RESEARCH ARTICLE

Molecular Characterization of Entomopathogenic Fungi *Aspergillus amstelodami* as a Biocontrol Agent of Tick Infestation in Cattle

Pratibha Jumade [1]*, S. Pal[2]

Abstract

Ticks and tick borne diseases affect the production performance of animals ultimately results in economic losses to livestock owners. Tick control using chemicals produces acaricidal resistance and environmental pollution. To overcome this, present study was conducted to introduce the biological control agents in the form of entomopathogenic fungi (EPF) isolated from naturally infected ticks. The molecular characterization of EPF species *Aspergillus amstelodami* isolated from naturally infected ticks of Durg district of Chhattisgarh was carried out by Polymerase Chain reaction (PCR) using internal transcribed spacer (ITS) gene as a molecular marker. The quality of DNA isolated from the culture was evaluated on 1.0% Agarose Gel with a single band of high-molecular weight DNA. Fragment of gene was amplified by PCR. The amplified PCR product was analyzed by agarose gel electrophoresis using 100 bp ladder as a marker showed a single intense band corresponding to a 543 bp indicating successful amplification of gene from template DNA. DNA sequencing reaction of PCR amplicon was carried out with ITS1 primer using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The gene sequence was used to carry out BLAST with the NCBI Genbank database. The fungal isolate showed high similarity with *Aspergillus amstelodami* based on nucleotide homology and phylogenetic analysis. The evolutionary history was inferred using the Neighbor-Joining method. The confidence probability (multiplied by 100) that the interior branch length was greater than 0, as estimated using the bootstrap test (500 replicates was shown next to the branches). The evolutionary distances were computed using the Maximum Composite Likelihood method and were in the units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding.

key words Acaricide, Biocontrol, Entomopathogenic fungi (EPF), Molecular marker, Resistance

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Introduction

Ticks and tick borne diseases produce huge economic losses to livestock owners. Chemical acaricides are commonly used for control of ticks all over the world producing acaricidal resistance. Use of chemical acaricides for the control of ticks was considered as one of the best methods, but ticks have developed resistance against a range of acaricides to all currently-used organophosphate-carbamates, synthetic pyrethroids and amidines (Martins *et al.,* 1995). The use of acaricides on livestock is toxic and hazardous, can remain in the environment for many years and may be transported over a long distance (Kunz and Kemp, 1994). The residues of them in soil and water are considered as significant environment threats and even classified as carcinogenic pollutants in many countries (Dich *et al.*, 1997). The excessive application of these compounds over the past half-century has posed serious risks to human health (Kolpin *et al*., 1998). Biological control of ticks using entomopathogenic fungus is proved to be most economical and safest method to overcome the risk of acaricidal resistance, environmental safety and human health (Samish *et al.*, 2004).The present study was aimed to confirm the species identification of entompathogenic fungi isolated from naturally infected ticks of Durg district of Chhattisgarh by molecular analysis.

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Materials and Methods

The fungal culture was procured from body surfaces of naturally infected ticks which were then cultivated on potato dextrose agar to obtain the pure culture. (Fig. 1 and Fig. 2). Fungal DNA was extracted from fungal isolates using the HiPurA™ Fungal DNA Purification Kit procured from Himedia following the manufacturer's protocol.

Fig. 1: Ticks naturally infected with *Aspergillus amstelodami*

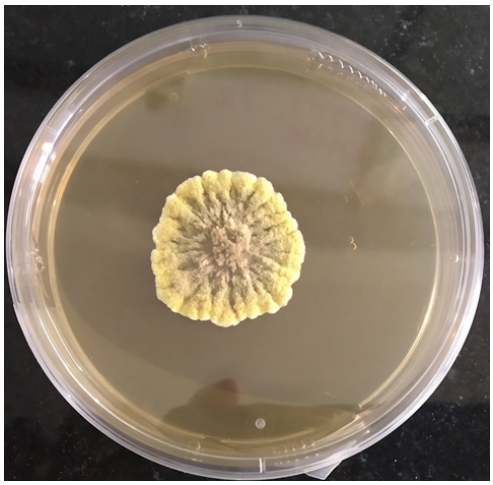

Fig. 2: Colony culture of *Aspergillus amstelodami*

The species of the fungi infecting ticks was identified using RAPD-PCR. The internal transcribed spacer region of chitinase2 gene of this fungal isolate was amplified by polymerase chain reaction (PCR) using universal fungal primers forward = ITS-1 (Gardes and Bruns, 1993) and reverse = ITS-4 (White *et al*., 1990). DNA from internal transcribed spacer (ITS) regions of the nuclear ribosomal repeat was extracted from pure cultures obtained from body surface of ticks and was amplified and sequenced. The quality was evaluated on 1.0% agarose gel, a single band of high-molecular weight DNA has been observed. The PCR amplicon was purified by column purification to remove contaminants. DNA sequencing reaction of PCR amplicon was carried out with ITS1 primer using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The gene sequence was used to carry out BLAST with the database of NCBI Genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software programs.

Results and Discussion

The fungal isolate showed high similarity with *Aspergillus amstelodami* based on nucleotide homology and phylogenetic analysis. The amplified PCR product analyzed by agarose gel electrophoresis using 100 bp ladder as a marker showed a single intense band corresponding to a 543 bp indicating successful amplification of gene from template DNA (Fig. 3).

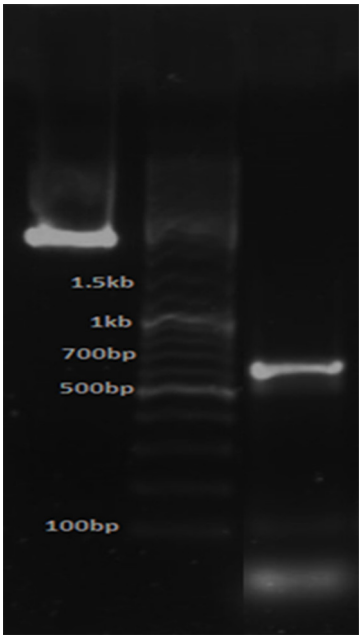

Fig. 3: Agarose gel electrophoresis of PCR amplicons of *Aspergillus amstelodami*

DNA Sequence Analysis of *Aspergillus amstelodami*

The sequences were confirmed by performing BLAST in NCBI database. Sequencing of PCR amplicon (543 bp) with Sequence ID: KX696387.1showed alignment with most coordinated ITS sequence with *Aspergillus amstelodami* strain E29 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence with maximum score of 741 and the percentage of sequence overlap with reference sequence was 100%. The species also found similarly aligned with *Eurotium amstelodami* strain SGE21 18S ribosomal RNAgene, partial sequence; internal transcribed spacer 1, 5.8 Ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence, *Aspergillus chevalieri* strain 1963 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, complete sequence and 28S ribosomal RNA gene, partial sequence, *Aspergillus amstelodami* isolate BAB-6506 small subunit ribosomal RNA gene, *Aspergillus pseudoglaucus* internal transcribed spacer 1, partial sequence, *Aspergillus chevalieri* genomic DNA containing ITS1, 5.8S rRNA gene and ITS2, isolate TUHT82 and *Aspergillus montevidensis* genomic DNA sequence contains ITS1, 5.8S rRNA gene, ITS2, isolate UTHSCSA:DI16-401 with maximum score of 730.

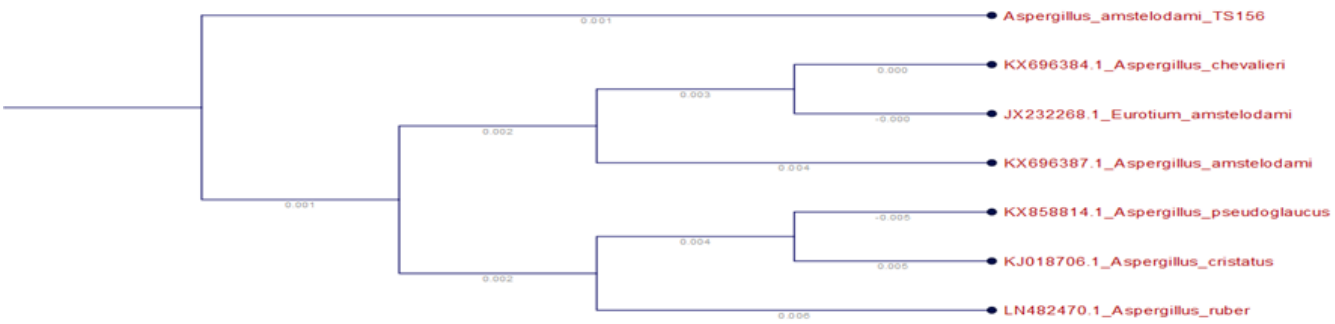

Fig. 4: Phylogenic neighbor- joining Tree of *Aspergillus amstelodami*

Evolutionary Relationships of Taxa of *Aspergillus amstelodami*

The sequences from the ITS gene region were used to generate a phylogenetic tree. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The confidence probability (multiplied by 100) that the interior branch length is greater than 0, as estimated using the bootstrap test (500 replicates is shown next to the branches (Dopazo, 1994, Rzhetsky A. and Nei, 1992). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated (Fig.4).

Thakur and Sandhu (2010), analyzed forty-eight isolates of indigenous strains of *Beauveria bassiana* from various insect hosts collected from Central India and characterized by RAPD analysis. Yayan *et al*. (2016), isolated entomopathogenic fungi from *Tetranychus kanzawai* Kishida (Tetranychidae: Acarina) using RAPD-PCR. They identified the internal transcribed spacer of 5.8s rDNA (ITS-5.8s rDNA) sequence of the fungal isolates which were amplified using two sets of universal primers for ITS. Very meager work on isolation and molecular characterization of EPF from naturally infected ticks in India and abroad are available. No work on molecular characterization of EPF from naturally infected ticks in India has been conducted, so more emphasis should be given to explore EPF as a biological control agent for tick control. This is the first report of isolation and molecular characterization of EPF *Aspergillus amstelodami* from naturally infected ticks.

Conclusion

Control of ticks using entompathogenic fungi *Aspergillus amstelodami* as a biopesticides will help to overcome the acaricidal resistance and will assure the environmental safety.

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