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Research Article
Monotypic PCR-RFLP Pattern of Circulating *Theileria annulata* Isolates from North India Based on HSP 70 gene

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Abstract

Background and Objective: Knowledge of local isolates and strains is a prerequisite for development of both effective mass vaccination strategy and a suitable molecular marker based diagnostic tool. The pathogenesis of bovine tropical theileriosis (BTT) caused by *Theileria annulata* in susceptible ruminants is known to vary depending upon the isolate and strain involved. The present study was designed to characterize *T. annulata* isolates from Northern India using PCR-RFLP technique. **Materials and Methods:** In the present study, HSP 70 gene was amplified from 28 naturally infected calves found in BTT endemic semi arid zone of Northern India. The amplified product was then digested with two restrictions enzymes viz., Taq I and Alu I to find out the pattern of restriction digests so as to have an idea of various strains of *T. annulata* involved. **Results:** With Taq I enzyme, two restriction digestion products were noticed with molecular weights of 100 and 175 bp, respectively. So far as Alu I enzyme is concerned, three restriction digestion products with molecular weights of 60, 90 and 125 bp, respectively, were noticed. **Conclusion:** Monomorphic pattern of restriction digest was noticed in all 28 studied *T. annulata* isolates, for both the enzymes, suggesting of presence of single strain of parasite involved in the studied area.

Key words: Characterization, genotypes, HSP 70, monotypic pattern, Northern India, *Theileria annulata*, PCR-RFLP

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Theileria annulata, an apicomplexan parasite involving three host tick-*Hyalomma anatolicum anatolicum* as its vector, causes a classical disease entity-Bovine tropical theileriosis (BTT) in ruminants[1,2]. It is known that the various isolates and strains of this parasite prevalent in an endemic belt showed variable levels of virulence in susceptible population[3,4]. Hence, it is justifiable to say that the pathogenesis of BTT is widely influenced by the strain/isolate of parasite involved. One of the most common ways for detection of parasitic strain variation is the sequencing of the respective gene involved although, it is not cost effective. Restriction digestion of PCR products using selective enzymes is an economic way of screening a large number of samples. PCR-RFLP technique is routinely used for specific detection and strain identification of *Theileria annulata* field isolates using a number of molecular targets like β-tubulin gene[5], SmI-2 gene[6], TAMS 1 gene[7,8] and HSP 70 gene[9].

Heat shock proteins are amongst the most conserved proteins across the organisms[10] and are vital in eliciting host response in stress conditions[11,12]. They are very significant in parasites involving vectors as when parasites are transmitted from their poikilothermic invertebrate vector to the homoeothermic vertebrate host, there is an enormous change in the habitat of parasite. Hence, it is appropriate to state that HSPs contribute an essential part in survival of parasite and or its developmental stages within the host by adopting it with various stress stimuli, in general and with variations in temperature, in particular[13]. HSP 70, a 70 kilo Dalton molecular weight heat shock protein, acts as a molecular chaperon preventing parasite aggregation and providing folding assistance to other polypeptides[14]. The present study was designed with the objective to characterize field isolates of *T. annulata* using PCR-RFLP targeting HSP 70 gene.

MATERIALS AND METHODS

Topology of studied area and various isolates of *T. annulata*: The studied area comprised of portion of semi-arid zone of Northern India. The studied area consisted of Mathura (Uttar Pradesh) and border areas of Rajasthan. The area is located at 27.49°N latitude and 77.67°E longitude axis, respectively, in semi-arid zone of India. The area is considered to be endemic for BTT[15] owing to the weather which consists of warm temperatures and high humidity with a little of rainfall favoring the abundance of tick vector involved in spread of BTT-*Hyalomma anatolicum anatolicum*[16].

Blood samples of 28 clinically confirmed BTT infected calves, (through microscopic examination and TAMS 1 based PCR), were stored in -20°C facility of the Department[17]. The samples were periodically collected over a period of 1 year (2018-19) from the animals that are brought for treatment at the Teaching Veterinary Clinical Complex (TVCC) of the parent University. Those samples were used for DNA extraction.

DNA extraction, primer selection and PCR: DNA was isolated from 300 µL of blood using commercial available DNA isolation kit (Wizard Genomics DNA Promega, USA) following manufacturer's protocol. Primers for HSP 70 gene of *T. annulata* were custom synthesized from Imperial Life Sciences Pvt. Ltd., Gurugram, India. The outer primer consisted of HSP 70 F 5'-TGTCAAGGAGGCCTCAAATTA-3' while the reverse consisted[9] of HSP 70 R 5'-TTTGACTTTGAATAGGCTGCC-3', spanning a 275 bp product. PCR was performed in a reaction mixture of 25 µL containing 12.5 µL green master mix (Fermentas) containing of 0.05 µL Taq DNA polymerase, 4 mM $MgCl_2$, 0.4 mM dATP, 0.4 mM dTTP, 0.4 mM dGTP, 0.4 mM dCTP), 1.5 µL of each primer (15 pmol), 5 µL of DNA template and nuclease free water upto a final volume of 25 µL. The reaction conditions included initial denaturation at 95°C for 4 min, followed by 36 cycles of denaturation at 94°C for 45 sec, annealing at 56°C for 45 sec and elongation at 72°C for 45 sec. A final extension of 72°C for 5 min was given before storing the products at 4°C until further use. The amplified products were visualized under a UV transilluminator following ethidium bromide incorporated 1.25% agarose gel (w/v) electrophoresis.

Restriction fragment length polymorphism (RFLP) analysis: After PCR amplification, the amplified PCR products were digested with Taq I and Alu I restriction enzymes (Fast Digest enzymes, Fermentas). The restriction digestion reaction was carried out in a total volume of 30 µL consisting of 10 µL of PCR product, 1 µL of respective restriction enzyme, 2 µL of 10X fast digest green buffer and 17 µL of nuclease free water. The restriction digestion was carried out at 65°C for 5 min for Taq I enzyme and 37°C for 15 min for Alu I enzyme, respectively. The restriction digests of Taq I were directly loaded on agarose gel while for Alu I enzyme restriction digestion was inactivated at 65°C for 5 min before visualization of digested products as per the manufacturer's protocol. The digested products were separated in ethidium bromide incorporated 2.0% agarose gel (w/v) following electrophoresis and visualized under a UV transilluminator.

RESULTS

PCR amplification of HSP 70 gene from *T. annulata* field isolates: The PCR on HSP 70 gene was firstly laboratory standardized on confirmed *T. annulata* DNA. The confirmation DNA was stored in the deep freezer facility of Department of Parasitology, DUVASU. Once standardized, the PCR was later performed on all 28 confirmed BTT affected blood samples. The confirmation of the samples was earlier done based on blood microscopy of the parasite and gene sequencing. A specific product of 275 bp was obtained using HSP 70 gene based PCR in all 28 confirmed BTT affected blood samples (Fig. 1).

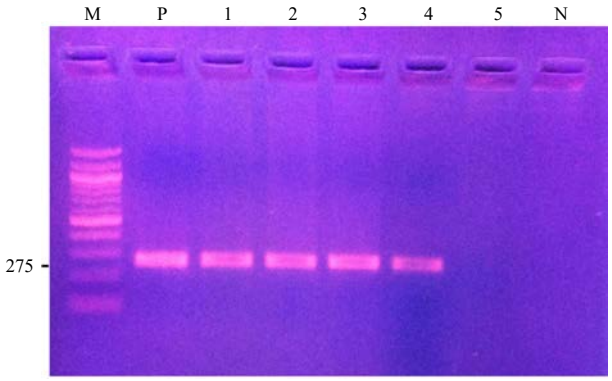

Fig. 1: PCR amplification of Hsp 70 gene
Lane M: 100 bp DNA ladder (Imperial Life Sciences), Lane P: Control positive, Lane 1-5: Amplified products, Lane N: Control negative

PCR-RFLP analysis: The amplified 275 bp product of 28 isolates were sequentially digested with Taq I and Alu I restriction enzymes. After digestion, the digested products were visualized on ethidium bromide incorporated agarose gel and specific bands corresponding to each restriction enzyme were noticed. When the amplified PCR product was digested with Taq I enzyme two restriction digestion products were noticed with molecular weights of 175 and100 bp, respectively (Fig. 2). On similar lines, when the amplified product was digested Alu I enzyme, three restriction digestion products, with molecular weights of 125, 90 and 60 bp, respectively, were noticed (Fig. 2).

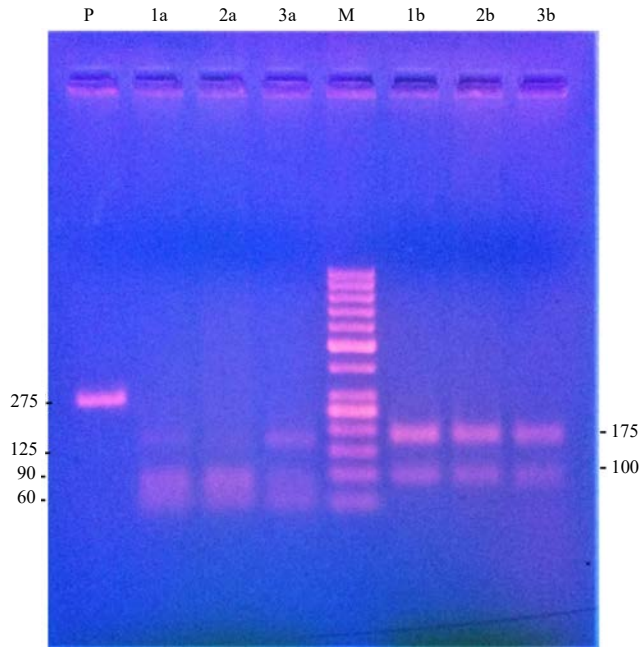

Fig. 2: RFLP digestion pattern with Alu I and Taq I enzymes
Lane P: Control positive, Lane M: 50 bp DNA ladder (Fermentas, Germany), Lane 1a-3a: Respective restriction digestion products of Alu I, Lane 1b-3b: Respective restriction digestion products of Taq I

Characterization pattern of *T. annulata* field isolates: Identical pattern of restriction digestion was noticed for all 28 samples using both Taq I and Alu I restriction enzymes. This monotypic uniform pattern of RFLP for both the enzymes suggested of no strain variation in all the studied 28 isolates. Hence, it was very much concluded that there was absence of strain variation in studied 28 isolates based on RFLP characterization of HSP 70 gene.

DISCUSSION

Present study revealed absence of strain variations in studied *T. annulata* field isolates at least on the basis of RFLP patterns of HSP 70 gene. Monotypic restriction pattern using two set of restriction enzymes justifies the same. Restriction fragment length polymorphism (RFLP) is a commonly employed technique in which parasites are differentiated based on analysis of patterns derived from cleavage of their DNA or specific gene. The dissimilarities in cleavage/digestion pattern often help in differentiation of closely related species and even strains from one another. PCR-RFLP is found to be suitable for easy and quick identification of genotypic variations in *T. annulata* isolates[7-9]. Genes like TAMS 1[7,8] and HSP 70[9] are often targeted for identification of strain variations in *T. annulata* both in vertebrate hosts as well as in tick vectors.

In the present study, HSP 70 gene was employed for restriction digestion using Taq I and Alu I enzymes. With Taq I enzyme, two restriction digestion products were noticed with molecular weights of 100 and 175 bp, respectively. On similar lines, Alu I enzyme yielded three restriction digestion

products, with molecular weights of 60, 90 and 125 bp, respectively. Same size of restriction digests were noticed in Iran[11] using these two restriction enzymes on HSP 70 gene products.

Similar sized products were earlier reported in PCR-RFLP based characterization of *T. annulata* from ticks affecting cattle in Iran[9]. Taq I was not able to digest all *T. annulata* isolates in that study and some isolates remain undigested. But all the isolates were found to be digested by Alu I enzyme[9]. This is in contrary to this study where all the isolates were digested with both these restriction enzymes and uniform restriction fragments were obtained suggesting to absence of strain variation.

Earlier work on PCR-RFLP on *T. annulata* from India employed TAMS 1 gene[8], where in 4 strains were noticed from three different parts of India using TAMS 1 PCR-RFLP employing Rsa I enzyme. Likewise, 4 different genotypes of *T. annulata* were again noticed in both cattle and tick vectors in Iran using TAMS 1 RFLP[7] employing Rsa I enzyme. Detection of a large number of circulation genotypes using TAMS 1 gene can be very well explained owing to presence polymorphism in the TAMS 1 gene[18]. Alternatively, a well conserved HSP 70 based PCR-RFLP was developed for detection of strain variations in *T. annulata*[9]. In the present study, only one type of genotype of circulating *T. annulata* isolates was observed. Similar trend was noticed in Iran where again only one genotype was detected[9].

So it can be firmly said that based on RFLP digestion patterns of HSP 70 gene of *T. annulata*, so strain variation was noticed. It may either be because of actual absence of strain isolates from the studied area of India. Or it may also be contributed to conserve nature of HSP 70 gene that facilitates absence of restriction patterns. However, it may be placed on record that HSP 70 gene has previously been used for RFLP based analysis of *T. annulata* isolates[11]. This very much takes the opinion of actual absence of field strains as well as inability of HSP 70 gene in identification of genotypes based on RFLP studies out of the equation. Hence, more studies involving a larger geographical domain alongside analysis involving much higher number of isolates are, thereby, warranted. However, for the area under study it can be very much said that a common mass vaccination and/or the effective diagnostic tool may be used in the studied area with good success owing to absence of multiple circulating genotypes leading to strain variation in *T. annulata* in the studied area.

CONCLUSION

The present study elucidated the presence of single circulating genotypes of *T. annulata* in cattle calves based on PCR-RFLP employing HSP 70 gene. More planned studies, are thereby warranted, throughout India in general as a basic prerequisite before developing either an effective mass vaccination strategy or a suitable molecular diagnostic tool in the area. Otherwise, there is every possible chance that the mass vaccination and/or the effective diagnostic tool may not work properly, due to possible presence of number of circulating genotypes leading to abundant strain variation. In the present study, uniform pattern was noticed suggesting similar strain in North India. So it can be concluded that a common diagnostic test or vaccine can be used on mass scale in the studied area.

SIGNIFICANCE STATEMENT

Absence of strain variation was noticed at HSP 70 locus for all the studied *T. annulata* isolates suggesting the possible success of diagnostic tool employing HSP 70 gene in the studied area.

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REFERENCES

1. Mehlhorn, H., E. Schein and J.S. Ahmed, 1994. *Theileria*. In: Parasitic Protozoa, 2nd Edn., Vol. 2, Kreier, J.P. (Ed.). Academic Press, San Diego, California, pp: 217-304.
2. Gupta, K.K., M. Srivastava, V. Sudan, S. Singh, S. Choudhury and D. Shanker, 2018. Variation in cardiac markers and electrocardiographic alterations in young calves naturally infected with bovine tropical theileriosis. Trop. Anim. Health Prod., 50: 1227-1230.
3. Darghouth, M.A., N.R. Boulter, M. Gharbi, L. Sassi, A. Tait and R. Hall, 2006. Vaccination of calves with an attenuated cell line of *Theileria annulata* and the sporozoite antigen SPAG-1 produces a synergistic effect. Vet. Parasitol., 142: 54-62.

4. Taylor, L.H., F. Katzer, B.R. Shiels and S.C. Welburn, 2003. Genetic and phenotypic analysis of Tunisian *Theileria annulata* clones. Parasitology, 126: 241-252.
5. Singh, H., P.S. Cheema, A.K. Mishra, A.K. Tewari and J.R. Rao *et al*., 2010. β-tubulin gene based PCR-RFLP method for specific detection of *Babesia bigemina* and *Theileria annulata* isolates. J. Applied Anim. Res., 37: 233-238.
6. Abari, J., J. Javanbakht, M. Tavassouli, M. Tabatabai and R. Shafiei, 2012. Molecular survey of *Theileria annulata* in cattle by PCR-RFLP method in Iran. J. Bacteriol. Parasitol., Vol. 3, No. 2. 10.4172/2155-9597.1000135.
7. Ghashgai, O., M. Yakhchali and S. Sohrabi, 2015. PCR-RELP for detecting of *Theileria annulata* infection in cattle and *Hyalomma* species in Kermanshah province, Iran. Arch. Razi Inst., 70: 7-12.
8. Manuja, A., D.V. Malhotra, V.K. Sikka, A.K. Sangwan and R. Sharma *et al*., 2006. Isolates of *Theileria annulata* collected from different parts of India show phenotypic and genetic diversity. Vet. Parasitol., 137: 242-252.
9. Tavassoli, M., M. Tabatabaei, B.E. Nejad, M.H. Tabatabaei, A. Najafabadi and S.H. Pourseyed, 2011. Detection of *Theileria annulata* by the PCR-RFLP in ticks (Acari, Ixodidae) collected from cattle in West and North-West Iran. Acta Parasitol., 56: 8-13.
10. Sudan, V., A.K. Jaiswal and D. Shanker, 2018. Heat shock protein 70 of *Trypanosoma evansi* is phylogenitically closer to salivaria than stercoraria homologs. Compa. Clin. Pathol., 27: 245-248.
11. Lindquist, S. and E.A. Craig, 1988. The heat-shock proteins. Ann. Rev. Genet., 22: 631-677.
12. Parsell, D.A. and S. Lindquist, 1993. The function of heat-shock proteins in stress tolerance: Degradation and reactivation of damaged proteins. Annu. Rev. Genet., 27: 437-496.
13. Lindquist, S., 1986. The heat-shock response. Annu. Rev. Biochem., 55: 1151-1191.
14. Hendrick, J.P. and F.U. Hartl, 1993. Molecular chaperone functions of heat-shock proteins. Annu. Rev. Biochem., 62: 349-384.
15. Sudan, V., D. Shanker, A. Jaiswal, A. Singh and V. Pandey, 2017. Standardization and validation of simple PCR, duplex PCR and RAPD in comparison to blood smear examination for diagnosing bovine tropical theileriosis. Biologicals, 46: 88-91.
16. Patel, G., D. Shanker, A.K. Jaiswal, V. Sudan and S.K. Verma, 2013. Prevalence and seasonal variation in ixodid ticks on cattle of Mathura district, Uttar Pradesh. J. Parasit. Dis., 37: 173-176.
17. Paliwal, S., D. Sanker, V. Sudan, S. Kumar, M. Srivastava and K.K. Gupta, 2019. Comparison of different PCR protocols and respective primer sets from pool of TAMS 1 gene for diagnosis of calf theileriosis from semi arid India. Biologicals, 57: 50-54.
18. Gubbels, M.J., F. Katzer, G. Hide, F. Jongejan and B.R. Shiels, 2000. Generation of a mosaic pattern of diversity in the major merozoite-piroplasm surface antigen of *Theileria annulata*. Mol. Biochem. Parasitol., 110: 23-32.