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Lysinibacillus fusiformis (*Bacillaceae*) isolated from Red Soil Reported for the First Time as Effective in Control of Mosquito Vectors

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ABSTRACT: Mosquitoes are the primary vectors for the transmission of human diseases, like dengue, chikungunya, and Zika transmitted by *Aedes* mosquitoes. *Culex* mosquitoes are vectoring pathogens for the diseases like lymphatic filariasis, West Nile virus, and Japanese encephalitis. Malaria is transmitted by *Anopheles* sp. Microbial control offers an alternative to chemical pesticides that have detrimental effects on the ecosystem. *Bacillus thuringiensis* is the most widely deployed mosquitocidal bacteria, offering sustainable alternatives to chemical insecticides. Exploration of novel biocontrol agents is crucial to understanding the bacteria-mosquito interaction, expanding the biopesticide industry, and countering the development of resistance. In the present study, different types of soils like red, clay, black, and alluvial soils from the agricultural fields were collected from 2021 to 2022 and examined for any mosquitocidal bacterial isolates. Microbiological examinations such as serial dilutions, plating, colony isolation, and bacterial culture were carried out in the laboratory from 310 samples collected from different locations. Mosquito toxicity (bioassays) was carried out against the mosquito larvae (*Anopheles stephensi, Aedes aegypti,* and *Culex quinquefasciatus*) to screen the potential bacterial colony. Thirteen isolates out of 310 samples showed promising results. Eight isolates were identified from red soil, four isolates were from black soil and one was from clay soil. The Phylogenetic tree from the 16S rRNA sequence of the isolate (TVL-KU23) showed that the potential bacterium was *Lysinibacillus fusiformis* (*Bacillaceae*). It is the first report that this strain was investigated from red soil with mosquitocidal properties.

Keywords: *Lysinibacillus fusiformis,* mosquito species, bioassays, red soil, 16S rRNA sequence.

INTRODUCTION

Soil plays a very important role in supporting many lives and the diversity of microorganisms. Microbial activity is essential to maintaining soil health for the growth of plants because microorganisms play a vital part in the biogeochemical cycles including carbon, nitrogen, phosphorus, iron, and other elements. These activities closely relate to crucial ecological processes including controlling greenhouse gas emissions, sequestering carbon, ensuring water quality, reducing erosion, attenuating pollutants, suppressing infections, and encouraging plant development. Examples of such commodities and services include food, fiber, and wood (Tecon and Or 2017). Soil microorganism has many important roles, and the most important role is to break down complex substances (dead plant and animals) into simpler form. Therefore, the soil is an essential part of the biosphere that can generate essential resources. In 1 gram of soil, billions of bacterial and fungal species are present (Rokas, 2022).

Vector-borne diseases affect public health and remain an important health hazard in many countries including India. Mosquitoes carry most vector-borne diseases in India. Many diseases affecting human beings and animals are caused by vector mosquitoes. Dengue, chikungunya, malaria, filariasis, and Japanese encephalitis are the major diseases spread by mosquitoes in the environment resulting in thousands of deaths every year (Anoopkumar and Aneesh 2022). In 2021, over 95% of malaria cases and 96% of malaria deaths were documented in Africa, with a considerable prevalence among children. Regardless of different preventive measures being taken globally, overall malaria cases have increased from 245 million in 2020 to 247 million in 2021(WHO, 2022).

Mosquitoes are hematophagous arthropods transmitting parasites and pathogens responsible for diseases with devastating impacts on human beings. To annihilate the

mosquitoes, people have utilized chemical insecticides. Since the continuous use of chemical insecticides, mosquitoes have developed resistance. The use of chemical insecticides not only developed resistance against mosquito vectors, but also it is not eco-friendly to the environment. Entomopathogenic bacteria are now a feasible and environmentally friendly alternative to chemical insecticides for controlling mosquitoes (Benelli *et al.,* 2016; Friuli *et al.*, 2022). Among several biocontrol agents, *Bacillus* species like *Bacillus thuringiensis israelensis* (*Bti*) and *Bacillus sphaericus* (*Bs*) are extensively used for mosquitocidal activity as they are highly effective for controlling mosquitoes (Virgillito *et al.,* 2022).

Since there are four poisons involved in *Bacillus thuringiensis*, there is less chance for the development of *Bti* resistance in mosquito populations (Bruhl *et al.*, 2020). *Bti* produces spores and a crystal mixture, which is effective against the larval population of mosquitoes (Ma *et al.,* 2023). *Lysinibacillus sphaericus* renamed from Bacillus sphaericus produces binary toxins, which can act at the midgut wall (Parmar *et al*., 2022).

The present study explores the possibility of screening the potential mosquitocidal bacteria from various types of soil, such as red, black, clay, and alluvial soils collected from the districts of Tamil Nadu during the study period, 2021 to 2022.

MATERIALS AND METHODS

A. Study sites

From 2021 to 2022, the research team from ICMR-VCRC, Puducherry underwent a field survey in the two districts namely, Tenkasi and Tirunelveli. The Tamil Nadu Agriculture and Farmer Welfare Department (TAFWD), and personnel (agricultural officers) coordinated the team. There are four types of soils, namely, red, black, clay, and alluvial soils found in the agricultural fields of 16 villages surveyed. The soil samples collected from these study sites were brought to the laboratory.

B. Sample collection

Based on soil topography data obtained from the TAFWD, various soil types (red, black, clay, and alluvial soils) were collected from the study sites. The areas to be sampled are cleared of all surface impurities, including small rocks, twigs, and trash. With the random sampling method (Wong *et al.*, 2020), the surface soil and 5 and 10-centimeter-deep soil layers were collected, pooled, and preserved in a 2 ml sterile microcentrifuge tube, labeled and transported to the laboratory (Unit of Microbiology and Immunology, ICMR Vector Control Research Centre, Pondicherry, India) and stored at 4ºC until further use.

C. Isolation of bacteria

The soil samples were processed using the serial dilution method. Soil samples collected were weighed for 1 gm and mixed in 9ml autoclaved distilled water in a test tube and serially diluted up to 10^{-3} fold under aseptic conditions. 100µl of the serially diluted sample was spread plated on Nutrient agar (NB) plates. The Petri plates were then incubated overnight at room temperature (28-30°C). A pure bacterium was inoculated into 10 ml of NB (pH 7.3±0.1) and incubated for 72 hours at 250 rpm in an orbital shaker.

D. Preliminary bioassay

Preliminary screening of mosquitocidal bacteria was carried out through bioassays. It was conducted in a 150 ml capacity wax-coated paper cup filled with 100 ml of tap water with each cup containing 25 laboratory-reared (Unit of Mosquito Rearing and Colonization, ICMR-VCRC, Puducherry, India) late 3rd instar larvae of three mosquito species (*Culex quinquefasciatus*, *Aedes aegypti*, and *Anopheles stephensi)*. Each bioassay cup was treated with 1 and10 µl of final culture (72-hour bacterial culture). Control cups were maintained and larval food (yeast and dog biscuits, 1:2) was given. The larval mortality was recorded after 24 hours. The bacteria showing larvicidal activity was further subjected to culture purity through quadrant streaking and thereafter, it was preserved in glycerol (50%) and stored at -30^{0}C until further use. A stock solution of 5 mg of Bti lyophilized powder was prepared and used for detailed/extensive bioassays against mosquito larvae. Lethal concentrations were determined, and percentage mortality was recorded after 24 hours of exposure. Each experiment was repeated four times on four different days to assess the reproducibility of the observations. The LC_{50} and LC_{90} values were calculated using SPSS (Version 16.0, Chicago, SPSS Inc.) through probit analysis (Manikandan *et al.,* 2023).

E. Identification of the Bacterial strain

Gen Elute™ Bacterial Genomic DNA Kits (Sigma-Aldrich, Germany) were used to extract genomic DNA from an overnight culture of bacteria. Agarose gel electrophoresis was performed to confirm the extraction of DNA, and polymerase chain reaction (PCR) was performed with universal forward and reverse primers (16S rRNA). Qiaquick PCR purification kit (Qiagen, USA) was used for amplified PCR products. Big Dye Version 3.1 sequencing was performed using an ABI-PRISM 3730 DNA Sequencer (Applied BioSystems). To build the contig sequences, Bio-Edit (Version 7.0.9.0) was used, along with Chromas (Version 2.01) to correct the ambiguous sequences. The 16S rRNA genome sequence was combined with the closely related sequence, and a next-generation phylogenetic tree was constructed using MEGA 5 using the K2P model and 1000 bootstrapping. The species identification was done using the BLAST tool (NCBI), which was used to find out the related sequences for the species identification (Poopathi *et al.,* 2014).

F. Microscopic studies

The morphological characteristics, including the size, colour, and opacity of the bacterial colonies on the nutrient agar plates, were observed and categorized into possible *Bacillus* species. Selected colonies were further cultured, smeared onto a clear slide, and fixed through heating. Simple staining (safranin) was carried out to visualize the shape of vegetative cells. Gram staining was carried out with primary stain (crystal violet) and counter stain (safranin) after 24 hours of

culture. Endospore staining was done after 72 hours of culture through the Schaeffer-Fulton method, in which heat-fixed smears are flooded with the malachite green solution and steamed to help the stain penetrate through the spore. The endospore was resistant to decolorization, whereas the vegetative cells were easily decolorized. Then counterstained with safranin, in which vegetative cells took the safranin (pink or red) and the endospore appeared green (Oktari *et al.*, 2017). The smears were visualized through phase contrast microscopy (Olympus CX41RF Binocular Microscope, Japan).

RESULTS AND DISCUSSION

The present study was carried out in the Western Ghats, covering 1, 59,000 square kilometers in the states of Tamil Nadu, Kerala, Karnataka, Goa, and Maharashtra, with diverse microhabitats. Tirunelveli (8°42′49″N 77°45′24″E) and Tenkasi (8.956400°N 77.315200°E) districts were selected as experimental sites in this study, located in the western Ghats of southern Tamil Nadu, India. These places have significantly varied annual rainfall (85 to 215 cm) and temperature (26 to 36 °C). Rainfall is observed as the effect of monsoon, South-West monsoon (SW) & North-East Monsoon (NE) extending from June to September (SW) as well as from October to December (NE) in a year. The predominant soils of the above districts comprise red, black, clay, and alluvial soils, with rice, pulses, banana, coconut, urad dal, cotton, other vegetables, and millets as major crops (Leninraja *et al.*, 2019).

Microorganisms, especially bacterial communities, are unexplored for their diversity in the Western Ghats region of Tamil Nadu particularly for mosquitocidal bacteria. This study has been specially carried out in the Western Ghats as it is the home of a variety of bacteria with multiple functional characteristics. Soil samples were collected from two districts of Western Ghats (Tenkasi and Tirunelveli) during the year 2021 – 2022. In each district, eight villages were selected for the collection of soil samples (Table 1). A Total of 310 samples (Tenkasi 160 and Tirunelveli 150) were collected from 16 villages where four types of soils were observed with specific plantations of paddy, cotton, coconuts, etc (Table 2 and 3). Fig. 1 showed the collection and preservation of red, black, clay, and alluvial soils from the field. The test samples have undergone a series of microbiological processes to isolate pure colonies (Fig. 2). As seen in Tables 2 and 3, totally13 mosquitocidal isolates from 310 soil samples were identified. In that, 8 and 5 isolates were investigated from the villages of Tirunelveli and Tenkasi districts. Red, black, and clay soils were the source of soil types for isolating the mosquitocidal isolates. No positive isolate was investigated from the alluvial soil. It may be the reason that the alluvial soil is not that much fertile for the cultivation of crops when compared with the other three soils. Indeed, it is not clear that the exact reason for the occurrence of the potential mosquitocidal isolate is only from red, black, and clay soils. Goldberg and Margalit (1977) isolated the *Bti* from the clay soils in Israel as a first report and it has revolutionized the control of mosquitoes. Here in this study, one isolate was identified from clay soil where groundnuts (*Arachis hypogaea*) plantations are in regular practice by the agricultural formers. Hence, the findings in this study are in agreement with pioneer workers of Goldberg and Margalit (1977). More interestingly, eight and four isolates were screened from red and black soils and it is the first report from India. Table 4 depicted the results of the preliminary screening of 13 isolates, wherein, >90% mortality of mosquito larvae was observed even in 1µl bacterial culture. It indicated that these isolates were the potential in controlling the larvae of *Cx. quinquefasciatus*, *Aed. Aegypti and An. stephensi*. The colony morphology of these isolates was studied extensively. Most of the isolates were circular, dry flat, rough, irregular margins with dull white color and the shape during the vegetative and sporulated stage was rod (filamentous) shaped and elliptical. Two of the isolates were Gram-negative and eleven others are Gram positive (Table 5). In general, these isolates are rod-shaped, facultative aerobic spore-forming, and Gram-positive/negative (Fig. 3).

To further segregate the most potential among the 13 isolates, an extensive bioassay was carried out with mosquito larvae. The isolate of TVL-KU-23 was taken into consideration based on the results from the preliminary bioassays. This isolate was morphologically dull brown, circular, glossy, Gram-positive, and round spores. Laboratory bioassay result analysis has dictated an LC_{50} and LC_{90} of the isolate for *Cu. quinquefasciatus*, were 0.031 and 0.069 mg/L respectively. Similarly, the LC_{50} and LC_{90} values for *Anopheles stephensi* was 0.348 and 0.644 mg/L respectively. For *Aed. aegypti*, these values were 0.605 and 1.246 mg/L respectively (Table 6). The result indicated that the isolate is the potential in controlling all three mosquito larvae. Consequently, identification of this isolate (TVL-KU-23) by constructing the phylogenetic tree through 16S rRNA sequencing was carried out. Fig. 4 depicted, based on 16S rRNA gene sequences the phylogenetic tree was constructed. It was observed from the highest similarity index that the isolate was identified as *Lysinibacillus fusiformis* (*Bacillaceae*). This strain is a naturally occurring bacterium, particularly in farming soils and wastewaters (Pinheiro *et al.*, 2022). It is considered as mildly alkaliphilic and moderately halophilic and growing well at pH of 6 to 9.5 and in the medium containing NaCl (He *et al.*, 2011). *L. fusiformis*, usually observed as a soil bacteria that can modify the architecture of biofilm live alongside *B. subtilis* (Arnaouteli *et al.*, 2021). Burgeoning literature showed that *Lysinibacillus sphaericus* was the only strain from the *Lysinibacillus* genus reported in mosquito control (Viersanova and Purwanto 2021). Whereas, *L. fusiformis* from the same genus (*Lysinibacillus*) was reported for the first time in mosquito control in this study. *Bacillus thuringiensis* has recently reported resistance in laboratory analysis and its effect on beneficial organisms (Steinigeweg *et al.*, 2023). The exploration of new isolates and further in-depth studies on the mode of action are needed to

find out alternatives to current biocontrol agents. Studies are also required to examine any adverse effect against the non-target aquatic organisms, such as damselfly larvae (*Zygoptera*), waterbugs (*Lethocerus)*, common snails (Physa), Tilapia (*Oreochromis mossambica*) backswimmers (*Notonectidae*) and tadpole (*Rana hexadactyla*) etc. Further in-depth studies are required to develop a suitable formulation from this mosquitocidal strain to carry out the product from laboratory to land for mosquito control.

Table 1: Study sites in the districts of Tamil Nadu.

Sr. No.	Tenkasi District (Villages)	Coordinates *(Geo location)*	Tirunelveli District (Villages)	Coordinates *(Geo location)*
1.	Karuvantha	8.9652° N, 77.4910° E	Moolakaraipatti	8.5454° N, 77.7683° E
2.	Uthumalai	8.9916° N, 77.5318° E	Chinthamani	13.4020° N, 78.0551° E
3.	Karuvantha	8.9652° N, 77.4910° E	Mannarkovil	8.7325° N, 77.4214° E
4.	Vadakku kavalakurichi	8.27865° N, 77.63565 ° E	Vagaikulam	8.7402° N, 77.8167° E
5.	Thenpothai	9.0038° N, 77.2481° E	Uvari	8.2759° N, 77.8889° E
6.	Pudhupatti	9.4361° N, 78.0016° E	Kuttam	8.3198° N, 77.9375° E
7.	Ayyapuram	9.0796° N, 77.3533° E	Narguneri	8.49583 ° N, 77.658 ° E
8.	Pudupatti	9.4361° N, 78.0016° E	Rajakkamangalam	8.1290° N, 77.3640° E

Table 2: Tenkasi district village with sample code, soil type and plantation.

Sr. No.	Sample code	Village	Type of soil	Plantation
1.	TEN-UTH-1	Uthumalai	Clay	Ground nut
2.	TEN-TP-12	Thenpothai	Red	Coconut
3.	TEN-TP-9	Thenpothai	Red	Coconut
4.	TEN-PUT-16	Pudupatti	Black	Coconut
5.	TEN-PUT-7	Pudupatti	Black	Coconut

Table 3: Tirunelveli district village with sample code, soil type and plantation.

Sr. No	Sample code	Village	Type of soil	Plantation
1.	TVL-UV-1	Uvari	Red	Coconut
2.	TVL-UV-12	Uvari	Red	Gooseberry
3.	TVL-KU-9	Kuttam	Red	Banana
4.	TVL-KU-16	Kuttam	Red	Coconut
5.	TVL-KU-7	Kuttam	Red	Mango
6.	TVL-KU-23	Kuttam	Red	Coconut
7.	TVL-MOO-15	Moolakaraipatti	Black	Cotton
8.	TVL-CH-20	Chinithamani	Black	Paddy

Table 4: Preliminary bioassay result with bacterial culture against mosquito species.

Sr. No.	Sample code	1 µl	10 µl	1 µl	10 µl	1 µl	10 µl
		Culex		*Aedes*		*Anopheles*	
1.	TVL-UV-**1**	19	24	20	25	12	19
2.	TVL-UV-**12**	10	19	17	22	11	18
3.	TVL-KU-**9**	9	15	11	21	13	23
4.	TVL-KU-**16**	19	24	21	24	17	21
5.	TVL-KU-**7**	15	18	18	22	15	18
6.	TVL-KU-**23**	11	20	7	13	5	10
7.	TVL-MOO-**15**	13	19	15	20	16	24
8.	TVL-CH-**20**	18	20	17	24	15	21
9.	TEN-UTH-**1**	5	11	9	15	7	13
10.	TEN-TP-**12**	20	24	22	24	21	25
11.	TEN-TP-**9**	18	22	21	25	20	24
12.	TEN-PUT-**16**	15	23	16	23	18	23
13.	TEN-PUT-**7**	17	24	17	22	18	24

Table 5: Colony morphology of mosquitocidal bacteria.

Sr. No.	Sample code	Colony morphology	Gram staining	Endospore
1.	TVL-UV-**1**	White circular, dry, flat, rough,	Gram positive	Oval shape
2.	TVL-UV-**12**	White, circular, dry, flat, rough,	Gram positive	Oval shape
3.	TVL-KU-**9**	White circular, dry, flat, rough,	Gram positive	Oval shape
4.	TVL-KU-**16**	White circular, dry, flat, rough,	Gram positive	Oval shape
5.	TVL-KU-**7**	White circular, dry, flat, rough,	Gram negative	Oval shape
6.	TVL-KU-**23**	Dull brown, circular, glossy	Gram positive	Round shape
7.	TVL-MOO-**15**	White circular, dry, flat, rough,	Gram positive	Oval shape
8.	TVL-CH-**20**	White circular, dry, flat, rough,	Gram positive	Oval shape
9.	TEN-UTH-**1**	Dull brown, circular, glossy	Gram positive	Round shape
10.	TEN-TP-**12**	White circular, dry, flat, rough,	Gram positive	Oval shape
11.	TEN-TP-**9**	White circular, dry, flat, rough,	Gram positive	Oval shape
12.	TEN-PUT-**16**	White circular, dry, flat, rough,	Gram positive	Oval shape
13.	TEN-PUT-**7**	White circular, dry, flat, rough,	Gram negative	Oval shape

Table 6: Toxicity values (LC_{50} and LC_{90}) of *Lysinibacillus fusiformis* TVL-KU-23 against mosquito vectors.

Bacterial strain	Mosquito species	LC_{50} (µg/ml) * (LCL – UCL)	LC_{90} (µg/ml) * (LCL –UCL)	Intercept	Slope	χ^2(df)
Lysinibacillus fusiformis TVL-KU-23	*Culex quinquefasciatus*	0.031 (0.027-0.034)	0.069 (0.063-0.076)	-1.041	0.008	9.459
	Anopheles stephensi	0.348 (3.11-3.83)	0.644 (5.90-7.19)	-1.510	0.004	11.151
	Aedes aegypti	0.605 (0.520-0.680)	1.246 (1.132-1.407)	- 1.723	0.002	8.025

*Lower confidential limit & Upper confidential limit

Fig. 1. Soil sample collections from the study sites.

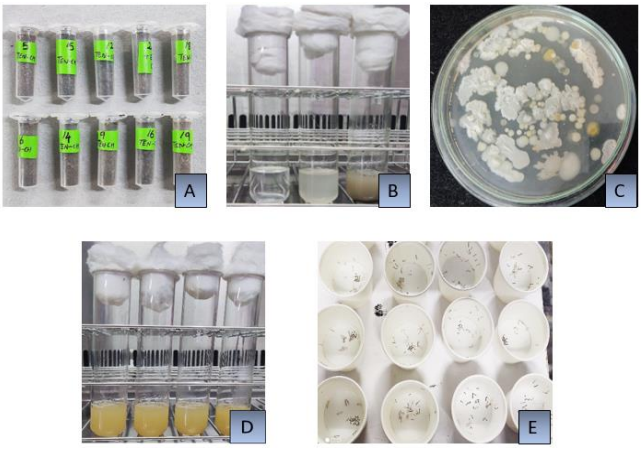

Fig. 2. Screening of bacteria a) Soil sample, b) Serial dilution, c) Spread plating, d) Culture, e) Bioassay**.**

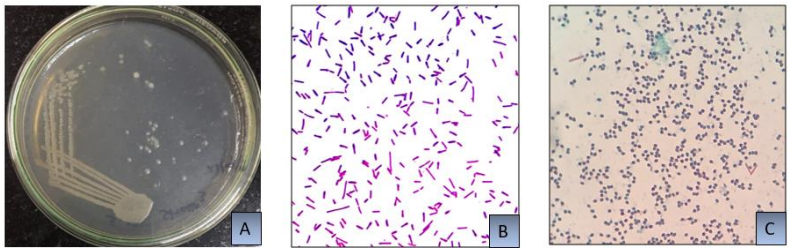

Fig. 3. Gram and spore staining of mosquitocidal bacteria a) quadrant streaking b) gram staining c) spore staining.

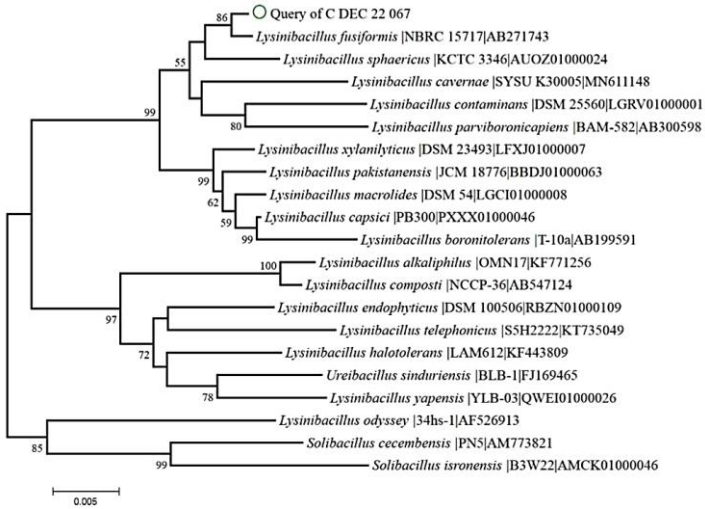

Fig. 4. Phylogenetic tree constructed based on 16S rRNA gene sequences of the isolates.

CONCLUSIONS

With no mosquito-specific medications or vaccinations, mosquito-borne diseases can be effectively countered only through vector control strategies. The microbial pesticide industry constantly demands the exploration of new effective mosquitocidal bacteria in terms of toxicity as well as environmental safety. As the soil is the primary source of microorganisms, we have explored red soil, black soil, and clay soil for mosquitocidal bacteria. This study examines the microhabitats of the Western Ghats region in Tirunelveli and Tenkasi districts in Tamil Nadu, India, focusing on their diverse mosquitocidal bacteria. This study reports mosquitocidal bacterial isolate *Lysinibacillus fusiformis* from red soils for the first time in India.

FUTURE SCOPE

The new isolate KU-23 (*Lysinibacillus fusiformis*) can serve as a major biocontrol agent in the future for countering mosquito vectors with the least effect on the environment, and this strain is an indigenous source for the pesticide industry and public health management.

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Conflict of Interest. None.

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