNase H was added to the reaction. The sample was incubated at 37°C for 20 min. The sample was micro centrifuged and obtained PCR products were examined through electrophoresis in a 1.0% agarose. PCR reaction was carried out by using BM 86 primers *(Table 1)* in a 25 µL volume in a thermal cycle(PCR-G-Storm Thermocycler–AG1972).

Conditions of PCR

- Heated Lid at 112°C
- Initial temperature at 94°C for 4 min
- Start Cycle
- Denaturation at 94°C for 30 sec
- Annealing at 59.4°C for 30 sec
- Extension at 72°C for 45 sec

} X 35 cycles

- End Cycle
- Last extension at 72°C for 10 min
- Store

Isolation of cDNA Product by Gel DNA Extraction Kit

cDNA band was separated from agarose gel with the help of sharp scalpel. It was transferred in a tube. For experiment, 300 µL of the agarose was used (as per manufacturer`s instructions). Re-suspended the Silica Suspension (Vial 1) until a homogeneous suspension was obtained. Then silica suspension amounting 10 µL was added in the sample. Ten min incubation was observed at 60°C along with vortexing after every 3 min. Centrifugation at 15000 rpm was applied for 40 seconds to samples and upper layer was discarded. cDNA containing matrix was taken in 500 µL Nucleic Acid Binding Buffer (Vial 3, green cap) and it was vortexed. Again, centrifuged and upper layer was discarded. The obtained pellet was briefly washed with washing buffer. For elution of cDNA, distilled water with pH 8 was used. To increase the elution effectiveness, more volume of elution buffer was taken. Then it was vortexed and 10 min incubation was given at 56°C then transferred the cDNA-containing solution to a new reaction. PCR products of *R. microplus* were sequenced along with the primers.

Table 1. *Primers used to target BM86 gene of R. microplus ticks*

BM 86 Primers	Sequence (5'->3')
F	ACGAGTGTTCTAGGGAGCCT
R	TGCGGTGACTGAAGTAGCTG

Primers were designed with bioinformatics tools by using reference sequence: KJ995910.1 Accession Number

Sequencing and Phylogenetic Analysis of Rhipicephalus (Boophilus) microplus BM86

One hundred and ten samples were analyzed by PCR. 18 PCR products of *Rhipicephalus (Boophilus) microplus* were sequenced along with the primers used in PCR. The Accession numbers are BankIt2333962 Seq1, MT344675 and BankIt2333962 Seq2 MT344676 *(https://www.ncbi.nlm.nih.gov/Genbank/update.html)*. Applied biosystems Genetic analyzer 3130 was used for sequencing at Centre of Excellence in Molecular Biology Lahore, Pakistan. The quality of the sequences was analyzed through Geneious software. Consensus sequences obtained from Geneious software were aligned with MUSCLE software and further confirmed manually by using MESQUITE software. Finally, the phylogenetic tree was generated by using the MEGA 7 *(Table 2)* through Neighbor Joining method. For phylogenetic tree, best model was selected showing the phylogenetic analysis of *R. microplus* through MEGA 7.0.

Analysis of Haplotype and Nucleotide Diversity

Genetic variability of Bm86 gene sequences was further analyzed in *R. microplus* through nucleotide and haplotype diversity. The number and the values for these diversities for each district were further calculated through software DnaSP 5.10 [36] *(http://www.ub.edu/dnasp/DnaSP_OS.html)*.

Results

Molecular Characterization of BM86

For the confirmation of 248 bp of BM86 gene, PCR products were run in 1.5% agarose gel marked with SYBER green safe dye and observed under the Ultra-violet light to declare positive against 100 bp ladder, as shown in *Fig. 1*.

Pairwise comparisons of nucleotide sequences showed nucleotide differences ranging between 0.007 and 0.01% *(Table 3)*. Furthermore, a comparison of BM 86 gene sequences determined herein and our sequences revealed that sequences as mentioned in *Table 2* from other parts of world were identical to BM 86 gene sequences.

Phylogenetic Analysis of Rhipicephalus (Boophilus) microplus Based on Maximum Likelihood Method

The presented phylogenetic analysis were carried out by

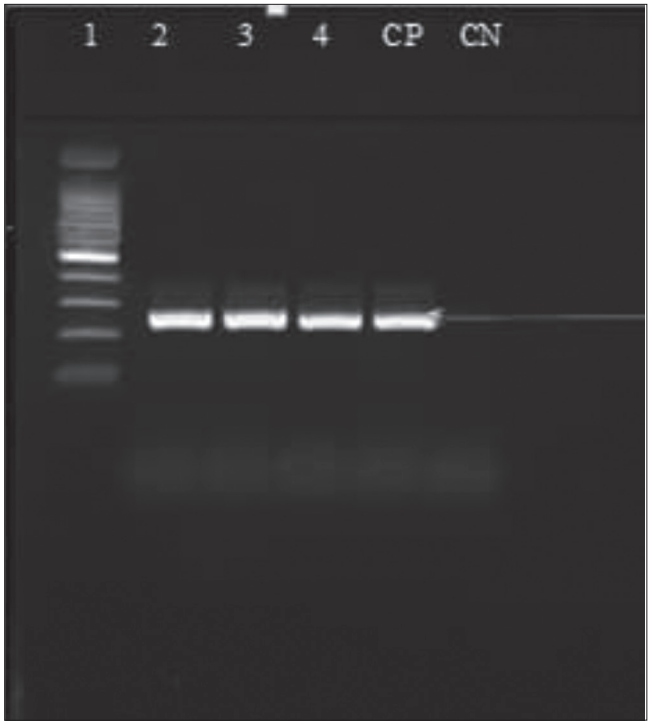

Fig 1. PCR results show the amplification of 248 bp. Lane 1 indicates 100bp ladder, lane 2-4 positive sample of *Rhipicephalus (Boophilus) microplus*. Lane CP is positive control while Lane CN negative control

Maximum likelihood method [37]. Preliminary tree for experimental search were got spontaneously by Neighbor join and BioNJ algorithms to a matrix of pairwise distances projected using the Maximum Composite Likelihood approach, trophy was selected with higher log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Twenty nine nucleotide sequences were analyzed. Gap positions and missing nucleotides were eradicated. There were a total of 1757 positions in the final dataset. Phylogenetic association showed the close resemblance of studied samples with the local strains of Pakistan, whereas it has also showed association with strains of Thailand. A common divergence point was observed with the local Pakistan *R. microplus* population with that of *R. annulatus* BA86 Israel. Other clades had more convergence for *R. microplus* BM86 gene from the USA, Spain, Australia, South Africa *(Fig. 2)*. Sequence alignment of current study revealed the conserved regions of studied gene with polymorphism among local population.

Evolutionary Divergence Between Sequences Estimates

The base replacements numbers per site from amongst sequences is presented. Twenty-nine nucleotides were part of analysis. All gaps containing positions including missing data were rejected. A total of 1757 positions were shown in the final dataset. And evolutionary analysis was carried out in MEGA7.

Local mean diversity = 0.005
Overall mean diversity = 0.038

Table 2. *Sequence identical to other parts of world*

Sr. No	Country	Accession Number	Gene
1	Thailand	GenBankKJ995910.1	*R. microplus*_NE14
2	Thailand	GenBank KJ995907.1	*R. microplus*_NE11
3	USA	HQ014394.1	*R. microplus* _ Zapata
4	USA	HQ014392.1	*R. microplu s*_ Zapata
5	USA	KX786647.1	*R. microplus*_Bm86
6	USA	M29321.1	*B. microplus*_BM86
7	South Africa	FJ809946.1	*R. microplus*_Bm86
8	Spain	FJ456928.1	*R. microplus*_bm86
9	Spain	EU191620.1	*R. microplus*_BM86
10	Spain	EU191620.1	*R. microplus*_BM86
11	Brazil	EU352677.1	*R. microplus*_BM86
12	Brazil	EU352677.1	*R. microplus*_BM86
13	Australia	MG002399.1	*R. microplus*_Bm86
14	Australia	MG002403.1	*R. microplus*_Bm86
15	Australia	MG002401.1	*R. microplus*_Bm86
16	Spain	EU191620.1	*R. microplus*_BM86
17	Spain	EU191620.1	*R. microplus*_BM86
18	Netherland	FJ809946.1	*R. microplus*_Bm86
19	Netherland	FJ809946.1	*R. microplus*_Bm86
20	Netherland	GU144589.1	*R. microplus*_bm86
21	India	DQ131539.1	*R. microplus*_BM86
22	India	DQ131539.1	*R. microplus*_BM86

Haplotype and Nucleotide Diversity

Haplotype and nucleotide diversities were found among different districts in Bm86 sequenced gene of *R. microplus*. Lowest haplotype diversity (0.88) was found in Okara district and highest (0.91) was found in Mandi Bahaudin district. Whereas, highest (0.012) nucleotide diversity was observed in Sahiwal district and lowest (0.010) was revealed in Mandi Bahaudin district *(Table 4)*.

Discussion

The present study provides the insights for the presence of *R. microplus* from bovines in three districts of Punjab, Pakistan and it confirms the already reported studies from Pakistan [16,38-44]. The tick-borne parasites i.e. *B. bovis* and *B. bigemina* are known to cause bovine babesiosis posing serious threat to the livestock health around the world [45-49]. In Indo-Pakistan, bovine ticks are frequently being diagnosed through classical methods i.e. microscopic observation of morphological features [22].

Genetic data shows that *R. microplus* is found in Thailand, USA, South Africa, Spain, Brazil, Australia, Netherland, India and many other parts of the world; this specie is assumed to be evolved from Asia and have been distributed mainly with cattle in all continents [50]. Previous studies on Phylogenetic

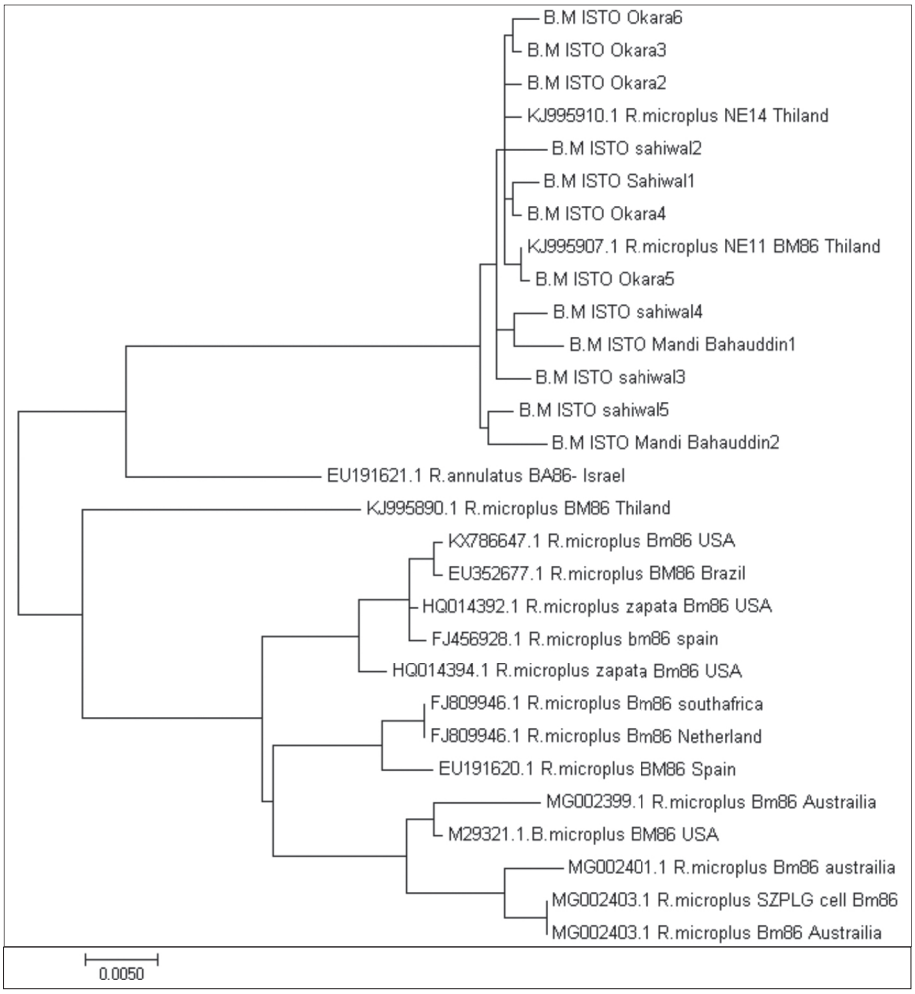

Fig 2. Phylogenetic analysis of *R. microplus* based on Maximum Likelihood method, comparison of BM 86 of local strain with other parts of world, identical to BM 86 gene sequences

analysis [51] show that *R. microplus* is present in every country of the Neotropical region apart from Chile [52] and has been reported to West Africa recently, probably from Brazil with Girolando cattle where it is supposed to be transferring local species of the alike subgenus [53]. It is supposed that East and Southern Africa got *R. microplus* from Asia, probably using the route of Madagascar [34]. Dispersed in savanna, including southern coastal strip of Kenya to the Cape Province of South Africa, as well as in Madagascar [35]. However all records for *R. microplus* including genetic, phenotypic and crossing studies with other *R. microplus* complicated taxa are desirable to check the findings [51].

The molecular-phylogenetic analysis revealed that *R. microplus* sequences are grouped into different clades with local mean diversity 0.005 and overall means diversity 0.038. Similar comments have been given in many renowned studies [54-60]. This study identified highly conserved regions with the help of alignment of sequences of BM86 gene from different isolates. Based on these findings, fully engorged female *R. microplus* ticks can be used to isolate cDNA encoding using transcriptomic studies on gut tissues [61]. Tick infestation has been significantly controlled by using *R. microplus* Bm86 antigen. But additional work related to tick-protective antigens is required to see the variable performance of BM 86 based vaccine due to local strain variation. Candidate protective antigens can be regarded as potential priority in vaccination studies based on gene knockdown on tick mortality and fertility. Though the evaluation of vaccine is subject to large scale field trials of recombinant antigens [62].

The current study confirmed that built on Bm86, R. microplus populations were homogeneous and showed high convergence among the different isolates studied by De la Fuente et al.[63], Sossai et al.[64], Guerro et al.[61], Canales et al.[65], Nijhof et al.[66], Freeman et al.[67]; strains of *R. microplus*-Thailand [68]; and Bm86 orthologs of *R. microplus,* such as *R. annulatus* (ABY58969) and *R. decoloratus* (ABY58970, ABG21130, ABG21131). Consequently, these results polymorphic sites peptides are more conserved whey they are compared with the Bm86 protein sequence.

Kamau et al.[69] described that cDNA of 4 *R. appendiculatus*

Table 3. *Estimates of Evolutionary Divergence between sequences*

Sr. No.	BM 86 Gene Sequences	Pairwise Comparison																											
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1	MG002403.1_R.microplus_SZPLG_cell_Bm86																												
2	MG002403.1_R.microplus_Bm86_Austrailia	0																											
3	MG002401.1_R.microplus_Bm86_austrailia	0.007	0.007																										
4	MG002399.1_R.microplus_Bm86_Austrailia	0.018	0.018	0.019																									
5	M29321.1.B.microplus_BM86_USA	0.012	0.012	0.013	0.008																								
6	KX786647.1_R.microplus_Bm86_USA	0.029	0.029	0.03	0.029	0.022																							
7	KJ995910.1_R.microplus_NE14_Thiland	0.058	0.058	0.059	0.062	0.055	0.059																						
8	KJ995907.1_R.microplus_NE11_BM86_Thiland	0.06	0.06	0.061	0.064	0.057	0.061	0.002																					
9	KJ995890.1_R.microplus_BM86_Thiland	0.05	0.05	0.051	0.049	0.042	0.043	0.047	0.048																				
10	HQ014394.1_R.microplus_zapata_Bm86_USA	0.028	0.028	0.029	0.028	0.021	0.007	0.054	0.056	0.036																			
11	HQ014392.1_R.microplus_zapata_Bm86_USA	0.026	0.026	0.027	0.026	0.019	0.003	0.056	0.058	0.04	0.006																		
12	FJ809946.1_R.microplus_Bm86_southafrica	0.028	0.028	0.029	0.029	0.022	0.018	0.059	0.061	0.042	0.02	0.019																	
13	FJ809946.1_R.microplus_Bm86_Netherland	0.028	0.028	0.029	0.029	0.022	0.018	0.059	0.061	0.042	0.02	0.019	0																
14	FJ456928.1_R.microplus_bm86_spain	0.027	0.027	0.028	0.027	0.02	0.003	0.056	0.058	0.04	0.006	0.002	0.021	0.021															
15	EU352677.1_R.microplus_BM86_Brazil	0.029	0.029	0.03	0.029	0.022	0.001	0.059	0.061	0.043	0.007	0.003	0.018	0.018	0.003														
16	EU191621.1_R.annulatus_BA86-_Israel	0.045	0.045	0.046	0.051	0.043	0.042	0.04	0.04	0.043	0.037	0.039	0.043	0.043	0.041	0.042													
17	EU191620.1_R.microplus_BM86_Spain	0.027	0.027	0.027	0.03	0.023	0.019	0.059	0.061	0.043	0.02	0.02	0.006	0.006	0.021	0.019	0.043												
18	B.M_ISTO_sahiwal5	0.06	0.06	0.061	0.064	0.057	0.061	0.005	0.005	0.048	0.056	0.058	0.061	0.061	0.058	0.061	0.04	0.061											
19	B.M_ISTO_sahiwal4	0.063	0.063	0.064	0.067	0.06	0.063	0.005	0.004	0.05	0.059	0.06	0.064	0.064	0.061	0.063	0.043	0.064	0.007										
20	B.M_ISTO_sahiwal3	0.06	0.06	0.061	0.064	0.057	0.061	0.004	0.004	0.049	0.056	0.058	0.061	0.061	0.058	0.061	0.04	0.061	0.006	0.006									
21	B.M_ISTO_sahiwal2	0.062	0.062	0.063	0.066	0.059	0.063	0.004	0.004	0.05	0.058	0.06	0.063	0.063	0.06	0.063	0.042	0.063	0.005	0.007	0.006								
22	B.M_ISTO_Sahiwal1	0.061	0.061	0.062	0.066	0.058	0.062	0.003	0.003	0.049	0.058	0.059	0.062	0.062	0.059	0.062	0.041	0.063	0.006	0.006	0.005	0.004							
23	B.M_ISTO_Okara6	0.061	0.061	0.062	0.066	0.058	0.062	0.003	0.003	0.05	0.058	0.059	0.062	0.062	0.059	0.062	0.041	0.063	0.006	0.006	0.005	0.005	0.005						
24	B.M_ISTO_Okara5	0.061	0.061	0.062	0.065	0.058	0.061	0.003	0.001	0.049	0.057	0.058	0.061	0.061	0.059	0.061	0.04	0.062	0.005	0.005	0.005	0.005	0.004	0.004					
25	B.M_ISTO_Okara4	0.06	0.06	0.061	0.064	0.057	0.061	0.002	0.002	0.048	0.056	0.058	0.061	0.061	0.058	0.061	0.04	0.061	0.005	0.005	0.004	0.004	0.002	0.003	0.003				
26	B.M_ISTO_Okara3	0.06	0.06	0.061	0.064	0.057	0.061	0.002	0.002	0.048	0.056	0.058	0.061	0.061	0.058	0.061	0.04	0.061	0.005	0.005	0.004	0.004	0.003	0.002	0.003	0.002			
27	B.M_ISTO_Okara2	0.06	0.06	0.061	0.064	0.057	0.061	0.002	0.002	0.048	0.056	0.058	0.061	0.061	0.058	0.061	0.04	0.061	0.005	0.005	0.004	0.004	0.003	0.003	0.003	0.002	0.002		
28	B.M_ISTO_Mandi_Bahauddin2	0.063	0.063	0.064	0.067	0.06	0.064	0.007	0.007	0.05	0.059	0.061	0.064	0.064	0.061	0.064	0.043	0.064	0.006	0.009	0.008	0.009	0.009	0.009	0.008	0.007	0.007	0.007	
29	B.M_ISTO_Mandi_Bahauddin1	0.063	0.063	0.064	0.069	0.061	0.065	0.006	0.006	0.053	0.061	0.062	0.064	0.064	0.063	0.065	0.041	0.064	0.008	0.006	0.007	0.008	0.006	0.007	0.007	0.005	0.006	0.006	0.01

The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model. The analysis involved 29 nucleotide sequences out of which 13 were from current study. All positions containing gaps and missing data were eliminated. There were a total of 1757 positions in the final dataset. Evolutionary analyses were conducted in MEGA7

Table 4. *Nucleotide and haplotype diversity of BM86 gene sequence analysis in R. microplus from different districts of Pakistan*

Districts	Observed Haplotypes in Number	Unique Haplotypes in Number	Diversity Haplotype (Bm86 gene)	Diversity of Nucleotide
Okara	5	2	0.88	0.011
Sahiwal	4	3	0.89	0.012
Mandi Bahauddin	3	1	0.92	0.010

field strains depicted genotypic polymorphisms and recommended that additional aspects like exposure during blood meal with innate immune components may be responsible for selection pressure which led to the observed polymorphism in these samples [64,70].

Various other studies reported the genotypic variations at molecular level between *B. microplus* isolates. To characterize habitat adoption and variant speciation in tick require more information following biogeographical separation among these species. Besides molecular approaches, morphological and physiological studies provide important information to achieve such goals which leads to the selection of useful tick antigens for anti-tick vaccine [63].

Considering the extensive sequence and functional polymorphism observed among strains of *R. microplus* from different geographical regions, we can conclude that it may be possible to achieve effective vaccination against these cattle ticks using a single universal Bm86-based antigen. With the advancement in genomic technologies in vaccine development sequencing of tick genome may help in identification of candidate tick strains for global application of anti-tick vaccine useful against different species of ticks [70].

Six single nucleotide polymorphisms were seen in sequences of BM 86 from indigenous tick population collected from the Punjab province. More interestingly out of these 6 polymorphism, we got 2 from district Okara (representative district of Northern irrigated Agro-ecological zone), 3 from Sahiwal (representative district of Northern irrigated Agro-ecological zone), and 1 in Mandi Bahauddin (representative district of junction of barani lands and Northern irrigated Agro ecological zone). Association of unique sequences to a particular geographical region supports the hypothesis of phylogeography proposed by different authors [71-73].

To our best knowledge this is only type of such study conducted in the area which identified the field strain as candidate specie for local tick vaccine, which in turn will increase the efficacy of tick control including reduction of economic burden on the farmer. New studies must concentrate on analyzing ticks in domestic and wild ruminants, covering diverse environmental areas of Pakistan and assessing the impact of diverse factors on its occurrence. Further research employing latest tools of genomics, proteomics and transcriptomics are necessary to depict the tick vector(s) involved in the spread of protozoal infections in Pakistan.

Author's Contribution

Higher Education Commission of Pakistan provided the financial assistance to Dr. M. Faiz Rasool for this research study, Dr. Nisar Ahmad, Dr. Haroon Akbar, Prof. Dr. Tahir Yaqub and Prof. Dr. Kamran Ashraf supervised this research study and Dr. M. Faiz Rasool is responsible for the provision of integration and writing of the article data. Acknowledgments to Prof. Dr. Aftab Ahmad Anjum, Dr. Muhammad Muddassir Ali and Dr. Mian Abdul Hafeez for their help in optimization of PCR, sequence analysis and estimation of genetic diversity.

Conflict of Interest

Authors declare no conflict of interest.

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