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RESEARCH ARTICLE

Ovicidal Efficacy of *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae) towards *Rhipicephalus sanguineus* (Acari: Ixodidae) Eggs

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ABSTRACT

Rhipicephalus sanguineus, commonly known as brown dog tick is a widespread species with considerable public health and economic importance. Tremendous efforts were performed to control the tick populations with the concern of resistance build-up and environmental issues. Alternative towards microbial control thus emerged as one option to reduce tick populations. In this study, the ovicidal efficacy of a native isolate entomopathogenic hyphomycetes fungi, *Metarhizium anisopliae* strain HSAH5 was evaluated against eggs of *R. sanguineus*. Spray applications with three different conidial concentrations of 10^5, 10^6 and 10^7 conidia mL^{-1}; 40 ppm of Flumethrin and a negative control. The *M. anisopliae* strain was found highly virulent to *R. sanguineus* eggs by reducing the hatching percentages to ≈30% compared with 8.9% in Flumethrin eggs. The result showed a significantly higher mortality in *M. anisopliae* group than those of the control groups ($F = 42.08$, $df = 32$, $P < 0.001$) at 30 days post-infection. However, there are no significant differences within the *M. anisopliae* group, in which the mortality between different conidial concentrations is almost the same. The estimated LC_{50} of *M. anisopliae* against eggs of *R. sanguineus* is 1.36×10^3 conidia ml^{-1}. Thus, these results suggest *M. anisopliae* strain HSAH5 could be a potential biocontrol agent of *R. sanguineus* in the integrated approach to managing ticks in the residential landscape by targeting on the eggs.

Keywords: Entomopathogenic Fungus; LC_{50}; Native strain; Tick eggs; Biological control.

INTRODUCTION

Rhipicephalus sanguineus (Arachnida: Ixodidae) or commonly known as brown dog tick is the most widespread cosmopolitan ticks in the world with great significance in the veterinary industry and human health. This tick is the vector of many important pathogens that caused high mortality and morbidity with considerable economic impacts (Dantas-Torres *et al*., 2012). *Rhipicephalus sanguineus* has three non-parasitic off-host periods which are relatively long compared to the on-host periods. Engorged females will oviposit clusters of large numbers of eggs in protected locations which are dark, humid and with moderate temperature; these eggs are difficult to eliminate, and the survival of the offspring eventually maintain substantial tick populations (Dantas-Torres, 2008).

As with many other major pests, the control of *R. sanguineus* is usually attempted with chemicals control, although this approach may present disadvantages such as the development of resistance, human and animal toxicity and environmental pollution. Therefore, a new interest towards biological control of ticks had been developed, particularly on entomopathogenic fungi (Fernandes & Bittencourt, 2008). Although a few entomopathogenic fungi are reported to be effective towards several tick species, the susceptibility of ticks to fungi might vary according to tick species and population as well as to fungal strain (Fernandes *et al*., 2012; Perinotto *et al*., 2012).

As the egg masses of ticks are often being laid in such conditions which are also suited for the development of entomopathogenic fungi, *R. sanguineus* eggs could be the key targets for controlling tick populations with mycoacaricides. Infectious conidia can either get into contact with tick eggs indirectly on substrates which are previously treated with the fungus or treated topically (directly) with conidia. Whereas fungal activity against *R. sanguineus* eggs, larvae, nymphs, and adults is well established (Dantas-Torres, 2008; Fernandes & Bittencourt, 2008; Gindin *et al*., 2009), the native

strains have been shown to be more virulent (Pajar *et al*., 2013). However, of the few studies available in the literature on the use of entomopathogenic fungi against *R. sanguineus*, none of the native strains of these fungi were tested against *R. sanguineus*. Therefore, the aim of this study was to investigate the *in-vitro* efficacy of a native strain of *Metarhizium anisopliae* (Ascomycota: Clavicipitaceae) towards eggs of *R. sanguineus*.

MATERIALS AND METHODS

Tick Samples

A total of 20 engorged female *R. sanguineus* collected from ticks infested dog in Kuala Lumpur, Perak and Kelantan were identified under a stereomicroscope using references from Abdullah *et al*. (2016) and Walker *et al*. (2014). These engorged females were surface sterilized by dipping in 0.1% sodium hypochlorite solutions to remove possible environmental fungi and bacteria and rinsed with sterile distilled water before placing in a sterile container with moist filter paper for oviposition. Tick specimens were maintained under controlled conditions of temperature (27 ± 2°C), relative humidity (RH 80 ± 5%) and photoperiod (12 h light, 12 h dark) to produce eggs.

Metarhizium anisopliae Origin and Suspension Preparation

A pure culture of *Metarhizium anisopliae* strain HSAH5 was obtained from Universiti Putra Malaysia (UPM). This strain was isolated from soil in Ayer Hitam Forest Reserve, Puchong, Selangor, Malaysia. It was then identified and archived in Institute of Bioscience, UPM (UPMC869) and was deposited in NCBI GenBank (Accession No.: KX279867).

The fungus was sub-cultured on sabourad dextrose agar (SDA) agar and passaged on sterilized *Tenebrio molitor* larvae before being maintained on SDA and kept at 4°C for further use. The conidial suspensions of *M. anisopliae* were prepared by growing the fungus on ten Petri dishes containing SDA for 2 weeks at 28 ± 2°C. Conidia were harvested by washing the Petri dishes with sterile distilled water containing 0.2% Tween 80 and filtered through a sterile filter paper to eliminate agar and mycelia. The concentrations of the conidia suspension were then determined by using the Neubauer chamber and adjusted to 10^5, 10^6 and 10^7 conidia mL^{-1} using dilution method. The negative control was a sterile 0.2% Tween 80 solution, while the positive control was a dilution of Flumethrin (Bayticol 6% E.C.) with distilled water into 40 ppm.

Laboratory Bioassay

Eggs laid during the first 4 days were collected for the bioassay. The eggs from each female tick were considered as a replicate and were randomly divided into five groups of equivalent number by counting them under a dissecting microscope and transferring them onto a sterile container with moist filter paper using a decontaminated fine camel brush (size 00). Egg clumps were first separated by agitating shortly with distilled water before the counting and transferring procedures to reduce the handling times. Filter papers in the plates were moisten from time to time to avoid desiccation. Plates were covered immediately after each transfer to reduce desiccation and avoid contamination as well.

A total of 10 replicates comprising of 456 eggs for each groups were used in this bioassay. The bioassay was composed of five Petri dishes for two were the control groups (positive & negative control) and three as the treatment groups (3 different conidial concentrations). The eggs were sprayed with 0.5ml of the treatment or control solution by using a cosmetic hand sprayer. The Petri dishes were sealed with parafilm and maintained under controlled conditions (see above). Eggs were examined under microscope and were considered hatched if a part of the larvae were seen out from the eggs shell (although it was not fully detached from the egg shell). The hatching rate was recorded every five days by observing the eggs under a stereomicroscope. The bioassay was terminated after 30 days of treatment.

Data Analysis

The results were reported as mean values in percentage. The eggs mortality of each treatment was indicated by the number of unhatched eggs after treatment and adjusted by using Sun-Shepard's formula (Püntener, 1981) prior analysis. The data were analysed using one-way analysis of variance (ANOVA) at 5% level of significance ($p \leq 0.05$). The lethal concentration at 50% and 90% (LC_{50} and LC_{90}) were calculated using probit analysis. All statistical analyses were conducted using SAS® Studio release 3.8 (SAS Institute, Inc., Cary, NC, USA). Meanwhile, for dose-response (LC_{50} and LC_{90}) graph was generated and visualized using Prism 7 for MacOS X ver. 7.0a (GraphPad Software, La Jolla, CA, USA).

RESULTS AND DISCUSSION

The *in-vitro* effect of *M. anisopliae* on eggs of *R. sanguineus* is shown in Figure 1. White fungal mycelium started to emerge and sporulate on the surface of the eggs of *R. sanguineus* started on day 3 post-treatment with the highest number of mycelia growth (42.7%) at 10^7 conidia mL^{-1}, followed by 10^6 conidia mL^{-1} (0.8%) and 10^5 conidia mL^{-1} (0.4%) (Figure 1). The formation of conidia began at day five post-treatment with the highest number of sporulation (84.6%) at 10^7 conidia mL^{-1}, followed by 10^6 conidia mL^{-1} (30%) and 10^5 conidia mL^{-1} (10%) (Figure 1). Larvae from both controls and treatment group hatched at day 13 post-treatment; in which the larvae from the treatment group (10^5 conidia mL^{-1}) and negative control were relatively strong and active compared to the positive control. The larvae from the positive control were weak as compared to the negative control in which larvae were not completely detached from the egg shell or limb were not fully extended, inactive whereby the movement were slow and die soon after hatching.

Eggs that unable to hatch were considered die and indicated eggs mortality. The treatment groups have shown that this *M. anisopliae* strain was highly virulent to *R. sanguineus* eggs and able to cause mortality up to 29.57–30.22% compared with only 8.97% of mortality in Flumethrin. The result showed a significantly higher mortality in treament groups than those of the control groups ($F = 42.08$, $df = 32$, $P < 0.001$) after 30 days of treatment. However, there are no significant differences within *M. anisopliae* group and within the control group, in which the mortality between different conidial concentrations and between positive and negative control are almost the same (Table 1). The estimated LC_{50} of *M. anisopliae* strain HSAH5 against *R. sanguineus* eggs was 1.36×10^3 conidia mL^{-1} while the LC_{90} value was 1.47×10^4 conidia mL^{-1} (Figure 2).

The present study shows, for the first time, that a native strain of *M. anisopliae* was found highly virulent towards *R. sanguineus* eggs. *Metarhizium anisopliae* has long been studied on its efficacy towards different species of soft and hard ticks (Gindin *et al*., 2009; Ren *et al*., 2016; Fernandez-Salas *et al*., 2017; Fischhoff *et al*., 2017; Prado-Rebolledo *et al*.,

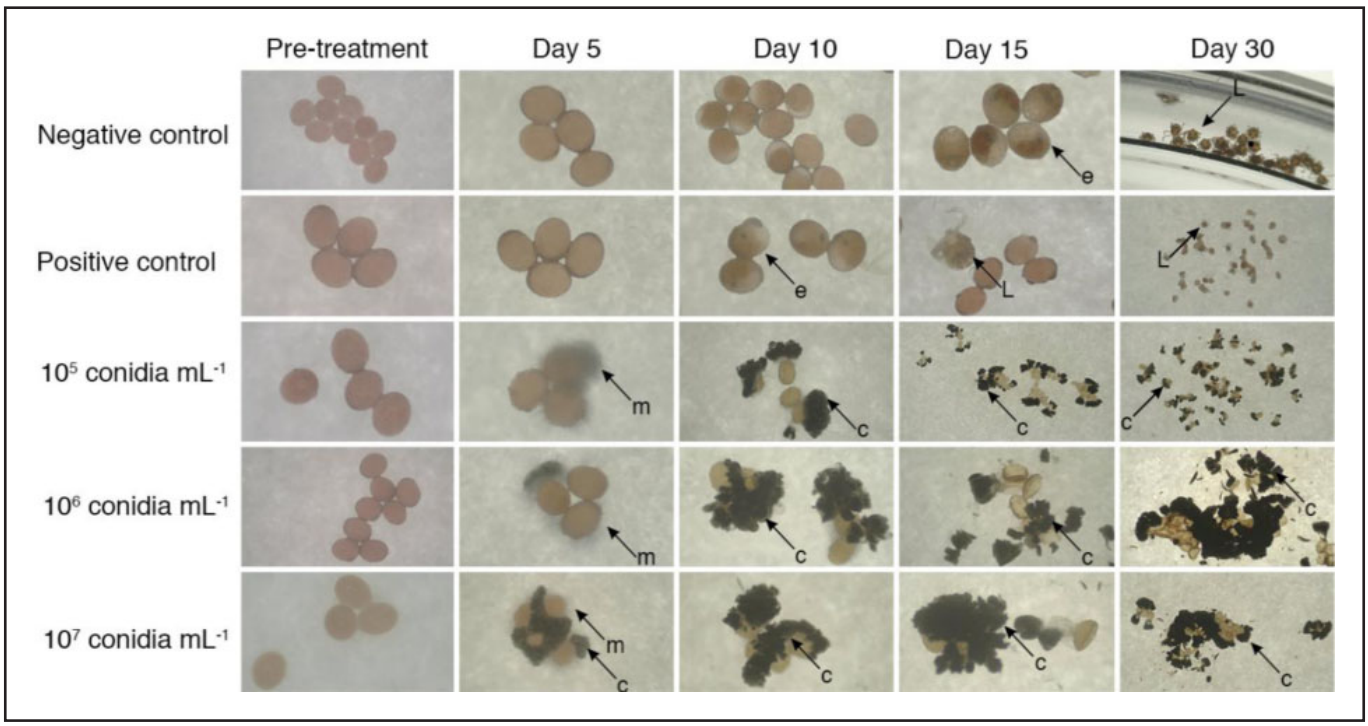

Figure 1. The *in-vitro* effect of different treatments on eggs of *R. sanguineus* at different period of time.
*Note: c = conidia; m = mycelia, L = larvae; e = embryo.

Table 1. Mortality of *R. sanguineus* eggs treated with different *M. anisopliae* concentration and positive control

Treatment	Mortality (% ± SD)
Positive control (Flumethrin, 40 ppm)	8.97 ± 2.16a
10^5 conidia mL^{-1}	29.57 ± 4.82b
10^6 conidia mL^{-1}	30.22 ± 4.90b
10^7 conidia mL^{-1}	30.22 ± 4.90b

[ab] Means followed by the same letter did not differ significantly ($p<0.05$, Tukey HSD Test = 6.45).

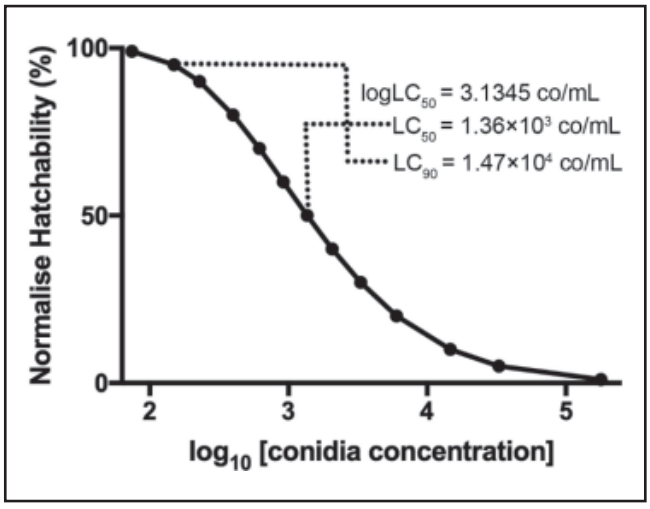

Figure 2. Dose-response curve of *R. sanguineus* eggs hatchability after treated with *M. anisopliae* conidial suspension.

2017). However, the effectiveness of fungus are subjected to many factors, one of the most important factors is the isolates of fungus. Indigenous isolates that have been adapted to local conditions are often found to be more effective in controlling the pest as they are more tolerant to the abiotic factors around (Pajar *et al*., 2013). Indeed, *M. anisopliae* strain HSAH5 containing 10^6 and 10^7conidia mL^{-1} were able to cause 100% mortality on local *R. sanguineus* eggs. This result is also in agreement with the findings by Gindin *et al*. (2009) where the study shows that *R. sanguineus* eggs are more susceptible to *M. anisopliae* compared to *R. annulatus*.

Surprisingly, the mortality caused by flumethrin exposure (positive control) was only 8.97%, which was subsided compared to eggs treated with *M. anisopliae* spore suspension. This result was contradicted by Haque *et al*. (2014) in which this commonly used acaricide are able to caused complete inhibition on *Rhipicephalus (Boophilus) microplus* eggs from hatching. Nevertheless, the difference may be deduced from the different species that have been tested on, as the susceptibility of different ticks species and even population may vary (Dantas-Torres, 2008). It might also due to the probability of acaricide resistance development of local tick population (Bandara *et al*., 2017). The bigger deviation on the results of mortality between the replicates of positive control indicates acaricidal resistance build-up might have happened on certain population groups of *R. sanguineus* as the ticks which were collected from different locations in Malaysia.

The ovicidal efficacy of *M. anisopliae* is dose-dependent, whereby the highest concentration used in this study having a better coverage and rapid mycelia growth following by sporulation. This was supported by Adames *et al*. (2011) that shows higher eggs mortality (55%) was achieved when exposed to 10^8 conidia mL^{-1} compared to the only 7%

when 10^4 conidia mL^{-1} was being applied. The difference might due to a higher conidial concentration increased the distribution of conidia on all eggs. This eventually enabled mycelium and conidia developed simultaneously on a high proportion of eggs and thus reduced the time of fungal development and increased the overall ovicidal activity (Luz *et al*., 2016). The LC_{50} and LC_{90} of this study corresponded with Luz *et al*. (2016), however, zero larvae eclosed from clustered eggs treated were not achieved in this study with the concentration of 10^5 conidia mL^{-1}. The reason might be due to uneven distribution of conidia on the eggs cluster when the lower concentration was used; it was then reduced the efficiency of the conidia.

CONCLUSION

Metarhizium anisopliae is one of the promising candidates of biocontrol even for the tick. In general, ticks controlling can either be done on or off hosts. The latter provides a means to reduce new infestation by killing off the moulting stages of ticks and more importantly the eggs. These results suggest that the native strain of *M. anisopliae* can be a potential biocontrol agent for reducing tick populations by killing off the egg masses of economically important ticks. Advantage such as sustainability of the fungus in controlling the ticks population can also be achieved when the application sites in which the ticks and egg masses present are also favourable for fungal development and persist for many months.

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Conflict of interest

The authors declare that they have no conflict of interest.

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