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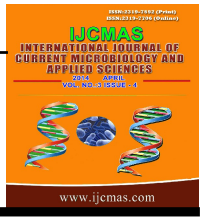

Original Research Article

Structural study of mosquito ovarian proteins participating in Transovarial transmission of dengue viruses

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K e y w o r d s

Aedes, Dengue transmission, Dengue virus blocking proteins, myosin, mass spectrometry

A B S T R A C T

Transovarial transmission has been reported as possible mechanism of maintaining dengue viruses across mosquito generations. Whether or not a mosquito can maintain dengue viruses need to be studied with respect to host-virus interaction studies. Larvae of *Aedes aegypti*, *Aedes albopictus* and *Aedes vittatus* were collected from urban and peri-urban settings of Jodhpur, Rajasthan and reared into adults in the laboratory. The head part of the mosquitoes was squashed and assayed for the presence or absence of virus employing the Indirect Fluorescence Antibody Test and confirmed by RT-PCR. Ovarian tissues of mosquitoes of all the three species (whose head squash was showing negative results for IFA Test) were dissected and subjected for proteomics studies employing SDS–PAGE technique. In total, 661 *Aedes* were examined for presence of dengue virus and corresponding presence of 200 kDa range protein in their ovarian tissues. Role of 200 kDa range protein in blocking virus to next generation was further confirmed in selected specimens. In majority of samples, presence of 200 kDa range protein was associated with the absence of virus in progeny. The protein identified was myosin heavy chain non-muscle/smooth muscle protein.

Introduction

Transovarial transmission has been reported as the possible mechanism of maintaining dengue viruses across mosquito generations (Rosen et al., 1974; Khin et al., 1983; Shroyer, 1990; Joshi et al., 1996; Joshi et al., 2002). The fact that all the progeny of experimentally infected mosquitoes do not show transovarial

transmission (Joshi et al., 2002), necessitates further research on mosquito host factors which determine passage or blocking of virus from parent to progeny of mosquito vectors. Our earlier studies have shown that majority of mosquitoes which do not show transovarial transmission of dengue viruses contain an ovarian protein of 200 kDa range (Angel et al., 2008). During course of present investigations we have confirmed the role of mosquito ovarian proteins among parent mosquitoes in allowing or blocking the virus passage and have made in- depth studies on composition and structure of observed ovarian proteins. The results of the studies could be of translational value in estimating transmission potential of vector fauna of a dengue endemic setting.

Materials and Methods

Collection of mosquito larvae and their rearing into adults

Mosquito larvae of *Aedes aegypti, Aedes albopictus* and *Aedes vittatus* were collected from their natural habitats in Jodhpur city, Rajasthan from urban and peri-urban settings. The observed breeding habitats were cement tanks, metallic, plastic and underground water tanks, etc. Larvae were collected with the help of a sieve or dropper in plastic containers and brought to the laboratory and were provided the larval feed of a mixture of yeast and dog biscuit powder. These were then reared into adult mosquitoes and maintained at ambient temperature of about 25-30°C and about 60-70% relative humidity. The adults emerged were provided with 4% glucose solution for 3-4 days. After 3-4 days, the adults were taken out and subjected for various assays.

Virus examination in mosquitoes

The head part of the mosquitoes was squashed and assayed for the presence or absence of virus employing the Indirect Fluorescence Antibody Test (IFAT) (Kuberski & Rosen, 1977). For performing IFAT, fluorescence microscope model BHL2 RFL-1 PM 10 ADS, manufactured by M/S Olympus, Japan, was used. For confirming the presence of virus, parts of ovarian tissues samples and rest of body parts of mosquitoes were subjected to Real Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) for confirming the presence of virus using the Dengue virus RT-PCR kit (m/s Primer Design, UK). The equipment manufactured by M/S Applied Bio Systems (ABI), USA was used.

SDS – PAGE and 2-D electrophoresis of Ovarian proteins

Ovarian tissues of individual mosquitoes of all the three species (whose head squash was showing negative results for IFA Test) were dissected and subjected for proteomics studies employing 11% SDS – PAGE gels. For digestion of samples, gel preparation, buffers and staining technique, the protocol as suggested by the manufacturer was adopted (m/s Mini 3 Protean system, Bio-RAD, USA). The protein bands which corresponded with the 200 kDa standard proteins (Broad range standard, m/s Bio-RAD, USA) as identified by the Quantity One software (m/s Bio-Rad, USA) were then sliced and transferred in eppendorfs containing deionized water for Mass Spectrometric analysis commercially.

Simultaneously, another set of individual ovarian proteins (whose head squash was showing negative results for IFA Test) were subjected to 2-Dimensional electrophoresis (m/s Bio-rad, USA) for identifying the isoelectric pH in which the 200 kDa protein bands will fall. The samples were assayed using the 2-Dimensional protein kit following the manufacturer's protocol (2-D starter kit, m/s Bio-RAD, USA).

Determination of amino acid composition of proteins and structure derivation

The commercial Mass Spectrometric analysis was performed by C-CAMP (Centre for Cellular and Molecular Platforms, NCBS, Bangalore). This involved in-gel trypsin digestion of the protein bands from all the three species followed by LC-MS/MS technique acquired using an Orbitrap Discovery System (Thermo) coupled to an Agilent 1200 series nano HPLC according to their lab protocol. The MS data obtained was then searched on Mascot using the Swis Prot database. Of the proteins identified, the protein which fell into the category of $\pm$200 kDa range were identified and further explored for its amino acid sequence using the NCBI website. For structural identification of the protein, the structure prediction tool i.e. uniprot database was searched and the structure showing close homology was selected.

Raising of progeny (F_1 generation) of selected mosquitoes

To confirm the role of 200 kDa range protein in blocking virus from passing to the next generation of mosquitoes, individual female mosquitoes were transferred to Barruad cage and allowed to blood feed on white Leghorn chicks. Petri plate with moist cotton were kept inside the Barraud cage for laying eggs. After the eggs were laid, the female mosquito was dissected. The paired ovaries was taken for SDS-PAGE to identify the 200kDa protein band, head was taken for IFA Test and remaining body part in PBS was subjected to RT-PCR assay for detection of virus. The eggs of the F_1 generation were allowed to hatch into larvae and then into adults and subjected to SDS-PAGE, IFA Test and RT-PCR as same as their parents. The blood feeding of parents, egg laying, surviving of parents and progeny were all recorded in a tabular format according to the Barraud cage number.

A total of 17 (1 p to 17 p) parents only were able to be maintained in the laboratory whose generation [coded as 1(Pr_1), 2(Pr_1) in table 2; 1 indicates female parent code, Pr in bracket indicates abbreviation for progeny, and subscripted number after 'Pr' shows the progeny mosquito i.e. 1, 2, 3 or 4] were also successfully maintained till subjection to different assays. Others, whose eggs were not viable or parents which did not survive after laying eggs were excluded from the study.

Results and Discussion

In total, 661 *Aedes* mosquitoes were individually examined for presence of dengue virus and corresponding presence of 200 kDa range protein in their ovarian tissues (Figure 1). Of the 158 *Aedes aegypti* examined, 91 showed 200 kDa range proteins and of these 91 mosquitoes only two mosquitoes showed presence of dengue viruses whereas 89 mosquitoes carrying 200 kDa range ovarian protein were virus negative. Of the 119 *Aedes albopictus*, 24 showed 200 kDa range

protein, of these only 6 mosquitoes showed virus presence while 18 were virus negative. Out of 384 *Aedes vittatus* examined, 172 showed 200 kDa range protein of which only 6 mosquitoes were virus positive whereas 166 mosquitoes containing 200 kDa range protein were virus negative (Table 1).

Role of 200 kDa range protein in blocking virus to go to next generation of mosquitoes was further confirmed in selected mosquito specimens. In sample no. 1(P) 200 kDa range protein was absent where corresponding dengue virus was present in mosquito. In F_1 generation ($1Pr_1$), dengue virus was present. While in mosquito sample 7 (P), 200 kDa range protein was present in ovary and mosquito also showed presence of dengue virus. In F_1 progeny of this mosquito, i.e. 7 (Pr_1); 7 (Pr_2); 7 (Pr_3) and 7 (Pr_4) virus was absent in all the samples. In another mosquito sample 11 (P), 200 kDa range protein was absent and virus was present in mosquito and also in all its F_1 progeny i.e. 11 (Pr_1); 11 (Pr_2); 11(Pr_3) and 11 (Pr_4). In mosquito sample 12(P), 200 kDa range protein and corresponding virus was present and in progeny of this mosquito i.e. 12(Pr_1); 12 (Pr_2) and 12 (Pr_4) virus was absent. However, in progeny sample 12 (Pr_3) virus was present despite presence of 200 kDa protein in the ovarian tissue of parent (12P) mosquito (Table 2).

This 200 kDa range ovarian protein related to blocking of transovarial transmission of virus from parent to progeny of mosquito was subjected to Mass Spectrometric profiling. For protein identification, the MS database was searched against the NCBInr database and a locally made database containing proteins from all species of *Aedes*. For protein identification, minimum 2 high confident peptides/proteins were considered. A decoy database was searched for (with p value:0.01) to minimize false protein identification. The Mascot search results were validated by Proteome Scaffold Analysis. Of the data generated, maximum % of sequence coverage was that of "Myosin heavy chain non-muscle or smooth muscle' protein consisting of 1963 amino acids. With the help of structural prediction tool 'uniprot', its probable structure was searched. A Swiss model of a closely related reference protein (code Q17L97) as "Myosin heavy chain, non muscle or smooth muscle [Aedes aegypti (Yellow fever mosquito) (Culex aegypti); EMBL EAT47478.1] appeared (www. uniprot.org/ uniprot/ Q17L97) (Figure 2).

The 2-Dimensional electrophoretic gels showed that in the range of 200 kDa, most of the proteins fell towards the acidic pH range i.e. of <7 pH value. (Figure 3). As these proteins are mostly showing the pH range of acidic nature we can infer that acidic nature of the mosquito membrane proteins, are transmission blocking proteins as far as their function in virus transmission across mosquito cells is concerned.

Dengue fever (DF) associated with dengue hemorrhagic Fever (DHF) has emerged as global problem in last one decade. In India, DF/DHF has affected thousands of people recently 770 deaths were reported since 2007 till March 2013 as per the official government records (www.nvdcp.org). Due to unavailability of vaccine against DHF, understanding of vector-virus interactions could contribute significantly in identifying transmission conducive areas of dengue. In present studies we have established that a non muscle /smooth muscle protein of 224 kDa

Table.1 Association of 200kDa range protein in Ovarian tissues with virus presence/absence

Species	Total mosquitoes tested	Mosquitoes displaying 200kDa range proteins	Mosquitoes positive for virus	Percentage of virus negative mosquitoes with 200 kDa range protein
Aedes aegypti	158	91	2	97.80
Aedes albopictus	119	24	6	75.00
Aedes vittatus	384	172	6	96.50
Total	661	287	14	95.12

Table.2 Relationship between 200kDa range ovarian protein and transovarial transmission of dengue viruses

Parent mosquito sample number	Parent mosquito details			Mosquito F_1 generation details		
	200kDa range protein in ovary	Virus in head squash (IFAT)	Virus in body parts (RT-PCR)	Mosquito progeny (F_1) sample number	Virus in head squash (IFAT)	Virus in body parts (RT-PCR)
1(P)	Absent	Present	Present	1(Pr_1)	Present	Present
2(P)	Present	Absent	Absent	2(Pr_1)	Absent	Absent
				2(Pr_2)	Absent	Absent
3(P)	Present	Absent	Absent	3(Pr_1)	Absent	Absent
4(P)	Present	Absent	Absent	4(Pr_1)	Absent	Absent
				4(Pr_2)	Absent	Absent
				4(Pr_3)	Absent	Absent
				4(Pr_4)	Absent	Absent
5(P)	Absent	Absent	Absent	5(Pr_1)	Absent	Absent
				5(Pr_2)	Absent	Absent
6(P)	Absent	Absent	Absent	6(Pr_1)	Absent	Absent
				6(Pr_2)	Absent	Absent
7(P)	Present	Present	Present	7(Pr_1)	Absent	Absent
				7(Pr_2)	Absent	Absent
				7(Pr_3)	Absent	Absent
				7(Pr_4)	Absent	Absent
8(P)	Present	Absent	Absent	8(Pr_1)	Absent	Absent
9(P)	Present	Absent	Absent	9(Pr_1)	Absent	Absent
				9(Pr_2)	Absent	Absent
10(P)	Present	Absent	Absent	10(Pr_1)	Absent	Absent
				10(Pr_2)	Absent	Absent
11(P)	Absent	Present	Present	11(Pr_1)	Present	Present
				11(Pr_2)	Present	Present
				11(Pr_3)	Present	Present
				11(Pr_4)	Present	Present

12(P)	Present	Present	Present	$12(Pr_1)$ $12(Pr_2)$ $12(Pr_3)$ $12(Pr_4)$	Absent Absent Present Absent	Absent Absent Present Absent
13(P)	Absent	Present	Present	$13(Pr_1)$ $13(Pr_2)$ $13(Pr_3)$	Present Absent Present	Present Absent Present
14(P)	Absent	Absent	Absent	$14(Pr_1)$ $14(Pr_2)$	Absent Absent	Absent Absent
15(P)	Present	Present	Present	$15(Pr_1)$ $15(Pr_2)$	Absent Absent	Absent Absent
16(P)	Absent	Present	Present	$16(Pr_1)$	Present	Present
17(P)	Absent	Absent	Absent	$17(Pr_1)$ $17(Pr_2)$ $17(Pr_3)$ $17(Pr_4)$	Absent Absent Absent Absent	Absent Absent Absent Absent

Figure.1 SDS- PAGE ASSAY OF OVARIAN TISSUES OF *AEDES VITTATUS* (L1-L5; L7: OVARIAN PROTEIN; L6, L8 & L9: DID NOT DISPLAY ANY BANDS. L10: STANDARD)

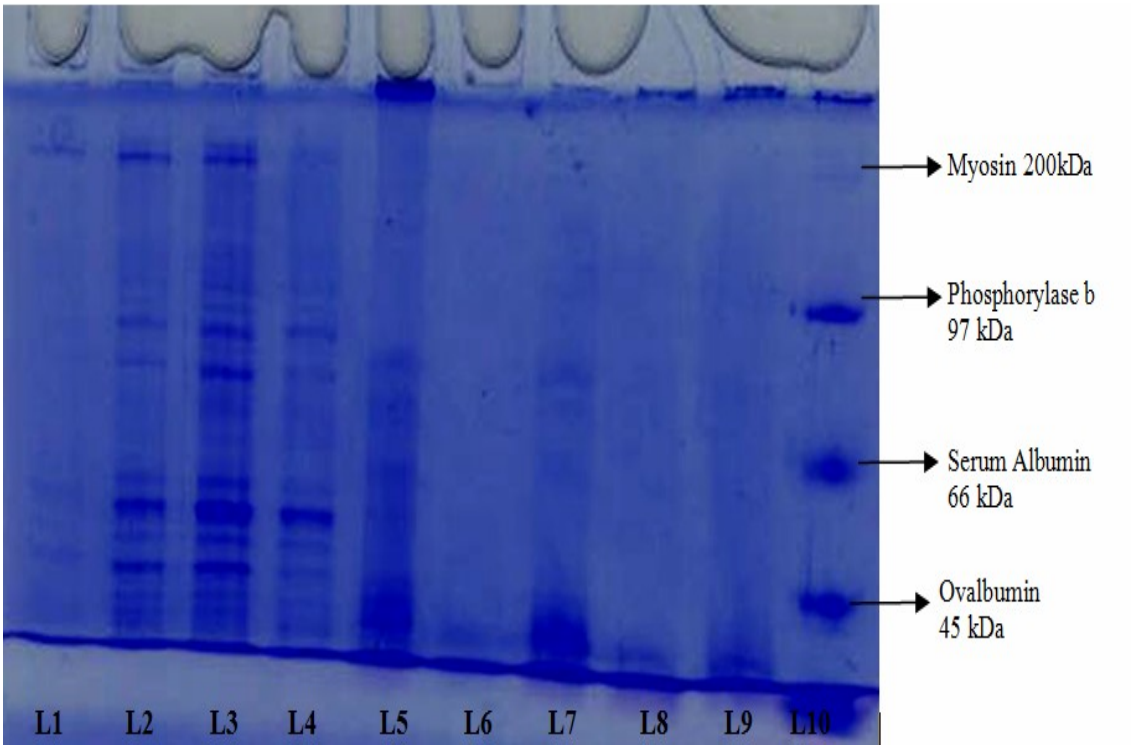


Figure.2 3-D Structure of Myosin heavy chain non muscle or smooth muscle protein

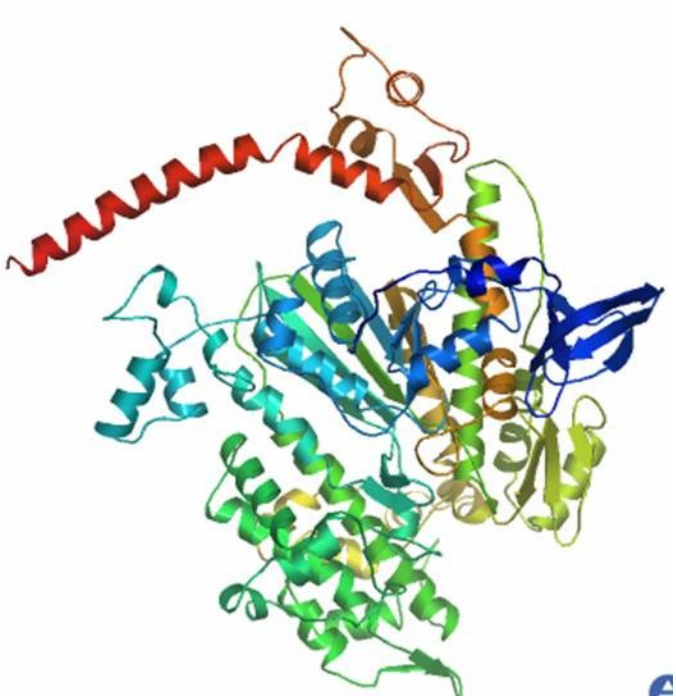
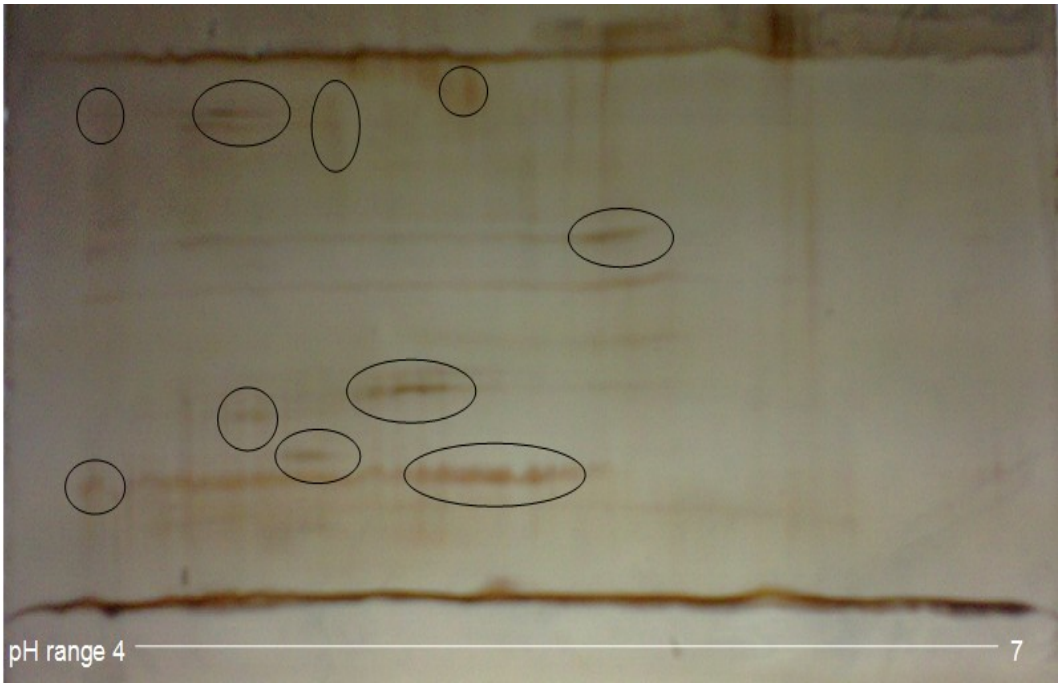


Figure.3 Two-dimensional Electrophoresis of Ovarian tissues of *Aedes aegypti*



with 1963 amino acids is capable of blocking passage of virus from parent to progeny of mosquitoes, through Transovarial route.

Vertical or Transovarial transmission of dengue virus has been reported first time in world by Khin et al., 1983. In India our group has established this mode of virus transmission (Joshi et al., 1996) and subsequently, serial passage of TOT was established (Joshi et al., 2002). Maintenance of dengue virus through TOT could act as interphase of virus retention in nature which may be responsible for reappearance of disease in dengue endemic settings. In present studies, implication of a mosquito ovarian protein of 224 kDa as a blocking molecule against TOT, may serve to act as an indicator whether mosquitoes of an area are competent to maintain dengue virus or not. The observations on this 'myosin heavy chain non-muscle/smooth muscle protein' can be translated into development of a probe to serve the virus maintenance capacity of mosquito fauna and corresponding risk of disease reappearance in an endemic setting.

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