PRELIMINARY FIELD TRIALS WITH THE ENTOMOPATHOGENIC HYPHOMYCETE *TOLYPOCLADIUM CYLINDROSPORUM* IN CENTRAL ALBERTA

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ABSTRACT. Conidia and blastoconidia of the pathogenic hyphomycete *Tolypocladium cylindrosporum* were applied to temporary and semi-permanent pools in central Alberta on 6 occasions. Infections were detected in the field only following application of blastoconidia; indications were that infections did not occur in the field following application of conidia. Most infections were detected only after holding field-collected immatures under laboratory conditions for up to 24 days. Five species of mosquitoes were found infected. Infections occurred in collections up to 29 days post-application with most mosquitoes dying as 4th instars. No infections were detected during subsequent years. Infection rates of up to 33% for blastoconidia and 55% for conidia were estimated. It is speculated that field-collected mosquitoes succumbed to the fungus as a result of the stress of transfer to laboratory conditions.

INTRODUCTION

Tolypocladium cylindrosporum Gams is a candidate as a microbial control agent of mosquitoes. It causes epizootics in larval mosquito populations in New Zealand (Weiser and Pillai 1981) and California (Sanders 1972,[2] Soares et al. 1979). To date all species of mosquitoes challenged with *T. cylindrosporum* have been susceptible (Gardner 1984,[3] Pinnock et al. 1973,[2] Soares 1979,[4] Soares et al. 1985, Weiser and Pillai 1981, Yu et al. 1980,[5] Goettel 1987[6]); they include 10 species in the genus *Aedes*, 6 in *Culex*, 2 in *Culiseta*, and one each in *Anopheles*, *Maorigoeldia* and *Opifex*. Introduction of blastoconidia (also referred to as "blastospores" or "hyphal bodies") of *T. cylindrosporum* into tree holes in California resulted in reductions of up to 71% in the emergence of *Aedes sierrensis* (Ludlow) (Pinnock et al. 1973[2]). Introduction of conidia into crab holes in Fiji resulted in reductions of 87% of immature *Ae. polynesiensis* Marks (Gardner et al. 1986), but applications of blastoconidia to ground pools in New Zealand were not as successful (Gardner and Pillai 1987).

This paper reports on field applications of *T. cylindrosporum* conidia and blastoconidia to semi-permanent and temporary ground pools in central Alberta conducted to further evaluate this fungus as a microbial control agent of mosquitoes.

MATERIALS AND METHODS

Inoculum preparation, storage and application. A Californian isolate of *T. cylindrosporum*, INRA 3 (Institut National Recherche Agronomique, La Miniere, France) = UAMH 4561 (University of Alberta Microfungus Collection and Herbarium, Edmonton, Canada) was used. Blastoconidia were produced by inoculating 500 ml of Sabouraud dextrose broth in 1 liter Nalgene® flasks with 1 ml of a 1×10^6 conidia/ml suspension. The cultures were incubated at 20°C and agitated at approximately 170 oscillations/min on a Burrell wrist action shaker for 4–5 days. Yields were typically in the order of 10^7 blastoconidia/ml.

For trials 1 through 5, conidia were produced on the surface of Pablum mixed cereal agar (Padhye et al. 1973) in large Petri dishes (14 cm diam) or in tin trays (36 x 26 x 4 cm). These were inoculated with a conidial suspension and were incubated at 20°C for 2 weeks. Conidia were harvested by washing off with sterile distilled water. These were then applied in the field within 4 hr of harvest.

For trial 6, conidia were produced on cellophane surface and a wheat bran medium according to the method of Goettel (1984). The cellophane, with adhering fungal mat, was rolled up, placed in plastic bags, and frozen at −20°C for

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[2] Initially described as *Beauveria tenella* but later confirmed as *T. cylindrosporum* (Soares et al. 1979).

[3] Gardner, J. M. 1984. Mosquito larvicidal potential of the New Zealand strain of *Tolypocladium cylindrosporum*. M.Sc. Thesis, University of Otago, Dunedin. 162 pp.

[4] Soares, G. G. Jr. 1979. A study of *Tolypocladium cylindrosporum* Gams, a new naturally occurring fungal pathogen of mosquitoes. Ph.D. Thesis, University of California, Berkeley. 177 pp.

[5] Initially described as *Culicinomyces* sp. but later confirmed as *T. cylindrosporum* (J. S. Pillai, personal communication).

[6] Goettel, M. S. 1987. Studies on microbial control of mosquitoes in central Alberta with emphasis on the hyphomycete *Tolypocladium cylindrosporum*. Ph.D. Thesis, University of Alberta, Edmonton. 198 pp.

Table 1. Field applications of *Tolypocladium cylindrosporum* against mosquitoes in central Alberta.

Trial no.	Date	Inoculum	Species composition (%)	Density[1]	Surface area (m^2)	Rate[2] (per m^2)	Temp °C	pH	Cond[3]
1	Jun. 24, 1982	blastoconidia	*Cs. alaskaensis* (90) *Cs. morsitans* (3) *Cs. inornata* (5) *Cx. territans* (2)	7	5	2.5×10^9	16	7.3	640
2	Jul. 1, 1982	blastoconidia	*Cs. alaskaensis* (8) *Cs. morsitans* (8) *Cx. territans* (83)	2	5	3.9×10^9	15	7.4	460
3	Jul. 17, 1982	blastoconidia	*Ae. vexans* (99) *Cx. territans* (1)	213	20	1.5×10^9	13	7.6	375
4	Aug. 23, 1982	conidia	*Cs. inornata* (90) *Cs. morsitans* (6) *Cx. territans* (4)	9	6	1×10^{12}	14	8.7	850
5	Jun. 23, 1983	conidia	*Ae. vexans* (100)	246	0.7	5×10^{12}	16	8.7	74
6	Jun. 14, 1984	conidia	*Ae. vexans* (100)	212	150	1×10^{12}	22	7.1	50

[1] Mean no. of immatures per 350 ml dip.
[2] Rate of application for portion of pool treated.
[3] Conductivity in micromhos/cm at 20°C.

later use. Conidia were stockpiled over an 11 month period using approximately 24 pans in continuous culture.

For field application, the conidia which had been frozen on the cellophane surface were thawed, scraped and then washed off. The resultant pulp was homogenized in a blender for approximately 5 min. The 262 pans yielded 18 liters of a 9×10^9 conidia/ml suspension which was the equivalent of approximately 1 kg dry weight of conidia. Prior to field application, samples of the inoculum were tested for viability and bioassayed (Goettel 1987[6]).[7] Viability was always above 80%.

The fungus was applied as a coarse spray to grassy roadside ditches on six occasions (Table 1) using a 20 liter back-pack sprayer equipped with a hand pump. Because of difficulties in culturing adequate amounts of inoculum, preliminary trials consisted of applications to portions of pools. Trials 1, 2 and 4 consisted of applications to different portions of semi-permanent pools (25 × 50 m for trials 1 and 4; 25 × 2 m for trial 2). Trials 3 and 5 consisted of applications to different portions of a temporary pool (30 × 50 m). Trial 6 consisted of application to the entire pool.

Larval monitoring and diagnosis. Approximately 12 pools and ponds 4 km NW of Devon, Alberta (114°47′W, 53°23′N) were continuously monitored over a three year period as part of a study to evaluate the incidence of naturally occurring mosquito pathogens in central Alberta. Some of these study pools were also used to evaluate field applications of *T. cylindrosporum.*

The pools were monitored from mid-April until the end of October. Ten to 30 samples were taken from each pool on a weekly basis using a 350 ml dipper. Sampling was increased to every 2 to 4 days following application of the fungus. During each field trip, pool parameters such as water temperature, pH and conductivity were monitored.

Field-collected immatures (i.e., larvae and pupae) were brought back to the laboratory, counted and placed into Bates' medium S (McLintock 1952) in trays or 500 ml plastic containers. They were reared at 20°C until emergence or death. Adults were held for at least 48 hr post-emergence and were identified using the key of Wood et al. (1979). Dead larvae were removed daily, identified using the same key, and then examined microscopically as whole wet mounts for mycosis (i.e., presence of mycelia in the hemocoel). At times, dead immatures were stored at 4°C for 24–48 hr prior to examination.

In 1982, 10 sentinel cages were placed into the water immediately after fungus application. Ten to 25 larvae that had been collected prior to treatment were then placed into each cage. Five cages were also placed in a control pool. The sentinel cages were constructed from 1 liter plastic tubs (10 cm diam × 13 cm depth). These had four −3 cm diam holes cut at equal distances in the side and one −6.5 cm diam hole each in the lid and bottom. All holes were screened with a nylon mesh (13 holes/cm). The cages were floated with Styrofoam® floats so that 8.5 cm of the cage was left submerged.

Sentinel cages were examined during each visit to the pools. The number of mosquitoes present was recorded and any dead immatures

[7] The assay numbers corresponding to the field trials were as follows: assay 07 for field trial 4 and assay 18 for trial 6. The assay for trial 4 was omitted due to very high mortalities at all concentrations on day 1.

were brought back to the laboratory and examined for mycosis. Pupae and missing larvae were replaced with freshly collected larvae from a control pool.

During the summer of 1982, all individuals with *T. cylindrosporum* mycosis were bathed in a 50 μg/ml sterile solution of chloromycetin for 5–10 minutes and then placed in Petri dishes containing potato dextrose agar supplemented with 60 μg/ml penicillin and 30 μg/ml streptomycin. These were incubated for 2 weeks at 20°C after which they were examined for the characteristic white, cotton-like growth of *T. cylindrosporum.* To verify the identity of the fungus, slide cultures were prepared for each field isolation. As diagnostic experience was gained, this procedure was discontinued after the first year.

Infection rate estimates. Because large numbers of mosquitoes were unaccounted for while reared under laboratory conditions, two estimations of infection rates of field-collected mosquitoes were made: (1) minimum estimated rate of infection and (2) maximum estimated rate of infection (Goettel 1987).

Where possible, the results of the field data were subjected to statistical analysis. Time-infection mortality responses were subjected to probit analysis (probit infection mortality as percent of total number infected and log time) (Finney 1971) using the computer program of SAS Institute Inc., Cary, NC, to obtain estimated ST_{50}s (survival time for 50% of total number infected). Statistically significant differences were judged by mutually exclusive 95% fiducial limits. Infection rate data were analyzed by the one-way analysis of variance using angular transformation of proportions infected and log transformed numbers of individuals infected. These were further analyzed using Scheffé's test at the 95% level of significance (Sokal and Rohlf 1969). Only the results of dip samples where the number of immatures were 25 or greater were used for statistical analysis (in trial 6, n = 12 for day 3, 11 for day 5 and 10 for day 8). In trial 6, only 4 samples were taken 12 days post-application, therefore the results of this collection were excluded from statistical analyses.

RESULTS AND DISCUSSION

Mosquito prevalence and composition. In 1982, the pools in which trials 1, 2 and 4 were carried out contained larvae from the first day of monitoring (June 15) until October 6. In 1983, larvae were present from May 5 until September 22. In 1984, larvae were present only between June 12 and July 10; thereafter, the pools dried up.

The pool in which trials 3, 5 and 6 were carried out was colonized by snow-melt *Aedes* in the early spring (April-May) of all three years. Subquently, large numbers of *Ae. vexans* (Meigen) hatched after heavy rains in early to mid-summer. In 1982 this occurred in the first week of July. Subsequently, the pool was recolonized by *Culiseta inornata* (Williston) and *Culex territans* Walker in early August until drying late that month. In 1983 the pool was flooded in the third week of June and the pool became colonized by *Ae. vexans* until mid-July. Thereafter the pool remained without mosquitoes until drying up in the first week of August.

In 1984 the pool was flooded between 9 and 6 days prior to treatment. A large hatch of *Ae. vexans* resulted. A second rainfall two days prior to treatment produced a second smaller hatch. At the time of treatment the mean water depth was 10 cm and it rapidly decreased so that by day 15, the pool was virtually dry.

Control estimates using mosquito densities. Tolypocladium cylindrosporum conidia are very slow acting with LT_{50}s of 3–14 days (Soares and Pinnock 1984, Goettel 1987[6]). Although long LT_{50}s are deemed a beneficial attribute of potential microbial control agents of mosquitoes (Barr 1985), such pathogens pose unique problems in their field evaluation. In the present study, the efficacy of *T. cylindrosporum* could not be determined based on comparisons of mosquito densities in treatment and control pools due to fluctuating mosquito densities, water volumes and uneven maturation rates. Detailed results of larval densities are therefore not presented.

Similar difficulties were experienced by Gardner and Pillai (1987) in field evaluations of *T. cylindrosporum* in New Zealand and by Sweeney (1982) with *Culicinomyces clavisporus* Couch, Romney and Rao in Australia. Sweeney (1982) experienced such large variability in mosquito densities even between mosquito populations in identical artificial ponds started simultaneously that he concluded "there would be little value in designating control sites for future field tests."

Control estimates using sentinel cages. Difficulties arose with the use of sentinel cages. These often became rapidly invaded by dense mats of algae, and at times, predators such as flatworms were also found in them. The cages were sometimes invaded by first instar larvae as the mesh size was not small enough to exclude them.

Mortalities in control and treatment sentinel cages were similar. In trial 3, however, 2 days post-application there was a significant reduction in the number of larvae in the treatment cages as compared to the control (30% reduction corrected by Abbott's (1925) formula for control mortality) ($F = 9.81$, $P = 0.0079$). Subsequently, there were no differences in the mortality rates between cages in control and treatment pools.

The use of sentinel cages demonstrated that

the application of *T. cylindrosporum* blastoconidia resulted in infections and mortality of mosquitoes in the field. Dead infected larvae were found in sentinel cages only in trial 3 as follows: 2 days post-application, 2 dead larvae (1% of mosquitoes accounted for in all traps); 4 days, 1 dead larva (0.5%); 6 days, 10 dead larvae (5%) (with 9 larvae found infected in a single cage) and 10 days, 1 dead larva (1%). Subsequently, no infected larvae were found for the 26 days that the cages remained in operation.

Control estimates using field-collected larvae. Because of high mortalities under laboratory conditions in both treatment and control pool-collected mosquitoes, control estimates could not be made using these parameters. Also, many immatures went missing while held in the laboratory. In collections where infections of *T. cylindrosporum* were noted, up to 66% of immatures collected were unaccounted for from the time of collection to the time the last individual either died or emerged as an adult (mean ± SE = 33 ± 4.7, n = 18). Similar rates of unaccounted immatures occurred in the collections from the control pools (max 56%, mean ± SE = 33 ± 6.1, n = 10). Presumably this was largely a result of cannibalism and rapid decomposition of dead immatures held in the laboratory.

Incidence of mycosis in field-collected immatures. Much information was gained by examining all dead individuals microscopically for mycosis. Applications of either blastoconidia or conidia under field conditions resulted in infections of 5 species of mosquitoes subsequently reared under laboratory conditions; *Cx. territans* and *Cu. minnesotae* Barr are new host records. Of the 937 mosquitoes diagnosed with mycosis, 0.1% were 2nd instars, 19% were 3rd, 80% were 4th, 10% were pupae and 0.2% were adults. Similar rates of infection at each stage occurred when 2nd instar larvae were exposed to conidia in the laboratory (Goettel 1987a).

In trials 1 to 5, infections occurred in collections up to 29 days post-application (Table 2). No infections were detected in the monitored pools during subsequent years. Estimated maximum infection rates for any collection date ranged from 0 to 33% for blastoconidia and 0 to 28% for conidia. Since applications of the fungus in these trials were made to portions of pools, it is felt that these estimates are conservative because of probable immigration and emigration of larvae from the spray zone area.

In trial 6, maximum infection rates for any collection date ranged from 17 to 50%. There were no significant differences in the maximum and minimum estimated rates of infection between the collections of 3, 5 and 8 days post-application ($F = 0.38$, $P = 0.69$; $F = 1.18$, $P = 0.32$ respectively). However, there were significant differences in the numbers of accounted mosquitoes that died in the laboratory ($F = 3.9$, $P = 0.03$) (mean ± SE on day 3 = 93 ± 9.0; day 5 = 82 ± 12.5 and day 8 = 65 ± 15.5%) with

Table 2. Effects of field applications of *Tolypocladium cylindrosporum* against mosquitoes in central Alberta.

Trial no.	Collection time[1]	Density[2] (instar)	Species infected (no.)	Infection rate[3]	Time of death[4]
1	29	0.1 (all)	*Cs. minnesotae* (1)	17–33	12
2	5	3 (all)	*Cx. territans* (1)	3	9
	7	2 (all)	*Cx. territans* (3)	14–18	11–18
	28	3 (all)	*Cx. territans* (1)	2–3	12
3	2	126 (3)	*Ae. vexans* (8)	2	2–16
	4	125 (3–4)	*Ae. vexans* (7)	1–2	0–7
	6	58 (4)	*Ae. vexans* (3)	1	5–6
	10	38 (4)	*Ae. vexans* (2)	1	3
4	2	16 (all)	*Cs. inornata* (1)	1	16
	4	8 (all)	*Cx. territans* (1) *Cs. inornata* (1)	2–3	4–9
	8	8 (all)	*Cs. inornata* (4)	3–4	8–13
5	5	45 (3)	*Ae. vexans* (1)	1	9
	12	46 (4)	*Ae. vexans* (28)	12–20	5–19
	19	21 (4-p)	*Ae. vexans* (1)	1	3
6	3	113 (2–4)	*Ae. vexans* (392)	14–35	3–16
	5	76 (2–4)	*Ae. vexans* (182)	10–23	4–24
	8	54 (2-p)	*Ae. vexans* (65)	6–17	2–19
	12	109 (2-p)	*Ae. vexans* (229)	30–50	2–9

[1] Days post-treatment.
[2] Mean no. of immatures per 350 ml dip.
[3] Infection rate of field-collected immatures reared under laboratory conditions; range of estimated infection = no. infected/total collected × 100 to no. estimated infected/total collected × 100: see Goettel 1987 for details.
[4] Death attributed to mycosis in days post-collection.

significantly less mortality on day 8 than day 3. On day 12, 86% (n = 468) of the accounted mosquitoes died in the laboratory.

In laboratory bioassays, I observed that large numbers of immatures died as a result of exposure to conidia of *T. cylindrosporum*, but without fungal colonization of the hemocoel; between 6 and 79% of dead immatures were found with infection at various doses (Goettel 1987[6]). In the present study, between 20 and 54% of the dead mosquitoes died in the laboratory with mycosis; there were no significant differences in these proportions between the collections of day 3, 5 and 8 (day 3 = 29 ± 5.3%; day 5 = 20 ± 4.6; and day 8 = 24 ± 7.7) (F = 0.62; $P = 0.54$). On day 12, the rate was 55% (n = 419 in one dip) for trial 6 and 28.2 ± 5.12 (n = 84) for trial 5. Based on the laboratory findings, it can be assumed that in the present study immatures also died as a result of exposure to *T. cylindrosporum* but without fungal colonization of the hemocoel. Therefore, the estimated infection rates in the field-collected mosquitoes are probably only a portion of the total mortality that could be attributed to the fungus.

Epizootiology. Natural epizootics of *T. cylindrosporum* have been reported occuring in mosquitoes only at relatively low temperatures. Weiser and Pillai (1981) observed the fungus in *Ae. australis* (Erichson) in supralittoral pools on the coast of Otago, New Zealand "in the same pool every year in July and August." At that time of year, pool temperatures are between 5 and 10°C (Gardner 1984[2]). In California, epizootics were reported in larvae of *Ae. sierrensis* (Ludlow) breeding in tree holes between March and April when the water temperatures are 11–13°C (Soares 1979[4]). Although epizootics resulting in up to 91% mortality have been reported (Sanders 1972), the epizootiology remains unclear as infections were diagnosed only after holding larvae in the laboratory. Weiser and Pillai (1981) reported "samples of population brought to the laboratory will develop the infection and larvae are killed." Soares (1982) reported "samples brought back to the laboratory in early March and incubated at 18–20°C often showed mortality due to this fungus in excess of 70%." In field studies in New Zealand, Gardner and Pillai (1987) were unable to witness larval mortality in the field after application of *T. cylindrosporum* blastoconidia against larvae of *Ae. subalbirostris* Klein and Marks at water temperatures of 4 to 10°C or against larvae of *Ae. australis* at water temperatures of 16 to 20°C. However, samples of larvae brought back to the laboratory and kept at 15°C or placed on Sabouraud agar plates at 25°C developed growth of *T. cylindrosporum*.

Although there have been no reports of *T. cylindrosporum* epizootics (i.e., large numbers of dead infected mosquitoes) occurring directly in the field, one cannot conclude that epizootics do not occur. For instance very few dead larvae were collected following application of *C. clavisporus* in Australia even though reductions of larval numbers in the order of 80–100% were recorded (Sweeney et al. 1983). It was speculated that many of the dead specimens sank to the bottom and were not recovered by the dipper. In the present study infections were detected in the field only following application of blastoconidia; in trial 3, two larvae were found infected on the day of collection 4 days post-application. Infections were also detected in sentinel cages.

Indications are that infections did not occur in the field following application of conidia; infections were detected only after holding the larvae for 2–24 days under laboratory conditions (Table 2). Further evidence of this can be found in the median survival times of the infected larvae (Table 3). Although there were statistically significant differences in the ST_{50}s between the different collection dates (Table 3), indicating that the infection had progressed to a certain degree in the field (i.e., in trial 6, day 12), these differences are probably insignificant since these ST_{50}s are within the same range as those of 2nd instar larvae exposed to conidia under laboratory conditions (Goettel 1987[6]).

In the present study, water temperatures were low (10–22°C). Median lethal times for conidia at these temperatures are in the order of 10 to 22 days (Goettel 1987[6]; Soares and Pinnock 1984). These low temperatures may be responsible for the lack of infection mortality in the

Table 3. Probit analysis of time-infection mortality responses of mosquitoes exposed to conidia of *Tolypocladium cylindrosporum* under field conditions and subsequently reared in the laboratory at 20°C.

Field trial	Collection time[1]	ST_{50}[2]	95% limits[3]	Range[4]
5	12	9.2	8.7–9.8	5–19
6	3	5.3	5.1–5.5	3–16
	5	6.6	6.2–7.0	4–24
	8	6.3	5.8–6.8	2–19
	12	4.3	4.2–4.5	2–9

Probit analysis of cumulative daily infection mortality as percent of total number infected and log time.

Infection mortality = no. individuals with diagnosed mycosis.

[1] Days post-application.

[2] ST_{50} = Survival time for 50% of total number infected.

[3] Fiducial limits.

[4] Range between first and last infection mortality in days.

field. Once larvae were brought back to the laboratory and placed at 20°C, the infection process was initiated.

For blastoconidia, LT_{50}s are between 1 and 5 days for *Ae. sierrensis* at temperatures encountered (Soares and Pinnock 1984); however, in the present study, larvae collected 28 and 29 days post-application of blastoconidia succumbed to the fungus after being held in the laboratory for 12 days. It is highly unlikely that blastoconidia survived for up to 29 days under field conditions for it is generally believed that they are short-lived (Ferron 1981, Roberts and Humber 1981). Soares and Pinnock (1984) observed that blastoconidia germinated and produced conidia after prolonged incubation at 25°C. It is possible that this also occurred in the present field trials. This would explain the length of persistence in the field as well as the time that it took for larvae to succumb in the laboratory, since conidia are slower acting. It is also possible that the larvae became infected much earlier in the field, but these infections remained latent until the larvae were brought under laboratory conditions.

Laboratory studies have shown that relatively high dosages of *T. cylindrosporum* are required to elicit a response in mosquitoes (Goettel 1987[6], Pinnock et al. 1973, Soares 1982). It is highly unlikely that such high doses would occur in the field under natural conditions. Sporulation on host occurs only on floating cadavers and only conidia are produced (Soares 1982). Blastoconidia are only produced in the hemolymph of infected larvae or in liquid broth culture.

Present evidence suggests that very few larvae succumbed to the fungus under field conditions even after application of massive doses. Since *T. cylindrosporum* is a common soil inhabitant (Bissett 1983), conidia may be washed into pools after heavy rains and then ingested by mosquitoes. Larvae containing these conidia in the gut and subjected to stress such as low temperature or transfer to laboratory conditions may then succumb to the fungus. In the present study, field-collected larvae placed under laboratory conditions suffered considerable stress as evidenced by the high mortality rates in the controls. Presumably, larvae in other studies also suffered the same type of stress.

Even if mosquitoes succumb to *T. cylindrosporum* only as a result of stress, this should not preclude its potential as a microbial control agent. With proper manipulation of stress factors, it might be possible to use microorganisms for pest control that are not normally highly pathogenic (Steinhaus 1960). Thorough understanding of the fungus-host relationships is required in order to further evaluate *T. cylindrosporum* as a microbial control agent of mosquitoes.

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