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Isolation, Characterization and Identification of *Bacillus* Species Toxic to Dengue Vector

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Abstract. The work reported in this article was carried out to screen different samples collected from Lahore to isolate bacteria with larvicidal activity against third instar *Aedes agypti* larvae.

Seventy-three bacterial isolates were collected from soil samples, water samples, soil conditioners and insects. Heat treated samples were used for the isolation of spore forming bacteria through spread plate technique. Isolated bacteria were identified as *Bacillus thuringiensis*, *B. laterosporus*, *B. circulans*, *B. sphaericus*, *B. megaterium*, *B. subtilis* and *B. alvei* by morphological and biochemical testing. Preliminary bioassays were conducted under laboratory conditions to assess the toxicity and efficacy of microbial isolates. Out of seventy three bacterial isolates only one showed ento-mopathogenic activity against *Aedes agypti* larvae. Isolate with larvicidal activity (CEPS-56) was identified as *B. thuringiensis* which was isolated from dead mosquito. Toxic isolate of *B. thuringiensis* (CEPS-56) may be further investigated at the molecular level and effective toxic concentration of CEPS-56 determined by conducting concentration bioassay. The results lead to the conclusion that ento-mopathogenic bacteria are present in natural environment of Lahore and screening of more number of samples may yield different and even more toxic strains of bacteria.

Importance. There are no two opinions about the fact that Dengue has caused a havoc in terms of both mortality and morbidity all over the globe particularly in the Asian countries. The social cost of its control is very high. Thus, it is obligatory for the global community to develop different techniques to eradicate it. The research presented here presents a newly discovered technique based on producing toxins by growing bacteria that kill Dengue virus. The study reports the discovery of a strain of *Bacillus subtilis* and signals towards further exploration of other bacterial strains that can be used to control dengue vector in different parts of the world.

Keywords: isolation, characterization, dengue, *Bacillus thruingiensis*, toxin

Introduction

Dengue is the most common infection transmitted by mosquitoes in human beings, which in recent years, has become a major international public health problem. It is usually encountered in tropical and subtropical regions around the world and has grown dramatically around in recent decades. Over 40% of the world's population is now at risk of dengue infection. World Health Organization (WHO) has reported that there may be 50 to100 million dengue infections worldwide every year WHO (2013).

The first outbreak of dengue fever occurred in Pakistan in 1994, but annual epidemic trend first occurred in Karachi in November 2005. Since 2010, Pakistan has been experiencing the epidemic of dengue fever that has caused 16580 confirmed illness cases and 257 deaths in Lahore and nearly 5000 cases and 60 deaths reported from the rest of the country. The three provinces facing the epidemic are Khyber Pakhtunkhwa, Punjab and Sindh. (WHO, 2013).

The earliest record of dengue fever was found in the year of 992 in Chinese Encyclopedia. Its incidence has increased over time and major factors that contributed to its increase are said to be the expansion of global shipping industry in 18th and 19th century causing its spread to new geographic areas, rapid unplanned urbanization in southeast Asia after World War 2 caused the increased transmission of dengue virus serotypes resulting in the hyper endemicity and lack of vector control measures. Additional major factors include lack of effective mosquito control in dengue endemic areas (Gubler, 1989; Gubler, 1987), increased number of mosquito larval habitats in the domestic environment

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and an increased air travel, which provides the ideal mechanism for the transport of dengue and other urban pathogens between population centers of the world (Gubler, 1996; Gubler and Trent, 1987). In 20th century dengue is most common infectious disease having same economic impact on community as malaria (Smith, 1956).

The mosquito borne diseases can be successfully controlled by implementing vector control measures by means of applying insecticides thereby reducing the breeding potential of mosquitoes.

Three management approaches have been used to control the dengue epidemic. These are environmental control, chemical control and biological control. World Health Organization Expert Committee on Vector Biology and Control described following environmental management method such as (i) Environmental modification (ii) Environmental manipulation and (iii) Changes in human habitations (WHO, 1980). Chemical insecticides such as Pyrethrins and DDT have been extensively used in the past, while chemical methods being currently applied for eradication of dengue vector include application of temephos as larvicide, methoprene as insect growth regulator and malathion, fenitrothin and pyrethroids as adulticides. Chemical insecticides cause threats such as contamination of food and water, damages to non-target species such as fauna and flora, development of resistance in mosquitoes and more importantly chemicals could be concentrated in the food chain and can be passed on to successive generations as described by Janisiewicz and Korsten (2002). Due to the insect resistance, there is a constant need for production of new more powerful chemical insecticides to combat the disease causing mosquitoes that leads to the ever increased cost for the production of chemical pesticides. Socially and ecologically they have caused death and disease in humans and have damaged the environment. That is why alternative methods of insect management offer adequate levels of pest control and pose fewer hazards. One such alternative is the use of biological methods to control insect vector. That is why discovery of new biological alternatives is currently an important research focus these days. It can limit ecological damage caused by the use of chemical insecticides including evolution of resistant mosquitoes, environmental pollution and destruction of beneficial insects (Vincent, 2000). Naturally occurring bacterial larvicides are effective in controlling immature stages of mosquito population because of absence of harmful effects on target flora and fauna and to counter the development of resistance in mosquitoes (Cetin and Yanikoglu, 2006).

Bacillus species are reported to have highly specific action against mosquitoes with no effect on other non-target insects, plants and domestic animals (Lima *et al*., 2002). A strain of *Bacillus sphaericus* named as H5a and isolated from diseased larvae of *Culex* species possesses high insecticidal properties. This strain has been reported to be promising against the fourth instar larvae of *Anopheles culicifacies*, *Anopheles stephensi*, *Anopheles subpictus*, *Aedes aegypti* and *Culex quinquefasciatus* (Gupta *et al*., 1991). The binary toxin (Bin) is the most important of the *Bacillus sphaericus* toxins owing to its predominant role in determining the overall toxicity of strains (Charles and Nielsen-Leroux, 2000). Mosquito pupicidal activity of two strains of *Bacillus subtilis* has been reported against *Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes aegypti* (Geetha *et al*., 2007). A strain of *Bacillus circulans* isolated from a larva of *Culex quinquefasciatus* showed larvicidal activity 107 times more toxic to *Aedes aegypti* as compared to *Bacillus sphaericus* strain (Darriet and Hougard, 2002).

The use of ento-mopathogenic bacteria like *Bacillus thuringiensis* as larvicide is a viable alternative for insect control (Lawrence, 2019). Water dispersible granules based on *Bacillus thuringiensis* serovar *israelensis* have been recommended by WHO for mosquito larvae control in container habitats (WHO 2009). These strains have been isolated from varied natural habitats such as soils (Amina *et al*., 2021; Abo Bakr *et al*., 2020; Kevita *et al*., 2020; Hastowo, 1992) dried tobacco residues and dead tobacco beetles (Kaelin *et al*., 1994), insect larvae (Itoua-Apoyolo, 1995), marine sediments (Maeda *et al*., 2000), flowers (Zhang *et al*., 2010) and leaves (Ohba, 1996).

Dengue is currently most important mosquito-borne viral disease. The emergence of dengue like symptoms was first recorded in China Dynasty of 265-420 AD which indicates that this disease has been present for centuries. In 1940 DDT was used to control dengue vector but after 1960 due to the development of resistance in mosquitoes against DDT, organophosphates and other chemicals became main focus for eradication of dengue vector. But hazards caused by those chemical insecticides to non-target organisms and environment created the need to move towards more environmental friendly and target specific control agents. Then in 1977 first toxic strain of *Bacillus thuringiensis* var *israelensis* was

isolated. Studies on this bacterium showed that *Bacillus thuringiensis* was target specific and highly toxic to immature stages of *Aedes* mosquito (Chilott and Kalmakoff, 1983). Then isolation of naturally occurring ento-mopathogenic bacteria became important research focus. Till now several ento-mopathogenic *Bacillus* species isolated from natural habitats such as *Bacillus sphericus*, *Bacillus thuringiensis*, *Bacillus circulans*, *Bacillus subtilis* and *Bacillus laterosporus* have been reported, while searching other than *Bacillus* species only *Serretia* strains were found to have toxicity against mosquitoes.

Keeping in view the natural habitats of different bacterial and other species, many studies (Ammouneh *et al*., 2011; Assaeedi *et al*., 2011; Geetha *et al*., 2011; Gonzalez *et al*., 2011; Hayes *et al*., 2011; Liang *et al*., 2011; Radhika *et al*., 2011; Zhang *et al*., 2010; Baruah *et al*., 2008; Martin *et al*., 2008; Geetha *et al*., 2007; Poopathi and Abidha, 2007; Yasutake *et al*., 2007; Ruiu *et al*., 2006; Monnerat *et al*., 2005; Ibarra *et al*., 2003; Dariet and Hougard, 2002; Cavados *et al*., 2001; Bhattacharya, 1998; Orlova *et al*., 1998; Kawalek *et al*., 1995; Asimeng and Mutinga, 1992; Gupta *et al*., 1991) have been conducted to isolate from environmental sources of different countries and subsequently isolate different strains of genus *Bacillus* and significant toxicity to dengue larvae have been reported. In some other study Bautista and Franco (2011) presented a review about pathogenicity of *Bacillus thuringiensis.* The reviewers pointed out that *Bacillus thuringiensis* was gram-positive, spore-forming bacteria that can act as a bio-insecticide to various mosquitoes due to the toxin it produced. Some recent studies on isolation and characterization are presented: Patil *et al*. (2012) evaluated *Bacillus thuringiensis* and *Serratia* species against early fourth instar larvae of *Aedes agypti* and other larvae and revealed that *Bacillus thuringiensis* showed 100% mortality against *Aedes agypti*, after 24 h. Foda *et al*. (2013) isolated three hundred and fifty nine *Bacillus* strains out of which 5 showed insecticidal activity againt *Culex pipiens*. Active isolates were identified as *Lysinibacillus sphaericus*. Maximum toxicity was observed in Ls-9B24. Omoya and Akinyosoye (2013) assessed the toxicity of *B. subtilis* and some other bacteria against second and fourth instar larvae of *Anopheles arabiansis*. Bioassay results showed that *B. subtilis* could be a potential bio-control agent with LC_{50} values of 0.865 mg/mL and 2.361 mg/mL for second and fourth instar larvae of *Anopheles arabiansis* respectively. Poopathi *et al*. (2014) isolated mosquito-cidal bacterium from marine soil of east coastal areas at Pondicherry in India and identified as *Bacillus cereus.* Bioassay results showed that this strain of *Bacillus cereus* had greater toxicity against *Culex quinquefaciatus* than *Aedes agypti* and *Anopheles stephensi*. Protein purification results from the cell mass of *Bacillus cereus* and SDS-PAGE analysis showed that a well perceptible polypeptide was responsible for mosquitocidal action (Nair *et al*., 2018). Soares-da-Silva *et al*. (2015) isolated 484 bacteria from twenty five soil samples from Amazon, Brazil. Out of 484 bacterial colonies fifty seven were identified as *Bacillus thuringiensis*. Six isolates named as IBt-03, IBt-06, IBt-07, IBt-28, IBt-30 and BtAM-27 showed toxicity against *Aedes agypti*. Lowest toxicity was showed by IBt-07 and IBt-28.

Taking into consideration the review of literature outlined above, the goal of the present study was to put forward a method for controlling life threatening disease caused by mosquito *Aedes agypti* by isolating some *Bacillus* species from various natural habitats non-harmful for humans and environment and using them against the said culprit for its eradication on the basis of their being toxic to it. The objectives were as below:

- Isolation of bacteria from natural habitats such as soil, water and insects
- Identification and characterization of isolated bacteria
- Assessing the toxicity of isolated bacterial strains against *Aedes agypti* by conducting bioassay.

Materials and Methods

Sample collection. Bacteria were isolated from different soil samples, water samples, insects and dead larvae. Soil samples were collected from PCSIR, Ferozpur Road Lahore, Pakistan. The lawn soil, pot soil, greenbelts in PCSIR and from nurseries from the surface to a depth of 5 cm with a sterile spatula. The collected samples were preserved in sealed plastic bags.

Isolation of spore forming *Bacillus* specie. Each 1g of the sample was suspended in 100 mL of sterile distilled water and shaken vigorously for about 10 min. The samples were heated at 80 °C for about 30 min in a water bath to destroy all non-spore forming bacteria for the isolation of *Bacillus* species (Daniel *et al*., 2018). To reduce the number of colonies per plate, six serial dilutions were prepared from the stock solution of each sample and placed on agar or LB (Luria broth) media plates. Plates were incubated at 37 °C in an incubator

until colonies appeared. Individual bacterial colonies varying in colour and shape were picked up and purified by streaking on nutrient agar slants and preserved for further testing (Palma, 2015).

Identification and characterization of bacterial strains. The bacterial isolates were identified on the basis of classification schemes published in Bergey's Manual of Determinative Bacteriology, reported by William *et al*. (1994). Following morphological and biochemical tests were carried out in order to characterize bacterial strains.

Gram staining. A small portion of colony from each streak plate was selected with the help of sterile inoculating loop and transferred to a clean slide having a drop of sterile water on it. The samples were air-dried and heat fixed by passing through flame several times. Each smear was first covered with crystal violet dye for one minute and washed with water. Then the smear was covered with gram iodine for thirty seconds and again rinsed with alcohol. At the end, smear was covered with counter-stain gram saffranin for one minute and washed with water. After drying, slides were examined by100X oil immersion microscopy. During this examination, grouping, gram stain results and morphology of the cells were recorded. Smears with violet colour were labeled as gram-positive and pink or red smears were labeled as gram-negative.

Spore staining. *Bacillus* species can form endospore in order to survive in hostile conditions. For spore staining, small portion of bacterial colony from pure culture was picked and transferred to a clean slide and heat fixed by passing through flame several times. The slide was placed over boiling water and malachite green was applied over the smear and heated for about fifteen minutes. After that slide was rinsed with water and counter-stain saffranin was applied for about one minute and again rinsed with water. The slide was air dried and examined under microscope. Endospores were green, whereas vegetative cells were pink.

Catalase test. For catalase test, colonies from streak plate were selected and transferred through a sterile inoculating loop to a clean slide. Hydrogen peroxide was applied to the bacterial colony on the slide. Catalase positive bacterial colony resulted in the appearance of bubbles within 5 to 10 s of hydrogen peroxide application. Bacterial colony with no bubbling was catalase negative.

Starch hydrolysis. Small portion of bacterial colony from a pure culture was streaked on starch agar plate using a sterile inoculating loop. The inoculated plate was incubated at 37 °C for 24 h. After 24 h, inoculated plate was flooded with iodine reagent. Presence of clear zone around the bacterial colony indicated the positive result, whereas blue colour indicated the absence of hydrolysis.

Lecithinase test. The bacterial colony from pure plate were streaked on to the egg yolk agar plate and incubated for about 24 h at 37 °C. After 24 h, inoculated plates were examined. Lecithinase positive bacterial colonies were surrounded by an opalescent halo whereas lecithinase negative colonies showed absence of clear zone around them.

Voges proskeur test. Voges proskeur stock solution was prepared and transferred to test tubes and autoclaved. The test tubes were inoculated with bacterial cultures and incubated at 30 °C. Inoculated tubes were examined for acetoin production after 2, 4 and 6 days. For VP test, 1 mL solution from test tube was transferred in another sterile test tube and then 18 drops of 40% KOH and a small amount of creatine was added. Tubes were allowed to stand for 15 min before interpreting the results. VP positive bacterial colonies gave pink or red colour at the surface of the medium showing the presence of acetoin whereas VP negative colonies gave yellow or no colour at the surface of the medium.

Indole test. Tryptone broth was prepared, transferred in test tubes and autoclaved. The test tubes were inoculated with a small amount of a pure culture and incubated at 37 °C for 24 to 48 h. After incubation 5 drops of Kovács reagent was added directly to the tube to test for indole production. Tryptone broth having indole positive bacterial colonies were indicated by the formation of a pink to red color in the reagent layer on top of the medium within seconds of adding the reagent. In indole negative culture the reagent layer was yellow or slightly yellowish green.

Glucose fermentation test. Phenol red glucose broth medium was prepared and autoclaved after transferring into test tubes. pH of the medium was adjusted to 7 before autoclaving. With the help of sterile loop inoculating bacterial culture was transferred to test tubes containing the sterile medium and incubated at 37 °C for 24 h. After a day, incubated culture was retrieved from the incubator and colour of the sample was observed. The appearance of yellow colour indicated

the positive glucose fermentation test, whereas magenta or hot pink colour indicated the negative test.

Motility test. Motility test agar medium was prepared by mixing enzymatic digest of gelatin, beef extract, sodium chloride and agar. Final pH of the medium was maintained at 7.3 and then medium was autoclaved at 121 °C for 15 min and transferred into the sterile test tubes. Tubes were inoculated by stabbing through center of the medium with inoculating needle to approximately one-half the depth of the medium. Inoculated tubes were incubated at 37 °C for 24 h. After 24 h diffused growth spreading from the line of inoculation was observed in motile bacterial cultures. Non-motile organisms grew only along the line of inoculation.

Preparation of bacterial inoculum. To evaluate toxicity against *Aedes aegypti* larvae, *Bacillus* strains were grown in nutrient medium. A loop full of bacteria was inoculated in 100 mL of nutrient medium and incubated for 5 days at 37 °C with 200 rpm. At the end of this incubation, the majority of the population was in the form of spores crystals mixture (Palma, 2015).

Bioassay. Bioassay was conducted against late third instar *Aedes agypti* larvae according to WHO (2012). Preliminary bioassay was conducted for the identification of toxic strains. One mL aliquots of the spore crystal suspensions were added to 120 mL cups containing 100 mL water and 25 late third instar larvae and kept at 25 °C for 48 h to assess toxicity. All strains were tested in duplicate and two containers without added bacteria were maintained as a control. Bacterial isolates causing the mortality of more than 50% of the mosquito larvae were considered toxic.

Results and Discussion

A total of seventy three bacteria were obtained all of which were *Bacillus* species. The %age distribution of their isolates was *Bacillus thuringiensis* (44%); *Bacillus laterosporus* (19%); *Bacillus sphaericus* (12%); *Bacillus megaterium* (10%); *Bacillus circulans* (6%); *Bacillus subtilis* (5%) and *Bacillus alvei* (4%).

The most frequently isolated bacterial species were *Bacillus thuringiensis* (44%) and *Bacillus laterosporus* (19%). *Bacillus thuringiensis* was isolated from all samples except wastewater samples but 75% of *Bacillus thuringiensis* isolates were obtained from soil samples. The soil collected from PCSIR colony exhibited highest microbial diversity (*Bacillus thuringiensis, Bacillus circulans, Bacillus megaterium* and *Bacillus subtilis*). The morphological and cultural characteristics of strains reported in Table 1 and staining and biochemical characteristics such as their being as gram-positive rod-shaped strains that mostly appeared as pairs, chains of 4-5 cells or single cells reported in Table 2, identified them as derivatives of the genus *Bacillus* (Table 1 and 2).

Thirty two *Bacillus thuringiensis* strains were isolated from root zone soil, moist soil, greenbelt soil, PCSIR Colony soil, nursery soil, leafy litter soil, garden soil, canal soil, agricultural land soil, water sample, honey bee, dead mosquito larvae and decayed leaves. These were named as CEPS-8, CEPS-10, CEPS-13, CEPS-16, CEPS-17, CEPS-19, CEPS-23, CEPS-25, CEPS-30, CEPS-34, CEPS-35, CEPS-36, CEPS-38, CEPS-43, CEPS-52, CEPS-53, CEPS-56, CEPS-62, CEPS-68, CEPS-69, CEPS-72, CEPS-74, CEPS-78, CEPS-79, CEPS-84, CEPS-85, CEPS-89, CEPS-92, CEPS-95, CEPS-96, CEPS-99, CEPS-100. All strains were grown on nutrient agar plates. The colonies appearing after 24 h incubation period were very variable in appearance. These were cream, pale white, off white or white in colour and varied in shape from circular to irregular with wavy, undulate or entire margins. Surface of most of the colonies were rough but some colonies also had dull or glistening surface (Table 1). *Bacillus thuringiensis* strains were gram-positive, catalase positive, motile, spore formers. The ellipsoidal spores were formed in a central or paracentral position without swelling the sporangium. The organism did not ferment mannitol and had a very active lecithinase production system. *Bacillus thuringiensis* isolates showed positive results for starch hydrolysis and voges proskeur tests and negative results for Indole production (Table 2).

Seven *Bacillus megaterium* isolates were obtained which were named as CEPS-40, CEPS-65, CEPS-73, CEPS-82, CEPS-86, CEPS-91, CEPS-98. Their agar colonies were circular or irregular shaped with entire, undulate or umbonate margins. Four colonies had dull surface, two of them had glistening surface and only one colony had rough surface. All rods were white in colour and opaque with convex elevation and butyrous or bristle texture (Table 1).

Bacillus megaterium isolates were motile and spore forming. The colonies of *Bacillus megaterium* showed positive characteristics of catalase but not oxidase. It interacted negatively with indole, Voges-Proskauer and

Table 1. Morphological characteristics of screened isolated *Bacillus* species

Isolate	Sources	Strain names	Colony morphology						
			Colony surface	Colony shape	Colony margin	Elevation	Colony colour	Colony texture	Opacity of colony
Bacillus thuringiensis									
Bacillus thuringiensis	Soil conditioner	CEPS-8	rough	Irregular	wavy	umbonate	Cream	Brittle	Opaque
Bacillus thuringiensis	Grassy soil	CEPS-10	Rough	Circular	Entire	Raised	White	Brittle	Opaque
Bacillus thuringiensis	Pot soil	CEPS-13	Rough	Circular	Entire	Raised	White	Brittle	Opaque
Bacillus thuringiensis	Lawn boundary	CEPS-16	Glistening	Irregular	Entire	Flat	Offwhite	Brittle	Opaque
Bacillus thuringiensis	Under tree young	CEPS-17	Glistening	Irregular	Entire	Flat	Offwhite	Brittle	Opaque
Bacillus thuringiensis	Under old tree	CEPS-19	Dull	Irregular	Undulate	Flat	Cream	Brittle	Opaque
Bacillus thuringiensis	Honey Bee	CEPS-23	Rough	Circular	Wavy	Raised	White	Brittle	Opaque
Bacillus thuringiensis	Soil conditioner	CEPS-25	rough	Irregular	Undulate	Raised	White	Brittle	Opaque
Bacillus thuringiensis	Park soil Wapda town	CEPS-30	Dull	Circular	Wavy	Flat	White	Butyrous	Opaque
Bacillus thuringiensis	Root zone	CEPS-34	Rough	Circular	Wavy	Flat	White	Mucoid	Opaque
Bacillus thuringiensis	Root zone	CEPS-35	Rough	Irregular	Undulate	Raised	White	Mucoid	Opaque
Bacillus thuringiensis	Moist soil	CEPS-36	Dull	Circular	Enitre	Raised	White	Mucoid	Opaque
Bacillus thuringiensis	Green belt	CEPS-38	rough	Irregular	wavy	umbonate	Cream	Brittle	Opaque
Bacillus thuringiensis	Green belt	CEPS-43	Rough	Irregular	Undulate	Raised	White	Brittle	Opaque
Bacillus thuringiensis	PCSIR colony	CEPS-52	Dull	Circular	Entire	Raised	White	Mucoid	Opaque
Bacillus thuringiensis	PCSIR colony	CEPS-53	Glistening	Irregular	Entire	Flat	White	Brittle	Opaque
Bacillus thuringiensis	Dead mosquito larvae	CEPS-56	rough	Irregular	wavy	umbonate	Cream	Mucoid	Opaque
Bacillus thuringiensis	Nursery	CEPS-62	Rough	Iregular	wavy	FLat	Offwhite	Brittle	Opaque
Bacillus thuringiensis	Decayed leaves	CEPS-68	Rough	Circular	Entire	Raised	White	Brittle	Opaque
Bacillus thuringiensis	Decayed leaves	CEPS-69	Rough	Circular	Entire	Flat	White	Butyrous	Opaque
Bacillus thuringiensis	Agricultural land soil	CEPS-72	Rough	Circular	Entire	Flat	Offwhite	Brittle	Opaque
Bacillus thuringiensis	Decayed leaves	CEPS-74	rough	Circular	Undulate	Raised	White	Brittle	Opaque
Bacillus thuringiensis	Nursery soil	CEPS-78	Rough	Circular	Undulate	Flat	Offwhite	Butyrous	Opaque
Bacillus thuringiensis	Nursery soil	CEPS-79	Glistening	Irregular	Entire	Flat	offwhite	Brittle	Opaque
Bacillus thuringiensis	honey bee	CEPS-84	Glistening	Circular	Entire	Raised	Pale white	Mucoid	Opaque
Bacillus thuringiensis	Nursery soil	CEPS-85	Rough	Irregular	Entire	Raised	Offwhite	Brittle	Opaque
Bacillus thuringiensis	Leafy litter soil	CEPS-89	Dull	Circular	Entire	Flat	White	Butyrous	Opaque
Bacillus thuringiensis	Garden soil	CEPS-92	Rough	Irregular	Entire	umbonate	White	Brittle	Opaque
Bacillus thuringiensis	Canal soil	CEPS-95	Glistening	Ir regular	Entire	Flat	White	Brittle	Opaque
Bacillus thuringiensis	Garden soil	CEPS-96	Rough	Circular	Entire	Raised	White	Brittle	Opaque
Bacillus thuringiensis	water sample	CEPS-99	Dull	Circular	Entire	flat	White	Mucoid	Opaque
Bacillus thuringiensis	Leafy litter soil	CEPS-100	Glistening	Circular	Entire	Raised	Pale white	Mucoid	Opaque
Bacillus megaterium									
Bacillus megaterium	Green belt	CEPS-40	Glistening	Circular	entire	Convex	White	Butyrous	Opaque
Bacillus megaterium	Decayed leaves	CEPS-65	dull	Circular	Umbonate	Convex	White	Butyrous	Opaque
Bacillus megaterium	Decayed leaves	CEPS-73	Glistening	Circular	Undulate	convex	white	bristtle	Opaque
Bacillus megaterium	PCSIR colony	CEPS-82	Dull	Irregular	Undulate	Convex	White	bristtle	Opaque
Bacillus megaterium	Nursery soil	CEPS-86	Dull	Irregular	Unbonate	Convex	White	bristtle	Opaque
Bacillus megaterium	Leafy litter soil	CEPS-91	Rough	Irregular	Unbonate	Convex	White	bristtle	Opaque
Bacillus megaterium	Garden soil	CEPS-98	Dull	Irregular	Entire	Convex	White	bristtle	Opaque
Bacillus sphaericus									
Bacillus sphaericus	Leafy litter soil	CEPS-15	smooth	Circular	Entire	Raised	Pale white	Butyrous	Opaque
Bacillus sphaericus	Leafy litter soil	CEPS-20	smooth	circular	Entire	Convex	Off-white	Muscoid	Opaque
Bacillus sphaericus	Green belt	CEPS-39	Rough	Circular	Wavy	Flat	Offwhite	Mucoid	Opaque
Bacillus Sphaericus	Green belt	CEPS-42	smooth	Circular	Entire	Convex	Pale white	Butyrous	Opaque
Bacillus sphaericus	water sample	CEPS-47	Rough	Circular	Entire	Flat	Offwhite	Mucoid	Opaque
Bacillus sphaericus	water sample	CEPS-54	smooth	Circular	Entire	Raised	Pale white	Butyrous	Opaque
Bacillus sphaericus	Green belt	CEPS-57	Rough	circular	wavy	flat	offwhite	butyrous	Opaque
Bacillus sphaericus	Leafy litter soil	CEPS-70	Rough	Irregular	Entire	Raised	Offwhite	Butyrous	Opaque
Bacillus sphaericus	Nursery soil	CEPS-75	Rough	Circular	Entire	Raised	Pale white	Mucoid	Opaque
Bacillus circulans									
Bacillus circulans	PCSIR colony	CEPS-50	Glistening	Circular	Wavy	Flat	Offwhite	Mucoid	Opaque
Bacillus circulans	Decayed leaves	CEPS-71	Glistening	Circular	Entire	flat	Offwhite	Mucoid	Opaque
Bacillus circulans	Leafy litter soil	CEPS-87	Rough	Circular	wavy	Flat	Offwhite	Butyrous	Opaque
Bacillus circulans	Leafy litter soil	CEPS-90	Glistening	Circular	Entire	Flat	White	Butyrous	Opaque
Bacillus subtilis									
Bacillus subtilis	Drainage water	CEPS-31	Rough	Irregular	Lobate	umbonate	Offwhite	Brittle	Opaque
Bacillus subtilis	Drainage water	CEPS-32	rough	Irregular	Lobate	Raised	Cream	Brittle	Opaque
Bacillus subtilis	Fc college parking soil	CEPS-37	Rough	Irregular	Lobate	umbonate	Cream	Brittle	Opaque
Bacillus subtilis	PCSIR colony	CEPS-51	Rough	Irregular	Lobate	umbonate	Offwhite	Brittle	Opaque

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Isolate	Sources	Strain names	Colony morphology						
			Colony surface	Colony shape	Colony margin	Elevation	Colony color	Colony texture	Opacity of colony
Bacillus laterosporus									
Bacillus laterosporus	Dead larvae	CEPS-1	Smooth	Circular	Wavy	Raised	White	Brittle	Opaque
Bacillus laterosporus	Soil conditioner	CEPS-9	Glistening	Circular	Entire	Raised	White	Mucoid	Opaque
Bacillus laterosporus	Nursery	CEPS-12	Glistening	Circular	Entire	Raised	White	Mucoid	Opaque
Bacillus laterosporus	Soil conditioner	CEPS-18	Rough	Circular	Wavy	Flat	Off-white	Brittle	Opaque
Bacillus laterosporus	Tap water	CEPS-21	Dull	Circular	Wavy	Flat	White	Mucosid	Opaque
Bacillus laterosporus	Soil conditioner	CEPS-24	Smooth	Circular	Wavy	Flat	off white	Mucoid	Opaque
Bacillus laterosporus	Soil conditioner	CEPS-26	Glistening	Circular	Entire	Flat	White	Mucoid	Opaque
Bacillus laterosporus	Soil conditioner	CEPS-27	Glistening	Circular	Entire	Flat	Offwhite	Butyrous	Opaque
Bacillus laterosporus	Soil conditioner	CEPS-28	Smooth	Circular	Entire	Flat	Offwhite	Butyrous	Opaque
Bacillus laterosporus	Park soil Wapda town	CEPS-29	Smooth	Circular	Wavy	Raised	Offwhite	Butyrous	Opaque
Bacillus laterosporus	Nursery soil	CEPS-55	Smooth	Circular	Entire	Flat	White	Butyrous	Opaque
Bacillus laterosporus	Nursery soil	CEPS-77	Smooth	Circular	Entire	Flat	White	Muscoid	Opaque
Bacillus laterosporus	Nursery soil	CEPS-81	Smooth	Circular	Entire	Raised	White	Butyrous	Opaque
Bacillus laterosporus	Soil conditioner	CEPS-83	Glistening	Circular	Entire	Flat	Offwhite	Butyrous	Opaque
Bacillus alvei									
Bacillus alvei	Honey Bee	CEPS-22	Rough	Circular	Undulate	Convex	White	Butyrous	Opaque
Bacillus alvei	Nursery pot soil	CEPS-76	Rough	Circular	Entire	Raised	White	Butyrous	Opaque
Bacillus alvei	Garden soil	CEPS-94	Rough	Circular	Entire	Convex	Pale	Butyrous	Opaque

lecithinase, while positively with starch hydrolysis. The species could not ferment mannitol (Table 2).

Nine *Bacillus sphaericus* strains were isolated from green belt soil, nursery soil, leafy litter soil and water sample. These were named as CEPS-15, CEPS-20, CEPS-39, CEPS-42, CEPS-47, CEPS-54, CEPS-57, CEPS-70 and CEPS-75. All strains grown on nutrient agar plates appeared after 24 h incubation period. The cells of the species were observed as motile rods with rounded ends presenting singly or in chains. All strains were stained positively. Colonies on nutrient agar were seen off white or pale white with entire or wavy margins. All isolates were opaque having smooth or rough surface with butyrous or mucoid texture (Table 1). The biochemical tests were performed for the identification of species and were observed strict aerobes having positive results for catalase and oxidase. It did not ferment mannitol and glucose but observed positive for starch hydrolysis. They observed to have negative interaction with Voges-Proskauer and Indole and do not produce lecithinase. Spores of *Bacillus sphaericus* isolates were sphaericus and terminal, swelling the sporangia (Table 2).

Four *Bacillus circulans* isolates named as CEPS-50, CEPS-71, CEPS-87, CEPS-90 were obtained from PCSIR Colony soil, leafy litter soil and decayed leaves. Colonies of all strains grown on nutrient agar plates appeared after 24 h incubation period. *Bacillus circulans* cells were seen as rods, arranged singly and in pairs and stained positively. The cultural characteristics recorded were flat butyrous or mucoid colonies with entire or wavy margins and glistening or rough surface. All colonies were opaque having off white or white colour (Table 1). Their cells were motile and spore forming. The colonies of *Bacillus circulans* showed positive characteristics of catalase but not oxidase. It interacted negatively with indole, Voges-Proskauer and lecithinase, while positively with starch hydrolysis. The species fermented glucose and mannitol (Table 2).

Bacillus subtilis colonies in agar plate were present as single cells. It grew in irregular, lobate, raised or umbonate and off-white or creamy pigmented on agar plates. *Bacillus subtilis* strains named as CEPS-31, CEPS-32, CEPS-37, CEPS-51 were isolated from water, PCSIR Colony soil and FC College parking (Table 1). These gram-positive rods were determined to be motile facultative anaerobes having positive results for Catalase and Oxidase tests. Lecithinase test was negative. Acid production from glucose and mannitol was positive. *Bacillus subtilis* isolates were observed positive for starch hydrolysis and Voges-Proskauer but negative for indole production (Table 2).

Fourteen strains were identified as *Bacillus laterosporus* on the basis of morphological and biochemical characteristics. *Bacillus laterosporus* isolates were named as CEPS-1, CEPS-9, CEPS-12, CEPS-18, CEPS-21, CEPS-24, CEPS-26, CEPS-27, CEPS-28, CEPS-29, CEPS-55, CEPS-77, CEPS-81, CEPS-83. The bacterium grew on nutrient agar plates as white or off-white colored rough, dull, glistening or smooth colonies

having wavy or entire margins. All colonies were circular with raised or flat elevation (Table 1). The gram positive rods were determined to be motile facultative anaerobes. The catalase test was positive. Voges proskeur and starch were negative and lecithinase test was positive whereas indole and mannitol test was positive (Table 2). Most *Bacillus laterosporus* strains were isolated from soil conditioner (Table 1).

Three *Bacillus alvei* strains named as CEPS-22, CEPS-76 and CEPS-94 were obtained (two from soil and one from honey bee). Colonies of all three strains were circular and opaque having rough surface and butyrous texture. Colony margins were either undulate or entire with convex or raised elevation (Table 1). The species were biochemically observed to be facultative anaerobes having positive results for catalase and oxidase. These did not ferment mannitol but showed positive results for acid production from glucose and were observed to have positive interaction with Voges-Proskauer starch hydrolysis and Indole and did not produce lecithinase (Table 2).

Evaluation of spore crystal mixtures of isolated bacterial strains for their insecticidal activity against 3rd and 4th instar *Aedes agypti* lavae for 48 h by screening bioassay showed out of 73 isolates only one *Bacillus thuringiensis* species named as CEPS-56 had insecticidal activity against 3rd and 4th instar larvae of *Aedes agypti*. The %age of active isolate against *Aedes agypti* larvae was 1.3%. The isolated *Bacillus thuringiensis* strain with ento-mopathogenic activity was obtained from dead mosquito. It must be emphasized that these tests were qualitative.

Bacillus thuringiensis isolate (CEPS-56) possessing ento-mopathogenic activity against *Aedes agypti* exhibited cultural characteristics such as irregular colony with wavy margin and rough surface. Colonies were cream in color with mucoid texture and umbonate elevation. Spore staining showed the presence of displaced spore and crystal staining showed the presence of single parasporal crystal in active strain (Table 2 and 3). The morphological characteristics were as below:

- Positive result for Voges Proskeur Test.
- Positive Lecithinase Test.
- Positive Catalase Test (Indicated by appearance of bubbles after applying H_2O_2).
- Positive Starch Hydrolysis Test (Indicated by a clear zone around colony).
- Positive result in glucose fermentation test (Change in colour from red to yellow).
- No fermentation of mannitol (no colour change).
- Negative result in Indole test (no red colour ring after adding Kovac's Reagent).
- Positive Motility Test.

The goal of the study was to isolate larvicidal bacteria from the natural soil of Lahore Pakistan and other sources to assess their activity against dengue vector under *Aedes agypti* larvae control program at laboratory level. Most frequently isolated bacteria here were *Bacillus thuringiensis* that had 44% occurrence in soil, decayed leaves, dead mosquito larvae, honey bee and water samples which is in agreement with the results of Balaraman (2005) who reported the isolation of larvicidal strains of *Bacillus thuringiensis* from these sources. Here, although *Bacillus thuringiensis* was found in almost all samples but its occurrence in soil was found to be relatively higher as compared to other samples. This observation is in accordance with the results of Bukhari and Shakoori (2010) who also reported

Table 2. Biochemical characteristics of microbial isolates

Identification tests	*Bacillus thuringiensis*	*Bacillus laterosporus*	*Bacillus sphaericus*	*Bacillus subtilis*	*Bacillus alvei*	*Bacillus megaterium*	*Bacillus circulans*
Gram staining	Gram positive	Gram positive	Gram positive	Gram positive	Gram positive	Gram positive	Gram positive
Spore staining	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Shape	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Motility	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Indole	Negative	Positive	Negative	Negative	Positive	Negative	Negative
Voges proskeur	Positive	Negative	Negative	Positive	Positive	Negative	Negative
Mannitol fermentation	Negative	Positive	Negative	Positive	Negative	Negative	Positive
Starch agar test	Positive	Negative	Positive	Positive	Positive	Positive	Positive
Catalase test	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Lecithinase test	Positive	Positive	Negative	Negative	Negative	Negative	Negative

relatively higher occurrence of *Bacillus thuringiensis* from soils of Pakistan as compared to the other samples but these results are in contradiction with those presented by Theunis *et al*. (1998) who reported 14% and 5%, incidence of *Bacillus thuringiensis* positive soil samples, respectively.

The isolated bacteria were identified as *Bacillus megaterium*, *Bacillus circulans*, *Bacillus alvei*, *Bacillus subtilis*, *Bacillus sphaericus*, *Bacillus laterosporus* and *Bacillus thuringiensis* but in preliminary bioassay out of 73, isolated bacteria only one isolate was found active against third instar *Aedes agypti* larvae. So, the percentage of active isolates was 1.4%. Same percentage of active bacterial isolates was reported by Foda *et al*. (2013) and Didamony (2014). A brief review of percentage of active bacterial isolates obtained in various studies is given in Table 3.

During a recent study, *Bacillus megaterium* isolates did not cause mortality in *Aedes agypti* larvae until 48 h. The results of this study are in favour of previous studies which reveal that *Bacillus megaterium* is ineffective against third instar *Aedes aegypti* larvae. The toxicity was not observed even after 48 h of incubation time (Walther *et al*., 1986). On the other hand our results are in disagreement with the findings of Radhika *et al*. (2011) which revealed toxic *Bacillus megaterium* strain with LC 90 value of 4.1±0.39 mg/mL causing 97% mortality of *Aedes agypti* larvae in 48 h. England *et al*. (1997) found that the toxic protein produced by some mutant type strains of *Bacillus megaterium* was effective against mosquito species (Table 3).

Bacillus laterosporus is an aerobic spore-forming bacterium with canoe shaped sporulating cells that distinguishes it from other spore formers (Favret and Yousten, 1985). Orlova *et al*. (1998) reported that crystal producing strains of *Bacillus laterosporus* had high toxicity to mosquito larvae and their toxicity level could be as high as that of *Bacillus thuringiensis*. During a recent study, the reason for the lack of insecticidal activity by *Bacillus laterosporus* isolates could be due to the absence of crystal formation during sporulation by the isolated strains. Favret and Yousten (1985) suggested that *Bacillus laterosporus* had less bio-control potential as compared to *Bacillus thuringiensis*.

Bacillus sphaericus is a naturally occurring soil bacterium that has been used as a biological control agent against insects. *Bacillus sphaericus* strain 2362 isolated from Simulium larvae has been demonstrated to have toxicity against *Culex larvae* (Cavados *et al*., 2001). *Bacillus sphaericus* was used to control *Culex pipiens, Anopheles stephensi* and *Culex quinquefasciatus* mosquito larvae (Surendran and Vennison, 2011). Here, isolated *Bacillus sphaericus* did not prove toxic to 3rd and 4th fourth instar *Aedes agypti* larvae but its strains reported as more efficient in controlling *Culex* mosquitoes (Barbazan *et al*., 1997).

Bacillus subtilis is a predominant endospore forming bacterium commonly recovered from soil, water, air and decomposing plants. In present study, none of the *Bacillus subtilis* isolates proved toxic to the *Aedes agypti* larvae. This observation was noticed after 48 h of infesting larvae with spore crystal mixture of *Bacillus subtilis*. The result is in disagreement with the study carried out by Geetha *et al*. (2010) in which the culture supernatant of a strain of *Bacillus subtilis* isolated from mangrove forests was found toxic to larvae and pupae of mosquitoes. Das and Mukherjee (2006) demonstrated that *Bacillus subtilis* secreted cyclic lipopeptides that had the larvicidal activity.

Table 3. Percentage of bacterial isolates active against mosquito in various studies

Source	No. of isolates	No. of Active isolates	Percentage of active isolates	References
Soil sample	881	13	1.40%	González *et al*. (2012)
Soil sample	1154	7	0.60%	Baruah *et al*. (2008)
Soil sample	384	5	1.30%	Didamony (2014)
Soil sample	210	6	2.80%	Monnerat *et al*. (2005)
Soil sample	88	2	2.20%	Yasutake *et al*. (2007)
Soil sample	64	1	1.50%	El-kersh *et al*. (2011)
Soil sample + animal waste + dry wheat straw	470	6	1.20%	Bukhari and Shakoori (2010)
Soil sample	359	5	1.30%	Foda *et al*. (2013)
Soil samples	484	6	1.20%	Soares-da-Silva *et al*. (2015)

Darriet and Hougard (2002) isolated a strain of *Bacillus circulans* from larvae of *Culex quinquefasiatus* which was toxic to *Aedes agypti* larvae during sporulation stage whereas in present study *Bacillus circulans* isolates did not cause mortality in *Aedes agypti* larvae. Another study demonstrated that *Bacillus circulans* was poorly toxic against larvae of *Aedes albopictus*, *Culex quniquefasiatus* and *Anopheles stephensi.*

Bacillus thuringiensis is a spore forming soil bacterium that shows unusual ability to produce different kinds of endogenous crystalline protein inclusions during its sporulation which contribute to its insecticidal properties (Jisha *et al*., 2013). During bioassay, only one *Bacillus thuringiensis* isolate showed toxicity against third instar *Aedes agypti* larvae and that toxic isolate was recovered from dead mosquito larvae. Cavados *et al*. (2001) also reported the isolation of strain of *Bacillus thuringiensis* from a dead insect that had toxicity against *Aedes agypti* larvae. Bioassay results here showed that the mortality rate gradually increased with the incubation time. CEPS-56 caused 25% mortality after 24 h and 50% mortality after 48 h of incubation. Similar trend of mortality of *Aedes agypti* larvae and incubation time was observed by Radhika *et al*. (2011).

The study was focused on the search of the presence of native bacterial isolates from the natural environment of Lahore active against *Aedes agypti*. Out of seventy three bacterial isolates obtained from different soil samples collected from Lahore and from dead mosquito larvae, water and soil conditioners samples, only single isolate proved toxic against *Aedes agypti* which was identified as *Bacillus thuringiensis*.

The results lead to the conclusion that ento-mopathogenic bacteria are present in natural habitats of Lahore and screening of more number of samples can yield different and even more toxic strains of bacteria. This study just reflects the identification of toxic bacterial strain (CEPS-56) which should be further investigated at the molecular level and effective toxic concentration of CEPS-56 should be determined by conducting concentration bioassay against *Aedes agypti* larvae.

The study recommends the following tracks for extension of the project

- Soil samples from different geographical regions of Pakistan should be extensively screened for the isolation of native entomopathogenic strains of bacteria against *Aedes agypti.*
- Isolated species should be studied at molecular level to identify the isolated strains at the subspecies level. Final evaluation should be based on field application to prevent significant economic loss.

Conflict of Interest. The authors declare no conflict of interest.

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