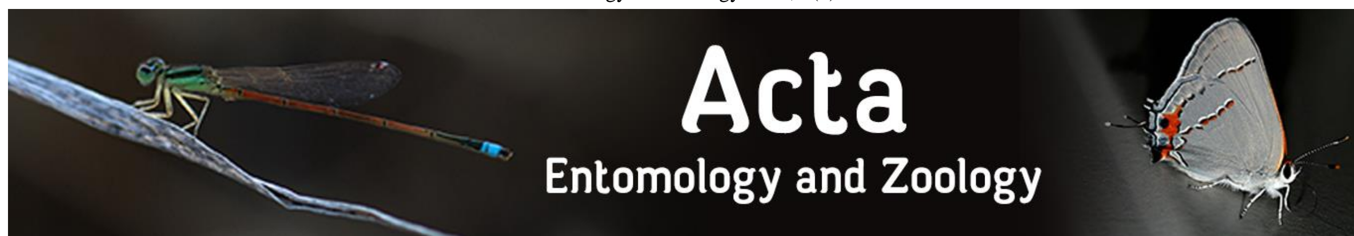

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In vitro mass culture technique of *Hexamermis vishwakarma* Dhiman

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Abstract
In order to create a laboratory culture, parasitized larvae were raised in the lab and the Nemas that emerged from the (*Leptocoris augur*) insect host bug were collected in Petri-dishes with damp sand. The entomophilic nematode *Hexamermis vishwakarma* Dhiman is considered a potential bio-control agent for the Schleichera oleosa pest *Leptocoris augur*. Before emerging from the host as fully formed, non-feeding postparasites, the nematodes pass through several nymphal and adult stages of *L. augur*. Nematodes departing the bug cause host mortality (*Leptocoris augur*).
Before becoming an adult, *H. vishwakarma* goes through three stages: pre-parasitic, parasitic, and post-parasitic. Only the parasitic juvenile stage of these parasitizes the host bug; the *L. augur* and remainder stages are not parasitic.
The objective of this study was to create a culture media that replicates the environment provided by the host in order to support *Hexamermis vishwakarma* throughout its parasitic phase *in vitro*. There is a description of the numerous ways cultures are started. The ability of various substrates to foster the nematodes' growth and development has been assessed, and the most recent *In vitro* techniques have been described. Five factors, including the choice and upkeep of test species and the culture of nematodes' pre-parasitic juveniles, have been examined in the current work.

Keywords: *Leptocoris augur*, *Hexamermis Vishwakarma*, *In vitro* mass culture technique, entomophilic nematode, parasitization, biocontrol agent

Introduction
It is necessary to create a culture media that replicates the environment provided by the host in order to support *Hexamermis Vishwakarma* Dhiman throughout its parasitic phase *in vitro*. Nematodes can grow and develop in the culture's hemocolloid environment. In order to create a laboratory culture, parasitized larvae were raised in the lab and the worms that emerged from the (*Leptocoris augur*) insect host were collected in Petri dishes with damp sand.
The bug initially becomes slightly annoyed and moves around a lot in the pre-parasitic juvenile stage, but as the parasitic stage develops inside the host, it appears sleepy and eventually, just before emerging, becomes quite sluggish. The parasitized bug's abdomen develops a biconvex form and swells up significantly. The parasitized bug exhibited flight incapacity due to severe impairment of the antennae, legs, and wings' ability to move. The nematode casts its cuticle and stylet during the second stage. The trophosome, which fills up with nutrients that have been stored, the stichocytes, and the vaginal primordia all develop concurrently during the third stage, which is characterised by an extended period of massive growth (Fig. 1, 2, 3).
Thus, the nematode is physiologically and structurally prepared to restart a free-living existence in water when the parasitic phase of its life cycle comes to a conclusion and it ultimately exits from its host. Before becoming an adult, *H. vishwakarma* goes through three stages: pre-parasitic, parasitic, and post-parasitic. Only the parasitic juvenile stage of these parasitizes the host bug; the *L. augur* and remainder stages are not parasitic.
After hatching from the eggs, pre-parasitic juveniles emerge in vast numbers from the damp soil. They are extremely small, ranging in length from 0.25 to 0.80 mm and width from 0.04 to mm (Plate-1). With the aid of a mouth stylet, transparent white infectious juveniles enter the host through weakly sclerotized body parts like the cervical membrane and wing axillaries, coxal joints of the legs, the underside of the wing pads, the genital area, and the joints of the abdominal sternite and tergal plates.

For penetration inside the host bug, 2-15 minutes are needed. Additionally, super-parasitism was seen when many pre-parasitic juveniles entered the same host. However, they do not get much longer, and more boys are typically born. It settles in the body cavity after penetrating. Initial growth is swift. After penetration, the second moulting takes place inside the host's haemocoel (*Leptocoris augur*) and transforms into the parasite form.

Younger parasitic juveniles vacuum bodily fluid through their mouths, and the muscular pharynx produces the suction force. The parasitic stage requires more nourishment as it becomes bigger. *H. vishwakarma* Dhiman consumes food from the muscles and hemolymph of insects (Dhiman, S.C. and Kumkum 2006) [2]. As a result, some enzymes are produced, lysing the body's fat, muscles, reproductive organs, etc., and the food that has been broken down is absorbed through the tiny pores in the skin's cuticle and body wall. A number of cells connect the pre-parasitic juvenile's intestinal to the base of the stichosome. The intestine grows anteriorly and overlaps the stichosome, but this connection breaks soon after the pre-parasitic stage enters the host's hemocoel. When the pre-parasites begin feeding in the artificial culture, the connective tissue breaks. While the posterior portion of the intestine has large cells filled with droplet and glycogen particles (trophosome), the anterior half of the intestine typically contains fluid. This dietary substance enters the intestine immediately and is transformed into a trophosome. The whole gut stores them. In addition, feeding is still done through the mouth using the pharynx's sucking power. The arthrodial membrane is punctured by the tooth of the parasitic stage in order to exit. Following perforation, it gently extrudes its body through peristalsis or wriggling motion. Weak body spots such the tergal joints, pleuron membrane of the abdomen, cervical membrane of the neck, coxal joint with the thorax, wing auxilliaries, and genitalia are where emerging organisms enter the body. These points are chosen at random using a system of trial and error.

Estimated time from penetration to emergence is 18 to 22 days, or 20 to 22 days on average. The host bug dies after emergence as a result of losing its hemolymph, biochemical changes and other essential internal organs. A post-parasitic stage is when the parasitic juvenile first appears. For the purpose of growing into an adult, it burrows into the soil between 10 and 30 cm deep. The life cycle ends at hatching, when the embryonated eggs release the infectious pre-parasites. The first moult occurs in the egg, the second occurs during the development of the parasite, and the remaining two moults occur in the post-parasitic juvenile. The 72-day life cycle is complete (Fig-4).

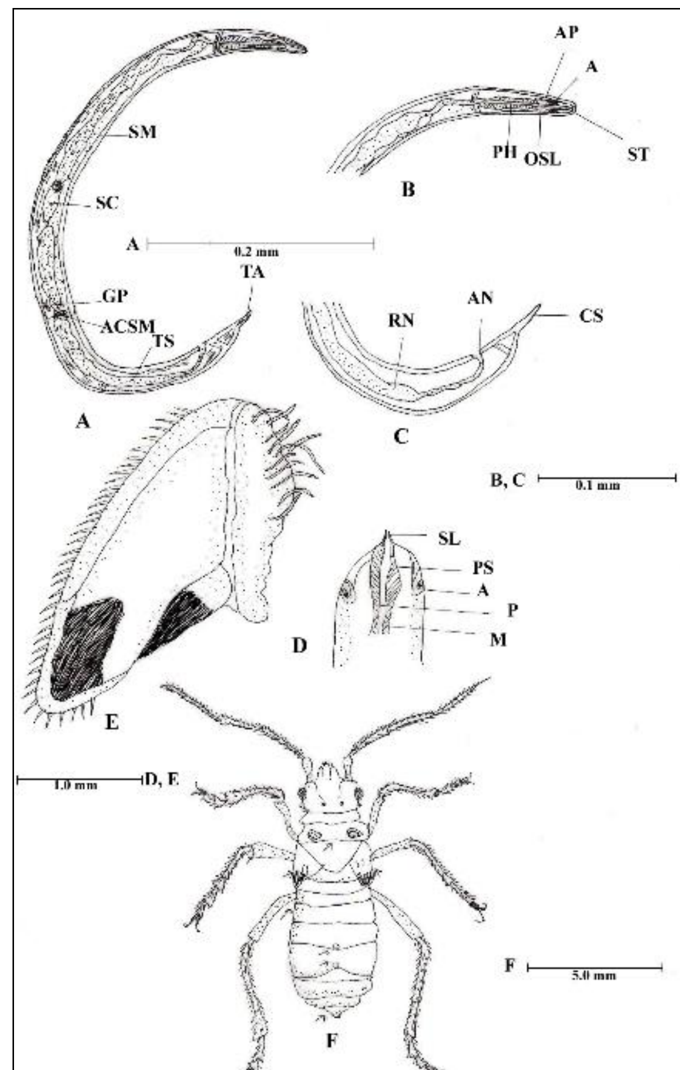

Fig 1: (a) Pre-parasitic stage before penetration. (b) Cephalic end showing stichosome and pharynx. (c) Penetration points for the entry of pre-parasitic juvenile under the wing bud of 5th instar nymph.

Abbreviation
AP-Amphidial pouch, ST-Stoma, OSL-Onchiostylet, PH-Pharynx, SC-Stichocyte, SM-Stichosome, GP-Genital primordium, I-Intestine, TS-Trophosome, AN-Anus, TA-Tail, A-Amphid, SLT-Stylet, MU-Mouth, L-Lip. PT-Pharyngeal tube, CS-Circular spike, PS-Phyaryngeal sheath, RT-Rectum, ACSM-Accessory stichosome, M-Muscle.

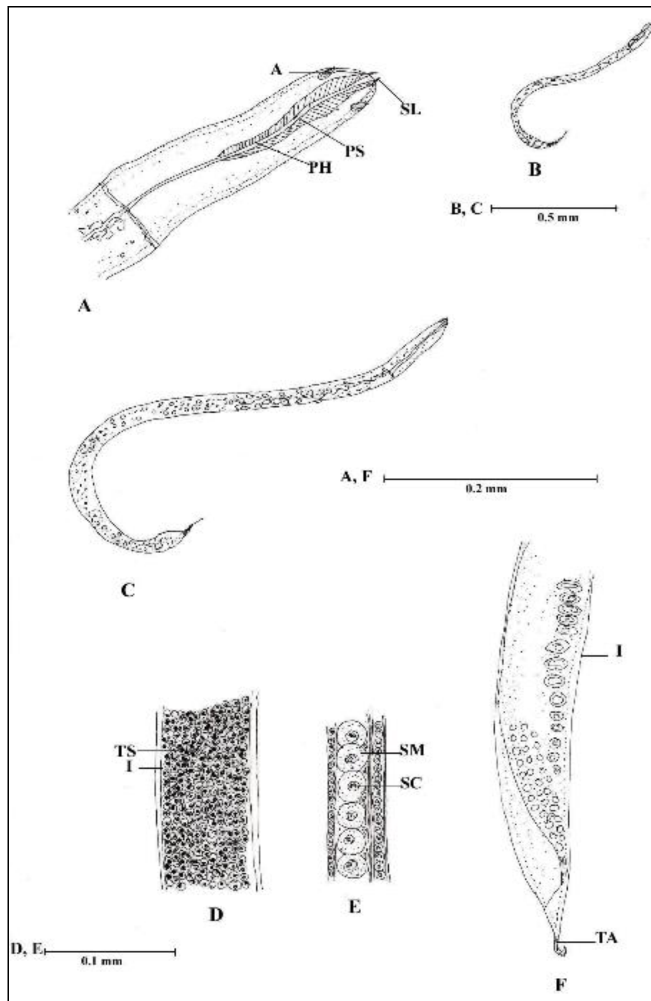

Fig 2: Different parts of Pre-parasitic juvenile just after entrance.

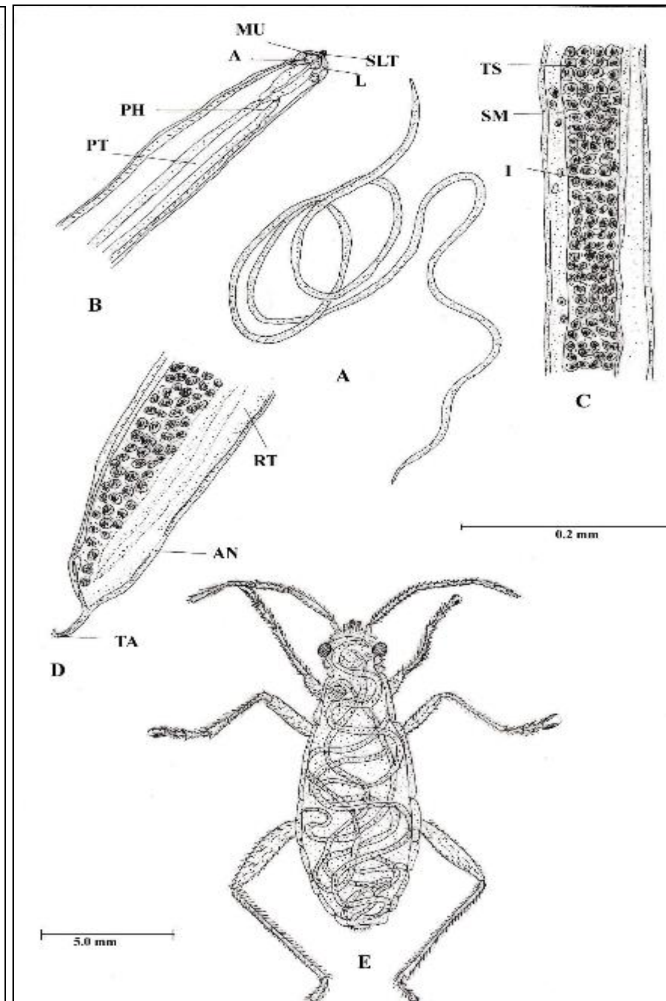

Fig 3: A, B, C & D Parasitic *Hexamermis* vishwakarma showing different parts, E. Parasitic stage within the body cavity

Abbreviation
PH-Pharynx, SC-Stichocyte, SM-Stichosome, I-Intestine, TS-Trophosome, AN-Anus, TA-Tail, A-Amphid, SLT-Stylet, PT-Pharyngeal tube, CS-Circular spike, PS-Phyaryngeal sheath.

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2. Materials and Methods

2.1. Selection and maintenance of test species: *Leptocoris augur* parasitized nymphs and adults were hand-collected from the field at the Horticulture Experiment and Training Centre in Saharanpur from July to September. They were also given fresh kusum plant leaves and seeds. In a lab, the collected bugs were raised in hurricane lamp chimneys and wooden wire gauze cages with fine muslin material covering the top.
A damp cotton swab was kept in a watch glass inside the chimney and cage to maintain the required R. H. (70%). With the help of a hand lens, the behaviour of the insects (*L. augur*) was observed both before and after the emergence of the parasitic juvenile stage.

2.1.1 Rearing of *Hexamermis* vishwakarma Dhiman (Infection technique)
During the rainy season, from July to September, parasitized insects, parasitized nymphs, and parasitized adults of the host, *L. augur*, were hand-picked from the field at the Horticulture Experiment and Training Centre in Saharanpur and placed in polyethylene bags. The samples were restored in hurricane glass lamp chimneys and carried to the lab at ambient temperature and humidity.

2.1.2 Rearing of parasitized bugs in laboratory: In a laboratory, collected insects were raised in hurricane glass lamp chimneys (24 x 36 cm) with a fine muslin fabric covering the top. These bugs were raised on freshly crushed kusam plant seeds, and old food was regularly changed. A cotton swab was inserted into a glass vial filled with water and kept there while the temperature was kept at 28 °C to maintain the required relative humidity (70%). *Hexamermis vishwakarma's* emergence was closely monitored, and regular inspections of the rearing Petri plates were made.

This is the emerging nema's post-parasitic stage. R.H. and temperature measurements were made using a dial hygrometer and a field thermometer, respectively.

2.1.3. Separation of emerged post-parasitic nematodes: Post-parasitic juvenile nematodes that had emerged from the host bug's body were collected using a pipette and kept in sterilised coarse sand (clean Petri dishes), which was then filled with water to drown the nematodes. In order to prevent the escape of post-parasitic nematodes as well as the ingress of ants, etc. (Double tray system), each Petri dish was now placed inside another large Petri dish filled with water.

2.1.4 Rearing technique for obtaining adults, eggs and pre-parasitic nematodes: After emerging from the host bugs, post-parasitic nematodes continue to be active for 24 to 48 hours. Some of these nematodes stayed on the coarse, sterile sand during this period of time. There are roughly 4-7 nematodes in each coil. They change into an inactive stage once they have reached their target location. This stage, which undergoes two moults, is known as the quiescent stage.

The development of the nematode till the pre-parasitic stage was closely examined under the stereoscopic binocular microscope when adults ultimately moulted from dormant post-parasitic worms. In order to track the development of incubation and hatching, the eggs were also retained, and the contents of the Petri plates were regularly inspected under a binocular microscope.

The water was added as needed to maintain the necessary wetness. The pre-parasitic stage's yield was also seen in Petri dish contents.

2.1.5 Rearing of pre-parasitic nematodes: The pre-parasitic nematodes were raised using a laboratory culture. Rearing culture media was made according to Cantwell's (1974) recommendations.

To avoid foaming, 170 grammes of dried whole egg solids were reconstituted in 500 ml of distilled water using a low-speed blender. A 250 ml flask was now filled with 50-100 ml of this medium, which had been steam sterilised. In test tubes, tiny amounts were used. Aseptically applied to the rearing medium and kept at 24°C for 20 minutes after being surface sterilised with 0.05% sodium hypochloride (HOCl). Using a 6-mm glass tubing pipette with a rubber teat that is 7 cm long and gradually tapers to an internal 3 mm diameter, pre-parasites are fed to the culture media. By removing the excess water, little pre-parasites can be stranded on a slide with a large hollow depression measuring about 2 cm in diameter.

Nematodes can be stored in this way without transfer for up to a year. The nematode was present in all of its stages at once in the media.

2.1.6 Culture of the pre-parasitic juvenile of nematodes: The contents of the raising Petri dishes should be daily decanted into a glass dish and placed on a dark surface, such as the lab bench, once the pre-parasitic juvenile nematode starts to emerge. The parasites stand out against the black background in this way, looking white. A pipette can then be used to transfer them to the culture jar. The 6.5 cm-diameter screw-top culture jars that were used worked well for 50 to 60 of the larger worms. These are filled with 5-8 centimetres of wet sand. With the help of a pipette, the pre-parasitic juvenile is deposited into the moist sand and begins to quickly burrow down.

2.1.7 Parasitisation of healthy bugs: 25 adult bugs were added to wooden wire gauze cages with moist soil and a hurricane glass lamp chimney, and proper R. H. and temperature were maintained. The help of a hand sprayer, 1.0% saline water was sprayed into the newly hatched pre-parasitic nemas, and the chimney's top was covered with fine muslin material. Gloves were utilised as a safety precaution as well.

These bugs were removed after three to four hours and raised independently on crushed kusum plant seeds. In addition, water was provided in tiny watch glasses. Now, it was carefully watched how parasitism affected the bug and its conduct. Additionally, the amount of time between the pre-parasitic stages' entry and their release from the host body was noted. In order to determine the impact of parasitism on the anatomy of the host bug, several dissections of the parasitized bugs were also performed under a binocular microscope. The bugs were raised until an *H. vishwakarma* post-parasitic juvenile emerged from the host bugs. Post-parasitic juveniles were raised using the technique developed by Petersen & Willis in (1972) [3]. The post-parasitic juveniles were collected, cleaned, and stored in Petri plates with sterile coarse sand water after they had left their hosts. The excess water was thrown off after about two weeks, taking with it any dead or dying nematodes that were on the sand surface. This technique produced vast numbers of pre-parasitic juveniles.

2.1.8 Release of *Hexamermis* vishwakarma, i.e., pre-parasitic juvenile in field: Large wooden wire gauge cages of 1.5 x I0 x 0.5 metres were made for this observation. Every cage had a wooden base, four sides covered in fine wire mesh, and a partially opening top. The removable lid and top were also covered with wire gauze. The bottom of the cage was devoid of any construction. To offer a natural setting, these cages were housed in a field area under a kusum tree from July to September.To make it simple to see the parasitic juveniles emerge, the bottom of each cage was set on a thick, white polyethylene sheet. Each cage included a particular number of host populations, including male and female adults and nymphs in their second to fifth instars, as well as a food source of crushed kusum seeds. Each cage also contained a large cotton swab to maintain the required R.H.

3. Results

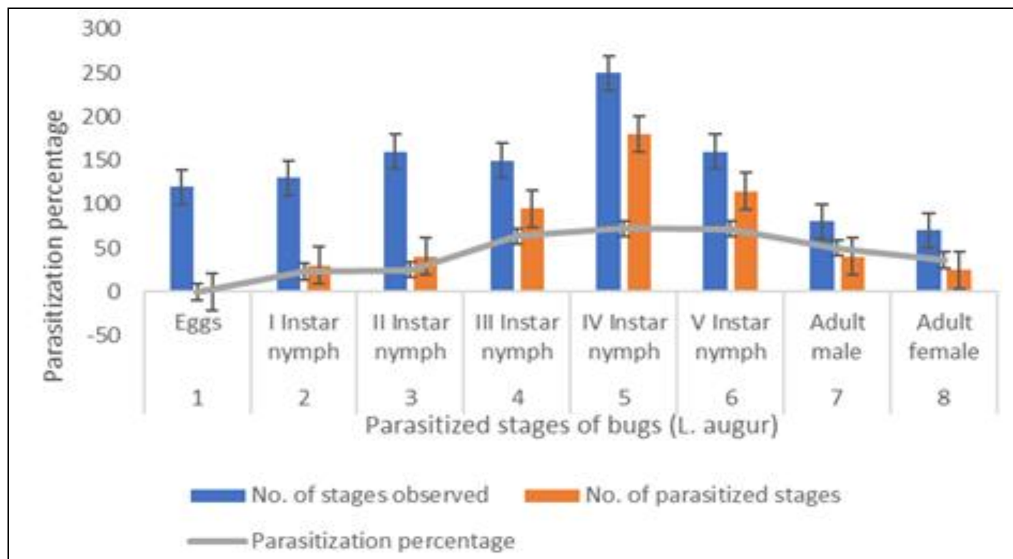
3.1 Stages of the bug found infested

Eggs from *L. augur* were not found to have nematode infestations, and this is likely because the eggs are not laid in the soil, but rather on the leaves of surrounding kusum trees or other shrubs. The sun town the plant on soil in search of the food falling seats of *S. oleasa* after having the first instar nymphs. The first through fifth nymphal instars, as well as adults, all graze heavily on the seeds. *Hexamermis'* pre-parasitic stage is currently penetrating these insects' bodies. Although parasitization has been noted in both nymphal and adult stages, the data in the table clearly shows that parasitization is highest in the third, fourth, and fifth instars. Sex is more parasitized in males than in females (Graph 1).

3.2 Number of parasitic or juvenile nematode in a single host: With just 1 to 3 hours of penetration into the host body, more than 100 pre-parasitic stages of nematodes have been seen in a single host, but only a few numbers of them have progressed to the post-parasitic stage, and the remainder are killed in the host body.

In the bug's body cavity, parasitic juvenile stage nematodes have been found in concentrations of 1 to 6.

3.3 Emergence of the post-parasitic stage from the host body: Following the conclusion of its parasitic life, the young nematode emerges as the post parasitic stage. Axis, external then Italia, or both, coxal membrane, scapal joint with head, pleuron of the abdomen, sternal and pleural joints, and actually through turgal of sternal plates have all been noted as emerging places on the host body. The parasitic nematode's head emerges first upon emergence, followed by the entirety of its long, cylindrical body. The length of the young worm determines how long it takes to do this behaviour. The longer one typically requires more time than the shorter one. For emergence, two to five minutes are needed.



Graph 1: Infestation percentage of various stages of *L. augur*

3.4 Effect of emergence on the host body: When a juvenile nematode emerges from the host, a hole is made in the body of the host, and as more juveniles leave the host, the number of holes grows correspondingly. One nematode only emerges from one pore. The insect starts to become fairly slow once it starts sleeping outside via the holes. After the nematode emerges, it perishes within 2 to 15 minutes. The host is to blame for the 100% mortality. It is further noted that none of the host's parasitic harbouring emerges. When dissection work is done under a binocular microscope, juvenile forms have occasionally been discovered in the body cavity following the death of the host.

3.5 Size of the post-parasitic nematode: The number of juvenile forms harbouring a host, the host's stage, and the nematode's sex all affect how big the post-parasitic stage is. Male post-parasitic stages range in size from 1.22, 4.9 to 19.4 cm, whereas female post-parasitic stages range from 4.3 to 19.4 cm. The length of the post-parasitic stage will be longer if a host is home to a single juvenile form, and it will be shorter if the host is home to multiple young forms. While the fourth and fifth instars and adult bugs contain long sized nematodes, the first two and third installing for all over give the development of short size nematode. Post-parasitic warming can last up to 19.4 cm in length and as little as 1.2 cm.

3.5.1 Release of pre-parasitic juvenile in laboratory on healthy bug population: (Stages of the bug found infested): By using this technique, several pre-parasitic juveniles were collected. These young pre-parasitic bugs were applied to healthy insects housed in different hurricane light chimneys. Pre-parasite to insect ratio was maintained at roughly 20:1. Using 0.1% saline water as the spraying medium, pre-parasites were sprayed; a tiny hand sprayer was employed for this. The bugs were dissected in 1.0% saline water under a binocular microscope after 5 days of pre-parasite exposure to confirm the parasitization of *H. vishwakarma*.

In the second set of experiments, insect mortality was noted following the appearance of the parasite stage. After 25 days, any leftover bugs were removed and dissected.

3.5.2 Parasitisation percentage in laboratory

In a laboratory setting, a high parasitization percentage benefits from high humidity (80-86%) and moderate temperature (29-31ºC). Following investigation, it was discovered that the percentage of parasitization in the lab ranged from 80.5 to 100 percent. The higher percentage of parasitization in the lab was mostly caused by the pre-parasites' ease access to hosts in the constrained area (Graph-1).

3.5.3 Observations after release of *Hexamermis* vishwakarma, i.e., pre-parasitic juvenile in field

The cages were then sprayed with freshly hatched pre-parasitic suspension in the 1.0% saline solution. A little hand sprayer was used to spray these nemas. Stale food was replaced with fresh food after two days. The percentage of parasitized bugs in the population was closely observed.

Through their undulating movements, the pre-parasitic nematodes locate potential penetration sites on the bug, such as weak body portions. Specifically, stylets are used to enter the host bug's body and settle in the haemocoelomic cavity after they have passed through the coxal joints of the legs, under the surface of the wing pads near the joint (in III to IV instar nymph), the genital region, the pleuron, between the abdominal sternal and tergal plates, the cervical membrane, and the wing auxiliaries.

These are known as parasitic juveniles and they expand in size at the expense of host tissue.

The Petri plate containing damp sterilised coarse sand was used to keep the post-parasitic nematodes that had emerged from the host body. These post-parasitic juveniles were collected using a fine camel hair brush, and their faculties were then transferred to another field with water using a double tray system to prevent the escape of post-parasitic nematodes as well as the ingress of ants and other pests. This Petri dish was now organised into a large tray that measured 45 cm by 30 cm and had a water-filled 3.75 cm border. To prevent the spread of microbes through the air, the entire tray was kept covered by delicate clothing. The Petri dishes were inspected daily (Plate 1).

3.5.4 Number of parasitic juveniles in a single host: The nematodes transitioned into sterile coarse sand between 24 and 48 hours into the active phase. Some of them continued to coil up on the sand surface. One to seven nematodes were detected in each coil above. Once they were where they wanted to be, they changed into an inactive stage. This stage is known as the quiescent stage and it sheds its skin twice every two to four weeks from July to September.

Within 4 to 10 hours after finally emerging from the quiescent stage of post-parasitic nematodes, adults copulated. Under the stereoscopic binocular microscope, the nematodes' copulation, oviposition, and hatching processes were kindly watched up till the pre-parasitic stage of development. In order to see the incubation and hatching, the eggs were also housed separately in a Petri dish containing moist coarse sand. The contents of the Petri dishes were regularly inspected under a binocular microscope and a light compound microscope. The water was added as needed to maintain the necessary wetness.

After hatching, moist crushed Kusum plant seeds and a few dried leaves were removed and placed in a Petri plate for the rearing of pre-parasitic nematodes. It was supplemented with the juvenile pre-parasitic organisms from cultured Petri dishes. These endured a week at 100% RH and temperatures between 24^0 and 30°C. Pre-parasitic nemas that had just emerged were sprayed onto adults and nymphs of healthy insects in their second to fifth instars. raised in wooden cages with wire mesh. It was carefully examined how parasitism affected the bug and its habits (Plate 2,3).

3.5.6 Emergence of post-parasitic stage from host body: The nematodes of its host, *L. augur,* emerged from the body by a hole in the pleural membrane, the wing auxiliaries, or the vaginal area. The post-parasitic juvenile was active for at least 24 to 48 hours following emergence before slowly migrating to a nearby safe area.

The Kusum plants' fresh, fragile leaves and damp, crushed seeds were placed in a Petri dish and covered with a hurricane glass lantern chimney, which was itself covered with a fine muslin fabric on top to allow air to circulate. The parasitized bugs were then raised there. Fresh food was substituted for the old every day, and the growing Petri plates were inspected every day (Plate 1).

3.5.7 Effect of emergence on the host insect: The host bug, *H. vishwakarma,* emerges while L. augur is dormant and nearly motionless. It takes 2 to 8 minutes for the host bug's stylets to allow emergence through weak body sections. Due to loss of vital bodily fluids, host insect death frequently occurs shortly after emergence. The time span between penetration and emergence is thought to be 18 to 22 days, on average 20 to 22. These stages are known as the post-parasitic stage and occur after the parasitic youngster emerges from the host bug..

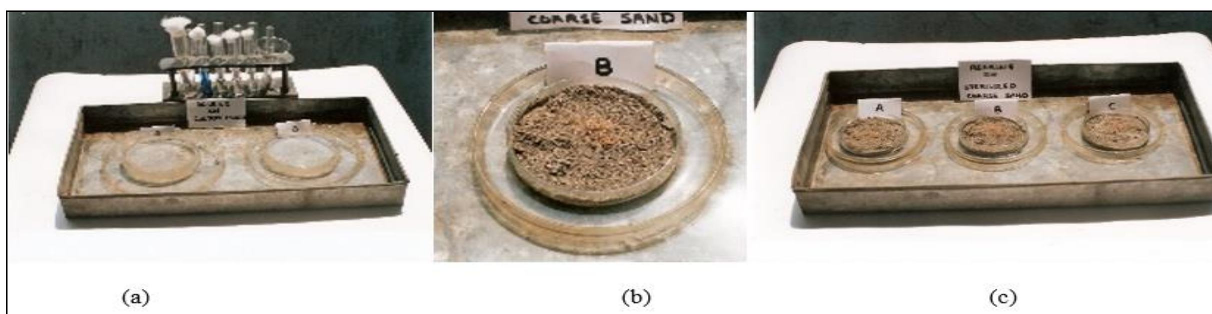


Plate 1: (a), (b) and (c) Double tray system used for rearing of H. vishwakarma on sterile coarse sand

Plate 2: (a) A hurricane glass lamp chimney for rearing, laboratory condition; (b) Post-parasitic stage of H. vishwakarma after emergence

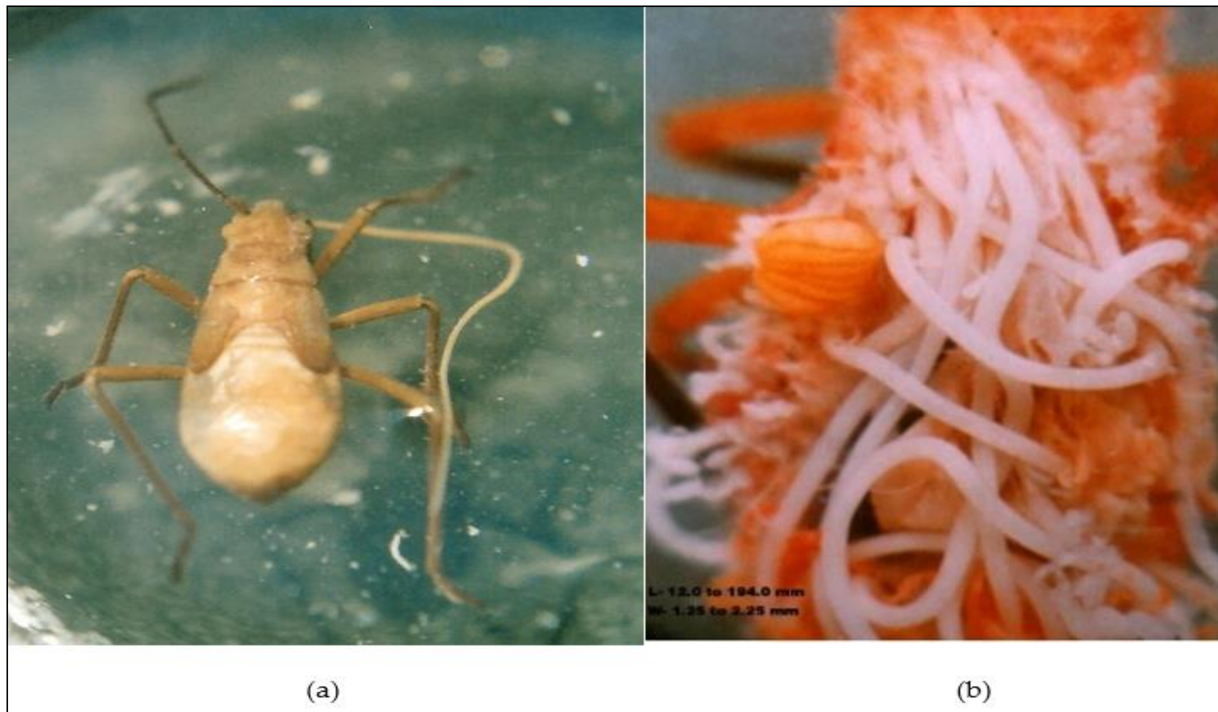
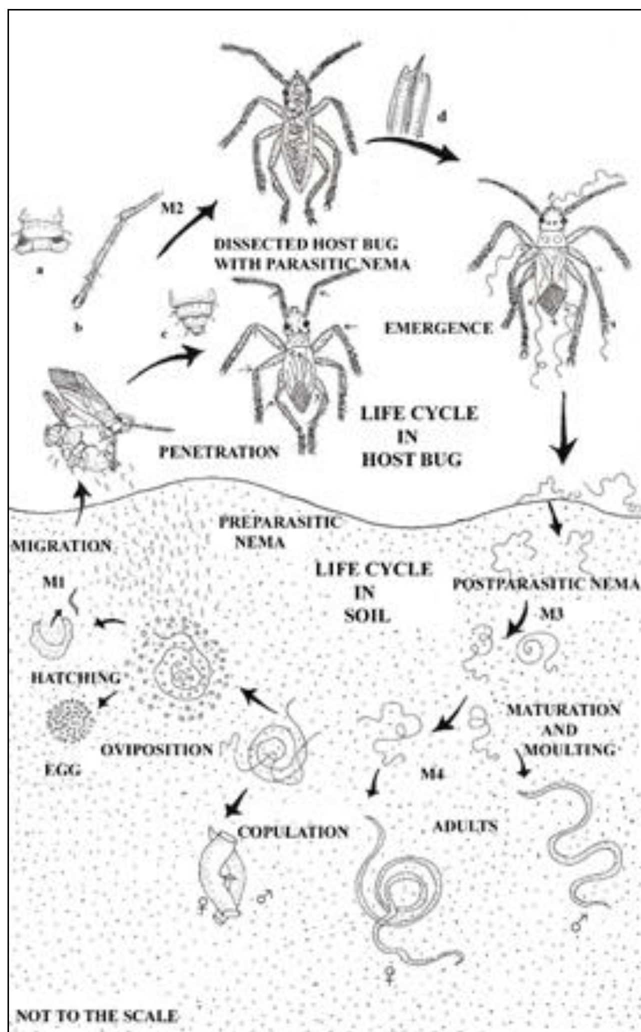


Plate 3: (a) An emergence of parasitic stage through head region. (b) Parasitized bug (L. augur) showing coils of parasitic juvenile (*Hexamermis* vishwakarma)



Abbreviation-M1-First mounting, M2-Second mounting, M3-Thirds mounting, M4-Fourth mounting

Plate 4: Life cycle of *Hexamermis vishwakarma* Dhiman in bug (complete 72 days)

4. Discussion

Detailed discussion of the current works on the "Mass cultivation (*In vitro*) of *Hexamermis vishwakarma* (Nematoda: Mermithidae) is still necessary in light of the research conducted in this area by other scientist.

According to reports from India, the genus *Hexamermis* parasitizes a variety of insects, including *Vanessa atalanta*, Polygonia comma, *P. interrogationis*, and *Isia isabella* (Puttler and Thewke 1971) [4], *Leptocoris augur* (Dhiman, 1984) [5], *Agrotis influsa* (Welch 1963) [6]. *Halys dentatus* (Dhiman and Yogesh, 2004) [7], *Cydia leucostema* (Subbiah, 1986 and 1989) [8, 9] and *Atritibialis spodoptera sp*.

With the aid of a mouth stylet, the infective stage of *H. vishwakarma* (pre-parasites) infiltrates the host and establishes itself in a bodily cavity; the initial development rate is rapid. The larval length has increased by half after four days and by twelve times after 11 days. Within a month, the full length of up to 30.0 cm is attained. The length of the parasitic stage does not reach its maximum length in super-parasitism, and more males are generated (Paine and Mullens, 1994) [10]. The distinctive digitate appendages of the post-parasitic larva are produced during the parasitic stage's growth. It is generally accepted that this appendage is the result of significant body expansion in which the tail tip is not involved.

In an effort to identify the nutritional needs of *R. culicivorax* and evaluate the physical requirements for its development, a variety of commercially available tissue culture mediums have been employed. To start cultures, sterilised eggs or pre-parasites were utilised. Throughout the culture period, sterility must be maintained since the introduction of a contaminant, particularly one that is slow-growing, can produce false results. This is especially important during the formative years of culture. Males in the *H. vishwakarma* species are typically smaller than females in size. Minimum 1 and maximum 5 Nema developed from a single host body in the case of *L. augur*.

Roberts and Van Leuken (26) [11] found that pre-parasites of *R. culicivorax* hatched from eggs surface sterilised with sodium hypochlorite did not grow but died in less than a week when cultivated in the following: Mitsushashi and Maramorosch's leafhopper medium, modified Grace's medium (Welch, 1963) [12], Schneider's Drosophilia medium plus foetal bovine serum (FBS) (4:1), Wolf and Quinby's amphibian culture medium, mycoplasma broth medium, Caenorhabditis briggsae medium, and water.

Myers (1975) [13] made reference to the nematode culture effort. Super-parasitism results in male *L. augur* (host bug) and the presence of a single nema results in female. According to Paine and Mullens (1994) [14], hosts harbouring numerous nematodes produced males, whereas a single worm typically produced females.

Maximum 20 and minimum 1 parasitic nematode have been found in the host body of *L. augur*. Additionally, *Strelkovimermis rubtsovi sp.* and *S. ozawindibi* sp.'s effects on chironomid adults (the host) were documented by Johnson and Kleve in 2002 [15]. A new record of nematode parasitism of meloid beetles was reported by Luckmann and Poinar in 2003 [16], and all previous records are compiled. He claimed that these beetles' poisonous substance cantheridin may have an impact on parasitism rates.

Sanders *et al.* (1973) [17], in contrast, injected pre-parasites into their experimental media after passing through an antibiotic solution. Even though the growth rate was only 1/2 to 1/3, nematodes developed through the first stage and grew in size. (In vivo cultivation) In Schneider's Drosophila medium with 10% FCS at pH 6.5-7.0 and 25 ^{0}C. The stylet shrank, but there was no evidence of moulting in the culture media. Stichocytes and trophosomes became increasingly noticeable when the third stage began, but no development of the testis or ovaries was seen. The effects of axenizing agents on the viability of treated eggs and preparasites were evaluated in light of the greater growth response observed after introducing sterilised preparasites into a culture medium, compared to that observed when sodium hypochlorite sterilised eggs were used to initiate cultures (Myers, 1975; Roberts, and Van Leuken. 1973) [13,11]. Sterilisation of embryonic eggs involved one or more washes in 0.05% sodium hypochlorite. The procedures that were subsequently used involved washing pre-parasites in sterile distilled water after passing them through a solution of penicillin, streptomycin, fungizone, and kanamycin (PSFK) (Hansen *et al.* 1975; Finney, 1977) [18,19]. In contrast to egg sterilisation, this procedure made sure that all nematodes were healthy when they were added to the culture media. Although mass sterilisation of pre-parasites was attempted in PSFK by centrifugation at brief intervals, individual sterilisation proved to be the more dependable technique despite being time-consuming.

The best results were obtained using Grace's culture medium, which contained 10% HIFCS and was maintained at 26°C with a pH of 6.4-6.5. The stylet was destroyed, and no cast cuticle was visible, yet the nematodes continued to grow and develop as if they were in stage three after passing through the first stage in this medium.

The physical characteristics of a good culture media are crucial. The *In vitro* culture of mermithid mosquito and black fly parasites was noted by Finny in 1976 [20]. *Romanomermis culicivorax* develops in *In vitro* culture, and the best temperature for *R. culicivorax* growth is 25 to 26 ^{0}C. Nematodes can also live in a pH range of 6.4 to 7.1. Experimental nitrogen or carbon dioxide gassing of Finney's (1977) [19] medium did not appreciably speed up or increase the amount of nematode development. *Reesimermis nielseni* exhibited axenic development in insect tissue culture conditions, as noted by Sanders *et al*. in 1973 [17]. Hydromermis conopophaga's preparasitic stage was only ever cultured once (Poinar, 1975) [21]. Bovine serum, rabbit serum, chick embryo extract, heated liver extract, peptone-yeast extract, Grace's tissue culture medium, Schneider's Drosophilia medium, blood from Galleria, and blood from the host *Tanytarsus* were among the culture media utilised. Pond water was also included. The best outcomes were obtained using a 3:1:1 mixture of pond water, bovine serum, and chick embryo extract.

Furthermore, scanty information is available on mass culture of mermithid nematodes persued by various workers, viz., Petersen, and Willis, (1972) Petersen, (1979) [3, 22] reported the Effect of temperature on longevity of laboratory cultures of *Romanomermis culicivorax*. Palely, (1990) [23] mentioned the mass culturing of *Romanomermis iyengari* mermithid nematode.

Biological work by mermithid nematodes has been done by various workers, viz., Simmons, and Edman (1978) [24], Poinar, and Otieno 1974) [25] mentioned the four molts in the Mermithidae. The effects of temperature on mermithid nematodes were studied by Thornton, *et al.* (1982) [26] mentioned the Effect of storage at low temperature on development and survival of post-parasitic juveniles of *Romanomermis culicivorx*.

Mermithid nematodes may show to be of considerable significance in the biological control of mosquitoes and other important insect vectors for medicine, such as blackflies, according to Welch (1960) [12] and Weiser (1963) [27] observations. Entomophilic nematodes works as a potential biocontrol agent and major contribution in this regard has been made by Finney, J. R. (1978) [28], *Romanomermis nielseni* for the control of Canadian mosquitoes, Petersen, *et al*. (1978) [29]. Puttler, and Thewke, (1971) [30] observed the Field and laboratory observations of *Hexamermis arvalis*. Petersen, and Willis, (1974) [31] experimental Release of *Romanomermis culicivorax* for the control of *Anopheles albimanus*.

Furthermore, Weiser (1963) [27] emphasises the need for the putative control agent to be widely popularised as a first stage in the evaluation of its potential.

5. Future Recommendations

Our current research intends to characterize the nematode's morphology down to the species level and develop mass-rearing techniques for the nematode for field releases and effective pest management, thereby enhancing this natural enemy as a potent biological control agent against *L. augur* (Kumkum, 2021) [32].

6. Significance Statement

The results from *H. vishwakarma* were encouraging, and within a month, 80-90% of the population of bugs in the cage died.

7. Conclusions

However, it should be noted that very little background knowledge on physiological and physical factors was

available when *In vitro* culture attempts were initially made, making it difficult to base medium selection or methodology for effective growth of mermithids.

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