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A new Bacterial strain *Streptomyces indiaensis* (LMG 19961) and its larvicidal and Histopathological effect against *Anopheles stephensi:* A Malaria Mosquito

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Abstract

The present study was carried out to establish larvicidal activity (LC_{50} determination) and mode of action of a soil bacteria Streptomyces indiaensis strain (LMG 19961) by taking midgut as a target tissue against third instar larvae of Anopheles stephensi (L.), the vector of malaria. LC_{50} of Streptomyces indiaensis was calculated by biotoxicity test. The LC_{50} (1.645 cfu/ml) treated third instar larvae showed dose dependent effect on mortality. The histoarchitecture of midgut revealed weak and disoriented epithelial cells with larger intercellular spaces and disruption of junctional complexes. The lysed gastric caecal cells were also noticed and reduced lumen further revealed the severity of bacterial strain against mosquito larvae. The peritrophic membrane was dislodged from the place. Since this strain of Streptomyces indiaensis was reported first time in our laboratory (NCBI Accession number KJ170314) the probability of ecosafe vector control agents from soil bacteria is quite encouraging.

Keywords: *Streptomyces indiaensis, anopheles stephensi,* histopathology, vector control.

Introduction

Throughout the world, mosquitoes are responsible for the transmission of several human diseases. WHO has described the mosquito as "public enemy number one" and reported mosquito borne diseases across globally infecting more than 700,000,000 people every year. In Indian scenario the data is alarming and gives that about 40,000,000 individuals are affected by this arthropod vector per year.

In South East Asia, the second most affected region in the world, India has the highest malaria burden (with an estimated 24 million cases per year), followed by Indonesia and Myanmar[1]. Historically, the highest incidence of malaria in India occurred in the 1950s, with an estimated 75 million cases and 0.8 million deaths. An estimated 6, 55,000 persons died of malaria in 2010 and the victims were mostly children under five years of age and major cases of malaria death occurred in African region[2].

Mosquitoes have a cosmotropical distribution between 30^0N and 20º S[3,4] and exhibit a distinct preference for human habitats, including artificial oviposition sites, e.g., tires, flower vases and water storage containers[5]. In the Kingdom of Saudi Arabia, different types of mosquito vectors spread all over the country[6-10].

Mosquito control is a necessary measure to improve environmental quality and public health. The controlling strategies are largely by synthetic chemical substances. The chief anti mosquito insecticides are organochlorine and organophosphate compounds. The spray or application of chemicals can pose many environmental sustainable problems such as harmful effect to human beings, toxic to non-target organisms and the nature of non-degradable compositions. Besides, the pesticidal residues enter into ecosystem, through food web it circulates and biomagnifies[11]. Biological control of immature stages now appears to be the most powerful tool of attacking target population. Microbial biopesticdes such as bacteria and fungi are widely used to overcome undesirable effect of synthetic chemicals[12].

The present study is an attempt to observe the LC_{50} treated third instar larvae of *Anopheles stephensi* (L.) by *Streptomyces indiaensis*. The insect midgut is the site of nutrient uptake and is the first line of defense against ingested pathogens and toxins. Further, midgut of mosquitoes is able to carry out major physiological processes like synthesis, secretion, absorption and transport[13]. It is the first tissue that parasite contacts during its migration hence, it was logically thought to take up this part to evaluate the effect of this potential bacterial biopesticide. The present study could be as important step towards the use of this newly discovered bacterial strain from soil as a potent means of mosquito control.

Material and Methods

Rearing of *Anopheles stephensi* (L.): Mosquito used in our research protocol is *Anopheles stephensi*. The larvae of *Anopheles stephensi* were collected from field then they were

reared as per WHO protocol[14] for several generations, in the insectary at the Department of Zoology, University collage of Science MLSU Udaipur, under controlled conditions at temperature of 27 ± 2°C, relative humidity 70 ± 10% and 12-12 light-dark regime. Larvae were fed daily with dog biskit and yeast powder in 3:1 ratio. This allowed them to reach maturity stage and females were offered blood meal for egg laying. Eggs laid on wet filter papers were transferred to water trays. Larvae were fed and sorted according to their developing stages first, second, third, fourth and pupa for bioassays. Freshly emerged third instar larvae were considered for the present study.

Bioassay and larval mortality: Larvicidal activity of *Anopheles stephensi* was assessed by using the standard method[15]. In brief, twenty 3rd instar larvae were taken and treated with *Streptomyces indiaensis* (strain LMG 19961). Similarly the uninoculated culture medium was used as control. The serial dilutions were done and doses were determined accordingly (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 of $6.5x10^9$cfu/ml). For each dose four replicates were maintained at a time. Additionally all the assay units were supplemented with larvae fed with a diet of finely ground brewer's yeast and dog biscuits (3:1) ratio. The LC_{50} value was calculated after 48 h by probit analysis[16].

Histological Studies: Third instar larvae treated with LC_{50} of *Streptomyces indiaensis* were considered for histopathological studies. Standard histopathological protocol was adopted. Only live larvae were examined. Then they were fixed in bouins solution for 24 h. After dehydration in a graded ethanol series, the material was embedded and cut with knives in a rotary microtome. Six microne thick sections were cut and stained in haematoxylin-eosin stain. Tissues were observed microscopically to prepare complete histomicrograph of midgut of third instar larvae of *Anopheles stephensi* (L) in control and treated stage.

Results and Discussion

When third instar larvae were treated with different concentrations 1.0, 1.5, 2.0, 2.5, $3.0\times6.5\times10^9$ cfu/ml of *Streptomyces indiaensis* the mortality trend was fluctuating. Higher doses depicted significant mortality as compared to middle and lower doses.

The values of mean mortality and standard deviation were 12.00±1.00, 12.67±4.73, 14.00±2.00, 20.67±4.73, 27.67±0.58 after 48 hours. The same observation after 48 hours for control was 4.40±0.55 (Table 1). Value of percent mortality at lower dose concentration of $1.0\times6.5\times10^9$ cfu/ml was 40.00 after 48 hours but when dose level increased from 1.5, 2.0, 2.5, $3.0\times6.5\times10^9$ cfu/ml mortality increased from 42.22, 46.67, 68.89, 92.22 after maximum treatment time of 48 hour. Present mortality in control group was 14.67 after 48 hours (table-1).

The probit equation for the present data was (probit = -1.128+0.686). The Medan lethal concentration LC_{50} value for the third instar larvae of *Anopheles stephensi* was 1.645 c.f.u/ml and LC_{90} value was 3.513 c.f.u/ml after 48 hour. When the Chi-square test conducted for the same data the value was X^2= 5.192 at df =4 which is non significant.

Table-1
Biotoxicity of *Streptomyces indiaensis* on the third instar larvae of *Anopheles stephensi* (L.)

	Mean Mortality ± Standard Deviation		Percent Mortality	
Dose Concentration	**24 hrs**	**48 hrs**	**24 hrs**	**48 hrs**
0.00	1.60 ± 0.55	4.40 ± 0.55	5.33	14.67
1.00	5.33 ± 0.58	12.00 ± 1.00	17.78	40.00
1.50	6.00 ± 1.73	12.67 ± 4.73	20.00	42.22
2.00	5.67 ± 0.58	14.00 ± 2.00	18.89	46.67
2.50	9.33 ± 1.53	20.67 ± 4.73	31.11	68.89
3.00	12.33 ± 1.15	27.67 ± 0.58	41.11	92.22

Probit Analysis: Probit Equation: Probit = -1.128 + 0.686 (Dose)

Table-2
Chi Square

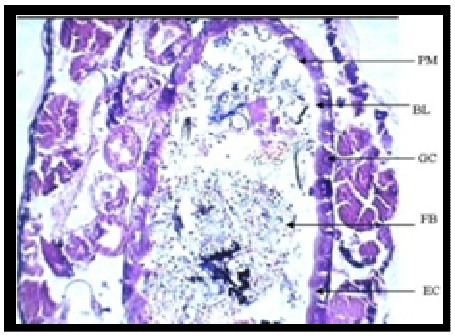
Chi-Square	Df	Result
5.192	4	NS

Table-3
Lethal Concentration

	Dose Concentration	95% CI	
		Lower Limit	Upper Limit
LC_{50}	1.645	1.334	1.945
LC_{90}	3.513	3.001	4.448

Histoarchitecture of midgut of *Anopheles stephensi* (L.) (Normal): The observations were taken in a series of cross sections of midgut tissues. The body section of *Anopheles stephensi* (L.) of third instar larvae passing through midgut normally revealed that midgut contains single layer epithelial cells dividing the whole body into two distinct sides. On outer side the midgut cells are bound with basement membrane or basal lamina facing the body cavity towards cuticular wall. This side is called as haemolymph side and the other side (inner) of midgut epithelium faces the gut lumen called as luman side (figure-1A). Both sides differ in morphology. The haemolymph side contains sections of trachea, muscles, fat bodies and connective tissues, whereas, towards the luman side, the

epithelial cells consist of columnar cells with cluster of small regenerative cells (figure-1A). The lumen is filled with food so it is called food bolus[17].The entire midgut is divided into two regions i.e. anterior midgut (figure-1B) and posterior midgut (figure-1C)



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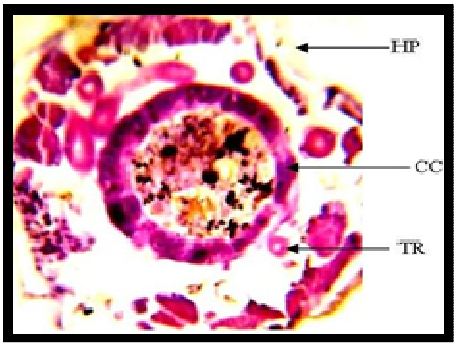

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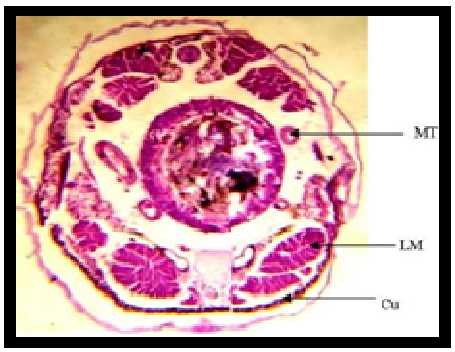

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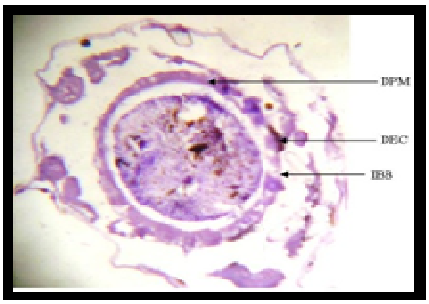
Figure-1
Histoarchitecture of whole body section passing through midgut of third instar larvae of *Anopheles stephensi* (L.) (Normal)

A: Whole Body Section: PM: Peritrophic Membrane; BL: Basal Lamina; GC: Gastric Caeca; FB: Food Bolus; EC: Epithelial Cells. **B:** Anterior Midgut: MT: Malphigian Tubule; LM: Longitudinal Muscles; Cu: Cutical. **C:** Posterior MIdgut: TR: Tracheole; HP: Haemolymph Part; CC: Columnar Cells.

Histology treated larvae showing anterior midgut and gastric caeca: The anterior midgut included flatter cells with clear cytoplasm. Well developed peritrophic membrane and lumen were also observed. On the haemolymph part, epithelial cells are seen to be highly bound with basal lamina (figure-1B). The anterior midgut is surrounded by gastric caeca at its anterior part and is divided into proximal and distal part by caecal membrane[18]. There are eight caecal chambers in mosquito midgut[19]. Gastric caeca consists of well developed layer of epithelial cells with ovoid nucleus in the centre of the cells. Brush border are normal in structure (figure-1A). When the treated larvae were observed for the mode of action analysis (histological) midgut was badly affected part. The epithelial part was swollen and vacuolated. The brush border was irregularly disposed. Gradually the epithelial cells started detaching with a blabbed tip. The peritrophic membrane was degenerated showing the infiltration into the lumen (figure-2A and B). The gastric caeca was observed with, serious morphological damage. The epithelial cells of gastric caeca were damaged severely. At places, the midgut epithelium was seen to be out pocketed in the gastric caeca mixing the two contents. Whole central area was seen occupied by rotten cellular mass. (figure-2C)



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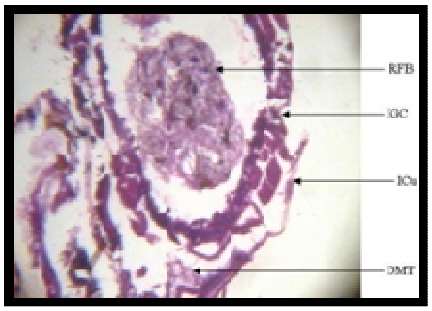

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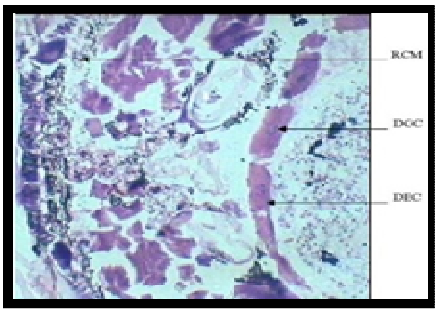

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Figure-2
Histoarchitecture of whole body section passing through anterior midgut of third instars larvae of Anopheles *stephensi* (L.) treated with LC_{50} of *Streptomyces indiaensis*

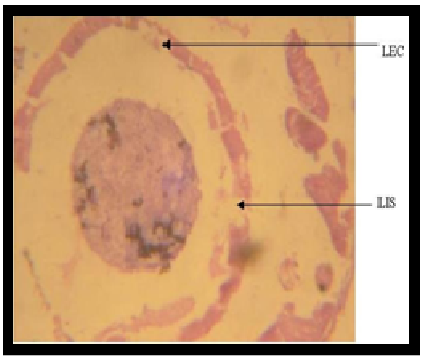
A: DPM: Degenerated Peritrophic Membrane; DEC: Disintegrated Epithelial Cells; IBB: Irregular Brush Border. **B:** RFB: Reduced Food bolus; ICG: Intercellular Gap; ICu: Infected Cuticle; DMT: Degenerated Muscular Tissue. **C:** RCM: Rotten Cellular Mass; DGE: Damaged Gastric Caeca; DEC: Disintegrated Epithelial Cells.

Histology of treated larvae showing posterior midgut: Next part of midgut is posterior part with dark cells showing normal intercellular contacts along with whole lateral plasma membrane, normal basal lamina and other structures as observed in control section. Epithelial cells have well defined ovoid nuclei at the centre of the cells. Well defined peritrophic membrane and brush border with apical extensions of epithelial cells are seen in normal cross section (figure-1C).

When treated with LC_{50} of *Streptomyces indiaensis*, dramatic changes were observed in the posterior mid gut tissues of *Anopheles stephensi* (L.) affecting mainly the gut epithelium.

The cells began to swell via a slight vacuolization with disorganized, shortened and confluent microvillai membrane (figure-3A). There is a slight hypertrophy of epithelial cells in apical regions of treated larvae were seen. Some cells do not have a nucleus. They have a very low volume of cytoplasm. Cytoplasm co-exists with hypertrophied cells. The very broad intercellular spaces are noted between the various cells. The inter-cellular space gives to the epithelium a non-uniform appearance. In some cases, cellular degeneration is accompanied by the disruption of the midgut (figure-3B). Other cytological abnormalities of the midgut included cellular swelling accompanied by cellular necrosis. The location of all detached cells is between epithelium and peritrophic membrane (figure-3C). Treated larvae show a gradual disappearance of cellular organelles. The cytoplasm is less homogeneous. There is a significant vacuolization and the cytoplasm lost was observed (figure-3C)

A: LEC: Lysis of Epithelial cells; LIS: Large Intracellular Space, B: CL: Cellular Lysis; ICu: Infected Cuticle, C: Large Vacuole; DOP: Damaged Outer Part



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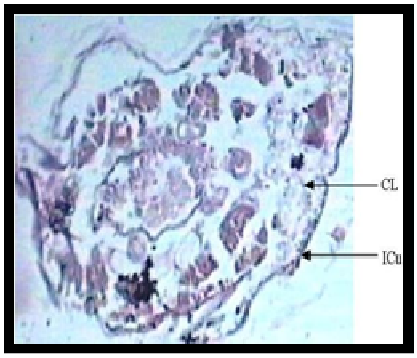

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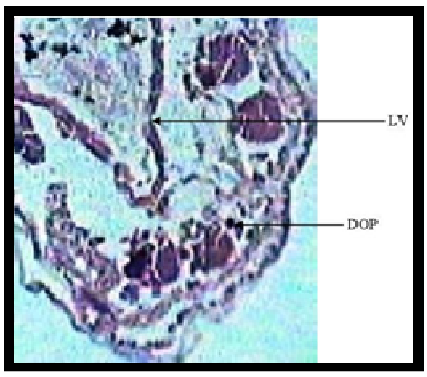

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Figure-3
Histoarchitecture of whole body section passing through posterior midgut of third instar larvae of *Anopheles stephensi* (L.) trated with LC_{50} of *Streptomyces indiaensis*

Discussion: The use of bacterial agents in controlling vector borne diseases has raised several concerns as to whether these microorganisms are highly effective, environmentally safe, non-toxic, and exert selective effects. Among the many tested bacteria, *Bacillus thuringiensis* (*Bti*) and *Bacillus sphaericus* (*Bs*) are the most promising bacterial larvicidal strains in malaria vector control.

Bacillus strains are cheap, can be locally manufactured, easily handled, and practically applied[20]. Compared to chemical insecticides, *Bti* and *Bs* showed faster spreading abilities. Within five years of their discovery, these bacterial strains rapidly colonized Europe and Africa, and methodically participated in routinely applied large-scale mosquito control operations in these regions[21].

The insect midgut is the site of nutrient uptake and is the first line of defense against pathogens and toxins. It is further the largest epithelial organ system and is the main site of uptake of ingested ions, water and nutrients[22]. Further the midgut is the main target organ for many xenobiotics which include not only dietary substances but also pathogens[23]. This is not surprising owing to major role of midgut in absorption.

All the above observations indicated that bacterial preparation has profound effect on the insect tissues. There are many reports available stating the toxic protein present in bacteria affecting the insect tissue.

Histopathological study revealed that *Streptomyces indiaensis* (strain LMG19961) treatment brought about a massive disintegration to the midgut epithelial tissues in *Anopheles Stephensi* larvae as compared to control. This disintegrated of midgut epithelial layer might be the main reason of cessation in feeding by 6h post-treatment then septicemia and finally death at 24h post-treatment as previously recorded in case of *B. thuringiensis*[24].

It has been reported that endotoxins of bacteria are capable of translocating a catalytic domain into the host cells and inhibiting protein synthesis by the ADP-ribosylation of cellular elongation factor[25]. The mode of action of *Streptomyces indiaensis* could have been occurred in the same manner as described for *B. thuringiensis*[26,27].

In our observations changes were seen in the anterior and posterior regions of the midgut, included separation of the epithelial cells from the basement membrane with damage of the peritrophic membrane. The mixing of the gut contents with the haemolymph caused the larval mortality.

Further histopathological studies of gastric caeca were also important because of the fact that this part of the midgut is directly in contact with the toxic elements[28]. Whereas the normal gastric caeca were with well developed epithelial cells and nuclei, the treated larvae of *Anopheles stephensi* (L.) revealed the sign of intoxication slowly proving the toxicity of bacteria right from the anterior most part of midgut.

The gastric caeca revealed total degeneration. The epithelial cells were destroyed with seriously damaged cellular components of midgut wall. At places out pocketing of midgut thereby mixing of whole contents were also observed. Maximum alkalization was observed in anterior part of midgut. The damage of these areas of gastric caecae may be due to the ambient pH for bacterial action.

The midgut is that part of alimentary canal that secretes digestive enzymes and absorbs nutrition. Infection of bacteria blocked both of these vital phenomenons of the insects. This was evident by loss of microvilli and brush border in the treated larvae. The well defined lining of brush border in control suggested physiologically active cells involved in both secretion and absorption of food[29]. These features lost gradually in treated insects. The important peritrophic membrane of posterior midgut revealed complete damage and mixing of gut contents and midgut lysed cellular mass were apparent at last stage of infection. A peritrophic membrane separates food from midgut epithelium[30].

This article is the first report of the histopathological effects of the *Streptomyces indiaensis* (Strain LMG19961) on the midgut of *Anopheles stephensi* larvae and the data obtained may contribute for better understanding the mode of action of this bacterial strain used as bio-insecticide against malaria vector.

Conclusion

Mosquito borne diseases are extensively spreads in the world population and influenced the global economy also. Consequently it should be eradicated from the world through the usage of the non-polluted mosquitocidal agents like microbial metabolites. *Streptomyces indiaensis* exhibited its effect against mosquito *Anopheles stephensi*. So it can be used as, an alternative insecticides because they are free from harmful effects on the environment. Further studies needed for identification the active compounds that can be used in broad spectrum for controlling insects and also determination the mode of action of these compounds.

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