Review Article **Biotechnology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

EFFECT OF OUTER MEMBRANE VESICLE PROTEINS OF *XENORHABDUS* BACTERIA AGAINST MALARIAL VECTORS

VANI C* AND LALITHAMBIKA B

Department of Biotechnology School of Biotechnology and Health sciences Karunya University, Karunya Nagar, Coimbatore-641114, TamilNadu, India

ABSTRACT

Native *Xenorhabdus Sp* was isolated from the Entomopathogenic Nematodes *Steinernema Sp* used as a biopesticide for the control of malarial vectors. The *Xenorhabdus* bacterium which is symbiotically associated with *Steinernema Sp* has a wider range of toxic proteins secreted in the medium. Most of the protein secreted by the bacteria are extra cellular proteins of the Outer Membrane Vesicles (OMVs) of this gram negative bacteria plays an important role in killing the host larvae. The molecular weight of the OMV proteins of the isolates of *Xenorhabdus Sp* ranges from 20-97 KDa. This bacterium is proved to be an insect pathogen with an excellent source of insecticidal agents of proven toxicity. The outer membrane vesicles of this bacteria act as natural bombs by transporting virulence factors into the host. The novel approach of our present study includes the biopesticidal approach of the OMVs proteins from the isolated strains of *Xenorhabdus Sp* from the sugarcane fields in Tamilnadu against third instar *Anopheles gambiae* mosquito larvae, which transmits *Plasmodium* causes a major infectious disease called Malaria.100 ng/ml of OMV protein treated against the larvae recorded maximum mortality (93.32%) with the isolates XBS21 and XNHS 12 (76%). This was the first report showing the biopesticidal property of the OMV proteins of the isolates of *Xenorhabdus Sp* against the malarial vectors.

KEYWORDS: ***Xenorhabdus Sp,*** **Outermembrane Vesicle Proteins, *Anopheles gambiae*, Malaria**

VANI C
Department of Biotechnology School of Biotechnology and Health sciences Karunya University, Karunya Nagar, Coimbatore-641114, TamilNadu, India

INTRODUCTION

The most common mosquito-borne communicable disease of human is protozoal infection that is caused by the organism protists of the genus *Plasmodium*. It involves high fever, shaking chills, flu-like symptoms and anaemia. It is more common in tropical and subtropical regions and transmits through mosquitoes. There are various families of mosquitoes but only *Anopheles gambiae* mosquitoes are responsible to cause malaria. Though a variety of medications are available against malaria[9] resistance has developed against many of the antimalarial drugs. Depending on the circumstances, source reduction (removing stagnant water), biocontrol (importing natural predators), trapping or insecticides to kill larvae or adults may be used. But the mosquitoes are still leading to death of many mammals including human. It has been found that fungi have blocked malaria transmission[10]. Insecticide-based control measures may be considered as the principal way to kill mosquito larvae. In agriculture, the use of insecticides has contributed to cause resurgence effects. Bio-insecticides possess many ideal attributes like high virulence, safety to non-target organisms, ease of mass production and exemption from registration in many countries. Hence there is a need to develop biopesticides. The aim of our present study is to develop biopesticide using *Xenorhabdus Sp* and *Photorhabdus Sp* is a symbiotic bacterium of the Entomopathogenic Nematodes (EPN) *Steinernema Sp* and *Heterorhabditis Sp*. Entomopathogenic nematodes belonging to the group of nematodes which is pathogenic to various insect pests. The non feeding stage of this EPN is called as infective juveniles (ijs), the natural habitat of these nematodes is fertile and humid cultivable soil. The ijs were symbiotically associated with the bacteria belonging to enterobacteriaceae family[12]. The life cycle of this ijs begins in the search of hosts. Mostly it infects the root pests of various crops and kills the host by entering through the natural opening into the hemocoel and releases the bacteria inside the host and causes septicemia and kills it. The development of ijs as adults is inside the host. The newly emerged ijs from the female EPN inside the host will re intake the bacterium and emerges out from the host through the natural openings in search of a new host in the soil. More than 80 species of this EPN were identified and 11 are commercialized as biopesticide[14]. The outer membrane of all gram negative bacteria has bleb like structures called Outer Membrane Vesicles (OMVs). This OMVs encapsulates complex of toxic proteins which causes death of the host. The primary form of this bacteria releases wider range of toxic proteins in the medium from their Outer membrane Vesicles (OMVs) causes septicemia and kills the host. The bacterial proteins of the *Xenorhabdus Sp* were found to be pathogenic to lepidopteran pests of important vegetable and cash crops[23]. Outer membrane vesicles (OMVs) of this bacterium is a lipid protein bi-layer that is regarded as the outermost layer of gram negative wall which contains complex toxic proteins secreted naturally in the environment that can be used as larvicides. 98 per cent mortality was recorded in *Helicoverpa armigera* by treating 100 µg/g of diet[17]. Our present study is focused on the isolation of this bacterium from the EPN and to study about the toxicity effect of the OMV proteins secreted by the bacteria against malarial vectors. It was interesting to note that the OMVs isolated has pathogenic effect against the malarial vectors. The isolate BS21 was observed to be an efficient strain in controlling the *Anopheles gambiae* larvae.

MATERIALS AND METHODS

The *Xenorhabdus Sp*, were isolated from the entomopathogenic nematodes collected from the soil samples from sugarcane fields in Tamilnadu were used for the present study. The isolates used for the present study were XAS 6, XVEL S6, AS 16, XNHS12, XBS 21, *X. poinarii*. The entomopathogenic nematode isolation was done as per[24].

(i) Isolation of symbiotic bacterium

Nutrient Bromothymol blue agar medium NBTA[1] was used for culturing the bacterium *Xenorhabdus Sp.*

(ii) Isolation of the bacterium, from the insect hemolymph

- 10 nos of final (IVth) instar *Galleria mellonella* larvae were placed in a petridish over fine wet sand and the same was infected with 100 IJs/ larva of Entomopathogenic nematode isolates.
- At the moment of larval death (after 48 hrs) the cadavers were washed in a cavity block with 70% alcohol for 5-10 minutes.
- The cadavers were opened with a scissors or a needle and a drop of hemolymph was streaked on NBTA with a sterile inoculation loop in the laminar flow for the growth of bacteria.
- The first colony of the bacteria was streaked in a fresh NBTA plate. The primary colony was identified and inoculated in YS broth kept in 25˚ C incubated in shaker for 24 hrs at a speed of 1000 rpm for further studies.

(iii) Identification of primary and secondary form of bacteria

The colony formed in the NBTA medium plates of the isolates were analysed for the identification of primary and secondary colony of the bacterium. The identification was done as per the procedure of [1]. The morphology and pigmentation plays an important role for the differentiation of different form of the bacterium.

(iv) Isolation of outer membrane vesicles (omvs) insecticidal toxic protein from the xenorhabdus isolates

The 24h broth culture of the primary form Xenorhabdus *Sp* isolates were used for the isolation of the Outer Membrane Protein. The isolation of the OMVs was done as per Khandelwal and Bhatnager, (2003). The culture supernatant was separated by centrifuging at 10,000 X g for 20 min and was filtered through 0.45 µm pore size filter and further centrifuged in ultra centrifuge for 2 hrs at 15,000 X g for 2 hrs (Ti 45 rotor with a Sorvall AH 650 model ultra centrifuge). The pellet obtained was washed and suspended in 50mM Tris –HCL buffer with pH 7. The samples were stored in -20˚ C until further analysis. The amount of protein was determined [18]. The molecular weight of the protein was determined by 12% SDS PAGE[22].

(v) Culturing of mosquitoes

Third instar *Anopheles gambiae* mosquito larvae were collected from National Center for Disease Control, Mettupalayam, India. The larvae were maintained at 27±2°C, 75±5 % RH and a 14 h/10 h (light/dark) photoperiod following standard procedures. The Larvae were fed with dog biscuits/yeast powder [4].

(vi) Bioassay against mosquito larvae

Xenorhabdus isolates were cultured in YS broth overnight**,** the cells were pelleted by centrifuged at 10,000 X g for about 20 min, and the supernatant was filtered through 0.45-µm pore size filter to remove the remaining bacterial cells. The cell free supernatant was lyophilized. The formulation was prepared by mixing the lyophilized powder with glycerine at 100g/ litre, and tested for activity against mosquito larvae 5 ml of chlorine free tap water and 25 larvae were introduced. The protein concentration of the formulation was estimated by Lowry's method[18]. Larval food containing yeast and dog biscuit (1:1 by weight) was added to the bioassay cups containing larvae. Larval mortality was scored after 24 hrs of exposure[7]. 100 ng/ml concentration of protein of the above isolates were treated. LD_{50} value was arrived by probit analysis[11]. Each treatment was replicated six times with 25 larvae per replication.

RESULTS

1. Isolation of *xenorhabdus* bacteria from entomopathogenic nematodes from sugarcane fields in Tamilnadu. The details of symbiotic bacteria isolated from nematode isolates [19] were presented in Table 1. The primary colonies were identified by blue / green color colonies with irregular margins on NBTA (Nutrient Bromothymol blue Agar) medium (Fig 1&1a).The isolates used in the study were, XAS 6, XVEL S6*,* XAS 16*,* XNHS 12*,* XBS 21*, X. poinarii.* (Table.1). The bacterium used in the present study were phase I variants.

Table 1
Entomopathogenic nematode isolates and its symbiotic bacteria

S.No	Entomopathogenic nematode species	Isolates	Symbiotic bacterial species	Isolates
1	*Steinernema* sp	AS 6	*Xenorhabdus sp*	XAS 6
2	*Steinernema riobrave*	VEL S6	*Xenorhabdus sp*	XVEL S6
3	*Steinernema carpocapsae*	AS16	*Xenorhabdus nematophilus*	XAS16
4	*Steinernema carpocapsae*	NHS12	*Xenorhabdus nematophilus*	XNHS12
5	*Steinernema siamkayai*	BS 21	*Xenorhabdus sp*	XBS 21
6	*Steinernema glaseri*		*Xenorhabdus poinarii*	

2. Biochemical characteristics of the bacterial isolates

Basic biochemical tests were performed to characterize the symbiotic bacteria isolated from the entomopathogenic nematode isolates. All bacterial colonies of *Xenorhabdus* and *Photorhabdus* comprised of distinct morphologies and biochemical reactions. Gram stained smears of the colonies of all isolates showed gram negative rod shaped bacteria. All bacterial isolates were found to be motile. The results of biochemical tests were presented in Table 2. . XAS 6, XVEL S6, XAS 16, XNHS 12, XBS 21, *X. poinarii* showed positive in motility and absoption of dye, and methyl red tests and negative in Bioluminescence, Indole, Voges Proskauer, Citrate, Oxidase, Catalase, Nitrate reductase and Urease.

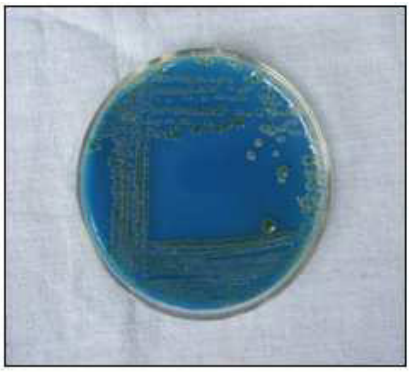


Figure 1
Xenorhabdus sp Isolate XBS21

Figure 1a
Xenorhabdus* sp. XN*HS12

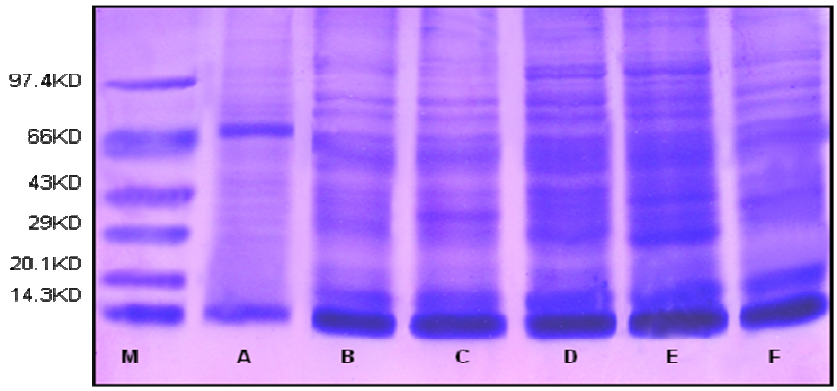
Table 2
Biochemical characteristics of xenorhabdus Sp.bacterial isolates

S.No	Tests	X AS 6	X VEL S6	X AS 16	X NHS 12	X BS 21	*X. poinarii*
1	Gram staining	-	-	-	-	-	-
2	Motility	+	+	+	+	+	+
3	Absorption of dye	+	+	+	+	+	+
4	Bioluminescence	-	-	-	-	-	-
5	Indole	-	-	-	-	-	-
6	Methyl Red	+	+	+	+	+	+
7	Voges proskauer	-	--	-	-	-	-
8	Citrate	-	-	-	-	-	-
9	Oxidase	-	-	-	-	-	-
10	Catalase	-	-	-	-	-	-
11	Nitrate reductase	-	-	-	-	-	-
12	Urease	-	-	-	-	-	-

3. Separation of the outer membrane vesicle (omvs) toxic protein from the xenorhabdus isolates

The OMV protein was isolated from XAS 6, VEL S6, XAS 16, XNHS 12, XBS 21 and *X. poinarii.* The amount of protein present in 1 ml of the culture supernatant was 20, 22, 18, 21, 15 and 22 mg/ ml of the culture. Fig 2 presented the OMV protein profile of the isolates XAS 6, XVEL S6, XAS 16, XNHS 12, XBS 21 and *X. ponarii*. The OMVs produced toxic proteins from 14.3KD to 97.4 KD. The preparation contains no proteins having molecular masses greater than 100 KD. The separation of protein was well observed in 12 % SDS PAGE analysis.

Figure 2
Outer membrane vesicular proteins of xenorhabdus Sp



M-Marker, A- XAS 6, B- XVEL S6, C- X. XAS 16, D- XNHS 12, E- XBS 21 and F- X.ponarii

4. Bioassay of OMV toxic proteins against the anopheles gambiae

Bioassay of the *Xenorhabdus* OMV toxic protein was studied against *Anopheles gambiae* larvae. The OMVs toxins were lethal to insects after treating 100 ng of protein/ml of 25 larvae per treatment. The bioassay of these toxins isolated from XAS 6, XBS 21, XNHS 12, XVEL S6, XAS 16 *and X. poinarii* caused mortality in *Anopheles gambiae* larvae with mortality per cent ranged from 20.47 to 100. The maximun mortality recorded was 90 – 100 per cent was due to the toxin effect of XBS21 in 24 hrs after treatment. The minimum mortality per cent of 20.47 – 57.12 was recorded due to the toxin effect of XVEL S6 (Table 2, Fig 3).

Table 3

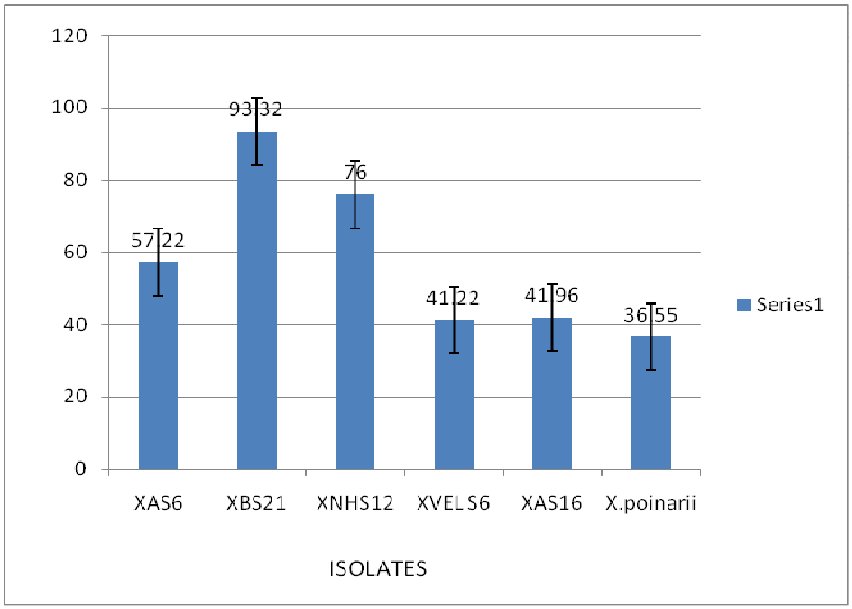
The effect of toxic proteins of the Xenorhabdus isolates against Anopheles gambiae

S.No	Isolates	Mortality % range	Mean Mortality %	LC_{50} (ng)	Fiducial limits (95%) Lower	Upper
1	XAS 6	56-63.60	57.22 ± 0.47 (28.634)[c]	78.79	42.52	115.05
2	XBS 21	90-100	93.32 ± 1.02 (37.696)[d]	56.31	13.12	99.50
3	XNHS 12	45.41-86.33	76.00± 0.52 (23.613)[a]	73.74	36.49	110.98
4.	XVEL S6	20.47-57.12	41.22 ± 1.81 (39.239)[f]	91.69	36.68	146.70
5	XAS 16	30.00-50.00	41.96±1.29 (38.149)[f]	60.00	30.70	112.00
6	*X. poinarii*	35.00 – 60.00	36.55±1.69 (26.707)[b]	60.72	40.10	100.32

Common superscribed letter are not significant at 5% level using DMRT analysis.
Values are expressed by mean ± SD of six replicates.
Figures in paranthesis are log transformed values respectively.

Figure 3

Mortality per cent of third instar larvae of Anopheles gambiae treated with OMV proteins.



Virulence of the protein was observed by calculating the LD_{50} value. The least LD_{50} was recorded in the XBS 21(56.31 ng/ml).The LD_{50} values of the other isolates recorded were XAS 16 (60.00 ng/ml), XNHS 12 (73.74 ng/ml), XAS6 (78.79 ng/ml), XVEL S6 (91.69 ng/ml) and *X. poinarii* (60.72 ng/ml).

DISCUSSION

Isolation and biochemical characterization of xenorhabdusand photorhabdus bacteria

Entomopathogenic nematodes are reported to harbour bacteria which exist in symbiotic association with it is *Xenorhabdus Sp.* which are carried in specialized vesicles formed by an out pocketing of the gut of *Steinernematids* [5].With a view to isolate and identify the symbiotic bacterial isolates XAS 6, XVEL S6, XAS 16, XNHS 12, XBS 21, *X. poinarii* associated with the nematodes, the hemolymph of *G. mellonella* was extracted. It was plated and incubated at 25°C for 24 h. Bacterial colonies were observed on NBTA medium plates. Since the NBTA media is an established media used to distinguish the bacterial colony, which differentiates the primary and secondary colony very clearly. All the primary colonies on the plates showed absorption of bromothymol blue dye on NBTA medium. Absorption of blue color dye on the medium was due to the reduction of triphenyl

tetra sodium chloride. This confirmed that they were primary forms of symbiotic bacteria of entomo pathogenic nematodes. This was in accordance with the report of [2]. The bacterial isolates, *Xenorhabdus* sp. used for the study were primary colonies. The isolated primary forms of symbiotic bacteria were further characterized by staining and biochemical tests. This could serve as potential tools to identify *Xenorhabdus*. Morphological characterization of the bacterial symbionts by staining techniques proved that all the EPN symbiotic bacterial isolates were gram negative motile and rod shaped. The results for all *Xenorhabdus* isolates in the present study confirmed the earlier reports of (Akhurst and Boemare 1988). They reported that only the primary forms of bacterial symbionts of EPNs were motile and rod shaped. *Further, the biochemical screenings were done for the bacterial isolates. XAS 6, XVEL S6, XAS 16, XNHS 12, XBS 21,* X. poinarii*, showed positive in motility and absoption of dye, and methyl red tests and negative in Bioluminescence, Indole, Voges Proskauer, Citrate, Oxidase, Catalase, Nitrate reductase and Urease. This affirmed the earlier report of [3,13, 16]. It was evident that the isolates were* Xenorhabdus Sp *and the EPN harboured them were* Steinernema Sp.

Isolation of outer membrane vesicles (Omv) toxic protein from Xenorhabdus isolates

In the present study the OMVs toxins formed a large protein complex ranging from 17.7 to 97 KD in the culture supernatant of XAS 6, XBS 21, XNHS 12, XVEL S6, XAS 16 and *X.poinarii*. The separation of proteins was well observed in SDS PAGE with molecular weight of 17 to 97 KDa. The above findings were in accordance with [20]. They reported the OMVs protein of *X. nematophilus* ranged from 17 KD to 97 KD and no protein was observed having the molecular masses greater than 100 KDa. Forst and Nealson, (1996)[12] reported that the OMVs of *Xenorhabdus Sp* bacteria contains a number of complex proteins, the high molecular weight (>700 kDa) native complex was composed of numerous protein subunits ranging in molecular weight from 23 kDa to 200 kDa [12].In our study the proteins of the Outer membrane Vesicles ranged from 17 to97 KDa, this may due to variation in the strains and the environmental impact of the isolates.

Bioassay of OMV toxic proteins against the Anopheles gambiae

The OMVs toxins were lethal to insects after the treatment of 100 ng of protein/ ml of water against IVth instar larvae of *Anopheles gambiae*. The OMV protein isolated from XAS6*,* XBS 21, XNHS 12*,* XVEL S6*,* XAS 16 and *X. ponarii* caused mortality with mortality per cent ranged from 20.47 to 100. The maximum mortality recorded was 90 – 100 per cent was due to the toxin effect of *Xenorhabdus Sp* XBS21. The minimum mortality per cent of 20.47 – 57.12 was recorded due to the toxin effect of *Xenorhabdus Sp*. XVEL S6. This results, confirmed that the pathogenicity caused by the OMV protein is much effective and the result was in accordance with the earlier report of [8]. They reported that the bioassay of the OMV toxic protein of *Xenorhabdus nematophilus* strain A24 when tested by an intra hemocoel injection bioassay killed a high percentage of *G.mellonella* and *H. armigera.* (Khandelwal and Bhatanagar 2003)[17] also reported that a dose dependent toxic effect was observed when *H. armigera* fed with OMV proteins in the diet. The mortality of the larva was observed after 24h. The virulence of the toxic protein was observed by calculating LC_{50}. The lowest value was observed in XBS 21 (56.31 ng protein / ml), and highest value was recorded in XVEL S6 (91.69 ng protein/ ml).The above observation was in accordance with the findings of [8]. They reported that *G. mellonella* was killed effectively by A24tox with a LC_{50} of 26 ng / g larva. (Kandelwar and Bhatnagar 2003)[17] reported that size of the outer membrane vesicle is 20-100nm which may be an important reason for the mortality of the larvae of *Helicoverpa armigera*, This result can be considered and can be used as biopesticide against *Anopheles gambiae* larvae because the size of the OMVs is nano meters, and the larvae can feed easily the toxic proteins may enter into the gut and may kill the larvae. They also reported that the OMVs of *Xenorhabdus nematophilus* secreted in the growth medium has bacteriocin activity and has adhesion proteins which help to transport the effector molecules inside the host. (Hinchliffe *et al* 2010)[15] reported that The “Toxin complexes” of *Xenorhabdus* bacteria are a major candidate for replacement of the BT toxins in transgenic crops. These have been heavily patented by

Dow Agrosciences and are a focus of their research. They also emphasized that the OMV toxins of *Xenorhabdus and Photorhabdus* bacteria are proving to a genomic goldmine with the genes encoding for important toxins, which are specific to target species and can be a potential source in crop protection. Our present study is the first report by identifying a new strain which can be used as an effective biocide for the control of *Anopheles gambiae.*

CONCLUSION

The OMV's can be used as bacterial bombs for the control of Mosquito vectors. Vesicles released from the *Xenorhabdus* bacteria serve as secretory vesicles for proteins and lipids of Gram negative bacteria. These vesicles act as toxin transporters. From the present study it is clearly understood that the isolate XBS21 and XNHS12 were found to be more virulent strains in killing the *Anopheles gambiae* larvae. More studies on these bacteria are required to understand the nature of the protein and can be used as a biopesticide for the effective control of Malarial vectors by formulating the OMV proteins. Work is in progress to characterize the proteins of OMVs of the isolates and to find out the mechanism in the transport of protein into the host.

REFERENCE

1. Akhurst RJ., Morphological and functional dimorphism in *Xenorhabdus* spp. bacteria symbiotically associated with the insect pathogenic nematodes, *Neoaplectana* and *Heterorhabditis* .*Journal of General Microbiology*, 121: 303-309,(1980)
2. Akhurst R.J. 'Saftey to economically important pests'. In: Laird, M., Lacey, L. A. and Davidson, E. W. (eds.), Safety is microbial insecticides, CRC press, Boca Raton Florida, pp: 223-240, (1986).
3. Akhurst RJ., and Boemare N.E., Numerical taxonomy study of the genus *Xenorhabdus* (Enterobacteriaceae) and proposed deviation of the subspecies of *X. nematophilus* to species. *Journal of General Microbiology*, 7:1835-1845,(1988).
4. Barik TK., Kamaraju R., Gowswami A., Silica nanoparticle: a potential new insecticide for mosquito vector control. *Parasitol Research*, 111:1075–1083,(2012)
5. Bird A F., and Akhurst R J., The nature of the intestinal vesicle in nematodes of the family Steinernematidae. *International Journal of Parasitology*, 13:599-606,(1983)
6. Boemare NW., and Akhurst, RJ., Biochemical and physiological characterization of clone form variants in *Xenorhabdus* spp. (Enterobacteriaceae). *Journal of General Microbiology*, 134: 751-761,(1988)
7. Brammacharry U., Paily K., Chitinase like activity of metabolites of *Pseudomonas fluorescens* Migula on immature stages of the mosquito, *Culex quinquefasciatus* (Diptera: Culicidae). *African Journal of Microbiology Research*, 6:2718-2726, (2012)
8. Brown SE., Cao AT., Hines E R., Akhurst R J., and East D., A novel secreted protein toxin from the insect pathogenic bacterium *Xenorhabdus nematophila*. *Journal of Biological Chemistry*, 279(15): 14595-14601, (2004)
9. Dondrop AM., and Day NP., The treatment of severe malaria. *Journal of American Mosquito control Association*, 23(2): 252-264, (2007).
10. Fang X L., Rodriguerz J., and Ghosh A k., using infections to fight infections: paratransgenic fungi can block malaria transmission in mosquitoes. *Future Microbiology*, 6(3): 851-853, (2011)
11. Finney DJ., Statistical methods in biological assay. In: Griffin, C. (eds.), Statistical Techniques', Charles Griffin and Co., Ltd., London, U.K, pp. 661,(1962)
12. Forst S., and Nealson K., Molecular biology of the symbiotic – pathogenic bacteria *Xenorhabdus* spp and *Photorhabdus* spp. *Microbiological Reviews*, 60 : 21-43, (1996)
13. Framer JJ., Jorgensen JH., Grimont PAD., Akhurst RJ., Poinar GO., Pierce GV., Smith JA., Carger, GP., Wilson K., and Hickman F.W., *Xenorhabdus luminescens* (DNA hybridization group 5)

from human clinical specimens. *Journal of Microbiology*, 27: 1594-1600,(1989)
14. Gaugler R., Grewal P., Kaya HK., and Fiola S., Quality Assessment of commercially produced EPN. *Biological Control,* 17: 100-109, (2000)
15. Hinchliffe SJ., Hares MC., Dowling AJ., and Constant RHF., Insecticidal toxins from the *Photorhabdus* and *Xenorhabdus* Bacteria. *The Open Toxicology Journal*, 3: 101-118,(2010)
16. Holt JG., Krieg NR., Sneath PHA., Staley TJ., and Williams ST., In: Williams, H. and Wilcons. J. (eds.), Bergey's manual of determinative bacteriology', Benjamin Publication, New york, U. S. A, pp, 188. (1994)
17. Kandelwal P., and Bhatnagar NB., Insecticidal activity associated with Outer membrane Vesicles of *Xenorhabdus nematophilus.* Applied and Environmental Microbiology, 69(4): 2032- 2037,(2003)
18. Lowry O.H., Rosenbrough N.G., Farr A.L., and Randall R.G. Protein measurements with Folin phenol reagent. Journal of Biological Chemistry, 193: 265-275,(1951).
19. Molyneux AS., The influence of temperature on the infectivity of Heterorhabtidid and Steinernematid nematodes for larvae of the sheep blowfly, *Lucilia cuprina* in Proc. IV. In: Bailey, P. and Swincer, D.(eds.)', Applied Entomological Research Conference, Adelaide, pp. 344,(1984).
20. Moureaux, N., Karjalainen, T., Givandan, A., Bourlioux, A. and Boemare, N., Biochemical characteristics and agglutinating properties of *Xenorhabdus nematophilus* fl fimbriae', Applied Environmental Microbiology, 61, 2707-2712.(1995)
21. Park JY., Baek MJ., Choi ES., and Woo S., Paramagnetic ultra small gadolinium oxide nanoparticles as advanced T1 MRI contrast agent: account for large longitudinal relaxivity, optimal particle diameter and in vivo T1 MR images', ACS Nano, 3:3663–3669, (2009)
22. Sambrook J., and Russel DW., SDS – Polyacrylamide gel electrophoresis of proteins, ed. In: Argentine, J., Irwin, N., Curtis, S. and Zierler, M. (eds.), Molecular cloning- A laboratory manual Cold Spring Harbour labouratory Press, Cold Spring Harbour, New York, pp. A8.40- A8. 49, (2001)
23. Singh J., and Banerjee, NB.,(2008),'Transcriptional analysis and Functional Characterization of a gene Pair Encoding Iron-Regulated Xenocin and immunity proteins of *Xenorhabdus nematophilus', Journal of Bacteriology*, 190(11): 3877-3885,(2008)
24. Vani C., and Rajalakshmi G., Antimicrobial activity of the symbionts of Entomopathogenic nematodes against *Bacillus thuringiensis and Serratia marcesens. Journal of Theoretical and Experimental Biology*, 6 (1): 7-16,(2009)