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Full Length Research Paper

Virulence of *Bacillus cereus* as natural facultative pathogen of *Anopheles subpictus* Grassi (Diptera: Culicidae) larvae in submerged rice-fields and shallow ponds

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Out of 4407 *Anopheles subpictus* larvae collected from submerged rice-fields and shallow ponds, 1412 were found to be unhealthy and 2.8% of unhealthy larvae were naturally infected by *Bacillus cereus*. *B. cereus* formed circular, white and flat colonies. Bacteria were gram positive, ellipsoidal/oval spore forming aerobic rods. Although the isolate was positive for catalase, urease, gelatinase, lipase, nitrate reduction and H₂S production, it was negative for indole production, Vogues-Proskauer test, oxidase test and acid/gas production from carbon sources. Through biochemical characterization and fatty acid methyl ester (FAME) analysis, the bacterial isolate was identified as *Bacillus cereus*. In the laboratory condition, *B. cereus* suspension resulted in 43.57% and 93.78% death of *A. subpictus* larvae within 3 and 6 h, respectively. The organisms were sensitive to recommended doses of kanamycin, gatifloxacin, gentamycin, levofloxacin, doxycyclin, tetracyclin, streptomycin, rifampicin, vancomycin, ciprofloxacin, but found resistant to ampicillin.

Key words: *Bacillus cereus*, fatty acid methyl ester analysis, scanning electron micrograph, biochemical characterization, pathogen, *Anopheles subpictus* larva.

INTRODUCTION

Anopheles subpictus Grassi 1899 is the most abundant anopheline in most parts of the Indian subcontinent (Rao, 1984) having widespread distribution eastwards to New Guinea (Suguna et al., 1994; Cooper et al., 2006), westwards to Iran, northwards to China and southwards to East Timor. It has been recognized as a vector of malaria (Panicker et al., 1981; Chatterjee and Chandra, 2000), west Nile virus in Asia (Manson-Bahr and Bell, 1991) and bancroftian filariasis (Manson-Bahr and Bell, 1991; Thenmozhi et al., 2006). *Bacillus* and *Pseudomonas* spp. are the most potent pathogens of mosquitoes (Lacey, 1997; Porter et al., 1993; Krattiger, 1997; Cooping and Menn, 2001; Wirth et al., 2004; Teng et al., 2005;

Chatterjee et al., 2008; Dangar, 2008). *A. subpictus* larvae were found to be abundant in submerged rice-fields and shallow ponds of Hooghly district of West Bengal, India. A part of larval population collected from shallow ponds and submerged rice-fields were found to be unhealthy and checked for bacterial infection to assay the natural virulence of bacteria against *A. subpictus* (Grassi) larvae. The present study was aimed to detect the cause of death and to characterize the pathogen of *A. subpictus* larvae to assess the impact on the environment.

MATERIALS AND METHODS

Bacteria isolation and characterization

A. subpictus larvae were collected from the submerged rice fields and shallow-ponds of Hooghly district, West Bengal, India and

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Table 1. Population of *A. subpictus* larvae invaded by *B. cereus* and other bacteria at two breeding habitats.

Breeding habitat	Larval instar	Collected larvae	Number of unhealthy larvae	<i>B. cereus</i> (Ts-25) infection (%)	Unidentified bacterial infection (%)
Shallow pond	2nd	770	155	2.58	0.64
	3rd	450	137	2.91	0.72
	4th	380	214	2.80	0.93
Submerged Rice-field	2nd	845	280	3.21	0.35
	3rd	975	276	2.89	0.72
	4th	987	350	2.28	0.85
Total/average*		4407	1412	*2.8	*0.70

brought to the Parasitology and Microbiology Laboratory, Department of Zoology, The University of Burdwan. It was recorded that a broad collar of dark chitin at the base of the head of younger instars decreased in width during further molting. This collar character was used to isolate fourth-instars from early stages. Although fourth instars had equal number of hairs as found in 3rd instars, the branch number of branched hairs was found to be high. The dead, paralyzed, moribund, irritable/sluggish, fragile, putrefied, blackish and fluid oozing larvae were recorded as the unhealthy insects (Poinar and Thomas, 1984). The moribund larvae were surface-sterilized by 70% ethanol (5 min) and washed three times with sterile distilled water. The gut was dissected out under the laminar air flow, the content was aspirated with a sterile syringe, diluted up to 10^{-2} level with sterile distilled water and pour-plated on 5 plates at 100 μ l/100 ml with nutrient agar (g/l: peptone 5, beef extract 3, agar 3, pH 7) media (Poinar and Thomas, 1984; Lacey, 1997). The predominant bacterial colonies obtained from the media were isolated, purified and characterized following morphological, physiological and biochemical characters following standard methods (Pelczar, 1957; Sneath, 1986; Lacey, 1997). Antibiotic sensitivity test was done with standard antibiotics discs following Brown (2007). The bacterial isolates were identified both phenotypically (Sneath, 1986) and on the basis of gas chromatographic (GC) analysis of extracted fatty acid methyl esters (MIS, MIDI Sherlock @ USA).

Preparation of scanning electron micrograph of bacterial smear

Smear preparation of bacterial suspension was done on a cover glass and heat fixed over a flame for 1 - 2 s followed by 2.5% glutaraldehyde (aqueous) for 45 min. The slides were then dehydrated passing through 50%, 70%, 90% and finally with absolute alcohol for 5 min each. The specimens were gold coated and observed under a scanning electron microscope (SEM).

Fatty acid methyl ester (FAME) analysis

For the identification of microorganisms, microbial Identification system (MIS, MIDI Sherlock @ USA) for fatty acid methyl ester (FAME) analysis is a standard method. Whole cell fatty acids were converted to methyl esters and analyzed by gas chromatography. The fatty acid composition of the bacterial isolate was compared to a Sherlock Library of known organisms in order to find the closest match.

Toxicity test

To determine the toxicity of *B. cereus* against *A. subpictus*, the

bioassay tests were carried out at $35 \pm 2^\circ\text{C}$ using 100 larvae (late third instar) kept in 1000 ml water in glass bowls. The larvae were exposed to the dose of 5 ml of *B. cereus* suspension (5.9×10^6 bacteria/ml) per liter of water. Each test was replicated three times along with a control and the mortality (%) was determined with the following Abbott's formula:

$$\text{Mortality (\%)} = \frac{(\% \text{ mortality in the experiment}) - (\% \text{ mortality in control})}{100 - (\% \text{ mortality in control})} \times 100$$

RESULTS AND DISCUSSION

Altogether, 4407 *A. subpictus* larvae were collected from submerged rice-fields and shallow ponds out of which, 1412 were found to be unhealthy and 2.8% of unhealthy larvae were naturally infected by *B. cereus*. The bacterial infection to different larval instars of *A. subpictus* is shown in Table 1. More infection caused by *B. cereus* (Ts-25) suggests that they would be the potent pathogens but limited infection caused by the unidentified bacteria suggest that it would be a secondary invader (Lacey, 1997; Dangar, 2008). The bacteria (Ts-25) formed circular, white and flat colonies. The bacteria were gram positive ellipsoidal spore (Plate 1) forming aerobic rods measuring $3 - 3.5 \times 1 - 1.23 \mu\text{m}$. The organisms were positive for catalase, urease, gelatinase, lipase, nitrate reduction and H_2S production but negative for indole production, Vogues-Proskauer test, oxidase test and acid/gas production from carbon sources (Table 2). The organisms were sensitive to recommended doses of kanamycin (30 $\mu\text{g}/\text{disc}$), gatifloxacin (10 $\mu\text{g}/\text{disc}$), gentamycin (10 $\mu\text{g}/\text{disc}$), levofloxacin (5 $\mu\text{g}/\text{disc}$), nalidixic acid (30 $\mu\text{g}/\text{disc}$), ofloxacin (5 $\mu\text{g}/\text{disc}$), doxycyclin (30 $\mu\text{g}/\text{disc}$), tetracycline (30 $\mu\text{g}/\text{disc}$), streptomycin (10 $\mu\text{g}/\text{disc}$), rifampicin (5 $\mu\text{g}/\text{disc}$), vanomycin (30 $\mu\text{g}/\text{disc}$) and ciprofloxacin (5 $\mu\text{g}/\text{disc}$), but resistant to ampicillin (10 $\mu\text{g}/\text{disc}$) (Table 2). Through the study of the phenotypic characters, biochemical properties and FAME analysis (Figure 1) (Library: RTSBA66.00, Sim-index-0540), the bacterial isolate (Ts-25) was identified as *B. cereus*-Gc subgroup B. In the laboratory condition, *B. cereus* suspension resulted to 43.57% and 93.78% death of *A.*

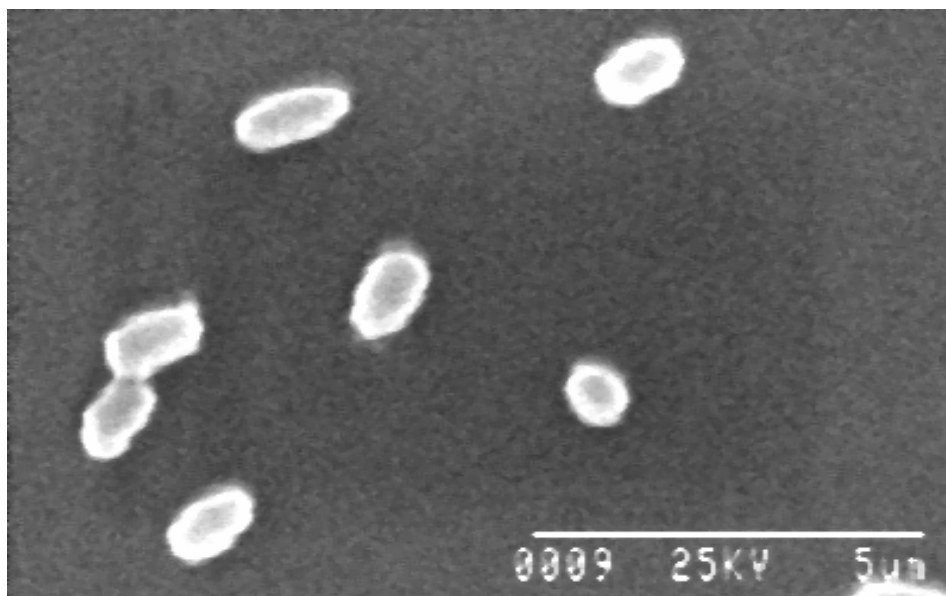


Plate 1. Electrons scan micrograph of spores of bacterial isolate (Ts-25).

Table 2. Phenotypic and biochemical characterization of the bacteria (Ts-25) isolated from the midgut of *A. subpictus* Grassi.

Character	Observation	Character	Observation
Colony character	Circular, Off-white, Flat, Entire	Urease production test	+
Bacterium (l x w, μm)	Rod shaped (3-3.5 x 1—1.23)	Oxidase	-
Spore (Dia., μm)	Ellipsoidal	H ₂ S Production Test	+
Spore position	Central	Citrate Test	+
NaCl tolerance	Upto 8%	Gelatinase	+
Acid and gas production	-	Casein hydrolysis	+
Catalase	+	Amylase	+
Indole production	-	Lipase	+
Methyl red test	+	Nitrate reduction test	+
Vogues-Proskauer test	-	Antibiotic sensitive (μg/disc)	Kanamycin (30)
Antibiotic resistant (μg/disc)	Ampicillin (10)		Gatifloxacin (10)
			Gentamycin (10)
			Levofloxacin (5)
			Nalidixic acid (30)
			Ofloxacin (5)
			Doxycycline (30)
			Tetracycline (30)
			Streptomycin (10)
			Rifampicin (5)
			Vancomycin (30)
			Ciprofloxacin (5)

subpictus larvae within 3 and 6 h, respectively. No larval mortality was recorded in control experiment during the 6 h. The results show that *B. cereus* is a natural facultative mosquito pathogen (Krattiger, 1997; Cooping and Menn, 2001; Wirth et al., 2004; Teng et al., 2005, Chatterjee et

al., 2008). *B. cereus* strains are able to colonize in the guts of the mosquito larvae (Plearnpis et al., 2001). Insecticidal activity of spores of *B. cereus* against *Aedes aegypti* has been determined (Dana et al., 1981). So, the present study is significant and depicts the role of *B. cereus*

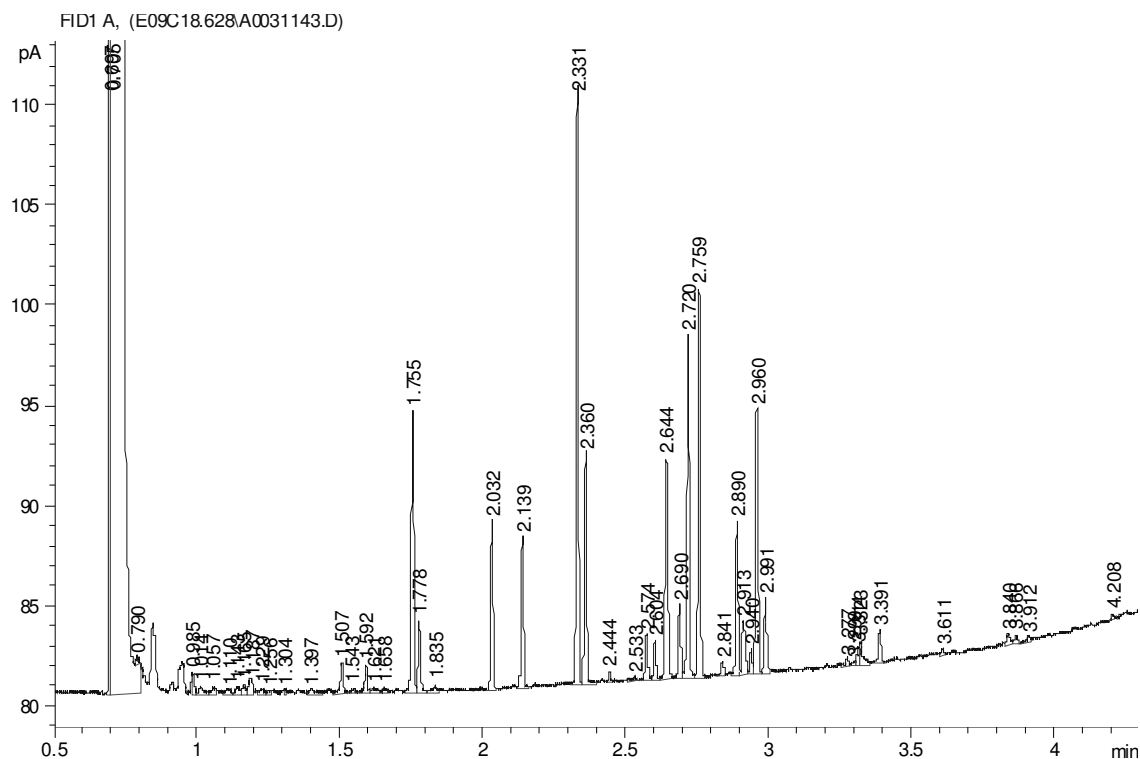


Figure 1. FAME analysis of bacterial isolate (Ts-25).

as a facultative pathogen of *A. subpictus* Grassi larvae in the natural environment.

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