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Metarhizium anisopliae as a Biological Control Agent Against *Hyalomma anatolicum* (Acari: Ixodidae)

[1]Elham A. Suleiman, [2]M.T. Shigidi and [2]S.M. Hassan
[1]Veterinary Research Institute, P.O. Box 8067, Alamarat, Khartoum, Sudan
[2]Faculty of Veterinary Medicine, University of Khartoum. P.O. Box 32, Khartoum North, Sudan

Abstract: In the Sudan, ticks and Tick-borne Diseases (TBDs) with subsequent costs of control and treatment are causing substantial economic loss. Control of ticks is mainly by chemical insecticides. The rising environmental hazards and problem of resistance has motivated research on biological agents as alternative methods of control. The present study aims at controlling livestock ticks using fungi for their unique mode of action besides their ability to adhere to the cuticle, to germinate and penetrate enzymatically. The study was conducted to evaluate the fungus *Metarhizium anisopliae* for tick control as an alternative mean to chemical acaricides. Pathogenicity of the fungus was tested on different developmental stages of the tick *Hyalomma anatolicum*. The fungus induced high mortality to flat immature stages. It, also, affected reproductive potential of the females. Egg laid, hatching percent, fertility and moulting percent of immature stages were significantly ($p \leq 0.05$) reduced. It was, also, shown that the fungus had ability to adhere to the cuticle and penetrate the integument of the tick. Conidia of the fungus were isolated from their internal tissues. This phenomenon is important in considering fungi as bioinsecticides. Infection of eggs laid by treated engorged female ticks, with the fungus might demonstrate suggesting transovarian transmission. The use of *M. anisopliae* to control ticks is discussed.

Key words: *Metarhizium anisopliae*, biocontrol, biotic potential, *Hyalomma anatolicum*

INTRODUCTION

Ticks are impermanent blood sucking ecto-parasite of mammals. They are potential vectors transmitted various diseases to both man and animals (Estrada-Pena and Jongejan, 1999). Over 70 species of ticks have been reported in the Sudan affecting domestic animals (Hoogstraal, 1956). *Hyalomma anatolicum* is the potential main vector of Theileriosis, Babesiosis and Anaplasmosis (Latif, 1984; Abdoon, 1985; Salih *et al*., 2005). Although control of ticks via pasture manipulation and chemical insecticides is a sensible method, the growing problem of resistance to acaracides; laborious technique of application besides high cost has stimulated research on biological agents as alternative methods of control. Biological control such as the predators, parasites and pathogens was applied against numerous pest insects (Samish *et al*., 2004). In Sudan, pathogenicity of fungi to different tick species was addressed (Elham, 2009; Suliman and Mohammed, 2012).

Metarhizium species are arthropod pathogens with broad geographic and host ranges. They are known to be saprophytic in soil and parasitic on insects (Maniania, 1991). *Metarhizium anisopliae*, is naturally grows in soil and has been isolated from various arthropods and decaying materials. It causes disease known as green muscardine disease in various insects acting as a parasite. Since the 19th century entomo-pathogenic fungi have been used as bio-control agents against agricultural pests. It was used against wheat grain beetle and sugar cane pest (Hall and Papierok, 1982).The potential use of such fungi for controlling vectors of human and animal diseases has been recently investigated with promising outcome. However, at present it has been produced as a commercial product and applied for control of different insect pests with no adverse effects to human or environment (Cloyd, 1999).

The fungus in USA was isolated from wild ticks of *Ixodes scapularis* (Zhioua *et al*., 1999). *M. anisopliae* has been used to control the cattle tick *Boophilus microplus* (Frazzon *et al*., 2000). Onofre *et al*. (2001), also, demonstrated its use as an effective biological control agent against *Boophilus microplus*. *M. anisopliae* var. *frigidum* and *M. flavoviride* were used as bioinsecticide (Bischoff *et al*., 2006). Furthermore, it has been used to control the Parasitoid, *Spalangia cameroni* (Nielsen *et al*., 2004), an adult African malaria vector *Anopheles gambiae* Scholte *et al*. (2006) and the

Corresponding Author: Elham A. Suleiman, Veterinary Research Institute, P.O. Box 8067, Alamarat, Khartoum, Sudan

subterranean burrower bug *Cyrtomenus bergi Froeschner* (Jaramillo and Borgemeister, 2006). The present study was conducted to investigate the possibility of using *M. anisopliae* spore suspension to control *Hyalomma anatolicum* under Sudan conditions in order to reduce hazards associated with the use of chemical acaricides.

MATERIALS AND METHODS

Engorged females ticks were collected from naturally infested cattle found in Gedaref area as well as University of Khartoum Farm in Khartoum State which lie entirely between latitudes 14° and 05'N; longitudes 35° and 39'E and latitudes 15° and 57'N; longitudes 32° and 54'E, respectively.

The study was conducted over 9 months from January to October 2011. Flat larvae and other developmental stages of *H. anatolicum* were obtained by maintaining engorged females (Bailey, 1960) in an incubator at 27+1° C and 75-80% RH. Ticks were identified according to Hoogstraal (1956).

Metarhizium anisopliae strain 62 was kindly provided by the International Centre of Insect Physiology and Ecology (ICIPE) in Nairobi, Kenya.

Preparation of spore suspension: Aseptic conditions were strictly followed. Propagules of *M. anisopliae* were formulated in a water carrier according to Goettel and Inglis (1997). Spore suspension was prepared according to method described by Maniania (1992). Young colonies (10-14 days old) of *M. anisopliae* were harvested by scraping colony surface culture. The harvests were collected in McCarteny bottles with glass beads. Ten mL of sterile water containing 0.1% tween-80 was added to the bottles. Glass beads were used to facilitate separation of the propagules and Tween 80 to reduce surface tension. Bottles were, then, agitated on vibrant shaker (Auto-vortex mixer, Stuart Scientific Co. Ltd., Great Britain) for 3-5 min to produce homogenous conidial suspension. The suspension was filtered through sterile glass wool packed into 10 mL disposable syringe.

Centrifugation of the suspension at 3000 rpm for 10 min was carried out. The supernatant was discarded and the sediment was washed three times in sterile distilled water. A test sample of the suspension was microscopically examined to confirm that it contained microconidia only. Later, the sediment was re-suspended in sterile distilled water and kept at 4°C until use.

Quantification of the propagules: Improved Neubauer chamber was used to quantify the number of propagules per unit volume according to Goettel and Inglis (1997) method.

Viability testing: Plating technique was used to provide a measure of viable propagules per unit volume. Spore viability was determined so that doses can be prepared on the basis of viable propagules according to method described by Lacey (1997).

Germination test: Spore viability was determined by germinating propagules on a translucent agar medium such as Sabouraud's Dextrose Agar (SDA). Propagules from the same batch to be used in the bioassay were immediately processed prior to the preparation of the inoculum. The spore suspension of *M. anisopliae* was prepared as above. A volume of 0.1 mL of 10^6 propagules was spread onto the media in 8.5 cm diameter Petri dish using rod sterile bent glass. Six sterile cover-slips were fixed onto the surface of the plates. The plates were, then, incubated in the dark at room temperature (30°C) for 18-24 h. Six fields were microscopically examined and at least 600 spores were calculated. Percentage germination was calculated as follows:

$$\text{Percent germination} = \frac{\text{Germinating spores}}{\text{Germinating+non-germinating spores}} \times 100$$

Pathogenicity of *M. anisopliae*: The developmental stages of *H. anatolicum* were subjected to infection with *M. anisopliae*. The parameters observed included percentage mortality, female fecundity, moulting and percentage hatchability of eggs.

Treatment of flat larvae: Three replicates each of a total of 150 *H. anatolicun* flat larvae were subjected to treatment with spore suspension of *M. anisopliae*. Recommended concentration of 1×10^7 spores mL^{-1} in distilled water was prepared according to Kaaya (1989) method. Inoculation was performed by dipping larvae into the spore suspension for 1 min according to the method described by Mwangi *et al.* (1995). Larvae were dried with sterile filter paper Whatman No. 4 and then, kept in sterile plastic test tubes covered with nylon mesh pounded to the rim of the tubes with plastic bands. Three replicates of 150 flat larvae served as control were treated in the same way with only sterile distilled water. Both treated and control *H. anatolicum* flat larvae were maintained at 27±1°C with 100%. Relative Humidity (RH) using plain water. Larvae were checked daily for development of infection and percentage mortality achieved.

Treatment of engorged larvae: One hundred of engorged larvae of *H. anatolicum* were treated by dipping into 1×10^7 spores mL^{-1} of the spore suspension of *M. anisopliae* following the same procedure applied for the flat larvae. Three replicates each of the same number of engorged larvae were performed. In addition, infection and percentage moulting were recorded for each group.

Treatment of nymphs: Both Flat and engorged nymphs of *H. anatolicum* were subjected to infection with 1×10^7 spores mL^{-1} *M. anisopliae* by dipping following the same procedure applied for larvae. Nymphs were transferred to sterile Petri dishes with filter paper to dry. They were kept in sterile test tubes covered with nylon mesh bounded with plastic bands and placed in desiccators in an incubator set at 27±1°C. Three replicates each of 100 flat nymphs or 50 engorged nymphs were used. Control groups were treated with sterile distilled water. Treated engorged nymphs were observed for moulting and development of mycosis and mortality. Dead engorged nymphs were surface disinfected according to method described by Mwangi *et al.* (1995) and these were, then, cultured into moist chamber kept at room temperature (30°C) and observed for fungal growth.

Treatment of adult *H. anatolicum*: Twenty flat female *H. anatolicum* were subjected to treatment with *M. anisopliae* following the procedure described above. Spores of 1×10^7 spores mL^{-1} were used. The control group was treated with sterile distilled water. The experiment was repeated three times. Mortality of treated ticks was recorded.

Experiment was conducted with engorged females against *M. anisopliae* spore suspension. Fifty engorged females were weighed on a sensitive balance before dipping. They were, then, treated by immersing for 1 min using 3.5×10^{-7} spores mL^{-1}. Control groups were immersed in sterile distilled water.

The treated female ticks were maintained under the same temperature and relative humidity for larvae and nymphs and observed for mortality, egg laying and hatchability of eggs.

Statistical analysis: SPSS (Social Package of Social Science) software program (Ver.16) was used to analyze the obtained data. The mean values were expressed as the Mean±Standard Deviation (SD) and were analyzed using one-way ANOVA. Significance t-test for continuous variables was applied to determine efficiency impact of the fungal treatment. The significance level was set at $p<0.05$.

RESULTS

Germination: Germination of *M. anisopliae* spores started after 8 h of incubation at room temperature (30°C). Germination percentage was found to be 95%.

The treatment effects on tick developmental stages

Treatment effects on larval stage: *Metarhizium anisopliae* caused high mortality (Table 1) to flat larvae of *H. anatolicum* 3-5 days post treatment. All treated larvae became black, with slugglished movement and eventually died. Flat larvae treated with *M. anisopliae* died three days post treatment after which fungal mycelium was seen covering their integuments. All the treated larvae died during 5 days. The effect was highly significant ($p<0.05$). Only 3% of the control group died.

M. anisopliae-treated groups of engorged larvae started moulting on day 7 post treatment. Moulting period was 10 days. Approximately, 60 engorged larvae out of 300 treated groups succeeded to moult into flat nymphs. Moulting per cent was significantly reduced in treated groups compared with the control (Table 1). Only 20% of the treated larvae moulted into nymph. Dead engorged larvae were colonized with *M. anisopliae* 3 days post infection.

Treatment effects on nymphal stage: The ability of fungi to infect and kill nymphs was high causing 100% mortality 17 days post treatment (Table 1). Infected nymphs developed fungal infection with mycelia covering their bodies.

Control and treated groups of *H. anatolicum* engorged nymphs started moulting 18 days post treatment. 121 out of 150 treated group succeeded to moult into flat adult. While 249 of the control group moulted. However, there was insignificant difference ($p>0.05$) in moulting percentage between both groups (Table 1).

Table 1: Effect of *M. anisopliae* spore suspension on flat and engorged stages of *H. a. anatolicum*

Developmental stage	% Mortality (Mean± SD)	% Moulting (Mean± SD)	Level of significance
Flat larvae	100.0±0.0		
Control	(3.07±2.11)		p<0.05
Engorged larvae		19.94±4.33	
Control		100.0±0.000	p<0.05
Flat nymphs	100±0.0		
Control	4.22±1.15		p<0.05
Engorged nymphs		80.67±5.94	
Control		83.00±4.24	p>0.05
Flat adult	86.30±1.04		
Control	3.77±1.26		p<0.05

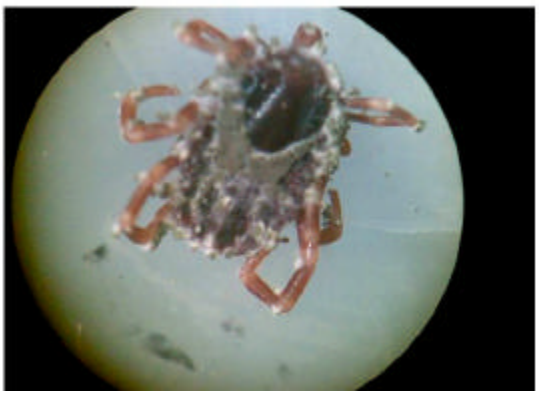

Plate 1: Flat adult *H.A. anatolicum* colonized with *M. anisopliae* 7 days post infection

Table 2: Mean±SD of the effect of *M. anisopliae* on engorged female *H. anatoilicum*

Group	Female fertility (%)	Egg mass (g)	Egg hatchability (%)
Treated females	39.39±6.52	0.06±0.06	0.00±0.00
Control	67.25±14.03	0.48±0.16	94.22±2.99
	p<0.05	p<0.05	p<0.05

Treatment effect on flat adult ticks: As shown in Table 1, the treatment of *H. anatolicum* flat adults with *M. anisopliae* induced high significant mortality rate ($p<0.01$). Fifty two out of the 60 treated group died. While only 3% of the control group died. Infected ticks developed fungal infection 7 days post treatment. Fungal hyphae were seen covering their bodies (Plate 1). All Infected ticks were seen colonized with *M. anisopliae* conidia. Microscopic examination of a slide mounted in lacto phenol cotton blue of surface of disinfected flat ticks' revealed conidia of *M. anisopliae* subsequently proved adhesion of conidia to tick cuticle. Moreover, *M. anisopliae* conidia were isolated from dissected treated *H. anatolicum*.

Treatment effect on engorged female ticks: *Metarhizium anisopliae*, spore suspension affected the biotic potential of the treated females. The female fertility, eggs laid and hatchability were significantly reduced $p<0.05$ compared with the control (Table 2). Fifty nine out 150 treated engorged female showed fertility of 39.39% while 100 of the control group showed fertility of more than 60%. *M. anisopliae*-treated groups and the control started oviposion 4 days from dropping time. *M. anisopliae* treated-groups produced eggs which showed infection 3-10 days after oviposition. Slides mounted in lacto phenol cotton blue revealed adhesion of conidia to egg cuticles. There was difference in oviposition period. It was 21 days in control *H. anatolicum* and 30 days in the treated group. Infection rate among *M. anisopliae*-treated groups was 100% and only 66% succeeded to oviposit. Ninety nine of the treated group had an average egg mass of 0.06 g that means significant reduction in eggs laid. There is significant difference $p<0.05$ between eggs laid by treated and control group. Moreover, 141 out of the control group succeeded to hatch their eggs while the treated group failed to hatch their eggs.

DISCUSSION

Ticks transmit a greater variety of pathogenic microorganisms than any other arthropod vector group. Tick-borne theileriosis, anaplasmosis and babesiosis are the most important diseases to livestock (Jongejan and Uilenberg, 2004) in the Sudan. They constantly pose higher risks to livestock industry. Control of tick vectors, hence tick-borne diseases, in the country is based mainly on the use of acaricides. However, the main threat to the success of this strategy is the growing problem of tick resistance to acaricides. Currently there is a rising interest in administration of entomo-pathogenic fungi presumably due to their selective activity against ticks and harmless effect to worm blood hosts including man. The present study was conducted to evaluate efficiency of *Metarhizium anisopliae* fungi (Maniania, 1994) under Sudan conditions as an alternative means for controlling tick of *Hyalomma anatolicum* the potential main vector of theileriosis, babesiosis and anaplasmosis (Latif, 1984; Abdoon, 1985; Salih *et al*., 2005).

In the current study pathogenicity of *M. anisopliae* to different developmental stages of *Hyalomma anatolicum* was assessed, the fungus proved to be highly pathogenic to the target tick species. This finding confirms previous result that considered the fungus as a promising biopesticide for tick control (Kaaya and Hassan, 2000).

In the present study treated ticks were maintained at high relative humidity (100% RH) which is optimal for growth of many fungi (Kalsbeek *et al*., 1995). Fungi were known to produce supplementary conidia under conditions of high relative humidity. Hence, such conditions might have lead to effective spread of the fungus covering ticks' body surface. Furthermore, the small size of unfed ticks and their incubation together might enhance physical transmission of infection by tick to tick contact; similar observations were reported by Kaaya and Okech (1990). In the present study, *M. anisopliae* treatment induced high mortality to the flat phases of different stages of *H. anatolicum* including larvae, nymph and adult ticks and colonization of such

ticks with fungal mycelia was obviously seen post infection. Obtained high mortality of *H. anatolicum* flat ticks might be due to invasion of their internal organs by the fungal hyphae as such fungus able to produce enzymes which facilitated its penetration via tick cuticles. *M. anisopliae* is known to produce hydrolytic enzymes such as chitinolytic enzymes when cultured with glycosamine as a source of carbon (Barreto *et al*., 2004). Since in this study, *M. anisopliae* was cultured in media with glucose as a source of carbon, thus, secretion of such enzymes is highly achievable. However, fungal hyphae from germinating conidia are able to penetrate the cuticle directly as established by Samish and Rehacek (1999). Altre and Vandenberg (2001) and Altre *et al.* (1999) confirmed adhesion of *M. anisopliae*, germination and proliferation in haemolymph of lepidopteran larvae.

Although in this work adhesion of *M. anisopliae* conidia to tick cuticle and germination was demonstrated and moreover, conidia were isolated from dissected treated ticks formed hyphae subsequently indicates that the fungus has the ability to penetrate the cuticles and invade treated ticks. Nevertheless, the high mortality of the treated flat ticks might be due to effect of toxins produced by *M. anisopliae* as observed by Huxham *et al.* (1989).

In the present study, the fungus was significantly found to alter moulting of the treated engorged *H. anatolicum* larvae although no significant difference in pre-moulting period was observed. Moulted treated larvae showed high infection rate due to high humidity which favored fungal infection (Hall and Papierok, 1982). However, significantly high proportion of the treated engorged nymphs succeeded to moult into adult. This might be due to moulting process, which prevent mycosis as the cuticle hardens within few days of engorgement, thus protecting the moulting tick. Mwangi *et al.* (1995) attributed the low susceptibility trend observed for immature stages of *R. appendiculatus* to *M. anisopliae* infection to moulting process.

Treatment induced significant reduction in weight and hatchability of eggs laid by treated engorged females of *H. anatolicum* compared with the control one, moreover larvae which emerged from infected eggs soon died. Decline of fecundity of the treated engorged ticks and egg hatchability scenario observed in the present work was reported by Kaaya *et al.* (1996) who used *M. anisopliae* against *Rhipicephalus appendiculatus* and *Amblyomma variegatum*. Since *M. anisopliae* is known to produce cyclic peptide lactone, destruxins (Suzuki *et al*., 1971; Huxham *et al*., 1989) thus the inhibition of reproduction achieved could be attributed to toxins effect.

Reduction of fecundity and egg hatchability significantly hold back the next generation of ticks; when considering the large number of eggs lay by females it has greater impacts on tick population than direct mortality of ticks which wipe out a few numbers of ticks Kaaya *et al.* (1996) thus inhibition of reproduction is very essential to consider when evaluate mycoinsecticides.

The high mortality of exposed flat stages of *H. anatolicum* attained in the present study has pointed out the potential use of *M. anisopliae* as a biological control agent since ticks are obligate temporary parasites spend 80% of their life span off the vertebrate animal hosts. Hence application of such fungi on grass in pasture in the early shower of rainy season can be drastically effective against questing ticks that ascent on vegetation to cling on passing host. Moreover, the capability of *M. anisopliae* to hinder the biotic potential of *H. anatolicum* via reducing fecundity and egg hatchability implying it's great prospective for tick control as the number of eggs laid and the proportion which hatches are crucial for the propagation of tick's population (Dipeolu, 1984).

CONCLUSION

It worth mentioning that the burden of ticks and tick-borne diseases mobilizing efforts which should be undertaken for controlling ticks in the Sudan.

The high cost, resistance development and adverse effect on environment are the main drawbacks for the existing tick control using acaricides.

In contrast specificity and cheaply mass production of the entomogenus fungi should encourage use of fungi as an alternative means to acaricides.

The high efficiency and promising results obtained in the present study against *H. anatolicum* using *M. anisopliae* should promote the use of such fungi under Sudan conditions to control ticks as alternative to chemical acaricides.

M. anisopliae potentcy to inhibit the biological performance of the treated fed and unfed free-living ticks is of great importance as it hold back the next generation and should encourage application of such fungi to tick breeding habitats as ticks spend 90% of their life off host.

As *M. anisopliae* is easily, inexpensively produced and efficient, hence an appropriate technology of production and application should be developed to allow putting it into practice.

Isolation and utilization of entomogenus fungi as bio-insecticides against hard ticks in the Sudan should be highly recommended.

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