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RESEARCH ARTICLE

Molecular Characterization and Phylogenetic Analysis of Babesia Species Isolated from Domestic Cattle

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A B S T R A C T

Babesiosis is a key tick born disease caused by the protozoal parasites belonging to the genus Babesia, cosmopolitan in nature and infecting enormous range of large ruminants. Study was intended to determine the presence of local isolates of Babesia species (*B. bovis, B. bigemina*) their molecular characterization and phylogenetic relationship in cattle (district Narowal) Punjab. Blood samples were collected from 200 suspected cattle, from seven villages of selected district. DNA was extracted by using DNA zole, Gene-all Kit, manual method and subjected to PCR for amplification using specific RLB and Universal Primers. Quantification of protozoan parasite was performed by RT PCR using Solis biodine kit. A total of 36 out of 200 animal samples, were found positive by microscopy. PCR positive samples (17) were amplified and bands of strength 520-bp and 800-bp with specific RLB and for universal primers respectively were obtained for Babesia species. These 17 samples were sent for sequencing and by using Clustal W; Bioedit software with Neighbor-joining method phylogenetic tree was established. Current study reported the presence of *B. bigemina* i.e. 18% (conventional method) in large ruminants of district Narowal, Pakistan, and confirmation by (molecular characterization) PCR (8.5%) and phylogenetic relationship. Phylogenetic results showed that our study has homology with *Babesia bigemina* strain.

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INTRODUCTION

The major constraints of livestock industry are tick borne diseases. In tick transmitted diseases, the imperative one is the tick fever, caused by intra-erythrocytic parasite of genus Babesia (Apicomplexa: piroplasmadia: babesidiae). Tick fever is most ubiquitous disease in its nature. Substantially backlog like reduced reproductive fertility and even deaths observed due to these tick borne diseases in livestock population (Vannier and Krause, 2009). Babesia after Trypanosome is the second most dangerous and prevalent haemo-parasite for mammalian host (Barandika *et al.,* 2006). Babesia species are classified according to their hosts, i.e. Cattle (*B. bovis, B. bigemina. B. divergens, B. major)* Buffalo (*B. bovis* and *B. bigemina*) etc. (Uilenberg, 2006). High temperature and anemia are the common clinical signs of babesiosis (Chisu *et al.,* 2019). Hemoglobinuria is a typical sign not commonly observed in other protozoan diseases. Other signs include anorexia, yellow mucous membrane, nasal and ocular discharge and reduction in milk yield in lactating animals (Muraleedharan, 2015). About 500 million cattle are at risk throughout the globe (Chaudhry *et al*., 2010; Oscar and Cristian 2018). Tick borne diseases cause 18.6 billion losses and deficiency of 3 billion pieces of hide in cattle per year (Terkawi *et al*., 2012). Economic losses of production by ticks are about 13-19 billion per year globally (De Castro and Newson, 1993; Oscar and Cristian, 2018). Diminazine aceturate and imidocorb dipropionate are used in the treatment of Babesiosis (Mosqueda *et al*., 2012).

Focus on the mitochondrial Cytochrome c Oxidase Subunit I (COXI) gene in the present study was due to its nature of being the most conservative protein-coding

genes in the mitochondrial genome of animals. COXI exhibit fast mutation rate and easily distinguished between closely related species (Hong *et al.,* 2019), preferred for the evolutionary time depths. Internal Transcribed Spacer primers reveal highest degree of variation along its genomic sequences via unequal crossing over and gene conversion (Eui *et al.,* 2016).

The molecular characterization and phylogenetic analysis was opted as such for the confirmation of parasites as by (Oliveira-Sequeira *et al*., 2005; Rajabi *et al*., 2017; Barbara *et al*., 2017; Jafarbeklo *et al*., 2018; Nasr *et al*., 2018; Pardeep *et al*., 2019). For prevention and immunization of calves, vaccines are used mostly imported ones. It is imperative to get idea about the prevalent strain and their genetic evolutionary relationship to be taken as candidate vaccinal antigen (local isolates). The purpose of present study is to know the genetic makeup of prevalent strains of Babesia with parasite of other areas to estimate the extent of disease caused by different strains.

MATERIALS AND METHODS

Sampling and identification: A total number of 200 blood samples were randomly collected from suspected cattle in district of Narowal. Sampling was performed on the basis of number of animals present in each station. Blood was collected from jugular vein into EDTA-coated vacutainers and transported. Staining was performed by thin and thick blood smear.

DNA Extraction and PCR: The preliminary screened samples were subjected to DNA extraction. For comparative study DNA extraction was performed by three different methods (DNAzol BD, Kit & Manual method) as per manufacturer's instructions.

Kit method: GeneAll Exgene kit was used for the purification of total DNA from blood samples. DNA was extracted by using kit by following the directions.

Manual method: DNA was extracted by manual method by using lysis buffer, TEN buffer and PCI buffer. The concentration of DNA was estimated by using the Nano Drop (Mtshali *et al*., 2013).

Two sets of primers were selected for the amplification of DNA sample. One set of primers 18s ribosomal DNA specific for Babesia species termed as RLB primers of product size 520-bp was used for amplification. Second set of primers was universal (COXI) i.e. 1202 and 400 primers which amplified a product of 800-bp size (Table 1).

PCR and conditions: To confirm the *B. bigemina* and *B. bovis* PCR was executed by using 25-µl reaction mixture, having 2µl of extracted DNA, 2.5µM of each primer and 15µl of Dream Taq Green PCR Master Mix. The PCR reactions were performed in an automated Thermal Cycler (Bio Rad, USA). Each cycle consisted of denaturation @ 94°C for 1 min., an annealing step of 1 min. at 55°C (with a bit modification) followed by 40 cycles and extension @ 72°C for 1.5 min. Preceding's the final extension step at 72°C for 10 min. (Gubbles *et al*., 1999).

The condition for universal primer was initial denaturation consist of 96°C for 10 minute followed by 94°C for 30 second annealing at 48°C for 30 second followed by 35 cycles extension at 72°C for 90 second and final extension at 72°C for 10 minute. Agarose gel 1.5% stained with Biotium GelRed Acid Stain was used to analyze the PCR-generated amplicons by electrophoresis. The gel electrophoresis was operated at 113 Volts for 35 minutes. DNA Ladder was also used as Molecular weight marker (Mtshali *et al*., 2013). A 100 kb molecular weight marker was run along with samples.

Sequencing: The confirmed PCR product was sent to laboratory Advance Bioscience International First base Malaysia for sequencing. Nucleotide sequences were calibrated and arranged by using Bioedit software. The consensus sequences which were obtained by using Bioedit software, was trimmed manually to the equivalent length for the construction of phylogenetic tree. The other software Mega-7 and neighbor-joining methods was used for the construction of phylogenetic tree (Criado-Fornelio *et al.,* 2003). The Basic Local Alignment of BLAST Search Tool was used to check the sequence similarity between our data and in database. Sequence homology helped to identify the putative gene. Sequences were checked on NCBI website by using URL Genbank. http://www.ncbi.nlm.nih.gov/nuccore.

RT- PCR: The Solis biodine kit was used for Real time PCR. Each cycle consisted of denaturation @ 94°C for 1 min., an annealing step of 1 min. at 55°C (with a bit modification) followed by 40 cycles and extension @ 72°C for 1.5 min. Preceding's the final extension step at 72°C for 10 min. The results for quantification of parasites were recorded in terms CT values.

Statistical analysis: The data pertaining prevalence was imperiled to chi-square test and univariate analysis for analyses using Statistical Package for Social Sciences (SPSS) version 20. P-value less than 0.05 were considered significant.

RESULTS

Samples of cattle (200 in number) were processed for the identification of Babesia under microscope after staining procedure. Thin blood smear was stained with Giemsa and observed under oil immersion lens of microscope i.e. 40x. Intra erythroctyic bodies were found in heavy concentration with Giemsa Stain. Blood sample was also stained with field stain B and intra-erythrocytic bodies were observed. These intra-erythrocytic bodies were found inside the red blood cells and these were horse shoe shape.

Out of 200 samples 36 were found positive i.e. they have intra erythroctyic bodies. Percentage of infection was 18% by microscopic examination. The PCR detected 17 samples positive (Table 2). PCR reactions targeting the 18S ribosomal DNA (RLB-Internal Transcribed Spacer Primers) of *Babesia bigemina* species was tested for its ability to amplify the target Babesia species with 100bp ladder. Exact 520 bp amplified product was retrieved. The amplified DNA used (Fig. 1) in PCR was of *Babesia.*

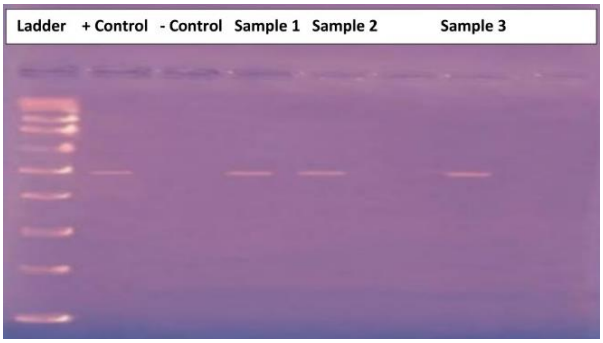

Fig. 1: PCR reactions targeting the RLB Primers of *Babesia bigemina* species that infect cattle & buffalo. Primer pair was tested for its ability to amplify the target Babesia species with 100bp ladder in lane #1. Lane#2 Positive Control, Lane #3 Negative Control, Lane #4, 5 & 7 Samples. Exact 520 bp amplified product was found.

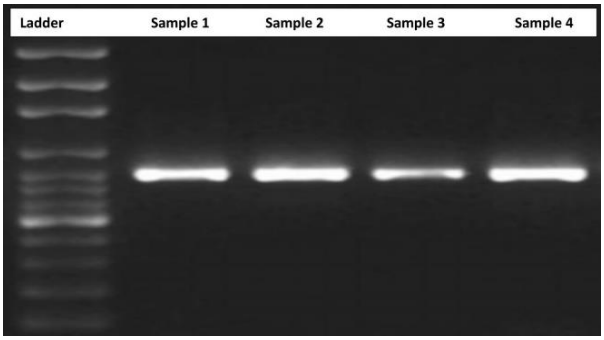

Fig. 2: PCR reactions targeting the Mitochondrial Cytochrome c Oxidase subunit I (COI) loci of Babesia *bigemina* species that infect cattle & buffalo. Primer pair was tested for its ability to amplify the target Babesia species with 100bp ladder in Lane #1. Lane # 2, 3, 4 & 5 samples. Exact 800 bp amplified product was instituted.

Babesia sp. WA1 isolate CA5 18S ribosomal RNA gene, partial sequence

Sequence ID: AY027815.1 Length: 1742 Number of Matches: 1

Range 1: 551 to 960 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
520 bits(281)	2e-143	373/415(90%)	16/415(3%)	Plus/Plus

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Query  1    GGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAATTGTTGCAGTTAA  60
Sbjct  551  GGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAA  610
Query  61   AAAGCTCGTAGTTGAATTTCTGCTGCATTG-C-TT-GTGTCCCTCTGGGGTCT-G-T-GC  114
Sbjct  611  AAAGCTCGTAGTTGAACTTCTGCCGCTTGGCCTTTCGTTCCCCT-TGGGGTTTCGTTCGC  669
Query  115  AT-GTGGCTTTTTTCGGACGG-AGTT-T-C-TTTGTCTGAATGTTTACTTTGAGAAAATT  169
Sbjct  670  CTGGTGGCTTACCTCTGGCGGTGGTTCTCCATTTG-C-CAGT-TTTACTTTGAGAAAATT  726
Query  170  AGAGTGCTCAAAGCAGGCTTTCGCCTTGAATAGTTTAGCATGGAATAATAAAGTAGGACT  229
Sbjct  727  AGAGTGTTTCAAGCAGGCTTTTGCCTTGAATACTTCAGCATGGAATAATAAAGTAGGACT  786
Query  230  TTGGTTCTATTTTGTTGGTTTCAGGTACCAAAGTAATGGTTAATAGGAACAGTTGGGGGC  289
Sbjct  787  TTGGTTCTATTTTGTTGGTTTCAGG-ACCAAAGTAATGGTTAATAGGAACAGTTGGGGGC  845
Query  290  ATTCGTATTTAACTGTCAGAGGTGAAATTCTTAGATTTGTTAAAGACGAACTACTGCGAA  349
Sbjct  846  ATTCGTATTTAACTGTCAGAGGTGAAATTCTTAGATTTGTTAAAGACGAACTACTGCGAA  905
Query  350  AGCATTTGCCAAGGATGTTTTCATTAATCAAGAACAAAAGTTAGGGGATCGAAGA  404
Sbjct  906  AGCATTTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGCTCGAAGA  960
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Fig. 3: Partial sequence of Babesia species WA-1 isolate from Narowal (18-S Ribosomal RNA gene)

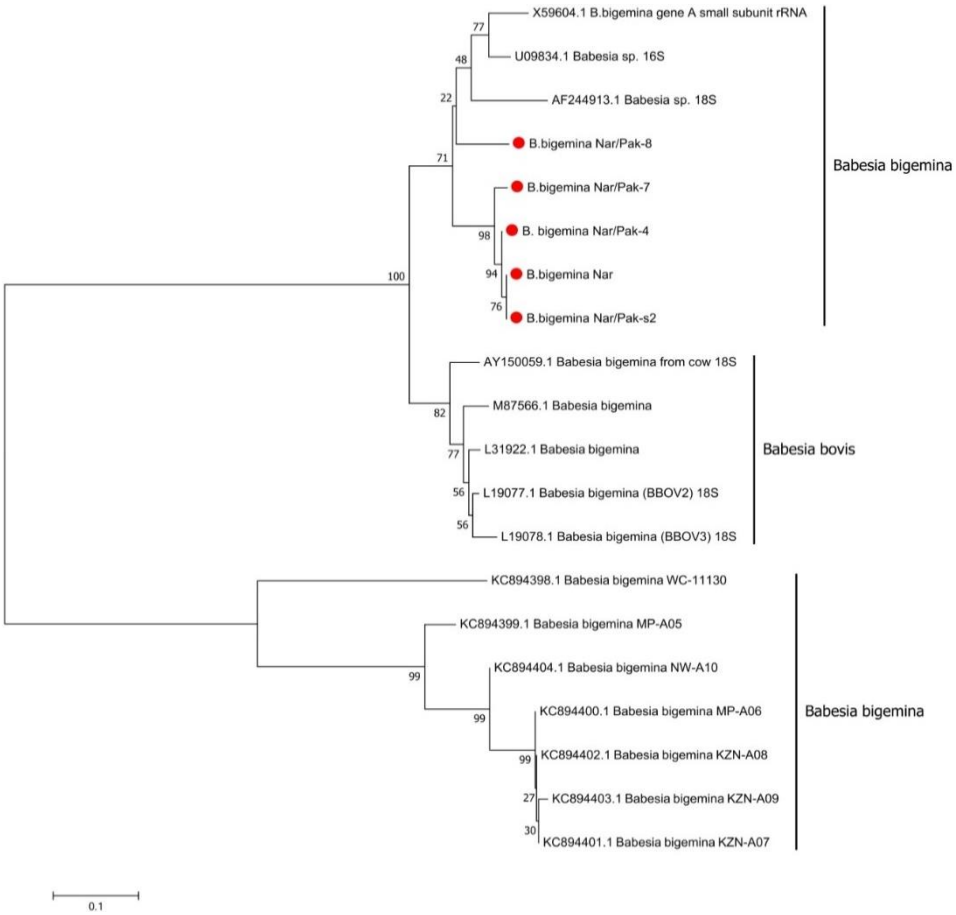

Fig. 4: Phylogenetic analyses sequences obtained from PCR positive sample. The Phylogenetic Tree reflecting the exact position of *Babesia bigemina* present the District Narowal, Pakistan.

Table 1: Two sets of primers were selected for the amplification of DNA sample. One set of primers 18s ribosomal DNA specific for *Babesia* species (RLB primers). Second set of primers was universal (COXI) i.e. 1202 and 400

Specie	Primer name	Primer sequence	Target region	Product size
Babesia	RLB(Forward)	5'- GAGGTAGTGACAAGAAATAACAATA-3'	18S ribosomal	520bp
	RLB(Reverse)	5'-TCTTCGATCCCCTAACTTTC-3'		
Babesia	400(Forward)	5'-GGDTCAGGTRTTGGTTGGAC-3'	COXI specific	800bp
	1202(Reverse)	5'-CCAAKRAYHGCACCAAGAGATA-3'		

(Ogedengbe *et al*., 2011; (Gubbels *et al.,* 1999).

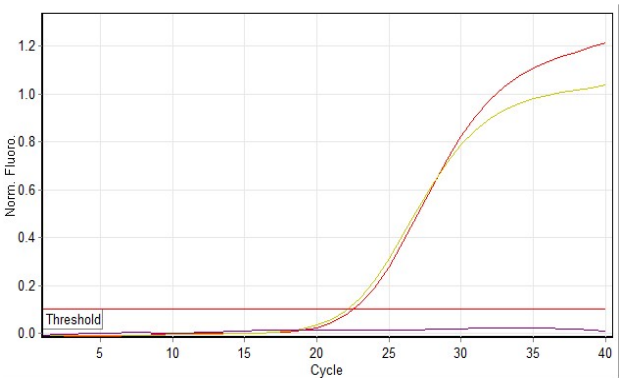

Fig. 5: Graphical representation of Real time PCR for *Babesia bigemina* as of X-axis number of cycles and on Y-axis Flourescence. The Ct value for *Babesia bigemina* samples showed fluorescence of sample R (purple) 22.6 and sample RM (yellow) 22.14.

Table 2: Percantage of Positive and Neagtive Samples by Micrometery & Molecular characterization of *Babesia bigemina* (Chi square)

	Microscopy	PCR
Positive	36	17
Negative	164	183
Total	200	200

P value=9.13E-060.000009 (Chi square was used to get the percentage).

Table 3: Estimates of net base composition bias disparity between sequences of babesia species prevalent in narowal and other areas of Pakistan

	SS2	SS3	SS4	SS7	Ikhlas_ pur.	Darman	Bara-mangh
SS2_RLBP							
SS3_RLBP	0.000						
SS4_RLBP	0.000	0.000					
SS7_RLBP	0.000	0.000	0.000				
Ikhlas_pur_Pak.	0.000	0.000	0.000	0.000			
Baramangh_Pak.	0.000	0.000	0.000	0.000	0.080		
Shakargarh_Pak.	0.000	0.000	0.000	0.000	0.000	0.028	
Noorkot_Pak.	0.811	0.811	1.255	0.722	0.448	0.189	0.415
Ikhlas_Poor_Pak.	0.000	0.000	0.038	0.000	0.000	0.009	0.000
Bhajna_Pak.	0.000	0.000	0.000	0.000	0.000	0.009	0.000
Shakargarh_Pak.	4.692	4.692	5.175	4.821	0.000	5.259	0.000
Narowal	0.425	0.425	0.608	0.325	2.637	0.368	2.882
SS5-RLPB	0.000	0.000	0.000	0.000	0.000	0.000	0.000

The Mitochondrial Cytochrome c Oxidase subunit I (COI) loci with universal Primers (1202 Reverse and 400 Forward) of Babesia *bigemina* species that infect cattle was experienced to amplify the target Babesia species. Precise 800 bp amplified product was obtained (Fig. 2).

Sequencing and phylogenetic: The tree (Fig. 4) showed a similar ancestor relationship of Babesia species with other areas as found in district Narowal. The sequences of the present study reflected to be distributed in same cluster of *Babesia bigemina* as well as same clade. But the constructed phylogenetic tree revealed that one sequence (*B. bigemina* Nar/Pak-8 Fig. 3) clustered in a different sub clade, denoted to be genetically different from other isolates collected from Narowal.

Disparity index per sites in comparison with area of present study for all sequences is presented in Table 3. The Zero value reflected the no difference while the values greater than zero indicate the higher difference in base composition. Evolutionary analyses were conducted in MsEGA7.

RT-PCR: The Ct value for *Babesia bigemina* was calculated by real time PCR and the samples showed fluorescence of sample R (purple) 22.6 and sample RM (yellow) 22.14 (Fig. 5). The NTC was used as thershold and two samples were amplified; named as R and Rm. AT cycle no. 20 the reaction shows flourecences.This shows that it starts amplification at cycle no. 20 and the maximum shows at ct value 22.55 and 22.14.

DISCUSSION

Tick borne diseases impose considerable constraints on cattle and buffalo health and economic development in temperate areas of the world (Barandika *et al*., 2006). Annually 500 million dollars economic losses are observed because of tick fever and most losses occur due to *B. bovis* infection (Ramos *et al*., 2012). These samples were screened for the phylogenetic analysis of *B. bovis* and *B. bigemina.* Although the distribution of *B. bovis* and *B. bigemina* was found all over the country but a notable observation was made during the screening of blood samples of district Narowal i.e. the distribution of B. *bigemina* was more than 18% i.e. in 200 suspected blood samples 36 were found as positive by microscopy which is in coherence with a previous study in which prevalence rate of *B. bigemina* was 20.66%. The high prevalence rate was found in low age group and in females.(Saad *et al*., 2015; Khan *et al*., 2016)

Another study showed a high prevalence rate of *B. bigemina* in which 24 samples were found positive out of 100 for *B. bovis* and *B. bigemina* by microscopy (Saad-Roy *et al*., 2015) which is in accordance with present study. The prevalence rate of *B. bigemina* around 19% and of *B. bovis* was 11% by Ahmad *et al*. (2014) is also in agreement with current findings. The prevalence rate in calves less than one year was higher than aged calves. As for as sex wise prevalence was concerned, female calves were more prone to Babesia than male calves (Ahmad *et al*., 2014; Abdelrasol *et al*., 2017).

Out of 36 microscopy samples only 17 samples got amplified. PCR reactions targeting the 18S ribosomal DNA of *Babesia bigemina* species was tested and exact 520 bp amplified product was retrieved which is in coherence with Gubbles *et al*. (1999).

The Mitochondrial Cytochrome c Oxidase subunit I (COXI) loci with universal Primers of *Babesia bigemina* species was experienced and 800 bp amplified product was instituted as that in case of Ogedenbge *et al*. (2011) and Hong *et al.* (2019).

These were sent for sequencing and by using Bioedit software extract the sequencing files. Mega 7 is software by align the sequences and Clustal W consensus sequences were formed (Jafarbeklo *et al*., 2018; Nasr *et al*., 2018; Pradeep *et al*., 2019). Sequences confirmed *B. bigemina* by using Neighbour-joining, Maximum likelihood method and Boot striping method a phylogenetic tree was constructed.

The sequences of the present study reflected to be distributed in same cluster of *Babesia bigemina* as well as same clade. But the constructed phylogenetic tree revealed that one sequence (*B. bigemina* Nar/Pak-8) clustered in a different sub clade, denoted to be genetically different from other isolates collected from Narowal. This tree was as similar as constructed within a study that conducted in South Africa in 2013 (Mtshali *et al*., 2013; Hajeel and Al-Fatlawi *et al.,* 2019). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 15 nucleotide sequences (Mtshali *et al*., 2013). All positions containing gaps and missing data were eliminated. There was a total of 212 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. This tree showed a similar ancestor relationship that found in district Narowal. This study was planned in first time in Narowal district and their evolutionary relationship in form of phylogenetic tree was constructed first time in Narowal.

In present study two samples of Babesial DNA was operated at same condition at which conventional PCR gave results. Same RLB primers were used. The Ct value at which it started to produce fluorescence was 22.41 and 22.31 respectively. The results were in comparison with the study carried out in 2007 by Chulmin Kim in National Research Centre for Protozoan disease and showed fluorescence at same Ct value (Kim *et al.*, 2007; Barbara *et al*., 2017).

Conclusions: The presence of *B. bigemina* i.e. 18% (conventional method) in large ruminants of district Narowal, Pakistan, and their molecular characterization by PCR (8.5%) and phylogenetic relationship. Phylogenetic results showed that our study has homology with *B. bigemina* strain.

Authors contribution: Conceived and designed the experiments: RF, MA Hafeez. Performed the experiments: RF, KK, FA, AR, SM & FB: Analyzed the data: KA, MO & NR: Contributed reagents/materials/ analysis tools: Wrote the paper: MAH & RF.

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