This review briefly focuses on the role of mosquito salivary glands; and on the biological processes and mechanisms relevant to transmission of malarial parasite (*Plasmodium*), the causative agent for malaria. A key requirement for transmission of the parasite is an infected blood meal which initiates parasite transmission cycle. The blood feeding is an organized biological mechanism which involves use of anticoagulants that cause severe immune reaction by the host, and minimizing the parasite load for its survival. The malarial parasite in the form of a sporozoite initially produced in the midgut-stage oocysts, travels to the salivary glands of blood-sucking female anopheline mosquito vector with a possible exploitation of specific receptors, if any, by it. During the development of *Plasmodium* in the mosquito midgut, sporozoites burst out of developing oocysts into the hemocoel (an open circulatory system in the insects) to locate and invade salivary glands prior to transmission to a vertebrate host. It is not only an obligatory step, but also seems to facilitate complete maturation of infection-competent sporozoites. The molecular events, especially recognition mechanisms between the sporozoite and salivary glands are poorly characterized, and a clear understanding is certainly expected to identify novel targets for further studies aimed at interrupting parasite transmission cycle.

Structure and gross anatomy of salivary gland

The paired salivary glands of mosquitoes are present in the thorax flanking the oesophagus (Figure 1). Each gland has three lobes, two lateral and one median. In the female mosquito the lateral lobes are formed by proximal, intermediate and distal regions. The median lobe on the other hand, is formed by a short neck region and a distal region. The extreme anterior part of each gland is innervated and the ingluvial ganglia situated at the junction of fore-gut and midgut (Figure 1), supply neurosecretory axons to the gland. Each lobe has a central duct constituted by a layer of epithelial cells that are bound externally by a basal lamina. The ducts from each lobe fuse so as to form a lateral salivary duct which runs forward and fuses with the one from the other gland to form a common salivary duct which opens at the base of the hypopharynx. The extracellular apical cavities of the posterior regions of female salivary glands are highly dilated with salivary secretions. It is interesting to note that the male salivary glands though tri-lobed, are much

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smaller than the female gland, and the protein profile of the male gland resembles that of the proximal region of lateral lobes of the female salivary glands. Equally interesting is the absence of whole median lobe and the intermediate region of the lateral lobes in non-blood-sucking mosquitoes like Toxorhynchites brevipalpis and the absence of polytene chromosomes in the adult mosquitoes.

**Salivary glands, blood feeding and immuno-modulation**

The salivary glands of mosquitoes perform the following role: (i) facilitate blood feeding; (ii) transmit parasites; (iii) minimize parasite infection; (iv) produce chemical stimuli like xanthuranic acid for completion of parasite life cycle; and, (v) have probable receptors for recognition of sporozoites.

During an insect bite, the salivary glands release components that include antihistamines, vasodilators like tachykinin, anticoagulants like thrombin- and Fxa-directed molecules and immunomodulators, in order to facilitate entry of inoculum containing pathogens. The salivary components of vectors have been implicated to be of importance in transmission of pathogens (viral, bacterial and protozoan) by ticks, mosquitoes, or other blood-sucking arthropods like star tick (Amblyomma americanum) bear proteaglandin E-2 (PGE-2) receptor which stimulates secretion of an anticoagulant in order to facilitate blood feeding. Similarly, another blood-feeding insect Rhodnius releases an anticoagulant, prolixin-S that binds to the smooth muscles of the blood vessels and relaxes them for efficient blood feeding. Certain studies have revealed that the salivary secretions of Phelebotomus duboscqui and Lutzomyia longipalpis influence the growth and development and transmissibility of Leishmania, while others reflect the immunosuppressive effect of salivary contents that affects the clinical manifestation of Leishmania infections in rodent models and in the natural hosts.

In Aedes aegypti, the saliva was first reported to have vasodilating functions and the presence of tachykinins was reported much later. Vasodilation is of major importance in facilitating efficient blood feeding, and thus for passage of pathogens and parasites. The tachykinins are peptides which act as vasodilators, with varied pharmacological functions on the central nervous system, cardiovascular and glandular tissues. Besides acting as a vasodilator, saliva inhibits the platelet aggregation and has anticoagulant properties. However, the biological functions of blood-feeding insects have evolved independently in the 13 different families of hematophagous insects. The vasodilators increase the diameter of blood vessels to allow greater blood flow and show the presence of vasoactive peptides, sailokinin I and II in Aedes. In vitro clotting assays have revealed the presence of thrombin-directed anticoagulants in Anophelines (malaria vectors), while Culicines had Fxa-directed anticoagulants. Besides these observations, the anticoagulants of Culex quinquefasciatus are twice more potent than those of the Ae. aegypti. Similarly, the saliva of ticks (Ornithodorous moubata) was reported to be heat labile and has thrombin-directed activity in the salivary glands, coxal fluid and egg extracts. Some of the activity was also Fxa-directed.

The hematophagous insects play a crucial role in the transmission of many parasites and pathogenic organisms. During the short time (seconds) of blood feeding, anophelines, for example, inject sporozoites (10–1000, exact number not known) along with other salivary-gland discharges. Once in blood circulation the sporozoites begin the malaria cycle in the susceptible host (Box 1). While facilitating the ingestion of blood meal of the mosquito, the salivary glands of the insect also express defence molecules for minimizing parasite infections for its survival.

Saliva also exhibits immunomodulatory activities by suppressing or enhancing the host immune response. Adenosine deaminase was found in the Lutzomyia sandfly. Sequencing of a subtracted cDNA library from the salivary gland of the sandfly revealed similarities to gene products of adenosine deaminase family. Other studies also reveal that the saliva protects the parasite, e.g. in Leishmania major. This was observed by comparing the inoculum numbers, wherein 1–100 parasites injected by the sandfly vector were efficient in causing leishmaniasis in experimental mice, compared to millions of parasites infected in hosts.

**Box 1. The malaria cycle**

Sporozoites invade the liver and undergo asexual schizogony (> 6–14 days), releasing thousands of merozoites which subsequently invade the erythrocytes to form rings, trophozoites, schizonts, male and female gametocytes, and thus complete the life cycle in the vertebrate host. Thereafter, the mosquitoes pick up gametocytes to form gametes that fertilize (within 20–30 min) and the zygotes transform into oocysts (16–30 h). The oocysts pass through the peritrophic membrane, surrounding the food bolus, cross through the epithelial cells and form oocysts on the midgut wall facing the hemocoele. The oocysts produce sporozoites (total time ~ 10–14 days after ingestion of the blood meal). Subsequently, the sporozoites released into the hemolymph invade the salivary glands and await introduction into a host during the next blood-feeding process. Mosquitoes thus acquire parasites as erythrocytic gametocytes and deliver parasites as sporozoites during the blood-feeding process.
needed by syringe delivery. Pre-exposure of mice to saliva of uninfected sandfly, on the other hand, was shown to protect animals against infectious L. major challenge.

Mosquito bites result in skin eruptions causing pruritic weals which could sometimes result in necrotic lesions. The immediate reactions consist of a pruritic weal with a surrounding flare or erythema which peaks at 30 min post-bite. It was also observed that IgE, lymphocyte, local IgG and immune-complex-mediated hypersensitivities are involved in allergy to Ae. vexans and Ae albopictus\(^{30}\). The hypersensitive responses could be classified into immediate (types I and III which depend on the interaction of antigen with humoral antibody, manifest within 30 min and disappear within 3 days), and delayed-type hypersensitivity (type IV hypersensitivity which involves cell-mediated immune responses and takes a longer time course to manifest). The impact of such activities on infectivity and transmission of malaria parasite has not been worked out.

**Salivary gland components with diverse functions**

**D7-like fragments of Anopheles**

Expression of salivary gland-specific genes was first characterized in A. aegypti\(^{25,31}\), and more recently in An. gambiae\(^{32}\). The A. aegypti D7 gene corresponds to a 37 kDa polypeptide present in the saliva, which is encoded in five exons separated by small introns\(^{33}\). Isolation and sequencing of 15 unique cDNA fragments from the salivary glands of An. gambiae (150–550 bp) following immuno-screening in COS-7 cells have recently been reported\(^{34}\). Three of these cDNAs, i.e. D7r1(dB1), D7r2 (iB6) and D7r3(iC5) show a high degree of resemblance to the D7 and apyrase genes of the salivary glands of A. aegypti. These clones hybridize closely to chromosomal positions on the right arm of the third chromosome in the division 30A (D7r2) and 30B (D7r1 and D7r3). The other three of the six D7-related cDNAs are new and have not been reported before. These clones are specifically expressed in the female salivary glands only. Although the exact functions of D7 are unknown, their stage-, sex- and tissue-specificity and location in the secretory cavities suggest their potential role in blood feeding and/or parasite transmission.

**Apyrase gene product**

It is a secretory protein which hydrolyzes ATP and ADP to AMP and P\(_i\), and has been shown to inhibit the ADP-induced platelet recruitment and aggregation. A few studies have revealed\(^{35,36}\) a relationship between sporozoite infection and the time of probing with a one-third decrease of apyrase activity in P. gallinaceum-infected A. aegypti. The apyrase activity is more confined to the distal regions of the female salivary glands only\(^{37}\). It has also been observed to facilitate mosquito feeding by inhibiting platelet recruitment and aggregation at the site of mosquito bite. The gene has evolved by duplication followed by divergent evolution from membrane-bound 5'-nucleotidase due to loss of carboxyl terminus domain involved in membrane-anchoring\(^{38}\). Recently, molecular cloning of An. gambiae homologue of Aedes apyrase has been described\(^{32}\).

**Defence molecules**

Induction of a 30 kDa protein in the salivary glands of An. stephensi in response to infection by Plasmodium yoelii yoelii has been suggested to impart tolerance to parasite infections in mosquitoes\(^{39}\). On the other hand, An. gambiae show an innate immune response after infection by malaria parasites. The molecular markers for such responses include a nitric oxide synthase (NOS) gene fragment and ICHIT (a gene encoding two putative chitin-binding domains separated by poly threonine-rich mucin region). Interestingly, the salivary gland shows the presence of six immune markers, which raises the possibility that these glands act as immune organs. They respond late in the infection, i.e. induction of NOS observed on day-9 post-infection, defensin, ICHIT and NOS on day-11, and between days 13 and 21 post-infection, respectively\(^{40}\). What is even more fascinating as well intriguing is the observation that the salivary glands did not show any immune induction following the second blood meal. The salivary gland of Ae. aegypti has been reported to show bacteriolytic lysozyme activity\(^{41}\). The production of defence molecules by salivary glands of other dipterans has been reported in the distal lobes of the salivary glands of Drosophila melanogaster. The glands express an anti-fungal compound called drosomycin\(^{42}\). It is possible that the expression of immune molecules from the salivary glands of mosquitoes might decrease microbial infection during feeding, which could also be beneficial for the malarial parasite.

**Molecular recognition between sporozoites and salivary glands**

**Parasite components**

Circumsporozoite (CS) and thrombospondin-related anonymous protein (TRAP) are two well-established molecules expressed in the sporozoites. They participate in a variety of processes ranging from maturation of sporozoites in the oocyst stage of the Plasmodium to gliding motility and subsequent invasion of salivary glands. Direct-
binding studies have revealed specific binding of recombinant CS protein to the salivary gland, and a peptide encompassing region I (highly conserved sequence found in all rodent and primate *Plasmodium CS* proteins) inhibited binding of CS protein to the salivary gland. This pointed to a receptor-mediated process for invasion of salivary glands by sporozoites, although the mechanism of invasion remains unknown. Targeted gene disruption studies with TRAP have revealed some role played by this parasite molecule in its motility leading to subsequent invasion of the salivary glands. This was revealed by the substitution of the conserved residue of the A-domain or a deletion in the TSP-motif of *P. falciparum* TRAP gene (*PfTRAP*) which resulted in an inability of the sporozoite to invade the salivary glands. The role of CS protein in motility and infectivity of sporozoites has also been shown by inter-strain replacement of CS protein gene in two versions at regions I and II (conserved motifs; disruption of region II impaired motility). Similarly, significance of MAEBL protein localized in the micronemes, in sporozoite invasion and attachment to the surface of salivary glands, was revealed by the targeted disruption experiments.

**Salivary gland components as putative receptors for *Plasmodium***

The fascinating series of biological studies by Rosenberg, directly suggested the involvement of highly species-specific (parasite and *Anopheles*) receptor–ligand interactions in the processes leading to invasion of salivary glands by *Plasmodium* sporozoites. A few studies published since this initial study, have attempted to characterize the process of sporozoite–salivary gland interactions.

A high invasion rate of *P. gallinaceum* sporozoites within 6 h was reported, which did not increase further after 24 h post-injection of sporozoites obtained from the oocysts of *A. aegypti*. Treatment of salivary glands with purified IgG from rabbit polyclonal antiserum against salivary gland extracts or monoclonal antibodies or lectins, blocked sporozoite invasion in *vivo*. Seven of the 19 lectins bound to salivary glands also include succinylated wheat germ agglutinin and wheat germ agglutinin. The authors suggested that the sporozoites interact with the glycosylated salivary gland surface molecules present in the salivary gland basal lamina, which might function as receptors for binding and invasion.

Using monoclonal antibodies raised against salivary gland proteins of female *An. gambiae*, Brennan et al. characterized two proteins of approximately 100 and 29 kDa molecular weights (non-reducing SDS–PAGE). The localization of these two proteins in the median and lateral female-specific lobes was demonstrated by the use of an indirect immunofluorescence and immunoelectron microscopy (Figure 2a–d). These proteins exhibited tissue- and sex-specificity as well as size polymorphism among various species and genera of mosquitoes. The inhibitory activity of the monoclonal antibody recognizing the 100 kDa protein was demonstrated in an *in vivo* assay by the authors.

The 100 kDa proteins (doublet) have recently been shown to exist as disulphide-bonded dimers of two immunologically identical subunits (Okulate and Kumar, unpublished, pers. commun.). Following an infected blood meal, the mosquitoes were subsequently fed antibodies nine days later followed by detection of sporozoites that invaded salivary glands (4–5 days after the antibody feed). The salivary glands from mosquitoes fed with antibody recognizing the 100 kDa protein had 73% fewer sporozoites compared to the glands from mosquitoes fed control or antibodies against the 29 kDa protein. The sporozoite invasion assay employed in this study emulates the natural course of malaria transmission (Figure 2e, f), as opposed to injection of purified sporozoites in almost all the previous studies†. These studies thus raise the possibility that molecules such as the 100 kDa protein identified by Brennan et al. might act as a putative receptor and warrant further characterization at the molecular level. It is highly possible that additional molecules might also participate in the processes leading to recognition of the sporozoite and subsequent invasion of the salivary gland. The recently completed genome sequence of *An. gambiae* will undoubtedly aid in detailed molecular characterization of such receptors. The genomic sequence information is already paving the way for the characterization of genes expressed in various tissues of the mosquito in response to a variety of physiological and pathological stimulations.

A phage display library was used to search for the ligands of malaria parasite on the epithelium of salivary glands and midgut. A 12 amino-acid peptide bound to distal lobes of salivary glands and to the luminal side of the midgut epithelium. It was observed that SM1 peptide strongly inhibited the *P. berghei* invasion of salivary glands and mid-gut. The peptide was identified by a phage display library from about 10⁹ different phages. In order to select phages displaying peptides having affinity to salivary glands or midgut, 10¹³ phages were injected into the hemocoel of mosquitoes (*An. stephensi*) and allowed to disperse for about 30 min. Subsequently, the salivary glands and midguts were dissected to ultimately characterize the peptide sequence displayed by the bound phage particles. A peptide with an unique amino acid sequence (PCQRAIFQSICN) was recovered following elutions from both the tissues, raising a possibility of a common ligand used by different invasive parasite stages (oocystes in the midgut and sporozoites in the salivary gland). In a recent study, transgenic mosquitoes expressing the peptide sequence have been found to exhibit reduced susceptibility to infection by *P. berghei*.  

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Concluding remarks

Salivary glands of mosquitoes perform several functions for effective survival of the insect, while maintaining the tenacity to harbour pathogens and parasites. The acute structural design and physiology of the salivary gland makes it an effective organ to perform various functions conducive for blood feeding and parasite transmission. At the histological level, the gland is made up of epithelial cells surrounded by the basal lamina so as to enclose a central canal that opens up in the hypopharynx, which results in direct inoculation or release of blood infected with parasites in the vertebrate hosts. Continuous blood feeding is possible due to the pharmacological attributes of the salivary secretions which are anti-hemostatic, anti-coagulatory and vasodialatory in nature. In the case of mosquitoes, the difference in the content of salivary proteins between the two sexes of *An. gambiae* indicates the importance of female-specific proteins in sporozoite recognition by the female. Expression patterns and persistence of such proteins in specialized anatomical locations have suggested their putative involvement in the recognition of sporozoites and subsequent invasion. It is not surprising that only a few select female-specific salivary gland proteins will ultimately function as putative receptors. Indeed, out of the two proteins identified by Brennan *et al.*, monoclonal antibodies directed against an epitope in only one protein (non-reduced molecular weight ~ 100 kDa) were effective in inhibiting sporozoite numbers in the invaded salivary glands, suggesting a potential role for this protein as a putative parasite receptor. Another significant information has been recently revealed about yet another likely candidate (*SM*1) common to the salivary gland and mid-gut, by use of phage display library. The interaction of *SM*1 peptide with both the malarial parasite and the distal lobes of salivary glands also suggests recognition of a common ligand. Invasion of the distal but not the proximal lobes of the gland compels one to believe that the parasite recognizes specific receptor/s of the gland.

Figure 2. Immuno-electron and indirect immunofluorescence microscopy of female. *An. gambiae* salivary glands. *a* and *b*, Binding of 2A3 and C26 monoclonal antibodies to the distal lateral lobes of the salivary glands respectively (× 15,600 magnification). *c* and *d*, Diffuse dispersion of the 29 kDa and 100 kDa proteins on female-specific lobes of salivary glands revealed by immunofluorescence assay. *e*, *In vivo* binding of fed monoclonal antibodies to salivary glands and *f*, *in vivo* blocking of sporozoite invasion of salivary glands; female *An. gambiae*-fed monoclonal antibodies in a blood meal and salivary gland-bound antibodies detected by ELISA. Inhibitory ascites factor, if any, was eliminated by performing a corresponding experiment using ammonium sulphate-precipitated mouse ascites (Modified from Brennan *et al.*; Copyright© 1993–2001, National Academy of Sciences, USA.).
transmission, physiology of blood feeding, specific molecular candidates participating in the interactions between parasites and different tissues of the mosquito, molecular changes associated with parasite infectivity, etc. Functional genomic analysis using micro-arrays is already providing valuable information about numerous physiological responses of the malaria vector, and such approaches would hopefully lead to novel strategies for intervention of malaria transmission.33 Similarly, gene expression analysis in sporozoites isolated from midguts and from those that successfully invaded salivary glands, has revealed specific molecular changes.34 Availability of genome sequences of humans, Plasmodium and anopheline mosquitoes opens up possibilities to decipher physiological, biochemical and molecular events involved in host–parasite–vector transmission cycles.


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