Laboratory evaluation of three strains of the entomopathogenic fungus *Metarhizium anisopliae* for controlling *Hyalomma anatolicum anatolicum* and *Haemaphysalis punctata*

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Abstract: **Hyalomma anatolicum anatolicum and Haemaphysalis punctata larval ticks were shown to be susceptible to different strains of entomopathogenic fungi Metarhizium anisopliae under laboratory conditions. To determine the susceptibility of H. anatolicum anatolicum and H. punctata to M. anisopliae, two suspensions of conidia were used (10^3 and 10^4 spores/ml). The treatments were conducted by immersing larval stage (at least 20 larvae/Petri dish) of H.anatolicum anatolicum and H.punctata in the spore suspension for 30 sec, followed by transferring to Petri dish containing moist filter paper. Control larval ticks were only immersed in 0.05% aqueous Tween 80. All treated and untreated ticks were observed by day interval up to day 18 to detect dead ticks and signs of mycosis. Comparative results of bioassays using three different fungal strains showed that all strains of M. anisopliae were highly pathogenic against two tick species used. The higher mortality rate was seen with strain of 689 when used in H. punctata and 685 in H. anatolicum anatolicum in comparison with the other strains($p<0.05$). The present study suggests that Metarhizium anisopliae has great potential for the control of H. punctata and H. anatolicum anatolicum.**

Key words: *Hyalomma anatolicum anatolicum, Haemaphysalis punctata,* tick, entomopathogenic fungi, *Metarhizium anisopliae.*

Introduction

Hyalomma anatolicum anatolicum is one of the world's most widely distributed and damaging tick. It transmits a wide range of debilitating, even fatal diseases of livestock including Theileria annulata, Babesia equi, Anaplasma marginale, and several arboviruses (Hooshmand_Rad and Hawa, 1973; Razmi, *et al*., 2003). It is also a vector of Crimean-Congo hemorrhagic fever virus to humans (Chinikar, 2002). Haemaphysalis punctata is also a widely distributed and harmful ectoparasite of livestock and vector of Anaplasma and Babesia as well as several viral diseases including tickborne encephalitis and Crimean-Congo hemorrhagic fever (Khalacheva and Kyurtov, 1981; Hashemi-Fesharahi, 1997).

Current tick control methods are still heavily dependent on the use of synthetic chemical acaricides but several problems which have been generated by these chemicals including resistance in tick populations, (Beugnet, and Chardonnet 1995) safety risks for humans and domestic animals, contamination of ground water, decrease in

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Table 1: Mean percentage mortality by day 2, 4, 6, 8, 10, 12, 14, 16 and 18 of *H. anatolicum anatolicum* larvae treated with three strains of *M. anisopliae*.

Strain	concentration conidia/ml	Day2	Day4	Day6	Day8	Day10	Day12	Day14	Day16	Day18
685	10^3	9.4	18.8	21.1	23.5	34.1	41.1	50.5	58.8	78.8
685	10^4	14.4	23.2	25.6	32	40.8	52	64	72	84
689	10^3	9.2	19.2	19.2	26.9	28.4	30.7	34.6	37.6	50
689	10^4	12.5	15	16.6	20.8	22.5	37.5	52.5	70	81.6
715C	10^3	11.5	24.8	26.6	27.2	28.4	30.9	39.3	48.4	57.6
715 C	10^4	12.6	29.8	32.1	37.9	42.5	51.7	59.7	72.4	88.2
Control	-------	9.5	21.4	21.4	21.4	21.4	23.8	23.8	23.8	23.8

Table2: Mean percentage mortality by day 2, 4, 6, 8, 10, 12, 14, 16 and 18 of H. punectata larvae treated with three strains of M. anisopliae.

Strain	concentration	Day2	Day4	Day6	Day8	Day10	Day12	Day14	Day16	Day 18
685	10^3	9	16.2	20.1	22	24	31.8	38.9	51.9	79.7
685	10^4	52.5	66.1	76.2	83	88.1	91.5	96.6	100	100
689	10^3	14.7	19.8	22.1	27.1	35.9	41	77	93.5	98.6
689	10^4	50.7	80.4	383.3	84.7	86.2	89.1	92	97.8	98.5
715C	10^3	19.5	31	34.4	36.7	39	41.3	43.6	67.8	74.7
715 C	10^4	20.8	33.5	36.9	40.4	45.6	56	60.6	87.2	90.2
Control	-------	7.4	8.9	10.4	10.4	11.9	13.4	14.9	14.9	19.4

biodiversity, and other environmental concerns (Lacey *et al.,* 2001), haveprompted interest in the development of more environmentally friendly alternative strategies such as biological control based on entomopathogenic fungi. Particular attention has been focused on the development of entomogenous fungi, such as Metarhizium anisopliae and Beauveria bassiana, for controlling a range of ticks including species of Ixodes (Zhioua *et al.,* 1997; Benjamin *et al.,* 2002; Ostfeld *et al.,* 2002; Hornbostel *et al.,* 2005), Boophilus (Frazzon *et al.,* 2000; Gindin *et al.,* 2001; Onofre *et al.,* 2001), Amblyomma (Kaaya *et al.,* 1996), Argas (Sewify and Habib, 2001), and Rhipicephalus (Mwangi *et al.,* 1995; Kaaya *et al.,* 1996; Samish *et al.,* 2001). Strains of fungi Metarhizium anisopliae have been extensively studied as a key regulatory organism for biocontrol (Frazzon *et al*., 2000; Dutra *et al.,* 2004) and they have been shown to be effective even under field conditions (Kaaya *et al.,* 1996; Hornbostel *et al.,* 2005).

The aim of this study was to investigate the susceptibility of the H. anatolicum and H. punctata to infection with three strains of the entomopathogenic fungi M. anisopliae and the possibility of using this fungus as a biological agent. To the best of our knowledge, the susceptibility of these ticks to entomogenous fungi has not been determined.

Materials and Methods

***Fungi*:** The *M. anisopliae* strains used in this study were 685 and 689, which were isolated from Ixodes ricinus in the UK and 715C which was isolated from a locust in Iran. The UK isolates were a part of the fungal culture collection at the University of Wales Swansea, while the Iranian isolate was maintained at Tehran University. The fungi were passaged twice through Argas persicus larvae before being cultured on potato dextrose agar (PDA) (Merck, Germany), at 25°C. Conidia were usually harvested 14 days post-inoculation and suspensions prepared in aqueous Tween 80 (Merck, Germany), as described by Butt and Goettel (2000). Two concentrations of Conidia including 10^3 and 10^4 conidia /ml were used in the bioassays.

Ticks: Ticks were collected from infested

animal's and transferred into dry plastic vials containing a few fresh grass leaves, and were covered by a lid containing several minute holes. Vials were properly kept under optimal condition at room temperature for a few days in order to maintain ticks survival, and then they were sent to the laboratory for taxonomic identification.. The ticks were identified by morphological characteristics according to standard taxonomic keys (Hoogstraal and Kaiser, 1959 and Estrada-Pena *et al.,* 2004). Engorged female H. anatolicum anatolicum and H. punctata were collected from the naturally infested sheep in Urmia, West Azarbaijan of Iran. They were surface sterilized by three second immersing in 70% ethanol. The females were then dried on sterile filter paper, transferred to Petri dishes containing one piece of filter paper moistened with 1 ml of distilled water and incubated at 28° C with 80% relative humidity (RH). Petri dishes were transferred to desiccators and humidity was provided using sutured salt solution. Females started laying eggs after 4 weeks. The eggs were transferred to separate tubes (2.5 × 15 cm) containing a piece of paper, sealed up by soft cotton band and incubated in desiccators at 28° C with 80% RH. The newly emerged larvae were used in bioassays.

Bioassay Procedure: To determine the susceptibility of H. anatolicum anatolicum and H. punctata to M. anisopliae, two suspensions of conidia were used (10^3 and 10^4 spores/ml). The treatments were conducted by immersing larval stage (at least 20 larvae/Petri dish) of H.anatolicum anatolicum and H.punctata in the spore suspension for 30 sec, followed by transferring to Petri dish containing moist filter paper. Control larval ticks were only immersed in 0.05% aqueous Tween 80. All treated and untreated ticks were observed by day interval up to day 18 to detect dead ticks and signs of mycosis. Three replicates were made for each suspension and the mortality percentages were calculated.

One way ANOVA test was used for analyzing statistical associations between the data results. Associations were statistically significant with a P-value of less than $p<0.05$. Statistical analysis of data was performed with computer software (SPSS windows 9.0).

Results

All three strains of M. anisopliae were pathogenic to H. anatolicum anatolicum and H. punctata, but the degree of virulence of fungal strains varied considerably ($p<0.05$). Strain 689 caused higher mortality rate when used for infecting H. punctata and strain 685 for H. anatolicum in comparison with other strains (Tables 1 & 2).

The mortality rate caused by M. anisopliae isolates for two tested concentrations of the fungi) was varied from 50 to 88.2% for 10^3 and 10^4 conidia /ml and 74.7 to100% for 10^4 conidia/ml against H.anatolicum anatolicum and H.punctata 18 days after post-infestation (Tables1 & 2). Tick mortality rate varied significantly with increasing spore concentration ($p<0.05$).

The mean percentage of mortality of H. anatolicum anatolicum and H.punctata using two different fungal suspensions 10^3 and 10^4 conidia/ml and no fungal (control) was shown in the Table 1 and 2.

Discussion

Although Strains of M. anisopliae have been widely used for the control of agriculture and forest pests (Ferron, 1981; Anderson *et al.,* 1988; Maniania, 1993), little has been reported on their use for the biological control of ticks. In the present study it was revealed that this fungus is highly infective to larval stage of H. anatolicum anatolicum and H. punctata and that there are differences in the infectivity amongst strains of M. anisopliae.

Fungal strains in general, seem to have same effect against different tick species and stages (Gindin *et al.,* 2002). In a study by Mwangi *et al*., (1994) isolates of B. bassiana and two of M. anisopliae were investigated for pathogenicity against R. appendiculatus. It has been revealed that the best isolates of B. bassiana and M. anisopliae induced mortalities of 73 and 35%, respectively, in unfed adults compared to 0% in controls. Pirali-kheirabadi *et al*., (2007) also in a study on the virulence of some entomopathogenic fungi including M. anisopliae

reported that these fungi can affect all developmental stages of Boophilus annulatus, however, their efficiency varied considerably based on the fungal species and strains. Nevertheless, in our study, all three strains of M. anisopliae caused mortality in the larval stages of H.anatolicum anatolicum and H. punctata, especially the strains 689 and 685 caused significantly higher reduction in the number of H. punctata and H. anatolicum anatolicum in comparison with the other isolates.

The mean percentage mortality of H. anatolicum anatolicum and H. punctata using different fungal suspensions (10^3 and 10^4 conidia /ml) was significantly different ($p<0.05$). Zhioua *et al*., 2002 reported the LC50 values of M. anisopliae for the organophosphorus susceptible and resistant strains of Boophilus microplus being 10^3 and 10^2 spores per ml, respectively.

In conclusion, our findings of a fungal strain pathogenic for two tick species increase the possibility to develop a commercial fungal anti-tick agent. The results obtained form this study showed that biological control of H.anatolicum anatolicum and H.punctata using M. anisopliae infection is feasible and can be considered an additional method of integrated tick control under field conditions.

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مجله بین المللی تحقیقات دامپزشکی، ۱۳۸۸، دوره ۳، شماره ۱، ۱۵-۱۱

استفاده از قارچ متاریزیوم آنیزوپلیه در کنترل بیولوژیکی هیالوما آناتولیکوم آناتولیکوم و همافیزالیس پونکتاتا در شرایط آزمایشگاهی

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چکیده

در این مطالعه، پاتوژنیسیته سه سویه قارچ انتوموپاتوژن متاریزیوم آنیزوپلیه بر روی مرحله نوزادی کنه های هیالوما آناتولیکوم آناتولیکوم و همافیزالیس پونکتاتا تحت شرایط آزمایشگاهی مورد ارزیابی قرار گرفت. بدین منظور، پس از کشت و جداسازی سه سویه این قارچ شامل ۶۸۵، ۶۸۹ و ایران C ۷۱۵ در محیط PDA، با افزودن آب مقطر استریل به استوک اصلی، غلظت های 10^3 و 10^4 اسپور در هر میلی لیتر از هر سویه تهیه شد. در گروه های مورد آزمایش نوزاد کنه ها با غلظت های 10^3 و 10^4 اسپور در هر میلی لیتر به مدت ۳۰ ثانیه آغشته شدند و سپس به داخل پتری دیش انتقال یافتند. در گروه شاهد نوزادها در توین ۰/۰۵ درصد غوطه ور شدند. گروه های آلوده و کنترل هر دو روز و به مدت ۱۸ روز مورد بررسی قرار گرفتند و درصد مرگ و میر ایجاد شده ثبت گردید. نتایج این بررسی، تأثیر بالای کشندگی در دو غلظت مورد بررسی در مقابله با مرحله لاروی هیالوما آناتولیکم آناتولیکم و همافیزالیس پونکتاتا نشان داد. همچنین مشخص گردید که سویه ۶۸۵ و ۶۸۹ به ترتیب بیشترین اثر کشندگی را در غلظت های اسپور به کار گرفته شده بر مرحله نوزادی هیالوما آناتولیکم آناتولیکم و همافیزالیس پونکتاتا دارند ($p<0/05$). این مطالعه نشان می دهد که قارچ متاریزیوم آنیزوپلیه، قابلیت بسیار خوبی برای کنترل نوزادی هیالوما آناتولیکم آناتولیکم و همافیزالیس پونکتاتا دارد.

واژه های کلیدی:

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