International Journal of Mosquito Research

ISSN: **2348-5906**
CODEN: **IJMRK2**
IJMR 2015; 2(3): 175-181

Received: 18-07-2015
Accepted: 20-08-2015

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Bacterial characterization in natural breeding habitats of *Aedes* mosquitoes and their role on ovipositional response

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Abstract
Aedes aegypti and *Aedes albopictus* play a major role in transmitting dengue, one of the most important resurging mosquito-borne diseases. *Aedes* are known for ovipositional attractants using bacterial cues and hence can be studied at grass root level to find out the association determining their role in ovipositional attractants. Water samples were collected from different natural breeding sites of *Aedes* species in Dehradun during December, 2013 to November, 2014. The water samples after serial dilution was processed for conventional bacterial characterization (morphology, biochemical, extracellular enzymes and antibiotic test). The bacteria present were characterized and processed for oviposition bioassay. A total of 17 isolates were found, among which four (DABH-1, DABH-5, DABH -6 and DABH-8) isolates were common throughout the year. The conventional characterization test confirmed the four isolates to be Bacillus species. The oviposition activity index (OAI) conferred DABH-5(0.96) > DABH-1(0.88) > DABH-6(0.76) > DABH-8 (0.68) on the following order. Present study envisages isolation and characterization of the bacteria found associated with *Aedes* breeding grounds. Further, the female *Aedes* uses semiochemicals emitted by certain bacteria in selecting oviposition sites.

Keywords: Bacterial characterization, natural breeding habitats, oviposition, *Aedes* mosquitoes

1. Introduction
Dengue fever is one of the most important resurging mosquito-borne diseases causing serious public health problem in tropical and subtropical countries worldwide, especially in urban and semi-urban areas, causing almost 50 million dengue infections every year [1]. *Aedes aegypti* and *Aedes albopictus,* playing an important role in transmitting dengue fever, are highly adaptive in nature and their distribution, relative abundance and survival are greatly influenced by human ecology and anthropogenic activity [2]. The choice of appropriate oviposition sites has a significant impact on the fitness of progeny, distribution of larvae, population dynamics and the overall maternal reproductive fitness and success [3]. Different physical, chemical and environmental cues are known to influence mosquito oviposition behaviour being attractant, repellent or stimulant and consequently integrate in their ultimate site selection [4]. Among the mosquitoes (*Culex quinquefasciatus, Culex restuans, Culex pipiens, Aedes aegypti, Aedes albopictus, Anopheles gambiae*) were affected by semiochemicals produced by bacteria [5]. The oviposition attractants of *Aedes* sp. include colour, odour, presence of larvae and eggs, semiochemicals *viz.,* different pheromones, allomones, kairomones; carboxylic acids and methyl esters produced by bacterial digestion of organic materials [6]. Today, studies focused on oviposition response of bacteria of natural breeding habitats on *Aedes* mosquitoes as attractants / repellents and stimulant / deterrent is well documented [7-12]. On the other hand studies using infusions of variety of plants are also frequently conducted [6, 13-19].
To control the *Aedes* mosquitoes, the application of chemical insecticides have been retarded [20] due to development of insecticide resistance and hence, the vector control strategy has shifted to biological control agents [21]. Further, natural mosquito ovitraps can be considered as a tool to monitor, detect and control *Aedes* populations [22] and also to act as an early warning signal [23].
The reports of Health Department, Uttarakhand, reflected the epidemiological pattern of dengue during the last decade in district Dehradun. As the known vector of dengue, *Aedes aegypti* and *Aedes albopictus*, it is mandatory to investigate the role of bacteria (attractant or

Repellent) in oviposition at grass root level. In the selected study area, microorganisms, especially bacteria (*Bacillus* sp.) have often been encountered in mosquito breeding sites [24, 25]. Hence, the present study was aimed to isolate and characterize the bacteria of *Aedes* breeding water and to determine their role as ovipositional attractant / repellent towards oviposition behaviour of *Aedes* mosquitoes.

2. Materials and Methods

2.1 Collection and Processing of Water Sample for Microbial Culture

Water samples were collected from different natural breeding sites of *Aedes* species in Dehradun during December 2013 to November 2014. In the laboratory the water samples were serially diluted (up to 10^{-4}) with sterile distilled water and plated on nutrient agar (peptone: beef extract: NaCl: agar at 5:3:3:1 g/l) plates and then incubated at 30 ±1 ºC in a BOD incubator for 24 hours to obtain the isolated colonies. Isolated bacterial colonies were made pure by quadrant streaking technique. Pure culture was maintained on Nutrient Agar (NA) slants and in Nutrient Broth (NB) and the isolate was plated in Mac'Conkey and EMB agar media, incubated at 30 ± 0.1 ºC for 72 hours and growth of the isolate was recorded.

2.2 Characterization of microbes

Morphological characters (shape, size, colour, margin and opacity) and physiological properties (temperature tolerance, pH tolerance, NaCl tolerance) were studied following the standard microbiological methods [26- 28]. Vegetative cells of the isolates were stained with gram staining. Morphology of the vegetative cells and spores were observed under a phase contrast microscope under a 100X objective.

2.3 Biochemical characterization

To characterize the isolate biochemically, Catalase Test, Citrate utilization Test, Nitrate reduction Test, Indole Production Test, Methyl red test, Voges-Proskauer Test, Urease Test, Oxidase Test and, Carbohydrate metabolism (acid-gas production) tests were performed [29].

2.4 Extracellular Enzymatic activity

The extracellular enzymatic activity of the strain was assayed by Starch Hydrolysis test, Lipase Test, Tween-80 hydrolysis Test, Protein Hydrolysis Test, Gelatin hydrolysis Test and Casein hydrolysis Test.

2.5 Antibiotic Sensitivity Test

Antibiotic sensitivity test of the bacterial strain was performed using antibiotic discs of various antibiotics like ampicillin (10mcg), chloramphenicol (30mcg), ciprofloxacin (5mcg), rifampicin (5mcg), penicillin G (10U), vancomycin (30mcg), doxycyclin hydrochloride (30mcg), kanamycin (30mcg), tetracycline (30mcg), nalidixic acid (30mcg), bacitracin (10mcg), levofloxacin (5mcg), polymyxin B (300U), Nystatin (50mcg), amoxicillin (30mcg) and erythromycin (15mcg). Some natural substances *viz*., neem oil, citronella oil, camphor oil, clove oil, goat bile and chicken bile were also used to assay the antibiotic activity against the bacterial isolates.

2.6 Mosquito collection and rearing

Larvae of *Aedes* sp. were collected from in and around Dehradun. Larvae were separated species wise using standard keys and catalogues [30-33] and reared in white enamel trays at 27±1 ºC and 80±5% relative humidity (RH). The adults emerged were maintained separately on sucrose solution soaked cotton pads and were fed on white rats at 27-28 ºC and 80 ± 5% RH in rearing cages.

2.7 Oviposition bioassay

For the oviposition bioassays, the purified bacterial isolates were grown separately in 100 ml nutrient broth at 30º±1 ºC for 72 hrs. Sterile nutrient broth media without bacterial inoculation was prepared for control. The test cups were filled either with 30 ml of each bacterial suspension or control media and randomly placed inside the experiment cages. A total of nine replicates were conducted for each treatment, each using a fresh female *Aedes* species. Oviposition activity index (OAI) was estimated following Kramer and Mulla [34]. OAI was determined by the formula: $OAI = (NT - Nc) / (Nt + Nc)$

[NT = number of eggs laid in test cups; Nc = number of eggs in control cups]

Index values lay within the range of +1 to -1 with 0 indicating no response and positive values > + 0.3 indicate that the material is an attractant, while negative values < - 0.3 indicate repellence.

3. Results & Discussion

Microbial analysis of water samples from *Aedes* positive breeding habitat (domestic containers, flower pots, tree hole and tanks) was done and, a total of 17 isolates were recorded. Of these, four common isolates named as DABH-1, DABH-5, DABH-6 and DABH-8 were present throughout the year in almost all breeding habitats. Morphologically the colonies of common four isolates were sticky and round shaped. DABH-1, DABH-5 and DABH-8 isolates were similar in having off-white colour, whereas DABH-6 found to have white colour. Smooth margins were featured in DABH-1 and DABH-6 in contrast to DABH-5 and DABH-8 where slightly serrated margins. The diameters recorded among the isolates as DABH-5 (6.1mm) > DABH-1 (6.0mm) > DABH-6 (5.6mm) > DABH-8 (4.1mm). The uncommon isolates were mostly non sticky, rounded and white to off white. The range of diameter is 3.8 to 6.5mm. While considering the seasonality of bacterial isolates, 13 isolates were found during monsoon season, followed by seven isolates in post monsoon and five in both summer and winter season. Isolates DABH-11 found to form highest colony of $1x10^5$ while lowest happened in DABH-5 ($5.3x10^4$). In monsoon season highest and lowest cfu value recorded in DABH-3 ($7.4x10^7$) and DABH-4 ($5.0x10^3$) respectively. In post monsoon period DABH-16 happened to have highest value of $3.2x\ 10^7$ while lowest value found in DABH-14 ($1.6x10^4$). Winter season recorded highest cfu value in DABH-17 ($7.4x10^7$) and lowest once again found in DABH-5 of value $5.8x10^4$. The average moving trend line depicts season wise prevalence of the bacterial isolates (Fig. 1).

The isolates showed no growth on Mac'conkey and EMB agar media. Gram staining characterized the bacterial isolates as gram positive showing purple-blue colour and green coloured vegetative spores were observed under microscope and rod shaped spores under scanning electron micrograph (Fig. 2). In the physiological test performed, all bacterial isolates were found to tolerate a temperature upto 65 ºC. DABH-1 and DABH-8 isolates had tolerance level up to 6% NaCl in contrast to DABH-5 and DABH-6 could tolerate upto 8% NaCl in the nutrient broth medium. The pH tolerance range was recorded highest in DABH-6 (5.5-7.7), followed by DABH-5 (5.8-7.7) and DABH-1 (6.0-7.1) whereas least tolerance frequency was found in DABH-8 (6.0-7.0) (Fig. 3).

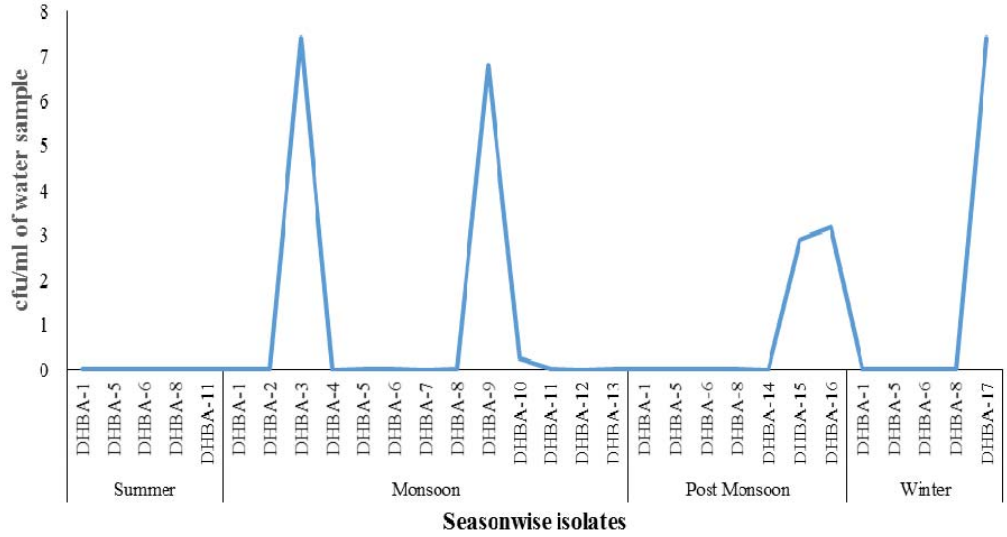
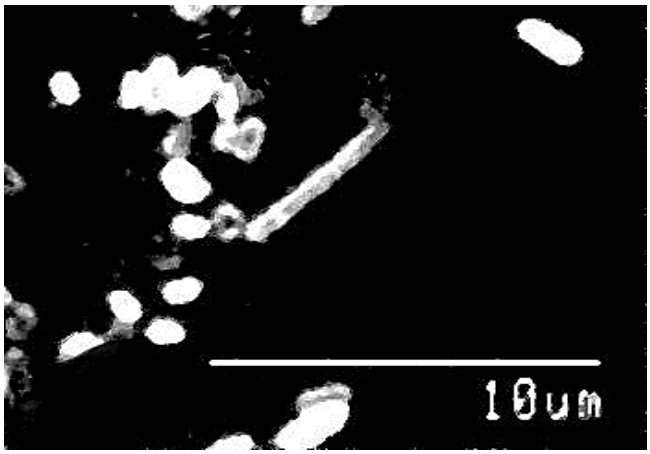
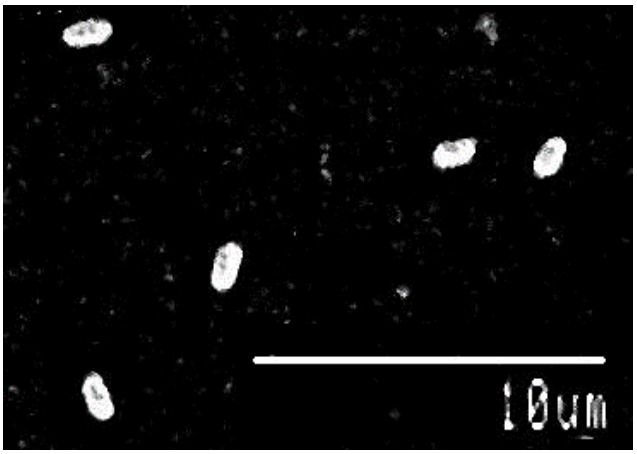


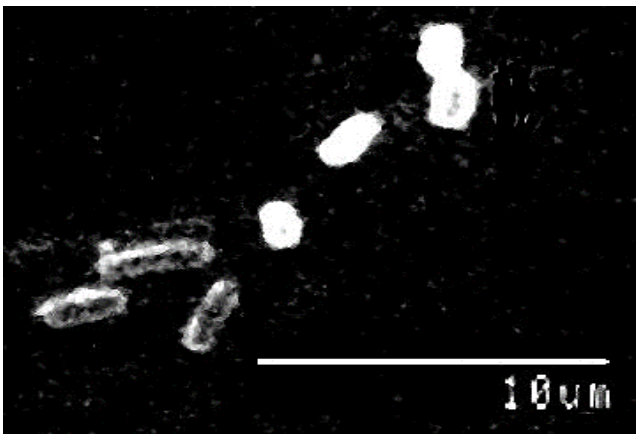
Fig 1: Different bacteria isolated from the breeding water of *Aedes* sp. in Dehradun



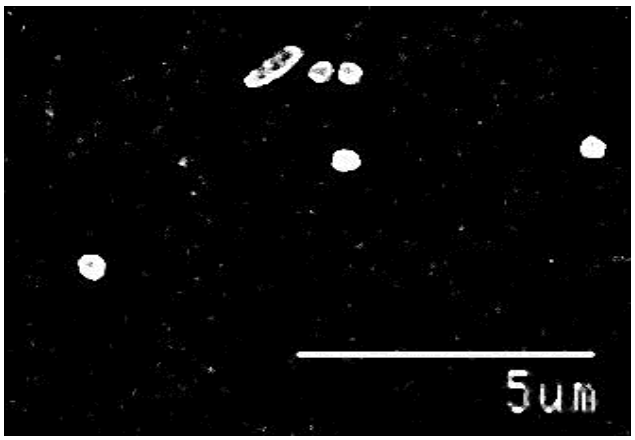
DABH-1



DABH-6



DABH-5



DABH-8

Fig 2: Scanning Electron Micrograph of the Bacterial isolates showing rod shaped. Bars marked in the image.

Biochemical tests depicted that all the four bacterial isolates were positive for catalase, MR and starch hydrolysis test and negative for citrate, indole, lipase hydrolysis, tween 20 hydrolysis and tween 80 hydrolysis test. Contrasts found in DABH-1 isolate was negative for VP test whereas DABH-8 & DABH-5 showed +ve result for urease test and gelation test. DABH-1 and DABH-5 isolates were positive for nitrate test. DABH-1 and DABH-8 isolates showed positive result for oxidase test. All the four isolates produced yellow slant and yellow butt (A/A) in TSI medium, determined to be lactose fermenter (Table 1). In fermentation tests, all isolates produced both acid and gas utilizing glucose, lactose, sucrose and

mannitol as a carbohydrate source but could not ferment aesculin in the medium. From mannose medium, DABH-1 produced both acid and gas and DABH-6 produced only acid. DABH-8 & DABH-6 produced only acid from salium and arabinose medium respectively (Table 2). In Antibiotic test, the four isolates showed sensitivity to chloramphenicol, ciprofloxacin, doxycycline hydrochloride, tetracycline, levofloxacin, erythromycin, neem oil and citronella oil. They showed resistant to polymyxin B, nystatin, camphor oil, goat and chicken bile (Table 3). Among the OD values, the highest was recorded in DABH-1 of OD value 0.398±0.007 at 40 ºC, followed by DABH-5 and DABH-8 0.389 ± 0.011 (35 ºC and 30 ºC respectively), while lowest peak value was recorded in DABH-6 (0.386 ± 0.006) at 30 ºC. The lowest negligible peak was found at 70 ºC for all the four isolates (Fig. 4).

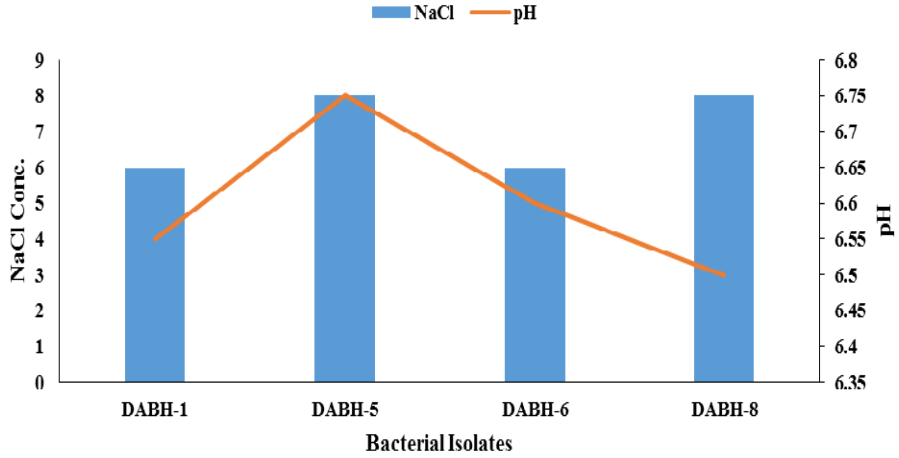


Fig 3: Physiological tolerance among bacterial isolates present throughout the year.

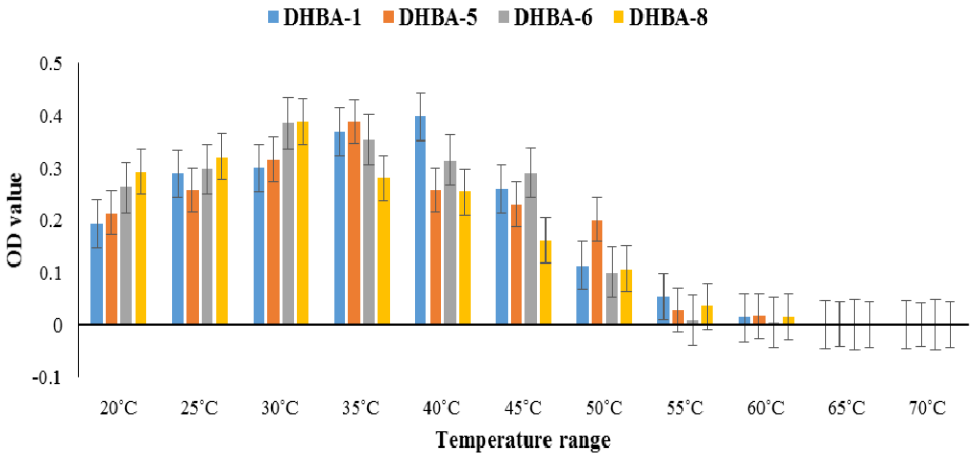


Fig 4: OD value recorded among the isolates among the temperature range. (Mean± SE)

Table 1: Biochemical test analysis among the bacterial isolates.

	DABH-1	DABH-5	DABH-6	DABH-8
Catalase	+	+	+	+
Citrate	-	-	-	-
Nitrate	+	+	-	-
MR	+	+	+	+
VP	-	+	+	+
Indole	-	-	-	-
Urease	-	-	-	+
Oxidase	+	-	-	+
Starch	+	+	+	+
Gelatin	-	+	-	+
Tween 20	-	-	-	-
Tween 80	-	-	-	-
TSI	A/A	A/A	A/A	A/A
Lipase	-	-	-	-

Note: A/A-Acid Assembled

Table 2: Fermentation test analysis among the bacterial isolates.

	DABH-1		DABH-5		DABH-6		DABH-8	
	A	G	A	G	A	G	A	G
Glucose	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+
Salicin	-	-	-	-	-	-	+	-
Aesculin	-	-	-	-	-	-	-	-
Mannose	+	+	-	-	+	-	-	-
Arabinose	-	-	-	-	+	-	-	-

Note: A=Acid; G= Gas

Table 3: Antibiotic Sensitivity test analysis among the bacterial isolates.

	DABH-1	DABH-5	DABH-6	DABH-8
Ampicillin	R	S	R	R
Chloramphenicol	S	S	S	S
Ciprofloxacin	S	S	S	S
Rifampicin	R	S	R	S
Penicillin G	R	S	S	R
Vancomycin	R	S	S	S
Doxycyclin Hydrochloride	S	S	S	S
Kanamycin	R	S	S	S
Tetracycline	S	S	S	S
Nalidixic Acid	R	S	R	R
Bacitracin	R	S	R	R
Levofloxacin	S	S	S	S
Polymyxin B	R	R	R	R
Nystatin	R	R	R	R
amoxicillin	R	S	R	R
Erythromycin	S	S	S	S
Neem Oil	S	S	S	S
Citronella Oil	S	S	S	S
Camphor Oil	R	R	R	R
Clove Oil	S	R	R	S
Goat Bile	R	R	R	R
Chicken Bile	R	R	R	R

Note: R=Resistant; S=Sensitive

Following Logan and de Vos [35] and featuring the morphology, gram positive staining and spore formation, biochemical analysis, fermentation test, tolerance test and antibiotic sensitivity test the four bacterial isolates *viz.,* DABH-1, DABH-5, DABH-6 and DABH-8 were characterized as *Bacillus* sp. While studying the oviposition attractant bio assay of the common isolates, it was found that oviposition activity index (OAI) of DABH-1, DABH-5, DABH-6 and DABH-8 isolates were 0.88, 0.96, 0.76 and 0.68 respectively which indicated that these four bacterial isolates acted as oviposition attractants to *Aedes* mosquitoes (Table 4).

Table 4: The oviposition attractant bio assay of the four bacterial isolates.

Nutrient broth medium		No of replicates	Total no. of eggs	Mean (± SE)	OAI
DABH-1 bacteria	with bacteria	9	739	82.11± 2.64	0.88
	without bacteria	9	46	5.11± 0.90	
DABH-5 bacteria	with bacteria	9	991	110.11±1.88	0.96
	without bacteria	9	34	3.77±0.46	
DABH-6 bacteria	with bacteria	9	709	78.77±0.59	0.76
	without bacteria	9	94	10.44±0.62	
DABH-8 bacteria	with bacteria	9	897	99.66±1.51	0.68
	without bacteria	9	167	18.55±1.29	

Note: OAI= Oviposition Activity Index

The oviposition activity of a female mosquito was mainly found intermingled with two factors; firstly, the female mosquito must be attracted to an oviposition medium, and secondly, by, some stimulants for stimulating the oviposition process [36]. Semiochemical cues resulted from fermenting or decomposition of organic material include volatile attractants / repellents or stimulants / deterrents [14, 37]. The selection of a place for oviposition requires a set of chemical, visual, olfactory and tactile cues that interact with the female before laying eggs, helping the localization of adequate sites for oviposition [12]. Moreover, mosquitoes exhibit a differential response to oviposition media based on the composition of microbial species and as well as on the concentration of the microbes present in the breeding water [14].

The findings and observations of the present study agree with the recent investigations on *Aedes aegypti* mosquitoes using different plants infusions [6, 17, 19]. Several studies [7-9, 11-13] have determined bacteria and its metabolites to be an effective oviposition attractant and / or stimulant in mosquitoes using different methods. Other studies using plants infusions also showed attractant property towards gravid *Aedes aegypti* in laboratory and field bioassays [14-16, 18].

Ikeshoji *et al.* [7] reported that *Pseudomonas aeruginosa* produced an oviposition attractant/stimulant for *Aedes aegypti* and *Culex pipiens.* Benzon and Apperson [8] isolated *Acinetobacter calcoaceticus* and *Entero-bacter cloacae* that attracted gravid *Aedes aegypti* while present in the larval rearing water. While undertaken studies on oviposition responses of *Aedes* mosquitoes in different types of water, it was observed that *Aedes aegypti* preferred to oviposit in well water that contained *Acinetobacter anitratus* [10]. In a study on bacteria as oviposition attractants for mosquitoes, differential oviposition responses by *Ae. aegypti* and *Aedes albopictus* to several bacterial species were observed [9]. Pavlovich and Rockett [11] reported *Bacillus cereus* as a significant oviposition attractant for *Aedes aegypti* and *Aedes albopictus*. The present findings of four bacterial isolates, characterized as *Bacillus* sp. elicited higher oviposition response which justified the previous studies.

Taken together, these studies support the theory that volatiles emitted by bacteria are utilised as semiochemicals by mosquitoes in oviposition behaviour. To control the mosquito population, the removal of breeding water bacteria may be an important part. Since various groups of bacteria are involved in eliciting the oviposition response, it is advantageous to the mosquito species, guiding them to the oviposition site and so ensuring their breeding. Thus the sensitivity of ovitraps are used to detect and monitor the activity of *Aedes* species and its populations.

In addition, the present study screened, though at preliminary level, some natural ingredients such as neem oil, citronella oil and goat bile which can be used to kill the bacterial isolates present in breeding site of *Aedes* mosquitoes in Dehradun, India. Henceforth, if the breeding water bacteria can be controlled by using natural substances, in spite of using harmful insecticides, it will surely be a success in controlling the *Aedes* mosquitoes without any environmental hazards

4. Conclusion

The present study clearly reflects that female *Aedes* uses the semiochemicals secreted by certain bacteria in selecting oviposition sites. These semiochemicals help to increase the number of eggs laid in target containers that in turn would likely enhance the sensitivity of ovitraps that are used to detect and monitor the activity of *Aedes aegypti* in endemic areas and thus may provide promising results in the control and monitoring of *Aedes* populations.

5. Acknowledgments
Authors are thankful to Council of Scientific and Industrial Research (CSIR), New Delhi for financial supports.

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