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Pathogenicity of Entomopathogenic Fungi *Fusarium beomiforme* against *Rhipicephalus microplus* Tick Infestation in Cattle

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ABSTRACT

Background: Tick infestation in cattle is commonly controlled using chemical acaricides producing acaricidal resistance, environmental pollution and hazards to human health. Biological control of ticks using entomopathogenic fungi can be an alternative to chemical acaricides and can be used in conjunction with chemical control in integrated pest management.

Methods: The isolation of entomopathogenic fungi was carried out from naturally infected ticks in Durg district of Chhattisgarh. The species confirmation of fungal isolates was conducted by Polymerase chain reaction (PCR) using ITS gene as a molecular marker. *In vitro* efficacy of entomopathogenic fungi at various concentrations against engorged (adult) female ticks and unfed (Nymphs), egg laying capacity and egg hatchability of treated *Rhipicephalus microplus* was assessed in laboratory.

Result: The entomopathogenic fungi isolated from naturally infected ticks was confirmed as *Fusarium beomiforme* by morpho-molecular analysis. The efficacy of *F. beomiforme* against adult female and nymphs of *R. microplus* at the concentration of $n \times 10^8$ spores ml^{-1} showed 100% mortality. The 100% reduction of egg laying capacity of treated female ticks and no hatchability of eggs treated with *F. beomiforme* at the concentration of $n \times 10^7$ spores ml^{-1} and $n \times 10^8$ spores ml^{-1} was observed.

Key words: Biological, Control, Entomopathogenic, Fungi, Pollution, Resistance, Ticks.

INTRODUCTION

Tick andtick borne diseases are one of the biggest public health and veterinary problems in the world (Salih *et al*., 2015). Ticks have an impact on the production and health of the animals either directly by the effect of their bites causing irritation, inflammation or hypersensitivity, localized dermatitis, secondary bacterial infections, and myiasis (Taylor *et al*., 2016) or by transmission of the infectious agents of viruses, bacteria, rickettsiae and protozoa (Eskezia and Desta, 2016; Kaur *et al.,* 2016; Diallo *et al.,* 2018). Massive tick infestations can cause anemia, as a result of blood loss causes stress which weakens its immune response affecting its productivity, increased morbidity mortality and indirect economic losses for farmers (Singh *et al.,* 2000). Use of chemical acaricides for the control of ticks was considered as one of the best methods, however ticks have developed resistance against a variety of acaricides to all currently-used organophosphate-carbamates, synthetic pyrethroids and amidines (Martins *et al*., 1995). The use of acarcides on livestock is toxic and hazardous, can stay behind 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. Thus many countries (Dich *et al.,* 1997) have 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). This fungus kills or seriously disables their host by their insecticidal toxicity. The mode of infection of these fungi includes adhesion, germination, appresorium formation by mechanical pressure, penetration of host cuticle, colonization of haemolymph, extrusion and sporulation (Kimberly and Seow, 2017).

The entomopathogenic fungi show minimal adverse effects on the animals and other non-target organisms. (Goettel *et al.*, 1990; Vestergaard *et al*., 2003, Margaret *et al.,* 2014). It can be used in integrated pest management replacing the conventional chemical insecticides (Pell *et al*., 2001). Although entomopathogenic fungi have been widely used for the control of agricultural and forest pests the present study was aimed to implement its application against animal pests *i.e*. against *Rhipicephalus microplus* tick infestation in cattle.

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MATERIALS AND METHODS

Isolation of entomopathogenic fungi from naturally infected ticks

About twelve to fifteen ticks (males, engorged females and unfed nymphs) were collected from body of six cattle using thumb and forefinger each from four villages (Anjora, Thanod, Mahamara and Siloda) of Durg district of Chhattisgarh. The identification of ticks under zoom stereoscopic microscope was carried out using morphological keys described by Walker *et al.* (2003). The ticks after collection were rinsed with sterile distilled water and were kept at room temperature in petri dishes and examined regularly for growth of fungus. The ticks showing fugal hyphae growth on their body surfaces were then isolated. Each tick carcass infected with fungus was plated on 2.5% potato dextrose agar (PDA) medium to assess the entomopathogenic fungi found in ticks. The plates with fungal culture were incubated at 29°C with 75% humidity in BOD incubator for 7 to 15 days to achieve full growth of fungi. The fungal colonies were sub cultured to obtain the pure culture.

Identification of fungal isolates by molecular analysis

DNA extraction from fungi

Fungal DNA was extracted from fungal isolates using the HiPurA™ Fungal DNA Purification Kit procured from Himedia following the manufacturer's protocol. Polymerase Chain reaction (PCR) was carried out 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.

Primer details

Primer name	Sequence details	Number of base
ITS1	5′ TCCGTAGGTGAACCTGCGG 3′	19
ITS4	5′ TCCTCCGCTTATTGATATGC 3′	20

PCR protocol

Polymerase chain reaction (PCR) was carried out in a 5 µL of isolated DNA in 25 µL of PCR reaction solution (1.5 µL of Forward Primer and Reverse Primer, 5 µL of deionized water, and 12 µL of Taq Master Mix). Taq Master Mix containing Taq DNA polymerase was supplied in 2X Taq buffer, 0.4 mM dNTPs, 3.2 mM MgCl2 and 0.02% bromophenol blue. PCR was carried out following thermal cycling conditions as initial denaturation at 95°C for 2 min., followed by 25 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 1 min. and a final extension at 72°C for 10 min. The reaction was held at 4°C. 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 evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Maximum Composite Likelihood method.

In vitro pathogenicity of entomopathogenic fungus

Efficacy of the entomopathogenic fungus after application of various fungal concentrations was assessed by observing mortality percentage of engorged female ticks and non-engorged nymphal stages of ticks, egg laying capacity of treated female ticks and egg hatchability in treatment group. The conidial suspension was prepared following the method described by Angelo *et al*. (2010) with some modifications. Conidia were harvested from culture plates by scraping the medium surface with a plastic loop. The fungal conidial suspension was prepared by dissolving 1 g of fungal culture into 10 ml of sterile distilled water. The conidial suspension was homogenized for 3 minutes using a vortex shaker. Conidial suspensions were quantified in haemocytometer chamber and adjusted to 1×10^8 conidia ml^{-1}. Four fungal concentrations of 10^5 spores ml^{-1}, 10^6 spores ml^{-1}, 10^7 spores ml^{-1} and 10^8 spores ml^{-1} were prepared by adjusting the volume of sterile distilled water.

In vitro efficacy of fungal isolates on engorged and non-engorged ticks, egg laying capacity of treated female ticks and egg hatchability of treated eggs

The engorged female ticks and non-engorged unfed nymph ticks were immersed in a 1% sodium hypochlorite solution for 1 min, rinsed with sterile distilled water and dried with sterile tissue paper for cuticle antisepsis as per the method described by Angelo *et al*. (2010). The ticks were grouped in six groups each containing six female ticks. Each female tick was maintained separately in covered petri plate. The ticks both engorged and non-engorged, were subjected to adult immersion technique described by Kaaya and Hassan, (2000) with slight modification. The petri plates were smeared with a suspension of four fungal concentrations of 10^5 spores ml^{-1}, 10^6 spores ml^{-1}, 10^7 spores ml^{-1} and 10^8 spores ml^{-1} and allowed to dry. The ticks were then placed in the petri plates in each concentration. The treatment of ticks was repeated on second day with same dose of conidial suspension. The ticks were incubated at 29°C temperature with 75% relative humidity in BOD incubator. The eggs were treated using same protocol with fungal suspension and incubated at 29°C temperature with 75% relative humidity. Mortality of unfed ticks, adult engorged female ticks, egg laying capacity of treated female ticks and egg hatchability of treated eggs were examined daily. The efficacy of entomopathogenic fungi was studied in comparison with commercially available synthetic pyrethroid @ 2% and control group treated with sterile distilled water. The ticks were considered as dead when it shows no movement even after pricking with dissecting needle to its appendages. The mortality data was tabulated and efficacy was worked out in terms of per cent mortality. The live ticks were maintainedto judge the egg laying capacityand compared with ticks in control group treated with distilled water.

RESULTS AND DISCUSSION

All the male, engorged females and unfed nymph of *R. microplus* ticks collected from Anjora village were found to be infected on third day of collection. The ticks were trapped by fungal hyphae showing growth on their appendages followed by the growth on their mouth partswith restricted movements. The mortality of nymphal stages of ticks occurred on third day of infection. The male and unfed female ticks were found dead on fourth day of infection with hyphae covered on whole body surface whereas the mortality in engorged female ticks was observed on fifth day post infection with very less number of egg laying. The fungus from cadaver of ticks cultured on potato dextrose agar showed full growth in seven days at 29°C temperature and 75% relative humidity. The species of fungi was identified morphologically as *Fusarium sp*. with hyaline filamentous hyphae and septet oval conidia (Fig 1).

Molecular analysis

The quality of DNA isolated from the fungal culture was evaluated on 1.0% Agarose Gel showed a single band of high-molecular weight DNA. Fragment of gene was amplified by Polymerase Chain Reaction. The gel electrophoresis showed a single intense band corresponding to a 553 bp indicating successful amplification of gene from template DNA (Fig 2). The fungal isolate showed high similarity with *Fusarium beomiforme* based on nucleotide homology and phylogenetic analysis.

Phylogenetic analysis

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) was observed next to the branches (Dopazo, 1994, Rzhetsky and Nei, 1992). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and were in the units of the number of base substitutions per site. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated (Fig 3).

Fusarium beomiforme strain CBS 100160 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene partial sequence:

Score	Expect	Identities	Gaps	Strand
784 bits	0.0	424/424	0/424	Plus
(424)		(100%)	(0%)	Minus

Sequence ID: MH862691.1; Length: 553; Number of Matches: 1; Range 1: 129 to 552.

Mechanism of action of entomopathogenic fungi on ticks after treatment

The adult and nymph stages of ticks after treatment with EPF showed the restricted and sluggish movement. On the second day after treatment the thin fungal hyphae were observed to grow on the body of ticks. The mycelial growth traps the appendages of ticks and makes them confined just like pray in the spider web. The hyphae grow so fast that the ticks appeared to be white cottony bundles (Fig 4 and 5). The mortality of ticks was confirmed by observing the pedal reflexes under the dissection microscope using dissecting needle.

Pathogenicity of EPF *F. beomiforme* against engorged (adult) *R. microplus* female ticks at different concentrations

The mean mortality percentage of engorged (adult) *R. microplus* female ticks after treatment with EPF *F. beomiforme* showed mortality of 16.66 and 33.55 per cent at 120 and 144 HAT respectively. The mortality of adult female ticks at the concentration of $n \times 10^6$ spores ml^{-1} was recorded as 16.66, 33.33 and 50 per cent at 96, 120 and 144 HAT whereas the concentration of $n \times 10^7$ spores ml^{-1} showed 0, 66.66, 83.3 and 100 per cent mortality at 72, 96, 120 and 144 hours after treatment (Table 1).

Pathogenicity against unfed (Nymphs) at different concentrations

The mortality of unfed (Nymphs) ticks at the concentration of $n \times 10^6$ spores ml^{-1} was observed as 0, 12.5, 25 and

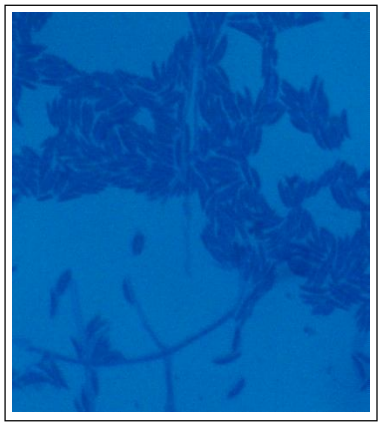

Fig 1: Hyphae and conidia of *Fusarium beomiforme.*

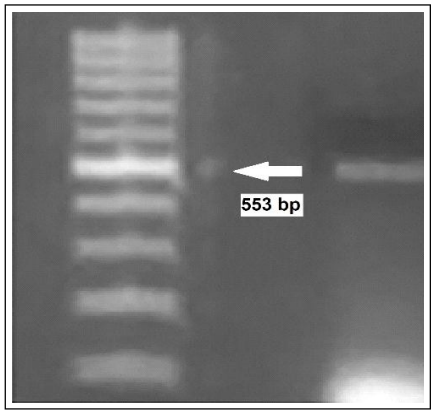

Fig 2: Agarose gel electrophoresis of PCR amplicons.

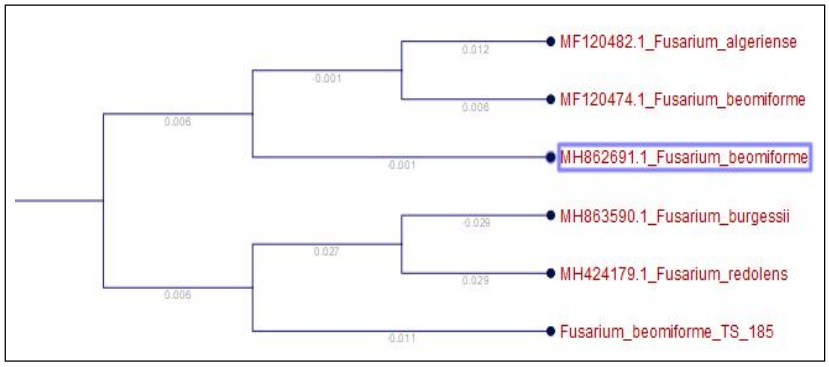

Fig 3: Phylogenic neighbour- joining Tree of *F. beomiforme.*



Fig 4: Engorged female of *R. microplus* infected with *F. beomiforme.*



Fig 5: Nymph of *R. microplus* infected with *F. beomiforme.*

50 per cent at 72, 96, 120 and 144 hours after treatment, respectively whereas the concentration of n × 10^7 spores ml^{-1} showed 0, 50, 62.5 and 75 per cent mortality at 72, 96, 120 and 144 hours after treatment respectively. The significant mortality of ticks at the concentration of n × 10^8 spores ml^{-1} was recorded as 50, 75, 100 and 100 per cent at 72, 96, 120 and 144 hours after treatment, respectively. (Table 1).

In vitro efficacy of fungal isolates on egg laying capacity of *R. microplus* female ticks

The mean egg laying capacity of female ticks treated with *Fusarium beomiforme* at the concentration of n × 10^5 spores ml^{-1} was recorded as 25.33 with percent efficacy of 3.59% as compared to egg laying capacity of females in control group. The reduction in mean egg laying capacity of female ticks was observed as 96.41%. At the concentration of n × 10^6 spores ml^{-1}, n × 10^7 spores $ml^{-1}$and n × 10^8 spores ml^{-1} the egg laying capacity of female ticks was observed as zero with 100% percent efficacy (Table 2).

In vitro efficacy of fungal isolates on egg hatchability of *R. microplus* female ticks after treatment with various isolates of EPF at different concentrations

Eggs treated with *F. beomiforme* at the concentration of n × 10^5 spores ml^{-1} and n × 10^6 spores ml^{-1} showed 18.80 and 12 per cent egg hatch, respectively. No egg hatching was observed at the n ×10^7 spores ml^{-1} and n × 10^8 spores ml^{-1}. The eggs laid by treated female ticks were showed no hatchability (Table 2). The entomopathogenic fungi *Fusarium* spp. produces the mycotoxin Fusaric acid as reported by Li *et al*. (2013) which is responsible for death of the host with the effects such as partial or general paralysis, decreased irritability, sluggishness in infested insects and behavioural symptoms that are persistent with the action of neuromuscular toxin as reported by Charnley *et al.* (1984). *In vitro* evaluation of pathogenicity of *Beauveria bassiana* and *Metarhizium anisopliae* isolates on eggs and larva of *Amblyomma cajennense* showed the significant reduction in egg laying and lower hatching rate in comparison with the control. *B. bassiana* was highly pathogenic against engorged *R. (B.) microplus* females with the concentration of 10^9 conidia/ ml significantly reduced the amount of ovipositioning with mortality of ticks before oviposit. *B. bassiana* strain tested on developmental stages of *R. sanguineus* under laboratory conditions with significantly higher mortality on eggs, larvae, nymphs and adults than those of the control groups at 5 days post-infection (Souza *et al.,* 1999; Campos *et al.,* 2010; Ming *et al.,* 2013 and Cafarchia *et al.,* 2015). *In vitro* treatment of *B. microplus* engorged females ticks with EPF *B. bassiana* at concentrations of 10^6, 10^7 and 10^8 conidia/ml resulted in the reduction in ovipositing period by 9.69-47.80%, egg mass weight by 4.71-53.87% and reproduction by 8.3-60.62%. Reduction in larval hatchability by 1.36-65.58% was noticed after immersion of tick eggs (0.25 g) in 1 ml of

Table 1: Efficacy of *Fusarium beomiforme* on engorged (adult) female and unfed nymph of *Rhipicephalus microplus* ticks at different concentrations.

Treatments	Mortality percentage of engorged (adult) *R. microplus* female ticks after treatment with EPF (N=8)															
	Concentrations															
	$n \times 10^5$ spores ml^{-1}				$n \times 10^6$ spores ml^{-1}				$n \times 10^7$ spores ml^{-1}				$n \times 10^8$ spores ml^{-1}			
	72 HAT	96 HAT	120 HAT	144 HAT	72 HAT	96 HAT	120 HAT	144 HAT	72 HAT	96 HAT	120 HAT	144 HAT	72 HAT	96 HAT	120 HAT	144 HAT
F. beomiforme	0.00	0.00	16.66	33.33	0.00	16.66	33.3	50.0	0.00	66.6	83.3	100	100	100	100	100
Pyrethroid @2%	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Control (D.W.)	0.00	0.00	0.00	16.66	0.00	0.00	0.00	16.6	0.00	0.00	0.00	16.6	0.00	0.00	0.00	16.66

Treatments are Significant at both 5% and 1% level of significance.

Critical difference at 5% = 8.547.

Critical difference at 1% = 11.348.

HAT- Hours after treatment.

Treatments	Mortality percentage of unfed nymph of *R. microplus* ticks after treatment with EPF (N=8)															
	Concentrations															
	$n \times 10^5$ spores ml^{-1}				$n \times 10^6$ spores ml^{-1}				$n \times 10^7$ spores ml^{-1}				$n \times 10^8$ spores ml^{-1}			
	72 HAT	96 HAT	120 HAT	144 HAT	72HAT	96 HAT	120 HAT	144 HAT	72 HAT	96 HAT	120 HAT	144 HAT	72 HAT	96 HAT	120 HAT	144 HAT
F. beomiforme	0.00	0.00	12.5	37.5	0.00	12.5	25	50	0.00	50	62.5	75	62.5	75	100	100
Pyrethroid @ 2%	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Control	0.00	0.00	0.00	16.66	0.00	0.00	0.00	16.66	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Treatments are Significant at both 5% and 1% level of significance.

Critical difference at 5% = 9.512.

Critical difference at 1% = 12.628.

Table 2: Per cent egg laying capacity and egg hatchability of *Rhipicephalus microplus* female ticks after treatment with *Fusarium beomiforme* at different concentrations.

Treatments	Per cent egg laying capacity of *R. microplus* female ticks after treatment with various isolates of EPF at different concentrations (N=6)							
	Concentrations							
	n × 10^5 spores ml^{-1}	% efficacy	n × 10^6 spores ml^{-1}	% efficacy	n × 10^7 spores ml^{-1}	% efficacy	n × 10^8 spores ml^{-1}	% efficacy
F.beomiforme	25.33±14.55	3.59	0.00±00.00	0.00	0.00±00.00	0.00	0.00±00.00	0.00
Pyrethroid@ 2%	0.00±00.00	0.00	0.00±00.00	0.00	0.00±00.00	0.00	0.00±00.00	0.00
Control (D.W.)	1200±68.24	0.00	1200±68.24	0.00	1200±68.24	0.00	1200±68.24	0.00

Treatments are Significant at both 5% and 1% level of significance.

Critical difference at 5% = 9.308.

Critical difference at 1% = 12.358.

Treatments	Per cent hatchability of eggs of *R. microplus* ticks after treatment with various EPF (N=100) Concentrations							
	Concentrations							
	n × 10^5 spores ml^{-1}	% efficacy	n × 10^6 spores ml^{-1}	% efficacy	n × 10^7 spores ml^{-1}	% efficacy	n × 10^8 spores ml^{-1}	% efficacy
F.beomiforme	18.80±3.12	18.80	12±2.34	12	0±0.00	0	0±0.00	0
Pyrethroid@ 2%	2.20±1.20	2.20	2.20±1.20	2.20	0±0.00	0	0±0.00	0
Control (D.W.)	85±5.60	85	85±5.60	85	85±5.60	85	85±5.60	85

Treatments are significant at both 5% and 1% level of significance.

Critical difference at 5% = 7.622.

Critical difference at 1% = 10.119.

a suspension of the different conidial concentrations for 1 min. Amongst the study of pathogenicity of EPF *B. bassiana, M. anisopliae, M. flavoviride* and *Paecilomyces fumosoroseus* to various developmental stages of *R. sanguineus* showed that *M. anisopliae* and *M. flavoviride* isolates prevented or reduced the ability of the ticks to lay eggs several days before their deaths. Female ticks infected by the fungi achieved only 11.3-60.8% of their egg-laying capacity compared with the controls (Onofre *et al.,* 2001; Paiao *et al.,* 2001 and Samish *et al.,* 2001).

CONCLUSION

Biological control of ticks using entomopathogenic fungus may proved to be the most economical and safest method of biological control to overcome the risk of environmental pollution and acaricidal resistance.

Conflict of interest

All authors declare that they have no confllict of interest.

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