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Three months of *Aedes aegypti* control with a novel infusion bag formulation of *Chilodonella uncinata* in domestic water-storage container in Delhi

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Abstract
The efficacy of *Chilodonella uncinata* formulation, a naturally occurring protozoan biolarvicide, was evaluated in 40 liter domestic water-storage tub, one of the most common and preferred larval habitat of the dengue vector *Aedes aegypti*, in NCT Delhi. The formulation used contained base material as sterilized sand to entrap ($3.5x10^4$ cells/ml) of the active ingredient, wrapped in infusion bag in dosages (10.0g; 20.0g) introduced in large water-storage tub during September 2016. Test mosquito larvae were added as and when available during >7 weeks period and all supplied by wild mosquitoes laying eggs in another disused plastic container kept nearby. Inhibition of adult emergence (IE) was measured in *Ae. aegypti* both in experimental and untreated control tub. While all pupae formed in untreated control tub metamorphosed into adult; only one pupa managed to emerge as viable adult mosquito out of 9 pupae formed in treated experimental tub over 3 months follow-up period (September to December 2016), indicating IE in treated tub fluctuated between 75% and 100% for this period. Thus 30g formulation of this biolarvicide provided control of *Ae. aegypti* in water-storage tub during dengue transmission season in Delhi that normally extends from August to November.

Keywords: *Aedes aegypti*, Biolarvicide, *Chilodonella uncinata*, Formulation, Inhibition of adult emergence, Mosquito breeding in container

1. Introduction
Aedes aegypti (*Ae. aegypti*) is now-a-days one of the most widespread mosquito species globally. It is primarily responsible for spreading mosquito borne viral diseases, viz.: dengue, Chikungunya and Zika. World Health Organization estimates that about 40% of world's population is at risk of being infected with dengue. All age groups are at risk and about 2.5% of those infected by severe dengue die. Dengue has emerged as a serious public health threat in India. It is endemic in all States/UTs which have reported dengue cases from time to time. During 2015, almost one lakh cases have been reported from the country which is much higher than the number of cases reported so far (Source: NVBDCP). In the year 2016 also both Dengue and Chikungunya have shown an increasing trend.
Since the disease has neither a drug nor a vaccine in sight, the disease management depends mainly on vector control aided by community involvement. The use of insecticides to target mosquitoes as a means of disease control can be effective, but is often prohibitively expensive, unsustainable and environmentally undesirable. This is particularly evident for anthropophilic species such as *Ae. aegypti*, which breed in densely populated urban and semi-urban areas [1]. Since 1980's temephos, an organophosphate compound is being used as a larvicide in urban areas in India to control the population of mosquito vectors viz. *Anopheles stephensi* and *Ae. aegypti*. Although there is no specific control strategy for the control of dengue vectors, temephos and *Bacillus thuringiensis var israelensis* (*Bti*) used under Urban Malaria Scheme are also now recommended for the control of *Aedes* mosquitoes [2]. However, *Bti* does not recycle in the environment requiring weekly application in most habitats [3], increasing the end cost in the process. *Ae. aegypti* is still susceptible to temephos as per earlier studies on insecticide susceptibility against dengue vectors from different parts of the country [4, 5, 6, 7].

Recent studies on insecticide susceptibility status revealed the possible development of resistance against temephos in the larvae of *Ae. aegypti* in some areas in Delhi [2]. Temephos was classified as highly toxic to *Daphnia magna* and pose a high environmental risk to this species. Mortality of *D. magna* was observed at concentrations lower than those used in the field to control *Ae. aegypti* larvae [8].

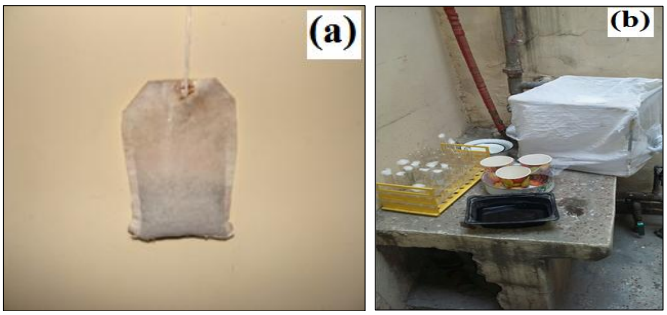
Thus, the need for alternative solutions has driven researchers to look for environmentally safe, cheap and non-toxic microbiological agents that are pathogenic to mosquito larvae including *Ae. aegypti.* An ideal biological system for control of *Ae. aegypti* breeding in water-holding containers must satisfy four basic criteria: i) be safe for humans and for the environment; (ii) facility to recycle in the environment; iii) be amenable to local production and maintenance; iv) be tolerant to desiccation.

One such microbial control agent is *Chilodonella uncinata (Ch. uncinata),* a naturally occurring facultative protozoan parasite of Japanese encephalitis (JE) vector larvae. These were produced on mass scale and stored easily in inactivated sand formulation [9-12]. A lower dose of this entomopathogenic protozoan formulation can be used as a potential biolarvicide to reduce vectors of malaria, dengue/Chikungunya and filariasis [13]. The present paper reports the results of a study on the use of infusion bag formulation of *Ch. uncinata* to control *Ae. aegypti* in large water-storage tub kept in a courtyard of Delhi during September to December 2016.

2. Meterials and Methods

2.1. Preparation of protozoan sample

In order to prepare fresh batch of *Ch. uncinata* formulation, *Cx. tritaeniorhynchus* larvae breeding in paddy fields were collected from Sonipat District, Haryana during 1st week of September 2016. These larvae were brought home, washed in water and subsequently examined using a compound microscope for the presence of *Ch. uncinata*. The infected larvae were transported to the laboratory facility of Department of Biosciences, Jamia Millia Islamia, a central University, New Delhi and these parasites (*Ch. uncinata*) were isolated from diseased *Cx. tritaeniorhynchus* larvae. Protozoan formulation used in the present study was prepared in inactivated sand formulation using *Ch. uncinata* BP 610-2016 strain ($3.5x10^4$ cells/ml) following the methodology [10-12] and packed in "infusion bag" (just as tea bag) in various dosages, viz.: 10.0g and 20.0g (Fig 1a).



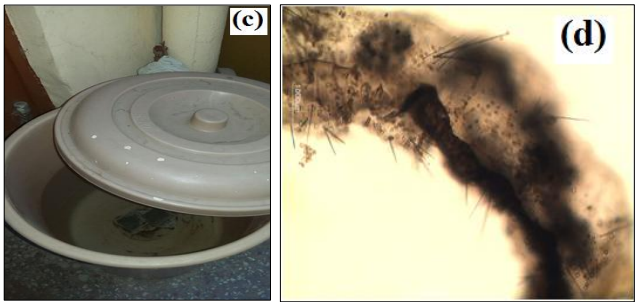


Fig 1: *Ae. aegypti* control with infusion bag formulation of *Chilodonella uncinata* in domestic water-storage container in Delhi. (a) Infusion bag (b) Black plastic tray used as ovitrap for wild mosquitoes (c) Plastic (Experimental) domestic water-storage tub (d) Photomicrograph of infected and dead fourth instar *Ae. aegypti* larva in magnification 100x, arrow to show degenerating gut.

2.2. Dengue transmission season in Delhi

Delhi is one of the states of north India that has been endemic for dengue from past several years [14, 15]. In Delhi, monsoon starts in early July and recedes in late September; post monsoon season continues till late October. Dengue cases usually start appearing from July/August and continue till November corresponding with the increased breeding of the vector species. *Ae. aegypti* mosquito is widely distributed in different parts of Delhi city and plays a key role in transmission of dengue fever [16]. Unprotected water storage practices in households, peri-domestic areas, building construction sites, hospital settings and schools, increasing use of water coolers in office buildings and at home facilitate increased breeding of this species in urban areas [17]. Larger containers that remained stable through time, a feature that make them more amenable to control with biological control agents and were more productive in terms of more pupal production [18]. Solid waste and plastic containers are amongst preferred breeding sites during transmission season in Delhi [19].

2.3. Larval collection for the study

In the absence of mosquito colony, *Ae. aegypti* larvae used in the study were those hatched from eggs laid by wild mosquitoes during a period of nearly eight weeks from mid-September 2016 to first week of November 2016 in a disused black plastic tray [Fig 1b] kept nearby the study site. No preference was given and all available larvae comprising of (I, II, III & IV) instar were used. During 1st week of November 2016, as there was less number of larvae available in the said tray, one fed female *Ae. aegypti* caught outdoor was kept in a test tube for egg laying. The eggs laid by the said mosquito hatched to larvae which were released in the treated experimental tub. Thereafter, no more larvae could be arranged for the study as climate became comparatively cool and egg laying by wild mosquito became a rare possibility.

2.4. Study design

Two plastic containers (round cylindrical tub of 45 cm height and 20 cm radius, one each for experiment and control), kept in peri-domestic area of one house, were used to study the biolarvicidal property of *Ch. uncinata* infusion bag formulation. The aim of the study was to record post treatment inhibition of adult emergence [IE] in *Ae. aegypti* induced by *Ch. uncinata* formulation applied in 40 liter

domestic water storage tub. Containers were half-filled with water (Fig 1c) and covered with its lid to prevent oviposition by wild female mosquitoes as well as the deposit of debris and were placed under a shelter to prevent direct exposure of rain and sunlight. The study was planned to end when the last larva of treated tub become pupa/emerged to adult/die or by 3 months post treatment. The later was chosen to reflect the extreme limit of dengue transmission season in Delhi.

To imitate field condition food was not provided in control as well as experimental set up. On day one, while a total of 21 larvae comprising of younger instars were added in control tub, only 12 larvae comprising mostly older instars were added in treated tub. Though initially started with a 10.0g infusion bag of *Ch. uncinata* formulation, another 20.0g infusion bag was added after a week as 2 pupae were observed in the treated tub. Thereafter larvae were kept on being added as and when available without further addition of formulation (Table 1). Study in control tub was concluded on 6th day as all larvae became pupae which were removed and kept safely in paper cup due to trans-ovarian transmission property of dengue virus in *Ae aegypti* [17]. Pupae formed in treated tub were not removed in order to observe the impact of formulation on its adult emergence. The study in treated experimental tub started on 19 September 2016 (peak dengue/Chikungunya season in Delhi) and ended on 19 December 2016 (after 13 week post treatment) with 4 larvae still alive. These larvae were kept in plastic cup for further observation during an extended follow-up period of >22 weeks.

The number of live pupae and the number of viable adults emerging from treated experimental tub were recorded on a daily basis. This procedure was repeated at two stages: i) till 13 weeks or 3 months (19 Dec 20016) when study in treated tub was concluded, and ii) till 24 Feb 2017 (>22 weeks) the day last larva died (Table 1). The inhibition of adult emergence (IE) was calculated by dividing the number of pupae failing to metamorphose into viable adults by the total number of pupae formed and multiplying by 100. Some of the dead larvae from the experimental tub were examined microscopically after a gap of 4-5 days for the protozoan (*Ch. uncinata*) infection. Re-isolation of the protozoan from the cadavers on artificial medium was also attempted to confirm infection.

Table 1: Efficacy (inhibition of adult emergence) of *Ch. uncinata* formulation against wild *Aedes aegypti* larvae in domestic water storage tub in Delhi, September-December, 2016

Date	Control/ Experiment (g/20 liters)	Nos. of new larvae introduced					Pupa (P)	Adult emerged	Remarks
		I instar	II instar	III instar	IV instar	Total			
19/09/16	Control	19	2	0	0	21	0	0	
25/09/16	Control	0	0	0	0	0	20*	1(M)	* (P) removed
27/09/16	Control**	0	0	0	0	0	0	20	13 (M); 7(F)
A total of 21 larvae were released in control tub: all emerged to adult in 8days									
19/09/16	10.0	0	4	3	5	12	0	0	
27/09/16	+ 20.0***	0	0	0	0	0	2	0	(P) not removed
29/09/16		0	20	14	0	34	0	0	
01/10/16		0	3	4	0	7	2	0	P) not removed
04/10/16		0	33	12	0	45	0	0	Many live larvae
05/10/16		10	0	0	0	10	0	0	Many live larvae
07/10/16		0	10	0	0	10	0	0	Many live larvae
10/10/16		0	7	9	0	16	0	0	Many live larvae
11/10/16		0	0	0	0	0	0	1 (M)	So far emerged
12/10/16		12	3	3	0	18	0	0	
13/10/16		5	2	0	0	7	0	0	dead larvae√
16/10/16		4	7	0	0	11	0	0	Many live larvae
19/10/16		17	20	1	0	38	0	0	Many live larvae
04/11/16		2	1	4	0	7	0	0	Many live larvae
09/11/16		1	1	0	5	7	0	0	Many live larvae
11/11/16		26	1	1	0	28	1	0	Many live larvae
13/11/16		0	0	0	0	0	2	0	Many live larvae
15/11/16		0	0	0	0	0	2	0	Many live larvae
20/11/16		0	0	0	0	0	0	0	√√
25/11/16		0	0	0	0	0	0	0	1 dead larva (IV instar) + only 4 live larvae: [3 (IV) + 1(III)] instar
28/11/16		0	0	0	0	0	0	0	
04/12/16		0	0	0	0	0	0	0	
09/12/16		0	0	0	0	0	0	0	
19/12/16	√√√	0	0	0	0	0	0	0	
A total of 250 larvae released in treated experimental tub						250	9	1	4 live larvae

NB: To imitate field condition food was not provided in control as well as treated tub

* Pupae removed, kept in paper cup covered with fine mesh netting held in place with rubber band

** Study in control tub concluded as all pupae emerged to adult

*** Another 20 g formulation (infusion bag) added as 2 larvae became pupae which were not removed

√ In addition to live larvae, many dead larvae (mostly IV & few III instar) which were not removed

√√ 5 dead larvae + remnants of dead pupae & larvae (only head) + approx. 10 live larvae

√√√ Experiment concluded on 19/12/16; 4 live larvae still remained & were kept in a plastic cup

- Only one adult male mosquito emerged from 9 pupae recovered from treated experimental tub
- After 15 weeks: 2 moribund larvae, one live pupa (which died in next 3 days) and one live larva that died after 22 weeks post treatment

3. Results

During the study period (September–December 2016), maximum and minimum temperature in Delhi ranged from 24 ^{0}C-37 ^{0}C and 8 ^{0}C-28 ^{0}C respectively. Though, there were intermittent monsoon showers during latter half of September, it hardly rained during the remaining part of the study. A total of 250 *Ae. aegypti* larvae were released in the treated tub over a period of nearly eight weeks. After 3 weeks post treatment many dead larvae were observed in the treated tub. After 4-5 days on examination under microscope, dead and transparent larvae were found to be infected with endoparasitic stage of the protozoan (*Ch. uncinata*); in some dead larva gut was seen in degenerating condition (Fig 1d). These protozoans were more predominant in the head capsule, siphon region of the host larva. Few dead larvae were kept with artificial media and protozoans were subsequently re-cultured following simple technology [10-11]. Of the four live larvae (which were kept in plastic cup after concluding the study in treated tub on 19 Dec 2016), one died after 4 days and was eaten by other 3 live larvae; one became pupa (after 15 weeks) which did not emerge into adult and died after 3 days; one larva died in 3rd instar stage after 15½ weeks and the last 4th instar larva died on 24th Feb 2017 (after 22 weeks post treatment).

It was observed that during the initial phase (day one) of the study, release of fourth instar larvae into treated tub resulted in pupa formation within a period of 7-11 days' time. 7-8 weeks after the treatment much shorter time (2-6 days) was required for the fourth instar larvae to develop into pupae (Fig 2). Three weeks after treatment, only one adult male *Ae. aegypti* mosquito emerged out of 4 pupae formed earlier in the treated tub. From week 4 until the end of the study in treated tub (week 13) a total of 5 pupae were recovered but none metamorphosed into a viable adult mosquito. In contrast, it took only 6 days' time for first instar larvae in the untreated control tub to develop into pupae and all became adult in 48 hrs. Three weeks after treatment in treated tub, the IE was 75%, from week 4 until the end of the study in treated tub (week 13) IE increased to 100% and remained as such till the extended follow up period of 22 weeks. In contrast, the IE in untreated control tub was 0%.

4. Discussion

In the present study, prolonged delayed development was observed in dengue vector larvae in treated experimental tub. At a target dosage of 30g infusion bag in 20 liters, the *Ch. uncinata* formulation provided control (75-100)% inhibition of adult emergence) in *Ae. aegypgti* in domestic water storage tub over a period of 13 weeks (3 months) study in Delhi. Apart from inducing inhibition of adult emergence, this protozoan formulation was capable of impacting effective mortality in both larvae and pupae of this species as only one larva out of a total of 250

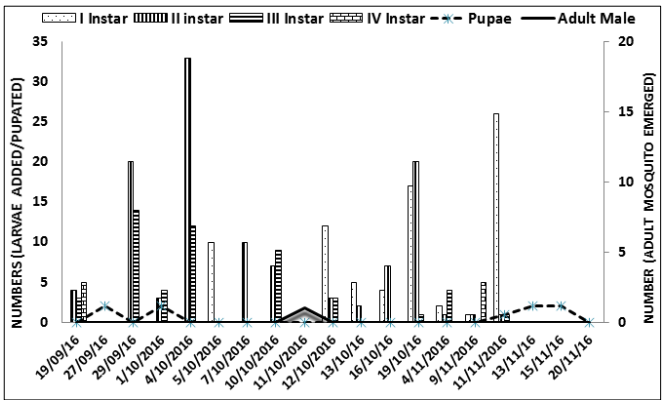


Fig 2: Efficacy (Inhibition of adult emergence) of *Chilodonella uncinata* formulation wrapped in infusion bag (10 + 20)g against *Aedes aegypti* larvae in 40 litre water storage tub

larvae released in the treated tub managed to survive without food all through the extended follow up period of over 22 weeks study. Delayed development was also reported earlier in colonized mosquito larvae exposed to *Ch. uncinata* formulation with 100% inhibition of adult emergence in *Cx. quinquefasciatus* for a period of 7 days, the time generally required for emergence of adult mosquitoes from 1st/2nd instar larvae [13].

Present findings corroborate with that of a study [20] by Chang who reported that single treatment of 5% controlled-release formulation of Pyriproxyfen, a growth regulator at a target dose of 0.03-0.04 mg of active ingredient provided control (80.4-100% inhibition of adult emergence) in *Ae. aegypti* in domestic water storage containers over a period of 34 weeks study in a village of Cambodia. Testing of an experimental controlled-release "chip" formulation of Pyriproxyfen, an insect growth regulator, under simulated field conditions in Cambodia resulted in nearly 90% inhibition of adult emergence (IE) of *Ae. aegypti* for more than 6 months in 200 liter concrete water storage jars [21]. Since Pyriproxyfen only prevents eclosion, larvae and pupae remain visibly active in breeding sites, conveying the false impression of a lack of efficacy, this intervention was not accepted by communities in Mexico [22].

Two novel approaches that have shown considerable promise in recent years are: i) the genetic control of *Ae. aegypti* mosquitoes and ii) the development of mosquitoes that are resistant to arbovirus infection. The first field-trailed genetic control strategy is known as RIDL (the Release of Insects carrying Dominant Lethal genes) and involves the mass rearing of *Ae. aegypti* that have been genetically modified so that the sperm cells of males carry a lethal gene. When those mosquitoes mate with females in the wild, their offspring die [23]. This approach is innovative and potentially powerful, but it could also be costly. To be effective on a large scale, it could be necessary to constantly release modified mosquitoes; otherwise, unmodified mosquitoes from surrounding areas would move into the area and replenish the population. The use of transgenic mosquitoes also faces strong opposition from critics of genetic modification.

An alternative approach is the use of *Wolbachia*, an obligatory intracellular endosymbiotic bacteria to prevent arboviruses replicating within the mosquito [24]. *Wolbachia* are

transmitted vertically from mothers to offspring, and also horizontally within or between arthropod taxa. Three *Wolbachia*-based control strategies have been proposed, viz.: i) Suppression of mosquito populations by large-scale releases of males incompatible with native females; this intervention requires ongoing releases; ii) Transforming wild mosquito populations with *Wolbachia* that shortens mosquito life, indirectly preventing viral maturation/transmission; and iii) Using *Wolbachia* that block viral transmission. Despite these manifold effects, relatively little is known about the underlying mechanisms [25], in part because *Wolbachia* cannot be cultured *in vitro*. Moreover, injecting mosquito embryos with the bacteria was a daunting task and the lead scientist compared the process with piercing a water balloon with a knitting needle and pulling it out without breaking the balloon [26]. What effect either RIDL or *Wolbachia* will have on arboviral transmission and epidemiology in the field remains uncertain but an important benefit of these environmentally friendly approaches is the reduced dependence they pose for insecticides—an increasingly important feature of future disease vector control [27]. But anticipating evolutionary changes of dengue–*Wolbachia*–mosquito interactions is important—and comparable to anticipating the evolution of resistance to pesticides and antibiotics [28].

In contrast, *Ch. uncinata*, was accidentally discovered in wild caught JE vector larvae in 1999 and within a few months these protozoans were isolated, purified, colonized and when formulation was prepared it was found to be more effective against *An. stephensi* [9, 11]. Efficacy of this protozoan (*Ch. uncinata*) biolarvicide is not dose dependent as least dose produced maximum mortality with minimum post exposure. Satisfactory efficacy was noted with LT_{50} and LT_{90} values 3.93 and 6.27 (in days) respectively against *Ae. aegypti* at 0.5 g tea bag formulation. *Ch. uncinata* tea bag (renamed as "infusion bag" based on type of wrapping paper used) formulation was easy to store, transport and evaluate with a shelf life of >18 months [13]. In the formulation the active ingredient (*Ch. uncinata)* remains inactivated when released in water gets reconstituted after a gap of two hours to few days and works as an effective biological control agent for mosquito vectors of human diseases (malaria, JE, dengue, Chikungunya and filariasis). It has many properties of a good biological control agent. These are: (i) the capability of infected female *Cx. tritaeniorhynchus* mosquitoes to spread the parasite to new habitats via trans-ovarian transmission; (ii) can be produced in large scale using simple technology; (iii) tolerant to desiccation; (iv) robust and not sensitive to ultra violet radiation of sun and vagaries of agricultural pesticides; (v) facility to recycle in the environment, vi) free-swimming trophozoites of the parasite are the infective stage, and these actively seek out and infect larval hosts by drilling through the host cuticle; vii) not harmful to larvivorous fish, viii) female *Cx. tritaeniorhynchus* mosquitoes infected with *Ch. uncinata* are significantly less responsive toward a vertebrate host as compared to uninfected females [10, 13].

Furthermore, *Ch. uncinata* is known to induce natural check on the abundance of infective JE vector population at Safiabad village (Sonepat District: Haryana state of India) with majority have developed inhibition in taking blood feed during peak JE transmission season and the area so far remained free from JE though other parameters for an impending outbreak of the disease remained the same with very high JE vector abundance, plenty of pigs and water birds in the area [12]. *Ae. aegypti* larvae are susceptible to *Ch. uncinata* prevalence of which is very high at Safiabad village, still several dengue cases are being reported every year from this village. It is most likely that *Ch. uncinata* are not accessible to *Ae. aegypti* larvae as breeding habitats of both the species (*Ae. aegypti* and *Cx. tritaeniorhynchus*) are entirely different thus preventing *Cx. tritaeniorhynchus* females infected with *Ch. uncinata* from laying these parasites in potential breeding habitats of *Ae. aegypti* including coolers, constructions sites, discarded containers in the area thereby not impacting population of dengue vector [13] for which *Ch. uncinata* formulation can be an option to control *Ae. aegypti* breeding. *Ae. aegypti* thrives in urban environments which provide it with numerous oviposition sites to lay eggs. Therefore, the distribution of this species is largely driven by human activities (e.g. storage of water outside) so control methods need to be directed at these factors [29]. Mosquito control programmes using a combination of control methods are suggested to be more effective against *Ae. aegypti* due to its strong urban preference and strong human feeding preference [19]. Present study indicates the prospect of providing *Ae. aegypti* control for three months during dengue transmission season following timely application of this biolarvicide targeted to these important and accessible vector breeding habitats in Delhi and would offer significant benefit to the Municipal Corporation in dengue vector control.

5. Conclusion

This (*Ch. uncinata*) entomopathogenic protozoan is natural as well as indigenous, further studies on its biosafety test and small scale field trials are urgently required to develop these parasites as a simple, cost effective potential bio-larvicide that can be produced and stored easily. Extensive field research in varied ecological condition would help better understand this protozoan-mediated control of mosquito vectors of public health importance.

6. Acknowledgement

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