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Field evaluation of *Chilodonella uncinata* formulation against *Aedes aegypti* in desert coolers and cemented tanks in Delhi

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Abstract
Infusion bag formulation of *Chilodonella uncinata*, a naturally occurring facultative protozoan parasite of *Culex tritaeniorhynchus* larvae that gets into the host body by piercing through host cuticle was evaluated for its residual efficacy to control *Aedes aegypti* breeding under field condition in a slum and a posh locality of Delhi during June to October 2018. The formulation (*Ch. uncinata* as *a.i* 3.5x 10^4 cells/ml) with a shelf life>18 months, available in easy to treat sealed pack was applied manually at doses 40.0, 60.0 and 80.0g in selected study sites. The impact was assessed by monitoring presence/absence of larvae by a dipper. In the present study, single application @ 80.0g in both coolers and cemented tanks in posh area resulted in 100% control of *Ae aegypti* breeding for 8-9.5 weeks. In slum area, limited study over a period of 3 weeks @ 80.0g in a cooler impacted 100% control of mosquito breeding. This formulation may be tested in large-scale field trials for further use in vector control programme.

Keywords: Efficacy, *Chilodonella uncinata*, Infusion bag formulation, Biocontrol agent, *Aedes aegypti*

1. Introduction
Dengue fever (DF)/Dengue hemorrhagic fever (DHF) is the most widespread vector-borne infectious disease of humans of tropical and subtropical countries worldwide [1]. Today, more than 2.5 billion people in these countries are at risk of infection [2]. In Southeast Asia, dengue is a leading cause of hospitalisation and death among children in most countries [3]. A recent study estimated that India had the largest number of dengue cases, with about 33 million apparent and another 100 million asymptomatic infections occurring annually [2].

In India, since the first confirmed outbreak occurred in Kolkata in 1963-1964 [4, 5], dengue/DHF has been also reported from other regions of the country [6-9]. Dengue is now endemic and almost hyper endemic in our population [10]. Principal factors which contribute to the spread of dengue in India include rapid and unplanned urbanization, human population growth, inadequate municipal services and increased use of non-biodegrable household products [11].

National Vector Borne Disease Control Program (NVBDCP) under the Directorate of Health Services, Government of India is the nodal agency for implementation of activities for prevention and control of vector-borne diseases including dengue across the country. According to the NVBDCP number of dengue cases in 2017 was the highest in a decade. From less than 60,000 cases in 2009, cases increased to 188401 in 2017 – more than 300 per cent spike (Fig 1, source NVBDCP) [12].

Larviciding using temephos, an organophosphate compound, is recommended by WHO since early 1970 for the control of container-breeding *Aedes* mosquitoes. In India, although there is no specific control strategy for the control of dengue vectors, since 1980 temephos and *Bacillus thuringiensis var israelensis* (*Bti*), a biological control agent used under Urban Malaria Scheme are recommended for the control of *Aedes* mosquitoes [13]. However, *Bti* does not recycle in the environment requiring weekly application in most habitats [14], increasing the end cost in the process. Study on insecticide susceptibility status revealed the possible development of resistance against temephos in the larvae of *Ae. aegypti* in some areas in Delhi [13]. Temephos was found to be mutagenic in two out of three assays at concentrations similar to those applied in household water reservoirs [15]. Although a number of existing different methods including space spraying of insecticide with ultra low volume.

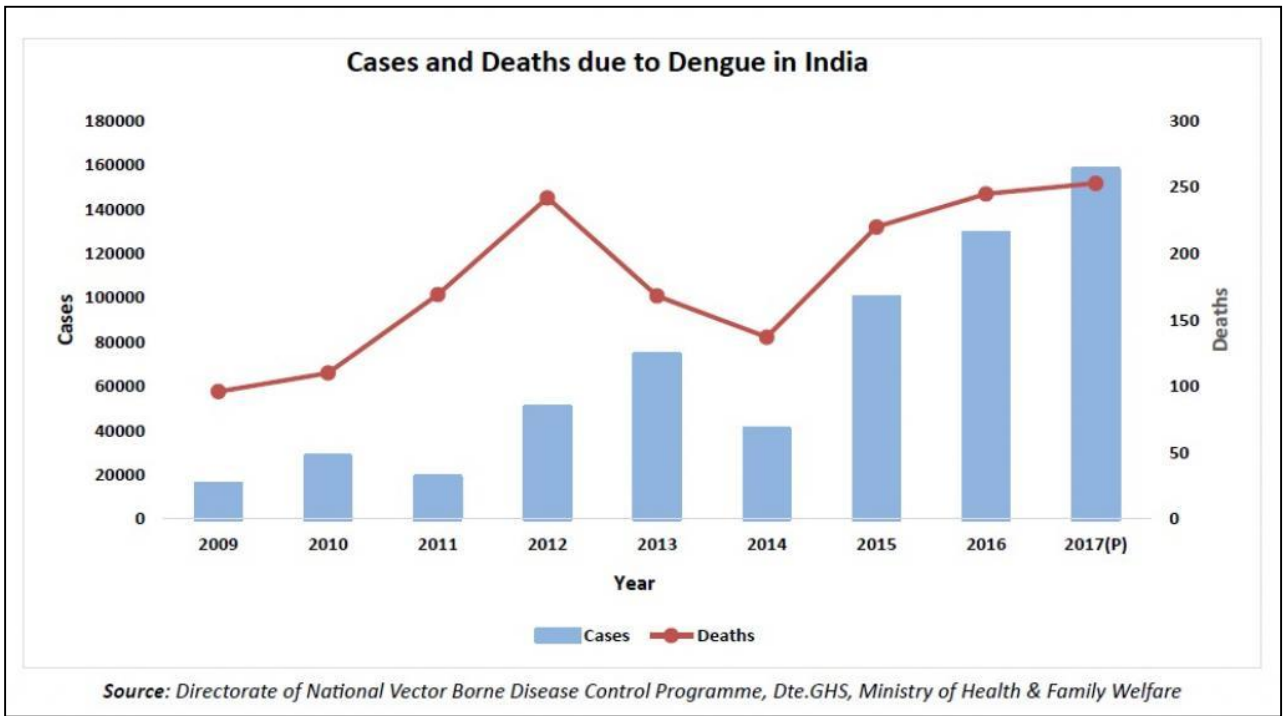

Fig 1: Cases and Deaths due to Dengue in India (2009-2017) *Source: NVBDCP*

technology are in use over the last 40 years, vector control has failed to prevent outbreaks from occurring and to avert an expansion of the geographical distribution of dengue [1,16]. Very few biological control agents are commercially available for the control of mosquito vectors of human diseases. Those exit, also failed to maintain "initial success" in reducing vector population. This could perhaps be improved if physiological and ecological attributes of potential biological control agent were determined prior field use.

In view of the above, researchers in recent years are looking for microbiological agents that are not only pathogenic to mosquito larvae including *Ae. Aegypti*, but also satisfy four basic criteria, viz. tolerant to desiccation; facility to recycle in the environment; be amenable to local production and maintenance; be safe for humans and for the environment. *Chilodonella uncinata (Ch. uncinata)*, a naturally occurring facultative protozoan parasite of Japanese encephalitis (JE) vector larvae is one such microbial control agent [17-22]. In this study, infusion bag formulation of *Ch. uncinata* was evaluated for its residual effectiveness to control *Ae. aegypti* breeding under field condition in a slum and a posh locality of Delhi during June to October 2018.

2. Materials and Methods

2.1 Preparation of *Chilodonella uncinata* formulation

In order to produce a stable formulation of *Ch. uncinata* with shelf life >18 months, the culture methodology developed prior to 2005 at the National Centre for Disease Control (NCDC) [17-19] was updated, modified in 2011 at the Department of Biosciences, Jamia Millia Islamia (JMI) University, New Delhi. The culture strain *Ch. uncinata* BP 610 maintained at JMI was deposited in 2012 with the International Depository Authority—ATCC, U.S.A. for which accession number: ATCC PRA-373 was allotted [20].

Ch. uncinata BP 610 formulation prepared in 2017 and stored in sealed pouch at room temperature was available from which 20.0g infusion bags (Fig 2a) were prepared and used in this study. The stalk formulation was prepared in Sept 2017 following standard methodology developed [20] at JMI using sterilized sand and *Ch. uncinata* BP 610 strain ($3.5x10^4$ cells/ml) isolated from infected *Culex tritaeniorhynchus* larvae collected during last week of August 2017 from paddy fields of Sonipat District, Haryana state of India (known area of influence of this protozoan). Towards the middle of the study period as the supply of infusion bags was exhausted, further supply of formulation was made available by dipping one 20.0g infusion bag in autoclave water for 48 hrs. The resultant culture strain *Ch. uncinata* BP 610 with cell concentration ($3.0x10^4$ cells/ml) was used for preparation of fresh formulation and used in this study.

2.2 Dengue transmission season in Delhi

Delhi is endemic for dengue since past several years [23,24]. Transmission season starts from July/August coinciding with beginning of rains in Delhi and continues till November corresponding with the increased breeding of the vector species (*Ae. Aegypti*). This mosquito is widely distributed in different parts of Delhi city and plays a key role in transmission of dengue fever. Unprotected water storage practices in households, peri-domestic areas, building construction sites, hospital settings and schools; use of desert coolers in office buildings and at home facilitates increased breeding of this species in urban areas [25].

2.3 Study design

Four desert coolers (two each located in a posh and a slum area) and three cemented tanks (all in a posh colony) of Delhi were used to study the residual efficacy of bio-larvicidal property of *Ch. uncinata* infusion bag formulation. The aim of the study was to record the duration (in weeks) of nil *Ae. Aegypti* breeding induced by *Ch. uncinata* formulation applied in coolers/cemented tanks. Two coolers installed at a taxi stand (both used as experimental)

Fig 2: *Ae. Aegypti* control with infusion bag formulation of *Chilodonella uncinata* under field condition. (a) Infusion bag (in sealed pouch) containing 20.0 g formulation (b) Cemented tank (at pump house located in a posh area in Delhi) maintained by Delhi Jal Board, a permanent mosquito breeding source of created by leaking tap of the said pipe line.

within a posh colony were selected as air conditioners were predominantly used by the residents in these areas. Of the two coolers selected in slum area one was included as experimental and the other as untreated control. When in use a cooler on an average filled with 30 litres of water. Owner of the coolers were advised not to clean his cooler but replenish with additional quota of water as when required. Cemented tanks located in a posh colony were part of a pump house maintained by Delhi Jal Board. Water in these tanks was accumulated by leaking taps of the pump house supplemented by additional quota of water as and when it rains. There were four rectangular tanks of which three were found positive for mosquito breeding (predominantly *Aedes* with few older *Anopleles* larvae). Of the three positive tanks, two: tank no.2 and 3 (195x145x135) cm^3 were used as experimental tanks as they were comparatively deeper, water level approximately 60 cm (Fig 2b). Tank no.1 was comparatively shallow with water level barely 5-10 cm, was used as untreated control. The study started during June 2018 and planned to end till re-infestation (mosquito breeding) starts/owner cleans his cooler or up-to 10 weeks post treatment.

Ch. uncinata infusion bag formulation was tested at three doses of (40.0g; 60.0g; 80.0g) and only one dose (80.0g) in coolers and tanks respectively. Except in cooler no.4 located in slum area, single application was adopted in two experimental coolers (cooler no.1 and cooler no.2) and two experimental tanks (tank no.2 and tank no.3). Application doses were achieved by simply removing the infusion bag from its protecting pouch and adding the required dose e.g. 60.0g (3 bags) into cooler no. 1 (Table 1). Prior treating the study sites, presence of mosquito larvae was estimated by dipper sampling method using a dipper of 150 ml capacity with 6.5 cm diameter. At pre-treatment stage both the experimental coolers (no. 1 and 2) at the posh area were found negative for mosquito breeding. Hence, a total of 50 mosquito larvae (*Ae. aegypti*) comprising all younger instars collected from neighbouring construction site were released in cooler 1 and three infusion bags were gently kept on three corners of water filled cooler. Similarly, a total of 45 larvae comprising mostly older instars were released in cooler no. 2 in which four infusion bags were added. No further larvae were introduced in these two coolers throughout the study period in order to observe the efficacy

Table 1: Residual activity of *Ch. uncinata* formulation wrapped in infusion bag against *Aedes aegypti* breeding in two coolers at Taxi stand (Posh area), June-Aug 2018, Delhi

Date	Formulation/water (g/30 liters)*/♣	Nos. of new larvae introduced/♣♣					Pupa (P)	Adult emerged	Remarks
		I instar	II instar	III instar	IV instar	Total			
Cooler 1 Experimental									
11/06/18	60 g (20gx3)	5	45	-	-	50			*Water in cooler replenished as & when required
18/06/18		-	-	-	-	-	-	-	
29/06/18		-	-	-	-	-	-	-	
16/07/18		-	-	-	-	-	-	-	
27/07/18		-	-	-	-	-	-	-	
06/08/18	The said cooler was emptied & cleaned by taxi owner; hence experiment was terminated								
Cooler 2 Experimental									
11/06/18	80g (20gx4)	5	-	40	-	45			
18/06/18		-	-	-	-	-	-	-	1 III L (D)
16/07/18		-	-	-	-	-	-	-	
27/07/18		-	-	-	-	-	-	-	
06/08/18		-	-	-	-	-	-	-	
16/08/18	Owner cleaned his cooler as it became smelly & experiment was terminated								

♣ Owner was told not to clean the said cooler ♣♣ Nos. unknown from wild mosquito breeding

and residual activity of the formulation to prevent dengue vector breeding in dengue transmission period which usually extend from July/August to November in NCR Delhi. However, after 8 weeks post treatment, owner of cooler no.1 cleaned his cooler and the study had to be terminated. Owner of cooler no.2 also cleaned his cooler after 66 days (>9 weeks) post treatment due to bad smell. In contrast, at pre-treatment stage both the coolers, viz.: no.3 (untreated control) and no.4 (experimental) at the said slum area were found to be heavily infested with *Ae. aegypti* breeding (Table 2). Though

initially started with 40.0g (two infusion bags) formulation in cooler no 4, another two bags were added after three weeks as re-infestation started. Subsequently, compelled by the situation the said cooler got cleaned and treated with 80.0g formulation. At pre-treatment stage all the three cemented tanks (1-3) were detected positive for mosquito (*Aedes* and *Anopheles*). Both the experimental tanks (no.2 & no. 3) were treated with 80.0g formulation each.

3. Results

During the study period (June–October 2018), maximum and minimum temperature in Delhi ranged from 34 ^{0}C-42 ^{0}C and 19 ^{0}C-26 ^{0}C respectively. The city witnessed its longest wet spell in the month of September in 22 years with 179 mm of rain registered during initial nine consecutive days (1-9), nearly 40% more than the normal for the entire month.

The efficacy of *Ch. uncinata* infusion bag formulation was evaluated against *Ae. aegypti* in desert coolers and cemented tanks in Delhi. A total of three coolers and two tanks were treated with different doses of *Ch. uncinata* formulation and one cooler and one tank were left untreated as control for comparison. It was observed that throughout the study period formulation bags remained hidden under a thick layer of mud at the bottom of each cooler and tank. This was mainly due to intense dust pollution in Delhi. Table 1 shows the efficacy and residual activity of one time application of *Ch. uncinata* formulation at two doses (60.0g; 80.0g) against *Ae aegypti* breeding in two coolers at a posh area over a period of 8-9 weeks. It was observed that both the coolers remained negative for mosquito breeding during the entire study period with only one dead III instar larva detected after one week of post treatment in cooler no. 2, indicating thereby the formulation applied not only impacted 100% mortality to all the larvae introduced at pre-treatment stage but also controlled *Ae. aegypti* breeding till the end of study period from June to August. Table 2 shows the result of using a lower dose (40.0g formulation) in cooler no. 4 at a slum area which impacted cent percent mortality in resident *Ae. aegypti* larvae followed by preventing further mosquito breeding for a shorter period of 3 weeks. However, no improvement in its residual efficacy was observed after addition of 2nd instalment of 40.0g formulation. Compelled by the situation (peak dengue transmission season) the cooler was emptied and study started with 80.0g formulation. Unfortunately its evaluation process had to be abruptly stopped as the owner preferred to run his cooler without water and residual efficacy study could not proceed beyond three weeks. Table 3 shows the residual efficacy of *Ch. uncinata* formulation (80.0g) in controlling mosquito breeding in two cemented tanks at the posh area. It was observed that one time application of 80.0g formulation impacted 100% mortality in resident mosquito larvae which were noted during pre-treatment stage thereafter preventing further mosquito breeding as both the tanks remained negative for mosquito breeding till the end of study period close to 10 weeks that extent from August to October.

Table 2: Efficacy and Residual activity of *Ch. uncinata* formulation wrapped in infusion bag against *Aedes aegypti* breeding in domestic cooler in a slum area, June-August 2018, Delhi

Date	Control/Pre-treatment/ Post treatment	Mosquito larvae detected					Pupa (P)	Adult emerged	Remarks
		I instar	II instar	III instar	IV instar	Total			
Cooler 3									
22/06/18	Control	+	+	+	+	-	+	-	
23/06/18	Control	+	+	+	+	-	+	-	
29/06/18	Control	+	+	+	+	-	+	-	
Cooler 4 Experimental									
22/06/18	**Pre-treatment**	+	+	+	+	-	+	-	
22/06/18	40 g (20gx2) Formulation (g/30 liters) ♣								
23/06/18						-	-	-	
29/06/18	-	-	-	-	-	-	-	-	
16/07/18	+40g (20gx2)■	+	+	+	+	-	+	-	(Total 80g)
27/07/18		-	-	-	-	-	-	-	
06/08/18		-	-	-	-	-	-	-	
11/08/18		+	+	+	+		+	-	►
Started once again strait away with 80g formulation (20gx4)									
16/08/18	80g (20gx4)							-	
24/08/18		-	-	-	-		-	-	
31/8/18		-	-	-	-		-	-	
06/09/18	Owner emptied the said cooler as climate became cool due to intensified monsoon in the city♥								

■ Total 80g formulation used

►Owner immediately advised to empty the said cooler as it was in peak transmission season

♥ Therefore sufficient period was not available to observe impact of 80 g post application

Table 3: Efficacy and Residual activity of *Ch. uncinata* formulation wrapped in infusion bag against mosquito breeding in cement tanks♣, Aug - Oct 2018, Delhi

Date	Control/ Experiment	Pretreatment/Nos. detected post treatment				Pupa (P)	Adult emerged	Remarks
		I instar	II instar	III instar	IV instar			
Control								
Cement tank 1								
07/08/18		+	-	-	+	+		
16/08/18		-	+	-	+	+	-	
10/09/18								Dried up
Experiment								
Cement tank 2								
07/08/18	Pretreatment	+	+	+	+	-	-	Mainly I/II instar; scanty III & IV
07/08/18	80g (4x20g)					-	-	
16/08/18		-	-	-	-	-	-	
10/09/18		-	-	-	-	-	-	
14/10/18		-	-	-	-	-	-	Study ended
Cement tank 3								
07/08/18	Pretreatment	+	+	-	-	-	-	Only I/II instar
07/08/18	80g (4x20g)						-	
16/08/18		-	-	-	-	-	-	
10/09/18		-	-	-	-	-	-	
14/10/18		-	-	-	-		-	Study ended

♣ Water accumulation in these tanks was created by leaking tap of the pipeline

4. Discussion

In the absence of a safe and effective vaccine for dengue viruses, vector control is the method to prevent viral disease. An effective vector control programme will require an increase in expenditure, new strategies to lower and limit the *Ae. aegypti* population [26]. This is particularly evident for anthropophilic species such as *Ae. aegypti*, which typically bite at dawn and dusk, breed in densely populated urban and semi-urban areas [27] in desert coolers of houses and in multi-storeyed building, discarded tyres, disposable cups, open tanks, water storage pots and construction sites.

In 1999 for the first time in science, pathogenic property of *Ch. uncinata* was accidentally discovered by one of us (BPD) in *Culex* mosquito larvae growing in paddy fields of Sonepat District, Haryana state of India. *Ch. uncinata* was isolated from these infected larvae, colonized and a basic culture strain including a preliminary sand formulation was prepared at National Centre of Disease Control (NCDC), Delhi [17-19]. Follow up studies by BPD in many districts of Northern India revealed: i) *Ch. uncinata* is a facultative protozoan parasite that is naturally found in *Culex* mosquito larvae growing in some paddy fields, village ponds, etc.; ii) These are maternally inherited that is these parasites are passed on from infected female mosquitoes to her offspring; iii) Surprisingly, these protozoan parasites are neither available in every paddy fields, nor they are available in man-made water reservoirs holding fresh or rain water (common breeding places of *Aedes aegypti* vector of dengue and Chikungunya) in urban and peri-urban areas. But wherever these parasites are present in very high densities, the area remain free from Japanese encephalitis (JE), a mosquito-borne disease due to which hundreds of death occurs in children each year in District Gorakhpur (eastern U.P.); iv) Unlike *Bacillus thuringiensis* var *israilensis* (the only microbial bio-larvicide used under Urban Malaria Scheme), that must be swallowed by the host mosquito larvae to cause death, *Ch. uncinata* gets into the host body by piercing through host skin (head, thorax, abdomen, siphon) to cause death in host larvae [17-19].

Ch. uncinata has many biological control properties, viz.: easy to colonize on artificial nutrition medium and can be mass produced in large scale using simple technology; recycle capability in aquatic habitat; tolerant to desiccation; adult female mosquitoes emerged from infected (*Ch. uncinata)* host larvae avoid blood feeding on human and other vertebrates thereby unable to transmit malaria/dengue/Chikungunyia/JE. Most of these biological properties of *Ch. uncinata* were demonstrated and included in National and International Patent Applications filed during 2001 on "Process for preparation of a microbial control agent" (Inventor: Bina Pani Das; Co-Applicants: Department of Biotechnology, Ministry of Science & Technology and National Centre for Disease Control, Erstwhile National Institute of Communicable Diseases). So far, 7 countries have granted these Patent Applications, viz.: USA [18], Bangladesh [28], Australia [29], Sri Lanka [30], Vietnam [31], Philippines [32] and India [33]. These protozoan parasites in dormant stage are available in sand formulation and packed in a sachet "infusion bag" (Fig. 2a) which on dipping in affected water will revive the organism and will kill pest mosquito larvae thereby controlling vector density. Earlier studies revealed delayed development in mosquito larvae (*Culex quinquefasciatus* and *Ae. aegypti*) exposed to *Ch. uncinata* infusion bag formulation which has a shelf life >18 months and is easy to store, transport and treat. Laboratory evaluation with both culture strain and infusion bag formulation carried out at four institutes including Vector Control Research Centre, Puducherry (VCRC) and NCDC revealed: *An. stephensi* larvae were most sensitive followed by *Cx. quinquefasciatus* and *Ae. aegypti.* 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 infusion bag formulation [21].

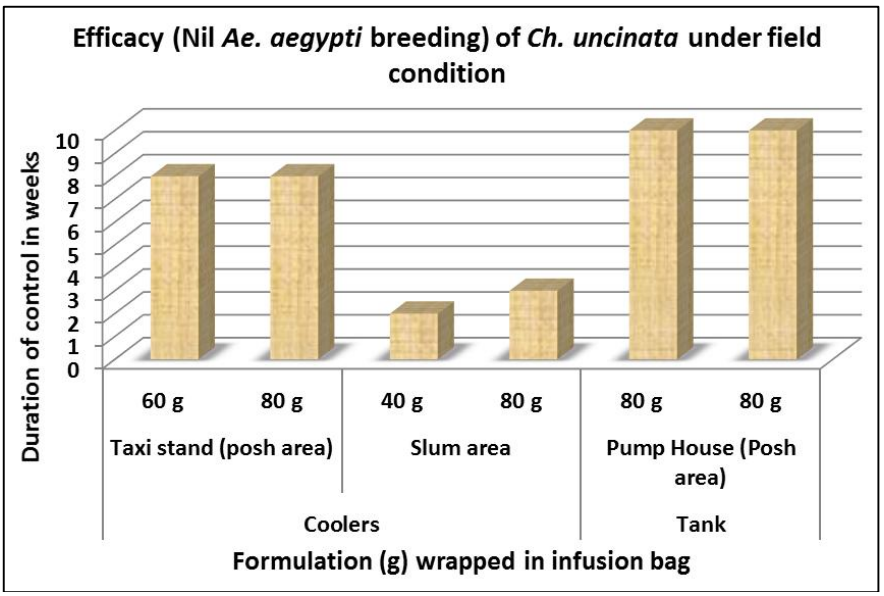

Fig 3: Residual efficacy (nil *Ae. Aegypti* breeding) of *Chilodonella uncinata* formulation under field condition. * Study ended as climate cooled down due to peak rainy season and the owner emptied his cooler and preferred to run his cooler without water

In the present study, one time application of 80.0g *Ch uncinata* infusion bag formulation showed 100% control of *Ae aegypti* breeding for 8-9.5 weeks in coolers and cemented tanks respectively in posh locality in Delhi during June to October coinciding with major period of Dengue transmission season in the city (Table 1,3; Fig 3). However in slum area, though started with a lower dose (40.0g), addition of 2nd dose of 40.0g formulation after a gap of 3 weeks did not stop re-infestation of *Ae aegypti*. That prompted us to start with 80.0g formulation which impacted 100% control of mosquito breeding for 3 weeks but further residual efficacy study could not be continued due to unforeseen situation created by continuous rainfall since day 1 till day 9 in the month of September 2018. In an earlier study, *Ch uncinata* infusion bag formulation impacted effective mortality, induced prolonged delayed development in dengue vector larvae and provided control (75-100% inhibition of adult emergence) in *Ae. aegypgti* in 40 litre domestic water storage tub over a period of 13 weeks in Delhi during dengue transmission season [22]. However, in the present study inhibition of adult emergence property of *Ch uncinata* could not be studied as: i) in majority of study sites post treatment re-infestation of *Ae aegypti* was not detected throughout the study period that extended from 8-9.5 weeks, ii) at target dose of 80.0g formulation study in cooler at slum area could not be carried out beyond three weeks post treatment due to adverse field condition. The study carried out by NCDC during 2008 in Delhi revealed that more than 50% of the breeding places of dengue vector are contributed by desert coolers because they hold water for long period. It appears currently these coolers are predominantly used by the residents in slum area in the city. As per report of "Urban slums in Delhi" Government of National Territory of Delhi, 6343 slums with approximately 10.20 Lacks households were estimated to be in existence in urban Delhi in 2012. The results of our study clearly show that a target dose of 80.0g infusion bag formulation of *Ch uncinata* is capable of inducing long duration of control of *Ae aegypti* larvae in coolers and cemented tanks in Delhi.

5. Conclusion

In conclusion, *Ch. uncinata* has shown long-term effectiveness against immature stages of *Ae. aegypti* under field condition during pre-monsoon and monsoon season. It appears to be one of the alternatives to conventional chemical insecticides such as temephos where *Aedes* larvae had been shown to develop resistance. Infusion bag formulation with a shelf life of >18 months is easy to store, transport and treat. In addition to Municipal Corporation, *Ch. uncinata* infusion bag formulation can also be used by individuals and community to control *Ae. aegypti* breeding in inaccessible desert coolers installed in multi-storied buildings (offices and apartments) and in other man-made water storage containers kept in their domestic and peri-domestic area. Further extensive field trial in varied ecological condition would help better understand this protozoan-mediated control of mosquito vectors of public health importance.

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