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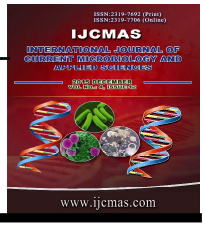

Original Research Article

In Vitro activity of the Nematophagous Fungi *Pochonia chlamydosporia* on *Rhipicephalus (Boophilus) microplus* Ticks

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Keywords

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A B S T R A C T

*Rhipicephalus (Boophilus) microplus*is a tick that parasites mainly cattle and serve as vector to many diseases including babesiosis and anaplasmosis that are transmitted during hematophagous feeding, bringing economic losses due to animal sickness and decrease in production. The arising of resistance to substances used for chemical control, along with the risk of environmental and food contamination by these chemicals, has raised interest in biological control for ticks. Engorged females of *R. microplus* were collected from naturally infested animals and divided into three treatment groups, which were bathed in solutions containing 5000, 10000 and 15000 chlamydospores, and one treatment group. The present study aimed to evaluate the *in vitro* effect of the nematophagous fungus *Pochonia chlamydosporia* on *R. microplus* engorged females. It was observed that *P. chlamydosporia* (VC4) was able to infect *R. microplus* ticks at concentrations of 5000, 10000 and 15000 chlamydospores, having statistical difference ($p<0.05$) between all treatment groups when compared to the control group but not when compared among themselves. These results suggest the possibility of VC4 to be used as a biological control for *R. microplus* ticks.

Introduction

The *Boophilus microplus*, currently *Rhipicephalus (Boophilus) microplus*, is a hard tick that parasites mainly cattle (Taylor et al., 2010), but that can eventually be found parasitizing other domestic and wild animals (Hoogstraal, 1956). It´s one-host life cycle can be divided into two phases, a parasitic and a free-living phase (Santos & Vogel, 2012). The parasitic phase begins at the moment the larvae fixates itself on the host and ends once the engorged female leaves the host to lay eggs on the environment. In its turn, the free-living phase occurs on the environment and it includes from oviposition to the moment the larvae settles on the host (Taylor et al., 2010).

The identification of the *R. microplus* ticks can be accomplished through its anatomic characteristics. The adults have a round to rectangular body, cream coloured legs and non-ornamented chitinous dorsal plate, or scutum. In females the dorsal plate is placed

only in a small portion of the scutum while in males it covers all dorsal surface of the plate (Taylor et al., 2010). Other differences between males and females are the caudal developed adanal shields and caudal process present only in males. In these ticks the eyes are present, the festoons absent, the capitulum is short with a hexagonal base (Monteiro, 2011) and the spiracular plate is round or oval shaped (Taylor et al., 2010).

The *R. microplus* tick is a vector to many diseases, such as *Babesia bovis* and *bigemina* and *Anaplasma marginale*that are transmitted through its hematophagous feeding (Gonzales et al., 1974; Taylor et al., 2010). These diseases can cause symptoms ranging from anemia, dehydration and weight loss to convulsion and death, causing economic losses due to abortions and reduction in milk production and weight gain (Taylor et al., 2010).

To control *R. microplus* populations, the use of chemical acaricides has been the main method of choice (Ojeda-Chi et al., 2010). However, cattle farmers tend to use these chemicals without a veterinarian´s orientation (Camillo et al., 2009), resulting in, many times, wrong management of pest control, which causes the ticks to develop resistance to these acaricides (Ojeda-Chi et al., 2010; Camargo et al., 2012). Another downside of chemical acaricides use besides causing resistance problems is its effect on human health and the environment. The use of these substances leads to the accumulation of toxic residues in the meat and milk of cattle causing the contamination of food and the environment becoming a health hazard (Castro-Janer et al., 2010; Ojeda-Chi et al. 2010; Camargo et al., 2012). In association with the problems already mentioned, the high cost of chemical control along with the risk of animal intoxication, has led to an increase in interest in using biological control as an alternative to tick control (Hajek & St. Leger, 1994; Gronvold et al. 1996; Monteiro, 2011; Camargo et al., 2012).

At livestock farms, biological control is used to keep endo and ectoparasites at sub-clinical densities, so it will not cause damage to the animals´ health or economic loss to the producers (Gronvold et al. 1996). For the biological control of ticks, entomopathogenic fungi have been the preferential choice (Angelo et al., 2010; Fernandes et al., 2012). These fungi are able to secrete enzymes that allows them to pass through the tick´s cuticle and reach its internal organs, causing its death (Monteiro, 2011; Fernandes et al., 2012). New research has shown that the nematophagous fungi are also effective when used as biological control for ticks (Braga et al., 2013).

The nematophagous fungi feed on both eggs and worms and can be divided in three groups: (1) trapping or predatory, that created traps with their hyphae to trap and kill the nematode; (2) endoparasites, that attack the nematodes with their spores, that can either be ingested or adhered to the surface of nematodes; and (3) egg-parasitic, that use an structure called apressoria to break through the eggs´ shell to feed on its interior content (Nordbring-Hertz et al., 2006).

Among the nematophagous fungi, the specie *Pochonia chlamydosporia* has attracted attention to its potential to be used as biological control of nematodes due to its capacity to pray on eggs (Araújo et al. 2009; Braga et al. 2010;Silva et al. 2010a,b). But Braga et al (2013) has recently opened a new door for biological control of ticks when he used a nematophagous fungus, *Duddingtonia flagrans*, to infect and kill engorged females of the tick *Amblyomma*

cajenense. Therefore, the aim of the present paper was to analyse the efficiency of the nematophagous fungus *Pochonia chlamydosporia* for the biological control of the tick *Rhipicephalus (Boophilus) microplus*.

Materials and Methods

Fungi

The nematophagous fungus *Pochonia chlamydosporia* (VC4) used was isolated and kept at a B.O.D. incubator at 25 ± 1° C and 80 ± 10% relative humidity. The number of chlamydospores in three aliquots of 10µl of the fungi solution was counted and the average was calculate to discover the amount of chlamydospores/ml.

Experimental Assay

Engorged females of *R. microplus* were collected from naturally infested animals and divided into three treatment and one control group, having eight ticks in each group. The ticks were first washed in distilled water and after in a 70% alcohol solution. They were then dried in paper towels and lastly fixated to Petri dishes.

The ticks from treatment group 1, 2 and 3 were bathed in a fungi solution containing 5,000 (VC4-A), 10,000 (VC4-B) and 15,000 (VC4-C) chlamydospores, respectively, for about 10 seconds before being fixated to a Petri dish. The control group, on the other hand, was bathed in distilled water for 10 seconds before being fixated. A wet cotton ball with distilled was placed in each Petri dish which were then wrapped in Pvc paper and incubated at 25 ± 1° C and 80 ± 10% RH.

The confirmation of the presence of *P. chlamydosporia* on the *R. microplus* was accomplished two ways. First, the hyphae present in the tick's cuticle was scraped off and inoculated into two Petri dishes, one containing agar-agar at 2% (WA2%) and one containing potato dextrose, and incubated at 25 ± 1° C and 80 ± 10% RH. The fungi colony was then placed into a test tube containing distilled water, the solution was stirred and aliquots of 10µ each were observed under a microscope to find chlamydospores. The same process was repeated but this time a tick with visible hyphae was placed in the test tube with distilled water to search for chlamydospores.

Statistical Analysis

The average and standard deviation of each treatment group was calculated for each day and total number of infected ticks. The data was interpreted statistically by analysis of variance at significance levels of 1 and 5% probability followed by the Tukey test (Ayres et al., 2003), using the program BioEstat 5.0.

Results and Discussion

During this experiment, it was observed that the first sign of the infection of the *P. chlamydosporia* fungus on *R. microplus* ticks is the manifestation of small white dots all over the ticks´ scutum, at this time no visible hyphae is present. As *P. chlamydosporia* continues to spread through the host, the presence of white, cotton like hyphae starts to be noticed, as well as a change in colour of the tick's body to a dark brown (Figure 1). It was also observed that after the tick changes colour and the hyphae becomes visible, it dies and its insides are digested, becoming a dark red/brown liquid.

On the treatment groups, only one tick in the VC4-B group showed visible hyphae without first having the presence of white dots in its scutum, this same tick was the only one that did not lay any eggs, showing

a higher predisposition to the fungal infection than the other ticks, which was also described by Perinotto et al (2012) . In their study, the authors observed statistical difference ($p>0.05$ and $p>0.01$) for the biological parameters evaluated between two different *R. microplus* populations with the same fungi concentration of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*, coming to the conclusion that the ticks´ predisposition to fungal infection was the probable reason for the results. None of the ticks in the control group presented white dots or visible hyphae in their bodies.

Table 1 shows the average number of *R. microplus* ticks that were infected by *P. Chlamydosporia* on the treatment and control groups considering all observation days. All treatment groups showed statistical difference ($p<0.05$) when compared to the control group, but did not show statistical difference when compared to one another.

Table 2, on the other hand, shows the average number of *R. microplus* ticks that were infected by *P. chlamydosporia* on the treatment and control groups for each observation day. On the first observation day that was no statistical difference ($p<0.05$) between the treatment and control groups, and among the control groups. However, from the second observation date to the sixth it was observed statistical difference when comparing the treatment groups to the control group, but not when comparing the treatment groups among themselves.

The results from Table 1 and 2 demonstrates that even tough *P. chlamydosporia* is a nematophagous fungus it is capable of infecting *R. microplus*. This ability may be due to the secretion chitinases and proteases (Esteves et al., 2009), which helps this fungus penetrate the scutum of the ticks and reach its internal organs. It also indicates that a solution with 5000 chlamydospore concentration is sufficient to successfully infect *R. microplus*. Braga et al. (2013) also suggested that the nematophagous fungus *D. flagrans* was able to infect *A. cajennense* ticks with the help of chitinases.

In Figure 2 the percentage of ticks with visible hyphae for each treatment group on different observation days is shown. Throughout the experiment, the ticks in the control group did not have any visible hyphae in their bodies, differently from the treatment groups, where all concentrations of VC4 showed ticks with visible hyphae. On the first observation day no ticks presented visible hyphae, while on the second day it was notice in only one tick in the VC4-B group. From the third day forward, all treatment groups had ticks presenting hyphae. As time progressed it was perceived that the higher the concentration of chlamydospores in the treatment group, the higher the percentage of the ticks in that group with visible hyphae in their scutum.

Table.1 Average and Standard Deviation of *Pochonia Chlamydosporia* Infecting *Rhipicephalus (Boophilus) Microplus* Ticks for Treatment and Control Groups

	Treatment	Control
VC4-A*	4.3 ± 2.2^{A}	0 ± 0^{B}
VC4-B**	5.3 ± 2.2^{A}	0 ± 0^{B}
VC4-C***	6.7 ± 3.3^{A}	0 ± 0^{B}

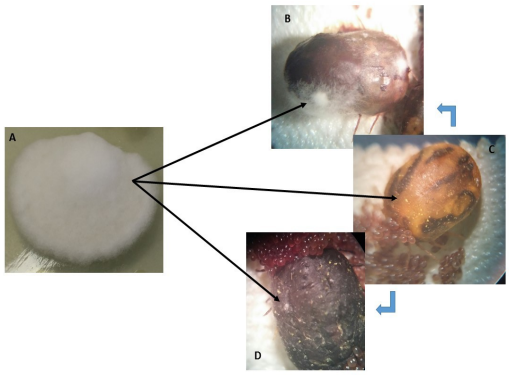
Values followed by the same letter in the same row and column do not show statistical difference ($p<0.05$). *concentration of 5,000 chlamydospores; **concentration of 10,000 chlamydospores; *** concentration of 15,000 chlamydospores

Table.2 Average and Standard Deviation of *Pochonia Chlamydosporia* Infecting *Rhipicephalus (Boophilus) Microplus* Ticks for Treatment and Control Groups on Different Observation Days

Days	VC4-A*	VC4-B**	VC4-C***	Control
1	0 ± 0^{A}	$0,1 \pm 0,3^{A}$	0 ± 0^{A}	0 ± 0^{A}
2	$0,6 \pm 0,5^{B}$	$0,8 \pm 0,4^{B}$	1 ± 0^{B}	0 ± 0^{A}
3	$0,6 \pm 0,5^{B}$	$0,8 \pm 0,4^{B}$	1 ± 0^{B}	0 ± 0^{A}
4	$0,6 \pm 0,5^{B}$	$0,8 \pm 0,4^{B}$	1 ± 0^{B}	0 ± 0^{A}
5	$0,6 \pm 0,5^{B}$	$0,8 \pm 0,4^{B}$	1 ± 0^{B}	0 ± 0^{A}
6	$0,8 \pm 0,4^{B}$	$0,9 \pm 0,3^{B}$	1 ± 0^{B}	0 ± 0^{A}

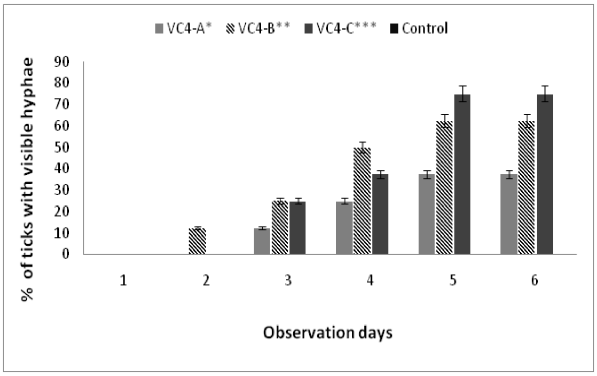
Values followed by the same letter in the same row and column do not show statistical difference ($p<0.05$). *concentration of 5,000 chlamydospores; **concentration of 10,000 chlamydospores; *** concentration of 15,000 chlamydospores

Figure.1 *P. Chlamydosporia* Parasitizing *R. Microplus* Ticks



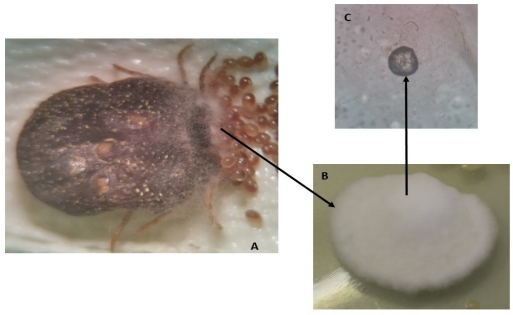
(A) *P. chlamydosporia* isolated from infected tick; (B and D) *R. microplus* with visible hyphae; (C) *R. microplus* without visible hyphae; Black arrows indicates ticks parasitized with *P. chlamydosporia*; Blue arrows indicates evolution from non-visible hyphae to visible hyphae

Figure.2 Percentage of Ticks with Visible Hyphae for each Treatment Group on Different Observation Days



*concentration of 5,000 chlamydospores; **concentration of 10,000 chlamydospores; *** concentration of 15,000 chlamydospores

Figure.3 *R. Microplus* Infected with *P. Chlamydosporia*



(A) *R. microplus* infected with *P. chlamydosporia*;(B) *P. chlamydosporia* isolated from infected tick; (C) Chlamydospore of *P. chlamydosporia* colony observed under a microscope. Bar = 30 µm

Since the visible hyphae also indicated mortality, at the sixth observation day a 37.5, 62.5 and 75 percentage of mortality could be observed for the VC4-A, VC4-B and VC4-C concentrations respectively. Studies with biological control of *R. microplus* using entomopathogenic fungi showed a higher control percentage when compared to the present one using a higher solution concentration. Athayde et al. (2001) observed a control percentage of female adults of *R. microplus* of 91% when using *M. flavoviride*, 86% when using *M. anisopliae*, and 89% when using *B. bassiana* at 10^8 conidia/ml concentration.

In their turn, Frazzon et al. (2000) described a control percentage of 10-29% for *M. flavoviride* and 38-49% for *B. bassiana* for engorged females at 10^7 and 10^8 conidia/ml concentration. Furthermore, Ojeda-Chi et al. (2010) detected an efficiency of 65-100% for 2 strains of *M. anisopliae* at concentrations of 10^6, 10^7 and 10^8 conidia/ml. Angelo et al. (2010) was the only one to obtain a lower control percentage of 3-41% for different concentrations of *L. lecanii* ranging from 10^5 to 10^8 conidia/ml for engorged females.

After isolating the fungi from infested tick on a Petri dish containing WA2%, it was observed that the colony that grew on the agar had similar cotton-like appearance from the fungi that developed on the ticks. From the fungi colony and distilled water solution seen under a microscope (Figure 3), it was also observed a chlamydospore measuring 30 µm (Braga, 2008) with similar characteristics to those found in the solution of isolated VC4, and that it was intact and viable, indicating that *P. chlamydosporia* was responsible for the infection of the tested *R. microplus.*

On the other hand, no chlamydospores were observed under the microscope for the tick and distilled water solution. The difficulty to find chlamydospore for both solution can be explained by the short time elapsed between the appearance of visible hyphae and inoculation of the fungi in the WA2%, and the day the test was performed, which was only 6 days. Therefore, it may not have been enough time to produce a high number of chlamydospores, making it difficult to be observed under the microscope.

In conclusion, the present study demonstrated that *Pochonia chlamydosporia* is able to infect and kill *Rhipicephalus (Boophilus) microplus* ticks, and therefore, has promising potential to be used as a biological control for these parasites.

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