Peritrophic membrane structure of *Aedes aegypti* (Diptera: Culicidae) mosquitoes after infection with dengue virus type 2 (D2-16681)

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Abstract

The peritrophic membrane (PM) is a non-cellular tissue involved in the protection of midgut epithelium from mechanical damage and insults from pathogens. This study was carried out to determine the involvement of PM in mosquitoes after infection with dengue virus. *Aedes aegypti* (Diptera: Culicidae) mosquitoes were fed sucrose and human blood with and without dengue virus type 2 (D2-16681), and collected at 0.5, 1, 6, and 12 h, respectively. Specimens were prepared for examination under light and electron microscopy. The results showed that PM was produced only in the blood-fed mosquitoes. The infected blood meal induced the mosquitoes to produce PM in their midgut earlier and thicker than in mosquitoes with blood alone. The initial evidence of PM occurred at 1 h post-blood meal (PBM) as a matrix-like structure. By 6 h PBM, PM had become a layer, which persisted at 12 h. Among mosquitoes fed with blood alone, this structure was found only from 6 and 12 h PBM. Dengue virus type 2 induced different modifications of mosquito PM construction and structures, confirmed under an electron microscope.

Key words: Flaviviridae; peritrophic matrix; ultrastructure

INTRODUCTION

Dengue virus is one of the most important resurging mosquito-borne diseases belonging to the Flaviviridae family of small enveloped viruses. It carries a single-stranded RNA virus, divided into four serotypes: DEN1, DEN2, DEN3, and DEN4. Dengue virus infection is prevalent in tropical areas of over 100 countries, with 2.5 billion people at risk of acquiring the infection. Fifty million estimated infections and 500,000 dengue hemorrhagic fever (DHF) cases occur annually (Gubler, 2002). Dengue infection is caused by the ingestion of viremic blood containing the virus by *Aedes* mosquitoes followed by passage to a second human host. *Aedes aegypti* (Diptera: Culicidae) is the most important vector for dengue transmission, which is initiated when the female mosquito ingests an infective blood meal (Gubler, 2002). The midgut of a

mosquito is the first site of interaction between the mosquito and pathogen and plays an important role in vector competence (Beerntsen et al., 2000). Midgut epithelial cells produce the peritrophic membrane, sometimes called the peritrophic matrix (PM), a non-cellular material that separates the midgut lumen containing the ingested food from the midgut epithelium (Jacobs-Lorena and Oo, 1996; Tellam et al., 1999; Ibrahim et al., 2000; Shao et al., 2001). The PM is composed of chitin, proteins, and proteoglycans (Wang and Granados, 2001) divided into two types: Type I PM is found in adult hematophagous insects and forms a thick bag-like structure that completely surrounds the ingested blood meal. Type II PM is produced in larval and adult (except hematophagous) insects, forms a thin open-ended tube-like structure and is produced in a specialized region located at the junction between the foregut and midgut called the

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cardia (Jacobs-Lorena and Oo, 1996; Tellam et al., 1999). Functions of the PM include protecting the midgut epithelium from mechanical damage, acting as a lubricant helping food pass through the gut, and preventing swelling and rupture of the midgut after the ingestion of high molecular-weight foods exerting high osmotic pressure. PM serves as a barrier against the entry of pathogens and toxins, prevents the rapid excretion of digestive enzymes and also limits the rate of food digestion. Moreover, it plays a role in the prevention of non-specific binding of undigested materials to midgut microvilli surfaces or binding to transport proteins at the midgut surface (Zhuzhikov, 1964; Terra, 1990; Lehane, 1997; Villalon et al., 2003). In addition, it helps in heme detoxification (Pascoa et al., 2002), and the assimilation and elimination of toxic ammonium ions (Kato et al., 2002). Dengue virus can invade and replicate in the midgut epithelium of *Ae. aegypti* mosquitoes. The morphology of the PM may change after the mosquitoes receive an insult from this virus.

The role of PM formation in dengue invasion of the midgut has not been examined; therefore, the purpose of this study was to examine the involvement of PM in midgut of *Ae. aegypti* mosquitoes after infection with dengue virus type 2 (D2-16681) at different time points.

MATERIALS AND METHODS

Rearing mosquitoes. *Ae. aegypti* mosquitoes (white-eyed Liverpool strain) were maintained in an insectary in the Department of Medical Entomology (Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand). Eggs were hatched and transferred to the rear of a plastic tray which was half-filled with well water and artificial food. After hatching from the pupa, the adults were maintained at 27°C with 80% relative humidity under a 14:10 h light/dark cycle. Adult mosquitoes were offered 10% sucrose. Three- to four-day-old female mosquitoes were collected for the experiment.

Virus propagation. Dengue virus type 2 strain (D2-16681) was obtained from the Armed Forces Research Institute of Medical Sciences (AFRIMS) (Bangkok, Thailand). The virus had been routinely passaged in C6/36 cells culture performed in a 25 cm² culture flask (Corning, USA). After adsorp-

tion for 90 min, 5 ml MEM medium (Gibco, USA) containing 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin was added. The culture flask was incubated at 28°C in a 5% CO₂ incubator. Aliquots of the culture medium were harvested seven days after infection and stored at -80°C until plaque titration and mosquito inoculation.

Viral plaque assay. LLC-MK2 cells were seeded in six-well cell culture plates (Corning) at a density of 5.0×10^5 cells/well and were incubated for three to four days at 37°C in 5% CO₂ to produce a confluent monolayer. Cell monolayers were inoculated with 10-fold serial dilutions of virus in a final volume of 0.2 ml. Viral adsorption (from extracted samples: negative media alone, sucrose, infected mosquito and infective blood meal) was allowed to proceed for 90 min at 37°C with plate rocking every 15 min. A 3 ml overlay of MEM, 5% FBS, and 0.6% SEQEM agar was added at the conclusion of adsorption. The infected monolayers were incubated at 37°C in 5% CO₂. Seven days after infection, a second overlay, similar to the first but with the addition of 1.5% neutral red (Sigma-Aldrich, UK), was added to the wells, and the plates were incubated at 37°C in 5% CO₂ overnight. The plaques were counted, and the viral titer was calculated and expressed as pfu/ml.

Infection of mosquitoes and verification of dengue infection. Insectary-maintained *Ae. aegypti* mosquitoes were infected by oral feeding with dengue virus type 2 (D2-16681) at 10^6 pfu/ml. Mosquitoes were maintained at 28°C and 70 to 80% relative humidity. Pools consisting of five mosquitoes were removed at different time points, 0.5, 1, 6, 12 h post-infection, and frozen for RT-PCR analysis. Dengue virus in mosquitoes was detected by RT-PCR and serotype-specific primers by nested-PCR.

RNA extraction. Total RNA was extracted with a QIAmp viral RNA kit (QIAGEN, USA), according to the manufacturer's suggested protocol. RNA was eluted twice in 40 µl nuclease free water. The extracted solution was stored at -70°C until evaluated.

Nested RT-PCR assay. Nested RT-PCR of dengue virus RNA was carried out with dengue virus consensus and serotype-specific primers, as described previously (Lanciotti et al., 1992). Modifications to the procedure were as follows: 5 µl of

RNA in 50 μ l reaction volume was used with the QIAGEN OneStep RT-PCR kit (QIAGEN). RT-PCR was carried out according to the manufacturer's instructions with 55°C annealing temperature. The resultant PCR product was diluted to 1 : 500 in water. Nested-PCR was carried out with 5 μ l of the diluted RT-PCR product in 50 μ l reaction volume with the TaqPCR Master Mix kit (QIAGEN). Initial denaturation of 10 min at 94°C was followed by 25 cycles, each consisting of 94°C for 30 s, 50°C for 30 s, and 72°C for 45 s, followed by a final extension step of 72°C for 10 min. Both the RT-PCR and nested-PCR products were analyzed by gel electrophoresis on 2% agarose gel containing ethidium bromide (0.5 μ g/ml). A band on the agarose gel of the correct size was visualized by the Bioimaging system (Syn-Gene, UK).

Mosquito preparation. One hundred and eighty female mosquitoes were starved for two days prior to feeding. Twenty starving mosquitoes were collected as a control group. One hundred and sixty mosquitoes divided into groups 1, 2 and 3 were fed with 10% sucrose and human blood without and with dengue virus type 2 (D2-16681), respectively. The infective blood meal was prepared by mixing 10⁶ pfu/ml of virus, pack cells of washed human erythrocytes, and 10% sucrose (4 : 1 : 1 ratio). This suspension was placed on non-wettable fine nylon mesh covering a small carton of mosquitoes for them to imbibe for 30 min. Fully engorged mosquitoes in each group were collected at 0.5, 1, 6, and 12 h (10 mosquitoes per time point) after feeding, and then anesthetized at -70°C. The legs and wings were removed before fixing in 2.5% glutaraldehyde in 0.1 M cacodylate buffer containing 5% sucrose, at pH 7.4, 4°C for 24 h. Finally, all specimens were prepared and examined by light and transmission electron microscopy.

Preparation for light microscopy. The fixed specimens of starved and human blood fed mosquitoes were dehydrated through a graded series of ethanol and embedded in paraffin. Five-micrometer-thick serial sections were cut and placed on glass slides, stained with Periodic Acid Schiff (PAS) (Luna, 1968) and examined under a Nikon Eclipse E600 light microscope.

Preparation for electron microscopy. After pre-fixation, the specimens were post-fixed in 2% OsO₄ in 0.1 M cacodylate buffer for 1 h, dehydrated by ethanol and embedded in Epon 812 (Electron

Microscopy Sciences, USA). Transverse sections of semithin (1 μ m thick) were cut by an ultramicrotome, placed on a glass slide, stained with 1% toluidine blue, and examined under a light microscope to study the entire tissue. When the area of interest was located, ultrathin sections were cut, picked up and placed on copper grids. They were then stained with uranyl acetate and lead citrate before examination under a transmission electron microscope (Hitachi, model H-7000, Japan) at 75 kV.

Statistical analysis. PM thickness and the size of midgut epithelial cells (in the anterior region of the midgut) were accurately measured from micrographs using the Image Frame Work program. The results are presented as the mean $\pm$ standard deviation (SD).

RESULTS

The present study showed that PM formation in the *Ae. aegypti* midgut after the infective blood meal with dengue virus type 2 significantly differed from in blood alone, sucrose, and starving groups, as described below.

Virus growth kinetics was determined in all samples by plaque titration performed in a confluent monolayer of LLC-MK2 cells as previously described (Miller and Mitchell, 1986; Huang et al., 2000). Only two groups, infective blood meal and infected mosquito, showed plaque titration at 10⁶ and 10⁷ pfu/ml, respectively.

We confirmed the specific dengue virus by RT-PCR and nested-PCR, as described previously (Lanciotti et al., 1992; Chien et al., 2006; Gomes et al., 2007). After amplification, 5 μ l of each product was analyzed by agarose gel electrophoresis utilizing 2% agarose gel, and the serotype was determined. The amplicon was approximately 119 bp, specific for dengue virus type 2 as described by Chien et al. (2006) (Fig. 1).

There was no PM in the starved (control) *Ae. aegypti* midgut when observed under light and electron microscopy. This result was the same as in sucrose-fed mosquitoes (group 1), in which PM was not observed in each time interval. The midgut of both groups consisted of a single layer of columnar epithelium lining the lumen (Fig. 2A, B). The apical region of the epithelium was characterized by a striated border of numerous microvilli projecting into the lumen (Fig. 2B, C) (image shown only at

Fig. 1. Agarose gel analysis of the DNA product from RT-PCR of RNA samples isolated from infective blood meal and infected *Ae. aegypti* mosquito. L₁, media alone; L₂, RNase-DNase free water; L₃, infective blood meal; L₄, infected mosquito; L₅, positive control dengue mix (dengue type 1–4); L₆, negative control; L₇, sucrose; L₈, DNA ladder.

6 h after sucrose feeding).

The results from PAS staining demonstrated that the PM formed only in blood-fed mosquitoes. The structure of PM was revealed as a fine delicate pink color between the epithelium and the food bolus, visible only at 6 and 12 h post-blood meal (PBM) (picture not shown). These results were confirmed by toluidine blue staining and electron microscopy.

Toluidine blue-stained midguts from human blood-fed mosquitoes (group 2) were observed under a light microscope. PM was not observed at 1 h PBM (Fig. 3A). Although a pale blue line similar to PM was observed between the epithelium and red blood cells, this was thought to be the tips of dense microvilli, demonstrating the first formation of PM at 6 h PBM which appeared as a pale blue thin layer between the epithelium and the ingested blood, approximately $0.73 \pm 0.28 \mu\text{m}$ in thickness (Fig. 3C). Subsequently (at 12 h, Fig. 3E), there was decrease in PM thickness ($0.42 \pm 0.06 \mu\text{m}$). When examined under an electron microscope, the PM structure at 6 h (Fig. 4C) was similar to at 12 h (Fig. 4E), a thin foamy layer.

The PM in the midgut after being fed human blood with dengue virus type 2 (group 3) was produced earlier and thicker than in mosquitoes fed blood alone. It first appeared at 1 h PBM, surrounding the red blood cells as a matrix-like structure and about $7.36 \pm 1.64 \mu\text{m}$ (Figs. 3B and 4B). At 6 h PBM, a light microscope image showed that the PM in the midgut had become a thick colorless layer of approximately $4.19 \pm 0.89 \mu\text{m}$ (Fig. 3D)

Fig. 2. Sucrose-fed female *Ae. aegypti* midgut at 6 h. (A, B) Transverse section light micrographs of toluidine blue staining, demonstrating a single layer of columnar epithelium (Ep) resting on the basement membrane (BM); high magnification of inset from A shown in B. (C) Transmission electron micrograph, showing numerous microvilli (M) at the apical pole extending into the lumen (L).

Fig. 3. Light micrographs from toluidine blue staining of adult *Ae. aegypti* midgut after feeding with human blood and infected blood at different times. (A) PM was not observed. (C) PM can be seen as a pale blue thin layer (arrows). (E) PM was decreased in thickness (arrows). (B) PM was observed as a matrix-like structure. (D) Thick colorless layer of the PM. (F) PM was decreased in thickness (arrows). Epithelium (Ep), red blood cells (RBC).

and the ultrastructure revealed multilayers of the PM in parallel (Fig. 4D). At 12 h (Figs. 3F and 4F), the general appearance of the PM structure was similar to at 6 h except that PM thickness had decreased ($1.76 \pm 0.38 \mu\text{m}$).

Before engorgement, the midgut was composed of a single layer of columnar epithelium. After blood ingestion, the morphology of the midgut changed both with blood alone (Fig. 3A, C, E) and infective blood (Fig. 3B, D, F). Marked distension of the midgut provoked flattening of the epithelium to a single layer of cuboidal epithelium. Changes to the morphology of the midgut continued and at 6 h

(Fig. 3C, D) and 12 h (Fig. 3E, F) PBM, blood digestion was visually evident as the presence of lysed erythrocytes.

The means of PM thickness and the size of midgut epithelial cells of mosquito group 1, 2, 3 and the control are shown in Table 1.

DISCUSSION

The digestive tract of insects is commonly shielded by the PM, which plays many important roles in protecting against various microbial, chemical, and physical challenges (Terra, 1990; Lehane,

Fig. 4. Electron micrographs of midgut sections from adult *Ae. aegypti* after feeding with human blood and infected blood at different time points. (A) PM was not found. (C, E) PM was visible as a foamy thin layer. (B) PM was observed as a matrix-like structure. (D, F) Multilayers of PM. Epithelium (Ep), lumen (L), microvilli (M), red blood cells (RBC).

1997). Many species produce different PM types at different life stages. In mosquitoes, type II PM is produced in the larval stage while type I PM is produced only in adult female mosquitoes (Stamm et al., 1978; Tellam, 1996; Tellam et al., 1999).

The present report contributes information to the histology and ultrastructure of PM in the midgut of *Ae. aegypti* mosquitoes. The PM is produced in di-

rect response to blood feeding; in contrast, it is not produced when mosquitoes receive sucrose or are starving, consistent with findings described by Perone and Spielman (1988), Jacobs-Lorena and Oo (1996), Tellam et al. (1999) and Shao et al. (2001). PM structure is composed of chitin fibers forming a strong and flexible framework to which proteins and proteoglycans attach (Wang and Granados,

Table 1. Means ($\pm$ SD) of PM thickness and the height of midgut epithelial cells (in the anterior region of midgut) of *Ae. aegypti* mosquitoes after feeding with sucrose (group 1), human blood (group 2), and human blood with dengue virus type 2 (group 3)

	No. of mosquitoes examined	PM	PM thickness (μ m)	Size of midgut epithelial cells (μ m)	Shape of midgut epithelial cells
Control	10	—	—	75.86 $\pm$ 13.81	columnar
Group 1	10	—	—	66.04 $\pm$ 14.01	columnar
Group 2					
0.5 h	10	—	—	11.58 $\pm$ 3.63	cuboid
1 h	10	—	—	9.32 $\pm$ 3.31	cuboid
6 h	10	+	0.73 $\pm$ 0.28	10.68 $\pm$ 3.11	cuboid
12 h	10	+	0.42 $\pm$ 0.06	11.23 $\pm$ 3.24	cuboid
Group 3					
0.5 h	10	—	—	11.67 $\pm$ 3.81	cuboid
1 h	10	+	7.36 $\pm$ 1.64	10.28 $\pm$ 2.70	cuboid
6 h	10	+	4.19 $\pm$ 0.89	13.15 $\pm$ 3.65	cuboid
12 h	10	+	1.76 $\pm$ 0.38	12.78 $\pm$ 3.13	cuboid

2001). Proteins and chitin play important functional roles and the number of proteins in the PM varies among species (Moskalyk et al., 1996). After a blood meal, protein and chitin synthesis is activated and results in PM formation (Bertram and Bird, 1961; Staubli et al., 1966; Peters, 1992; Morlais and Severson, 2001; Shao et al., 2005). Among female adult *Ae. aegypti* mosquitoes, PM formation in the midgut was almost completely absent when chitin synthesis was disrupted; PM proteins alone failed to form this structure (Wang and Granados, 2001; Kato et al., 2006). In addition, the inhibition of protein synthesis resulted in disturbed PM formation (Zimmermann and Peters, 1987; Terra, 2001).

In adult *Anopheles aquasalis*, *An. albitarsis*, *An. bellator*, *An. homunculus* (Chadee and Beier, 1995), *An. gambiae*, *An. stephensi* (Freyvogel and Staubli, 1965), *An. darlingi* (Okuda et al., 2005), the PM developed 18, 30, 30, 36, 13, 32, 18 h PBM, respectively. Compared to other mosquitoes, such as *Culex tarsalis* (Freyvogel and Staubli, 1965), *Cx. quinquefasciatus* (Okuda et al., 2002), and *Ae. vigilax* (Wijffels et al., 1999), this barrier occurred at 10, 12, and 4 h PBM, respectively. The PM of adult *Culiseta melanura* mosquitoes first appeared at 6 h PBM and reached maximum thickness at 12 h (Weaver and Scott, 1990). The kinetics of PM formation and degradation were found to be related to the ingestion and time of digestion of the

blood meal (Secundino et al., 2005).

In this study, PM formation was first observed 6 h after feeding *Ae. aegypti* mosquitoes with blood alone; this finding is in line with previous studies (Freyvogel and Staubli, 1965; Perrone and Spielman, 1988). Interestingly, this barrier was observed as early as 1 h and was fully formed at 6 h post-blood with dengue virus type 2 (D2-16681) meal, which induced the mosquitoes to produce this structure earlier and thicker than in mosquitoes fed blood alone. This may be a defense mechanism of mosquitoes to protect their midgut from virus. Lehane (1997) reported that the degree of microbial contamination of the liquid diet may be a more important factor determining the presence or absence of the PM in most cases. Insects feeding exclusively on a largely sterile, or at least a less infected liquid diet, tend to lack a PM while those that feed on liquid diets likely to be heavily contaminated with microorganisms tend to retain their PM. Billingsley and Rudin (1992) described that the infectivity of *Ae. aegypti* by *Plasmodium gallinaceum* was reduced when PM thickness increased, indicating that the PM does act as a partial barrier to *Plasmodium* development.

Dengue virus can invade and replicate in the midgut epithelium of *Ae. aegypti* mosquitoes. How do viruses invade the midgut epithelium of blood-sucking insects after a blood meal? Three strategies include: 1) it may invade before PM formation; 2)

it may persist in the remnants of the blood meal until the PM dissolves and then attach to the epithelium; 3) it may disrupt and penetrate the PM. Dengue virus may fall into the first category, due to a previous study stating that most arboviruses penetrate the mosquito gut soon after blood meal ingestion and before the PM has formed (Devenport and Jacobs-Lorena, 2005). *Litomosoides chagasfilhoi* microfilariae can invade the midgut epithelium during PM development of *Culex quinquefasciatus*, but when the PM was fully formed, microfilariae were no longer able to cross it (Santos et al., 2006). On the other hand, it may belong to the third strategy as Mitsuhashi et al. (2007) demonstrated that fusolin, the constitutive protein of the spindles of entomopoxvirus, may bind with chitin in the PM, thus enhancing disruption in the host insect. In larval *Ae. aegypti*, the motility caused by *Derris urucu* extract (used as an insecticide) related to disruption of the PM structure. PM disruption can facilitate the transport and enhance the insecticidal activity of pathogens (Gusmão et al., 2002).

From this study, we can conclude that there are changes in the construction and structure of the PM in the midgut of *Ae. aegypti* mosquitoes during the ingestion of blood with dengue virus type 2. Some compartments or products of this virus may enhance the process of PM production in the midgut of mosquitoes. This event is one of the most important defense mechanisms of mosquitoes to protect their midgut from virus infection; therefore, the relationship of virus molecules and other mosquito innate immune responses related to PM should be explored through further experimentation.

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REFERENCES

- Beerntsen, B. T., A. A. James and B. M. Christensen (2000) Genetics of mosquito vector competence. *Microbiol. Mol. Biol. Rev.* 64: 115–137.
- Bertram, D. S. and R. G. Bird (1961) Studies on mosquito-borne viruses in their vectors 1: the normal fine structure of the midgut epithelium of the adult female *Aedes aegypti* (L.) and the functional significance of its modifications following a blood meal. *Trans. R. Soc. Trop. Med. Hyg.* 55: 404–423.
- Billingsley, P. F. and W. Rudin (1992) The role of the mosquito peritrophic membrane in bloodmeal digestion and infectivity of *Plasmodium* species. *J. Parasitol.* 78: 430–440.
- Chadee, D. D. and J. C. Beier (1995) Blood-digestion kinetics of four *Anopheles* species from Trinidad, West Indies. *Ann. Trop. Med. Parasitol.* 89: 531–540.
- Chien, L. J., T. L. Liao, P. Y. Shu, J. H. Huang, D. J. Gubler and G. J. Chang (2006) Development of real-time reverse transcriptase PCR assays to detect and serotype dengue viruses. *J. Clin. Microbiol.* 44: 1295–1304.
- Devenport, M. and M. Jacobs-Lorena (2005) The peritrophic matrix of hematophagous insects. In *Biology of Disease Vectors* (W. C. Marquardt, ed.). Elsevier Academic Press, Amsterdam, pp. 297–310.
- Freyvogel, T. A. and W. Staubli (1965) The formation of peritrophic membrane in Culicidae. *Acta Trop.* 22: 118–147.
- Gomes, A. L., A. M. Silva, M. T. Cordeiro, G. F. Guimarães, E. T. Jr. Marques and F. G. Abath (2007) Single-tube nested PCR using immobilized internal primers for the identification of dengue virus serotypes. *J. Virol. Methods* 145: 76–79.
- Gubler, D. J. (2002) The global emergence/resurgence of arboviral diseases as public health problems. *Arch. Med. Res.* 33: 330–342.
- Gusmão, D. S., V. Páscoa, L. Mathias, I. J. C. Vieira, R. Braz-Filho and F. J. A. Lemos (2002) *Derris* (*Lonchocarpus*) *urucu* (*Leguminosae*) extract modifies the peritrophic matrix structure of *Aedes aegypti* (Diptera: Culicidae). *Mem. Inst. Oswaldo Cruz.* 97: 371–375.
- Huang, C. Y., S. Butrapet, D. J. Pierro, G. L. Chang, A. R. Hunt, N. Bhamarapravati, D. J. Gubler and R. M. Kinney (2000) Chimeric dengue type 2 (vaccine strain PDK-53)/dengue type 1 virus as a potential candidate dengue type 1 virus vaccine. *J. Virol.* 74: 3020–3028.
- Ibrahim, G. H., C. T. Smartt, L. M. Kiley and B. M. Christensen (2000) Cloning and characterization of a chitin synthase cDNA from the mosquito *Aedes aegypti*. *Insect Biochem. Mol. Biol.* 30: 1213–1222.
- Jacobs-Lorena, M. and M. M. Oo (1996) The peritrophic matrix of insects. In *The Biology of Disease Vectors* (B. J. Beaty and W. C. Marquardt, eds.). University Press of Colorado, Colorado, pp. 318–332.
- Kato, N., R. Dasgupta, C. Smartt and B. M. Christensen (2002) Glucosamine: fructose-6-phosphate aminotransferase: gene characterization, chitin biosynthesis and peritrophic matrix formation in *Aedes aegypti*. *Insect Mol. Biol.* 11: 207–216.
- Kato, N., C. R. Mueller, J. F. Fuchs, V. Wessely, Q. Lan and B. M. Christensen (2006) Regulatory mechanisms of chitin biosynthesis and roles of chitin in peritrophic matrix formation in the midgut of adult *Aedes aegypti*. *Insect Biochem. Mol. Biol.* 36: 1–9.
- Lanciotti, R. S., C. H. Calisher, D. J. Gubler, G. J. Chang and A. V. Vorndam (1992) Rapid detection and typing of dengue viruses from clinical samples by using reverse

- transcriptase-polymerase chain reaction. *J. Clin. Microbiol.* 30: 545–551.
- Lehane, M. J. (1997) Peritrophic matrix structure and function. *Annu. Rev. Entomol.* 42: 525–550.
- Luna, L. G. (1968) *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology*. McGraw-Hill Book Company, New York. 158 pp.
- Miller, B. R. and C. J. Mitchell (1986) Passage of yellow fever virus: its effect on infection and transmission rates in *Aedes aegypti*. *Am. J. Trop. Med. Hyg.* 35: 1302–1309.
- Mitsuhashi, W., H. Kawakita, R. Murakami, Y. Takemoto, T. Saiki, K. Miyamoto and S. Wada (2007) Spindles of an entomopoxvirus facilitate its infection of the host insect by disrupting the peritrophic membrane. *J. Virol.* 81: 4235–4243.
- Morlais, I. and D. W. Severson (2001) Identification of a polymorphic mucin-like gene expressed in the midgut of the mosquito *Aedes aegypti*, using an integrated bulked segregant and differential display analysis. *Genetics* 158: 1125–1136.
- Moskalyk, L. A., M. M. Oo and M. Jacobs-Lorena (1996) Peritrophic matrix proteins of *Anopheles gambiae* and *Aedes aegypti*. *Insect Mol. Biol.* 5: 261–268.
- Okuda, K., A. de Souza Caroci, P. E. Ribolla, A. G. de Bianchi and A. T. Bijovsky (2002) Functional morphology of adult female *Culex quinquefasciatus* midgut during blood digestion. *Tissue Cell.* 34: 210–219.
- Okuda, K., A. Caroci, P. Ribolla, O. Marinotti, A. G. de Bianchi and A. T. Bijovsky (2005) Morphological and enzymatic analysis of the midgut of *Anopheles darlingi* during blood digestion. *J. Insect Physiol.* 51: 769–776.
- Pascoa, V., P. L. Oliveira, M. Dansa-Petretski, J. R. Silva, P. H. Alvarenga, M. Jacobs-Lorena and F. J. Lemos (2002) *Aedes aegypti* peritrophic matrix and its interaction with heme during blood digestion. *Insect Biochem. Mol. Biol.* 32: 517–523.
- Perrone, J. B. and A. Spielman (1988) Time and site of assembly of the peritrophic membrane of the mosquito *Aedes aegypti*. *Cell Tissue Res.* 252: 473–478.
- Peters, W. (1992) Peritrophic membranes. In *Zoophysiology* (S. D. Bradshaw, W. Burggren, H. C. Heller, S. Ishii, H. Langer, G. Neuweiler and D. J. Randall, eds.). Springer-Verlag, Berlin, pp. 1–238.
- Santos, J. N., R. M. Lanfredi and P. F. P. Pimenta (2006) The invasion of the midgut of the mosquito *Culex* (*Culex quinquefasciatus* Say, 1823 by the helminth *Litomosoides chagasfilhoi* Moraes Neto, Lanfredi and De Souza, 1997. *J. Invertebr. Pathol.* 93: 1–10.
- Secundino, N. F., I. Eger-Mangrich, E. M. Braga, M. M. Santoro and P. F. Pimenta (2005) *Lutzomyia longipalpis* peritrophic matrix: formation, structure, and chemical composition. *J. Med. Entomol.* 42: 928–938.
- Shao, L., M. Devenport and M. Jacobs-Lorena (2001) The peritrophic matrix of hematophagous insects. *Arch. Insect Biochem. Physiol.* 47: 119–125.
- Shao, L., M. Devenport, H. Fujioka, A. Ghosh and M. Jacobs-Lorena (2005) Identification and characterization of a novel peritrophic matrix protein, Ae-Aper 50, and the microvillar membrane protein, AEG12, from the mosquito, *Aedes aegypti*. *Insect Biochem. Mol. Biol.* 35: 947–959.
- Stamm, B., J. D'Haese and W. Peters (1978) SDS gel electrophoresis of proteins and glycoproteins from peritrophic membranes of some Diptera. *J. Insect Physiol.* 24: 1–8.
- Staubli, W., T. A. Freyvogel and J. Suter (1966) Structural modifications of the endoplasmic reticulum of midgut epithelial cells of mosquitoes in relation to blood intake. *J. Microsc.* 5: 189–204.
- Tellam, R. (1996) The peritrophic matrix. In *Biology of the Insect Midgut* (M. J. Lehane and P. F. Billingsley, eds.). Chapman and Hall, London, pp. 86–108.
- Tellam, R. L., G. Wijffels and P. Willadsen (1999) Peritrophic matrix proteins. *Insect Biochem. Mol. Biol.* 29: 87–101.
- Terra, W. R. (1990) Evolution of digestive systems of insects. *Annu. Rev. Entomol.* 35: 181–200.
- Terra, W. R. (2001) The origin and functions of the insect peritrophic membrane and peritrophic gel. *Arch. Insect Biochem. Physiol.* 47: 47–61.
- Villalon, J. M., A. Ghosh and M. Jacobs-Lorena (2003) The peritrophic matrix limits the rate of digestion in adult *Anopheles stephensi* and *Aedes aegypti* mosquitoes. *J. Insect Physiol.* 49: 891–895.
- Wang, P. and R. R. Granados (2001) Molecular structure of the peritrophic membrane (PM): identification of potential PM target sites for insect control. *Arch. Insect Biochem. Physiol.* 47: 110–118.
- Weaver, S. C. and T. W. Scott (1990) Peritrophic membrane formation and cellular turnover in the midgut of *Culiseta melanura* (Diptera: Culicidae). *J. Med. Entomol.* 27: 864–873.
- Wijffels, G., S. Hughes, J. Gough, J. Allen, A. Don, K. Marshall, B. Kay and D. Kemp (1999) Peritrophins of adult dipteran ectoparasites and their evaluation as vaccine antigens. *Int. J. Parasitol.* 29: 1363–1377.
- Zhuzhikov, D. P. (1964) Function of the peritrophic membrane in *Musca domestica* L. and *Calliphora erythrocephala* Meig. *J. Insect Physiol.* 10: 273–278.
- Zimmermann, D. and W. Peters (1987) Fine structure and permeability of peritrophic membranes of *Calliphora erythrocephala* (Meigen) (Insecta: Diptera) after inhibition of chitin and protein synthesis. *Comp. Biochem. Physiol.* 86: 353–360.