VETERINARSKI ARHIV 78 (6), 545-551, 2008

Randomly amplified polymorphic DNA-polymerase chain reaction fingerprinting of *Babesia bigemina* isolates of India - short communication

Reghu Ravindran*, Ashok Kumar Mishra, and Jammi Raghavendra Rao

Division of Parasitology, Indian Veterinary Research Institute, Izatnagar, Bareilly, U. P. India

RAVINDRAN, R., A. K. MISHRA, J. R. RAO: Randomly amplified polymorphic DNA-polymerase chain reaction fingerprinting of *Babesia bigemina* isolates of India. Vet. arhiv 78, 545-551, 2008.

ABSTRACT

This short communication describes the randomly amplified polymorphic DNA fingerprinting of *B. bigemina* from two geographically distant locations of the Indian subcontinent viz, Wayanad (Kerala state, Southern India) and Izatnagar (Uttar Pradesh State, Northern India). A total of four isolates, two each from Wayanad (Kerala, South India) and Izatnagar (North India) were used in the present study. RAPD-PCRs were carried out using four random oligonucleotide primers. Depending on the parasite isolate-primer combination, between one and five fragments in the range of 257 bp and 1948 bp were amplified. DNA fragments which can differentiate the two isolates were identified.

Key words: randomly amplified polymorphic DNA (RAPD) PCR, *Babesia bigemina,* cattle, India

Introduction

Randomly amplified polymorphic DNA-PCR (RAPD-PCR) is a technique widely used for studying the DNA polymorphism between closely related species without the requirement of prior knowledge of the genome (WILLIAMS et al., 1990; WELSH and McCLELLAND, 1990). This technique uses single oligonucleotide primers at low stringency to generate random amplified polymorphic DNA. The pattern generated by this technique provides a rapid method for detecting the polymorphism/ genetic variation. The technique, also known as arbitrary primer-polymerase chain reaction (AP-PCR), has been extensively used for elucidation of intraspecific variation between Indian isolates of various parasites viz, *Trypanosoma evansi* (BASAGOUDANAVAR et al., 1998; OMANWAR

*Contact address:

Dr. Reghu Ravindran, Assistant Professor, Department of Veterinary Parasitology, College of Veterinary and Animal Sciences, Pookot, Lakkidi, P.O., Wayanad, Kerala, India-673576, Phone: +91 4936 256380; +91 9447 713422; Fax: +91 4936 256390; E-mail: drreghuravi@yahoo.com

ISSN 0372-5480
Printed in Croatia

et al., 2001), *Toxoplasma gondii* (SREEKUMAR, 2001), *Eimeria tenella* (KULKARNI, 1997), *Fasciola gigantica* (GUNASEKAR, 2000), *Theileria annulata* (SARAVANAN, 2000).

Bovine babesiosis caused by *Babesia bigemina* transmitted by *Boophilus microplus* is an economically important disease in tropical and subtropical countries of the world including India. Even though the disease is widespread throughout the Indian subcontinent, molecular characterization of isolates from geographically distant regions has not been carried out before. This short communication describes the RAPD fingerprinting of *B. bigemina* from two geographically distant locations of the Indian subcontinent *viz*, Wayanad (Kerala state, Southern India) and Izatnagar (Uttar Pradesh state, Northern India).

Materials and methods

Animals and samples. A total of four isolates, two each from Wayanad (Kerala, South India) and Izatnagar (North India) were used in the present study. Healthy male bovine crossbred calves of three months of age were procured from the Division of Livestock Production and Management of the Indian Veterinary Research Institute. Animals negative for *B. bigemina* by IFAT (RAVINDRAN et al., 2002) and blood smear examination were used for infecting with Wayanad isolate of *B. bigemina*. Larvae of adult *Boophilus microplus* ticks engorged on two *B. bigemina* infected cattle from two different villages of the Wayanad district, Kerala were allowed to feed on the ear of the susceptible bovine calves. These animals were splenectomised and then immuno-suppressed to achieve peak parasitaemia (RAVINDRAN et al., 2006) of Wayanad isolate. Similarly, two IFAT positive or blood smear positive animals were directly splenectomised and then immuno-suppressed for isolation of *B. bigemina* Izatnagar isolate. *B. bigemina* rich erythrocytes were purified from leucocytes by CF-11 Cellulose chromatography (RAY et al., 1998). Genomic DNA was extracted from *B. bigemina* piroplasm rich erythrocytes and bovine leucocyte DNA from a 4 day-old calf free from *B. bigemina* infection (SAMBROOK et al., 1989).

RAPD-PCR reactions. RAPD-PCRs were carried out using four random oligonucleotide primers (Table). The RAPD -PCR reactions were set up in 25 μL reaction volume in 0.2 mL thin walled PCR tubes (Axygen). The reaction mixture was as follows: One microlitre of the template DNA (10 ng/μL), 2.5 μL of 10x PCR buffer (Bangalore Genei with 15 mM $MgCl_2$), 5 μL of 1 mM dNTP mix, 1 Unit of *Taq* DNA polymerase (Bangalore Genei) and 1 μL of primer (15 pmol or 50 ng). A control sample containing bovine leucocyte DNA was run along with every reaction.

Table1. List of random primers used

Nº	Code	Sequence (5'-3')	Length	GC%
1.	BG79	TCACGATGCA	10	50
2.	OPM12	GGGACGTTGG	10	70
3.	P5	GCATGCGATC	10	60
4.	P6	GTGGGCTGAC	10	70

PCR was performed in a thermal cycler with a heated lid (Perkin- Elmer Gene Amp2400). The cycling conditions used were initial denaturation at 94 °C for 5 min followed by 45 cycles each of 1 min denaturation at 94 °C, 45 seconds annealing at 36 °C and 1 min elongation at 72 °C. This was followed by a final extension for 5 min at 72 °C.

After amplification reactions, five microlitres of loading dye were added to each tube and 15 μL of the product was run on 1.5% agarose gels. Standard molecular weight markers were also run with the sample. Electrophoresis was performed at 60 V for 1.5 h. After the run, the gel was transferred to a gel documentation system and documented.

Molecular sizes of scorable RAPD bands were estimated by comparing their mobility with respect to that of the standard molecular size marker using the Alphaimager™ gel documentation system. The RAPD patterns were scored (BOWDITCH et al., 1993). DNA fragments which can differentiate the two isolates were identified.

Results

The arbitrary primed polymerase chain reaction (AP-PCR) profiles of *B. bigemina* isolates using four primers are shown in Figs. 1-4. Depending on the parasite isolate-primer combination, between one and five fragments in the range of 257 bp and 1948 bp were amplified.

Using the primer BG79 (Fig. 1), three fragments ranging in size from 506 bp to 1071 bp were amplified. The 754 bp and 506 bp fragments were monomorphic, but the 943 bp fragment appeared only in the Izatnagar isolate while 1071 bp fragment appeared only in the Wayanad isolate.

The primer OPM12 (Fig. 2) generated 1-3 fragments ranging from 578 to 1628bp. The 768 bp fragment was seen only in Wayanad isolates.

A maximum of 5 bands were amplified by the primer P5 (Fig. 3) ranging in size from 257 to 1948 bp. The 386 bp and 1591 bp fragments were monomorphic. The 867 bp fragment was Izatnagar isolate specific while the 708 bp fragment was Wayanad isolate specific.

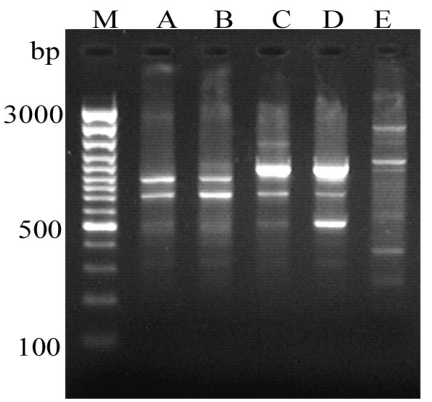

Fig. 1. The primer BG79

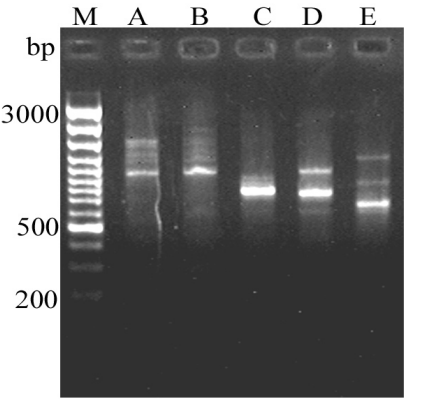

Fig. 2. The primer OPM12

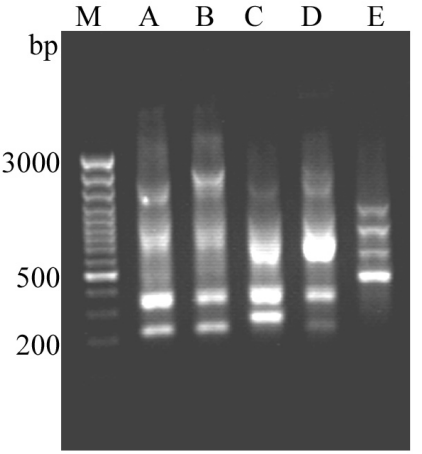

Fig. 3. The primer P5

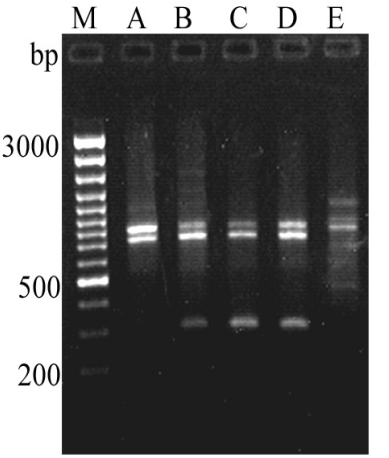

Fig. 4. The primer P6

Figs. 1-4. RAPD-PCR fingerprints obtained using primer BG79 (Fig. 1), OPM12 (Fig. 2), P5 (Fig. 3) and P6 (Fig. 4). M - GeneRuler™ 100bp DNA ladder plus; A - Izatnagar 1 isolate; B - Izatnagar 2 isolate; C - Wayanad 1 isolate; D - Wayanad 2 isolate; E - Bovine leucocyte DNA.

The primer P6 (Fig. 4) also produced 3 fragments ranging in size from 327 bp to 930 bp. The 930 bp and 816 bp fragments were monomorphic. Apart from the Izatnagar 1 isolate, all isolates showed a fragment of size 329 bp.

Discussion

According to the Annual report of the Project Directorate of Animal Disease Monitoring and Surveillance (PD-ADMAS) of the Indian Council of Agricultural Research (2005-2006), bovine babesiosis is the top ranking disease among the top ten important parasitic diseases affecting domestic livestock in India. The disease is transmitted by one host tick, *Boophilus microplus*. With the changing trends in tick epidemiology, by gradual replacement of multi-host tick *Hyaloma anatolicum anatolicum* by one-host tick, *Boophilus microplus* (SANGWAN et al., 2000), there is an urgent need to monitor the status of this disease in the country.

In the present study, the RAPD-PCR method clearly detected the genetic variation among *B. bigemina* isolates in India. The RAPD-PCR technique was used previously for differentiation of *B. bovis* from *B. bigemina* stock*s* (CARSON et al., 1994). LEW et al. (1997) found that the random primer, oligonucleotide ILO-525 5'-CGGACGTCGC-3' could differentiate *B. bigemina* from *B. bovis.*

These observed variations could be due to various factors. The variations in the geographical location, virulence of the parasite, and the strains of vectors are some of the factors that can lead to micro-heterogeneity among *B. bigemina.* The variation in the rhoptry associated protein gene of *B. bigemina* has been well documented (HOTZEL et al., 1997). Recently, the analysis of the internal transcribed spacer 2 (ITS2) of the rRNA genes *B. bigemina* isolate (Switzerland) revealed high polymorphism, not only among the isolates but even within the isolates (HILPERTSHAUSER et al., 2007).The present study is in conformity with MADRUGA et al. (2002) who found that in Brazil the Northern, Eastern and Mid-western isolates of *B. bigemina* showed genetic diversity and the South-eastern and Southern isolates were genetically closest. The study also identified potential DNA fragments which could be developed as *de novo* isolate/parasite specific probes or PCR primers.

Acknowledgements

The authors are thankful to the Director, IVRI for the facilities provided and for the award of Senior Research Fellowship to the first author.

References

BASAGOUDANAVAR, S. H., J. R. RAO, R. K. SINGH, G. BUTCHAIAH (1998): Random amplification of polymorphic DNA fingerprinting of *Trypanosoma evan*si. Vet. Res. Commun. 23, 249-255.

BOWDITCH, B. M., D. G. ALBRIGHT, J. G. K. WILLIAMS, M. J. BRAUN (1993): Use of randomly amplified polymorphic DNA markers in comparative genome studies. Methods Enzymol. 224, 294-309.

CARSON, C. A., H. M. BRANDT, J. B. JENSEN, C. W. BAILEY, G. K. ALLEN (1994): Use of random amplified polymorphic DNA analysis to compare *Babesia bovis* and *B. bigemina* isolates. Parasitol. Res. 80, 312-315.

GUNASEKAR, K. R. (2000): *Fasciola gigantica*: Random amplified polymorphic DNA-polymerase chain reaction and immunoblot analysis. M.V.Sc. Thesis, Deemed University, Indian Veterinary Research Institute, Izatnagar, Bareilly, U.P., INDIA.

HILPERTSHAUSER, H., P. DEPLAZES, M. L. MELI, R. HOFMANN-LEHMANN, H. LUTS, A. MATHIS (2007): Genotyping of *Babesia bigemina* from cattle from a nonendemic area (Switzerland), Vet. Parasitol. 145, 59-64.

HOTZEL, I., C. E. SAUREZ, T. F. MCELWAIN, G. H. PALMER (1997). Genetic variation in the dimorphic region of RAP-1 genes and RAP-1 loci of *Babesia bigemina*. Mol. Biochem. Parasitol. 90, 479-489.

KULKARNI, R. R. (1997): Molecular characterization of a strain of *Eimeria tenella* selected for precocioussnesss. M. V. Sc. Thesis, Deemed University, Indian Veterinary Research Institute. Izatnagar, Bareilly, U. P. India.

LEW, A. E., B. P. DALRYMPLE, P. J. JESTON, R. E. BOCK (1997): PCR methods for the discrimination of *Babesia bovis* isolates. Vet. Parasitol. 71, 223-237.

MADRUGA, C. R., C. R. B. LEAL, A. M. T. FERREIRA, F. R. ARAUJO, A. L. V. BONATO, R. H. KESSLER, M. A. M. SCHENK, C. O. SOARES (2002): Genetic and antigenic analysis of *Babesia bigemina* isolates from five geographical regions of Brazil. Pesquisa Veterinária Brasileira, 22, 153-160.

OMANWAR, S., J. R. RAO, R. K. SINGH, G. BUTCHAIAH (2001): DNA polymorphisms in *Trypanosoma evansi* isolates amplified by randomly amplified polymorphic DNA-PCR. Vet. Rec. 148, 244-246.

RAVINDRAN, R., A. K. MISHRA, J. R. RAO (2002): On the high prevalence of bovine babesiosis in Wynad district of Kerala. J. Appl. Anim. Res. 22, 43-48.

RAVINDRAN, R., A. K. MISHRA, J. R. RAO (2006): Methodology for raising high parasitaemic *Babesia bigemina* infection in donor bovine calves. J. Appl. Anim. Res. 29, 59-60.

RAY, D., G. C. BANSAL, B. DUTTA (1998): Purification of intraerythrocytic piroplasms of *Theileria annulata* from infected bovine blood. Indian J. Anim. Sci. 68, 1167-1168.

SAMBROOK, J., E. F. FRITSCH, T. MANIATIS (1989): Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring, Harbor, New York.

SANGWAN, A. K., N. SANGWAN, M. C. GOEL (2000): Progressive displacement of *Hyalomma* ticks by *Boophilus microplus* in Haryana. J. Parasitic Dis. 24, 95-96.

SARAVANAN, B. C. (2000): Characterization of *Theileria annulata* isolates by an arbitrary primer -polymerase chain reaction and macroschizont proliferation assay. M.V.Sc. Thesis, Deemed University, Indian Veterinary Research Institute, Izatnagar, Bareilly, U.P. India.

SREEKUMAR, C. (2001): PCR based diagnosis and genotyping of *Toxoplasma gondii*. Ph.D. Thesis, Deemed University, Indian Veterinary Research Institute. Izatnagar, India.

WELSH, J., M. McCELLAND (1990): Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. 18, 7213-7218.

WILLIAMS, J. G. K., A. R. KUBELIK, K. J. LIVAK, J. A. RAFALSKI, S. V. TINGEY (1990): DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic Acid Res. 18, 6531-6535.

Received: 2 May 2007
Accepted: 24 November 2008

RAVINDRAN, R., A. K. MISHRA., J. R. RAO: Fingerprinting analiza lančanom reakcijom polimerazom nasumce umnožene polimorfne DNA izolata protozoona *Babesia bigemina* izdvojenih u Indiji. Vet. arhiv. Vet. arhiv 78, 545-551, 2008.

SAŽETAK

Ovo kratko priopćenje prikazuje rezultate fingerprinting analize nasumično umnožene polimorfne DNA izolata protozoona *Babesia bigemina* izdvojenih u različitim indijskim državama: Wayanad (Kerala i Južna Indija) i Izatnagar (Država Uttar Pradesh, Sjeverna Indija). Ukupno su istražena četiri izolata (dva iz države Kerala i dva iz države Uttar Pradesh). Umnažanje odsječaka DNA provedeno je uporabom četiriju oligonukleotidnih početnica. Ovisno o kombinaciji početnica umnožen je od jednog do pet odsječaka u rasponu od 257 i 1948 parova baza. Identificirani su odsječci pomoću kojih se mogu razlikovati dva izolata.

Ključne riječi: nasumce umnožena polimorfna DNA, *Babesia bigemina*, govedo, Indija