Chapter 2.

REVIEW OF LITERATURE
2. REVIEW OF LITERATURE

2.1. Malaria: Brief History

Man-malaria relationship started somewhere in tropical Africa at the dawn of humanity (Livingstone, 1958). With the Neolithic revolution, followed by further growth of humans and their migration, the infection spread and established itself in most of the tropical world and much of the land in the temperate climates via, riverain civilization in Mesopotamia (India), South China, Nile valley and in the Mediterranean shores (Bruce-Chwatt, 1965). Indians suffered from a number of epidemic diseases including malaria for thousands of years before recorded history, as surmised from many references to deadly fevers mentioned in the writings of the Vedic (1500-1800 BC) and the Brahmanic (800BC to 100 AD) periods.

As a milestone in the history of malaria, the parasitic agent of the malaria was discovered by Laveran in 1880, in a drop of blood from a young soldier admitted with intermittent fever. He named the parasite *Oscillaria malariae*. In 1885, Marchiafava and Celli assigned the term ‘Plasmodium’ to the malaria parasite. The natural transmission of malaria remained a mystery until Sir Ronald Ross observed and identified oocysts in the midgut of *Anopheles* mosquito in 1897 in India. Just after Ross’s work, complete developmental cycles of the *Plasmodium vivax* and *P. falciparum* were described (Grassi et al., 1899).

2.1.1. Malaria History: Control Vs Eradication

Quinine, the wonder drug was discovered around seventeenth century much before the first description of *Plasmodium* in 1880 and was successfully used for malaria control in 1900 for the first time (Koch, 1900). Besides drug administration, other control strategies used during that period were the use of larvivorous fishes in breeding habitats (Howard, 1901), source reduction (Darling, 1910, Gorgas and Henderick, 1924), mosquito larvicidal oil (Howard, 1930), space spraying of Pyrethrum (Covel, 1941) or reduction of man-mosquito contact by personal protection (Russel, 1955).
Dichloro Diphenyl Trichloroethane (DDT) was prepared in 1874 by Zeilder, a Swiss Chemist while looking for a substance active against cloth moths. The DDT remained obscure till the discovery of its insecticidal properties in 1939 and was soon used in agriculture (Muller, 1946). DDT signaled a new era of malariology (Pampana, 1948). World Health Organization (WHO) recommended nation-wide indoor residual spraying (IRS) of DDT supplemented by benzene hexa chloride (BHC) and dieldrin in 1948, to eradicate malaria (WHO, 1948a). Malaria Control Detection Teams (MCDTs) were set up by WHO in 1948 to train the local staff in DDT spraying and to carry out field research (WHO, 1948b). By the end of 1949, seven MCDTs were operating in Asia out of which four were functional in India (Pampana, 1951). In India, malaria control programme worked in analogy with WHO. Just after the introduction of DDT in malaria control, it was released for use in public health in 1944 (Afridi, 1962). Initial tests showed that DDT was the most potent and safe insecticide for use in public health. It was also a very cheap alternative to the control of insects of public health importance. Further developments in malaria control were based on the indoor residual spraying of DDT. One spray round of DDT adequately protected the target population for about 6 months. DDT was successfully used in Delhi (Afridi and Singh, 1947) and in Bombay (Visvanathan, 1950). In 1953, Government of India launched the National Malaria Control Programme (NMCP) to tackle the formidable challenge of malaria control in the country (Rao, 1955, Singh et al., 1957). The spectacular success in malaria control by DDT indoor residual spraying (IRS) paved the way for possibility of malaria eradication. NMCP was converted to National Malaria Eradication Programme (NMEP) in 1958 (NMEP, 1960). DDT acquired the reputation of a miracle insecticide and became popular almost instantly throughout the world.

The indoor residual spraying was coupled with proper surveillance and anti-malaria drug distribution in 1956 throughout the world (Pampana, 1963). The following two decades saw a tremendous improvement of the malaria situation. Malaria was eradicated from 18 countries and was nearly eradicated from other countries of Asian continent. In India, the annual malaria incidence fell from 75 million cases to an all time low of 0.1 million cases. Deaths due to malaria were completely eliminated from India. It
Figure 1. Malaria Profile of India 1961-2004.  
[Source: NVBDCP Data]

- **Pv**: Plasmodium vivax
- **Pf**: Plasmodium falciparum
- **Pv %**: Percent *Plasmodium vivax* positive
- **Pf %**: Percent *Plasmodium falciparum* positive
- **ABER**: Annual Blood Examination Rate
- **SPR**: Slide Positivity Rate

*Pv & Pf Cases in million*
was estimated that in one year alone i.e. 1965, NMEP prevented nearly 100 million episodes of malaria and 1 million deaths. NMEP successfully eradicated malaria by the mid-sixties from 3/4th of the country, and the programme was heading towards eradication. Various technical, logistic and financial problems coupled with the developing insecticide and drug-resistance led to an epidemiological degradation. Eventually the resurgence of malaria was witnessed during mid 1970s (Bruce-Chwatt, 1979). There were serious setbacks to the NMEP of India from 1968 onwards. Malaria, which had been nearly eradicated, started to raise its ugly head throughout the country. Epidemiological investigations revealed that in addition to the reverses in rural malaria control, malaria cases were multiplying in the urban areas. To control the rising trends of urban malaria, the Government of India launched the Urban Malaria Scheme (UMS) in 1971-72. A total of 132 towns were identified for UMS. Re-emergence of malaria continued during the late 1960s and early 1970s, and in 1976, 6.45 million parasite positive cases were reported by NMEP, highest since its resurgence (Figure 1). First chloroquine-resistant strains of *P. falciparum* were detected in 1973 in Assam (Sehgal *et al.*, 1973). Deaths due to malaria that had been completely eliminated became a regular feature of the programme. In order to reduce malaria morbidity and mortality, and protect the green revolution and industrial belts of the country, in 1977 the Government of India converted the malaria eradication programme to control programme under the Modified Plan of Operation (MPO) (Pattanayak and Roy, 1980). During 1977-1988, *Plasmodium falciparum* Containment Programme (P/CP) financed by the Swedish International Development Agency was launched to contain the spread of *P. falciparum* and drug-resistant parasite strains (Ray *et al.*, 1988). P/CP was terminated after 11 years of efforts to contain the diseases but at the end of the programme *P. falciparum* and drug-resistant parasite strains were widely spread in the country (Sharma, 1996, 2000). DDT at one time considered a miracle insecticide had lost all its effectiveness in vector control (Sharma, 2003). Malaria situation continued to deteriorate throughout India. In 1994 malaria epidemic in Rajasthan covered four western districts and in 1995 another four districts in the eastern side (Tyagi, 1997). Similar epidemics were reported from other parts of the country and an uncounted numbers at a smaller scale (Sharma, 1998).
World declaration on malaria, adopted in October 1992, committed all the member states to the worldwide intensification of control efforts against the disease. Accordingly, a Global Malaria Control Strategy (GMCS) emphasized the need to develop sustainable control programmes adapted to local needs. Indian malaria eradication programme, NMEP adopted Global Malaria Control Strategy of the WHO in 1995. In the same year, revised malaria control strategy was also launched (Anonymous, 1995). In 1997, the Government of India strengthened malaria control in predominantly tribal areas with the financial support of the World Bank (WB). This programme, known as Enhanced Malaria Control Project (EMCP) was launched in 1,045 primary health centers (PHCs) (100 districts, 8 states). In 1998, World Health Organization, the World Bank, the United Nations Development Program (UNDP) and United Nation Child Education Fund (UNICEF) launched ‘Roll Back Malaria’ (RBM) program with an aim to reduce the malaria morbidity and mortality to its half by 2010 and again halve by 2015. (Nabarro and Taylor, 1998, Narasimhan and Attaran, 2003). In 2000, NMEP of Government of India was re-christened as the National Anti Malaria Programme (NAMP). National health policy of India was reformulated on the principals of RBM in 2002 (NAMP, 2002). In 2003, based on the policy decision to integrate the control of all vector-borne diseases, NAMP was again re-named as the National Vector-Borne Disease Control Programme (NVBDCP). At present NVBDCP is the largest national programme of the Government of India (NVBDCP, 2004).

2.2 Malaria: Current Scenario

According to the first comprehensive report of Roll Back Malaria, 2005, some 3.2 billion people living in 107 countries and territories were at risk of malaria transmission in the year 2004. Globally, 300-500 million people contract the disease each year and it accounts for 1.7-2.5 million deaths annually. Africa alone contributes around 100 million clinical cases and more than 90% of malaria deaths per year (WHO, 2006). Besides Africa, malaria is a serious problem in countries of South East Asian Region like, Thailand, Nepal, Bhutan, Bangladesh, Mayanmar, Indonesia, Sri Lanka and India. South-East Asia has the highest rate of drug resistance in the world, and multi-drug resistance has contributed to the re-emergence of malaria in many areas, especially along
international borders (RBM, 2005). Among member countries of South East Asian Region Office (SEARO) of WHO, India contributes >85% cases annually. India is endemic for malaria except for some healthy areas in the mountainous regions above 1800 meter sea level and well-drained coastal areas along with Western and Eastern Ghats. In the island territories, Lakshadweep Islands are free from malaria while in Andaman and Nicobar Islands malaria is confined to the coastal areas only. More than 990 million population out of total 1050 million population in India are at risk of malaria. There are 2.5 to 3 million reported cases and 1000 deaths each year due to malaria (WHO, 2003). These malaria cases were highly under-reported; the estimates of WHO indicate about 81 million cases and 20,000 deaths in India in the year 2004 (Korenromp, 2005). This has also been brought out by several independent studies and the in-depth evaluations of the NMEP. Malaria epidemics have started spreading to the endemic areas. DDT is ineffective in most parts of the country due to resistance, hexachlorohexane (HCH) has been banned and malathion has high refusal rates due to its pungent smell. Wherever malathion has been sprayed, resistance in malaria vectors develops rapidly. Deltamethrin or cyfluthrin or lambda cyhalothrin (synthetic pyrethroid, SP) insecticides have been pressed into service particularly in areas showing rising trend of malaria, but the vectors have started showing resistance to SP insecticides (Raghavendra et al., 1991, Singh et al., 2002). Malaria vectors are either resistant to insecticides or they are primarily exophilic in nature e.g., vectors in north-eastern states. Either way indoor residual spraying (IRS) is not fully effective in the control of malaria. Resurgent malaria has entered new ecotypes requiring local knowledge of malaria epidemiology for cost-effective control (Pattanayak et al., 1994). The proportion for *P. falciparum* cases has increased from about 12% to 45% over the last two decades (Figure 1) and along with it the problem of drug-resistance in *P. falciparum*. Resistance to anti-malarial drugs in *P. falciparum* has spread in different geographical area and intensified from RI and RII levels to RIII level, and currently progressing from mono-to-multi-drug resistance (Dua et al., 2003). To estimate the burden of a disease, World Development Report, 1993 used a measure expressed as Disability Adjusted Life Years (DALY) (Murray, 1994). DALY is a measure that combines healthy life years lost because of premature mortality with those lost as a result of disability. DALY for malaria in India was estimated
approximately 0.47 million for women and 0.48 million for men (total DALY ~0.95 million) annually (WHO, 1999).

2.3. Human Malaria Parasites

*Plasmodium*, the malaria parasite belongs to the *Phylum*: Protozoan, *Class*: Telospora, *Order*: Hemosporidae and *Family*: Plasmodidae. Four species of the genus *Plasmodium* viz., *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale* are responsible for different types of human malaria. *P. falciparum* is responsible for hyper-endemicity in Africa where ~ 90% of malaria deaths occur each year (WHO, 2006). *P. vivax*, *P. falciparum* and *P. malariae* are reported from Indian subcontinent (Sharma, 1999). *P. vivax* predominates the malaria scenario in India with 60-65% of total number of cases followed by *P. falciparum* with 30-40% of cases while only a few malaria cases i.e. 2-3% of *P. malariae* are reported from the foothills of Orissa. The proportion of *P. falciparum* in India has been increasing since 1977 (Figure 1).

2.4. Anophelines

The menace of mosquitoes can be traced back to fifth century BC in the works of great ancient Indian sage ‘Sushruta’ in his doctrine ‘Sushruta Samhita’ (Russel, 1955). Mosquitoes, including anophelines have been known for a long time also in the west and in China. Christophers (1933) in his book ‘the Fauna of British India’ gave the systemics of 42 species and 10 varieties of anophelines in India. In the subsequent years, the number of anophelines discovered in India reached to 51 species and 7 sub-species by 1984 (Rao, 1984). So far, nearly 58 anopheline species have been reported from India (Nagpal and Sharma, 1995).

The *Plasmodium* parasite requires mosquito vector especially, anopheles for transmission (Ross, 1897). All anophelines are not vectors of malaria. There are a total of nearly 420 species of anophelines reported all over the world, out of which approximately 70 are known vectors of malaria (Nagpal and Sharma, 1995). In South Africa from where maximum cases of malaria deaths are reported, *Anopheles gambiae* is the most important
malaria vector. *An. gambiae* is responsible for 100 million clinical cases each year (Suh *et al.*, 2004). Out of the total 58 anophelines reported from India, ten *Anopheles* species are vectors of human malaria (Sharma, 1999). Among ten malaria vectors of India, six viz., *Anopheles culicifacies*-the rural and peri-urban malaria vector, *Anopheles fluviatilis*-the hilly and foothill vector, *Anopheles stephensi*-the urban vector, *Anopheles dirus*-forest vector, *Anopheles minimus*-vector of North-Eastern hills, and *Anopheles sundaicus*-the coastal vector, are primary vectors. Remaining four vectors viz., *Anopheles philippinensis*, *Anopheles annularis*, *Anopheles varuna* and *Anopheles jeyporiensis* are of secondary importance.

### 2.5. Sibling species complex

Presence of sibling species is a common phenomenon encountered among anophelines. Sibling species may be defined as a group of morphologically identical but reproductively isolated evolutionary units. Among the genus *Anopheles*, species *maculipennis* is the first species complex identified (Mayr, 1970). Members of a species complex, commonly known as sibling species or isomorphic or cryptic species.

#### Table 1. Sibling species of primary malaria vectors of India

<table>
<thead>
<tr>
<th>S.No</th>
<th>Species</th>
<th>Sibling species identified</th>
<th>Identified in India</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>An. culicifacies</em></td>
<td>5 (A, B, C, D and E)*</td>
<td>5 (A,B,C,D,E)</td>
</tr>
<tr>
<td>2</td>
<td><em>An. fluviatilis</em></td>
<td>3 (S, T and U)*</td>
<td>3 (S,T,U)</td>
</tr>
<tr>
<td>3</td>
<td><em>An. dirus</em></td>
<td>7 (A*, B*, C*, D*, E*, <em>An. nemophilus</em> and <em>An. takasagoensis</em>)</td>
<td>2 (D,E)</td>
</tr>
<tr>
<td>4</td>
<td><em>An. minimus</em></td>
<td>4 (A, B, C and D)*</td>
<td>1 (A)</td>
</tr>
<tr>
<td>5</td>
<td><em>An. sundaicus</em></td>
<td>3 (A, B and C)*</td>
<td>None</td>
</tr>
</tbody>
</table>

* Provisionally designated

are reproductively isolated evolutionary units with distinct gene pools, and hence, differ in biological characteristics that determine their potential in the transmission of disease. The discovery of this complex has resolved the epidemiological paradox that prevailed in the 1930s in Europe and North America. In some areas in southern Europe, there was no malaria in spite of the presence of *An. maculipennis*, which led to the famous saying in malariology "anophelism without malaria". Detailed studies on biological and cytogenetic characters of these populations have identified eight sibling species in this taxon in Europe. Species complexes are of common occurrence among different anopheline taxa. About 24 *Anopheles* taxa have been identified so far as species complexes and they are important vectors of malaria in different parts of the world (Subbarao, 1998). In India all the reported vectors except *An. stephensi* exist as a sibling species complex (Table 1).

2.5.1. *Anopheles culicifacies* Complex

*An. culicifacies*, the most important malaria vector of India is responsible for approximately 60% of the total malaria. For the first time *An. culicifacies* was colonized in laboratory by Ainsley (1976) in Pakistan and by Ansari *et al.* in 1977 in India at Malaria Research Center (In 2005 renamed as National Institute of Malaria Research). Following colonization of *An. culicifacies*, various basic studies regarding the biology, genetics and bionomics were initiated. The most important finding from these studies was the evidence of the existence of two biologically distinct species, species A and species B within the taxon *Anopheles culicifacies* Giles, reported by Green and Miles in 1980. Subsequently three more species, species C (Subbarao *et al.*, 1983), species D (Vasantha *et al.*, 1991) and species E (Kar *et al.*, 1999) have been reported within the taxon.

Many studies were intensified to devise the identification techniques for the diagnosis of the morphologically indistinguishable members of *An. culicifacies* complex. Green and Miles (1980) used diagnostic inversion- $X^{+a+b}$, $2^{+gi+hl}$ for the identification of species A and $X^{ab}$, $2^{gi+hl}$ for identification of species B. Structural differences of Y chromosome on mitotic karyotype were also observed and were used for distinguishing species A and species B (Vasantha *et al.*, 1982). Species C had inversion on h region of
Figure 2. The distribution of members of the *Anopheles culicifacies* complex in India.

Source: A Profile of Malaria Research Center (2002) published by Malaria Research Centre on completion of 25 years (1977-2002).
chromosome no 2 as compared with species A (Subbarao et al., 1983). The mitotic karyotype of species A and species C was identical. Cuticular hydrocarbon profiles of species A, species B and species C were also used to distinguish these species (Milligan et al. 1986). Further, electrophoretic variation of lactate dehydrogenase (Ldh) isozymes of the members of the species complex was used to distinguish species A and species D from species B and species C (Adak et al., 1994b). DNA probe hybridization assay-based on highly repetitive DNA sequences was also employed to distinguish species A from species B and C (Gunasekera et al., 1995). Subsequently, various PCR assays were developed for the identification of the members of An. culicifacies complex. Recently, allele-specific polymerase chain reaction (ASPCR) assay (Singh et al., 2004) and PCR-restriction fragment length polymorphism (PCR-RFLP) assay (Goswami et al., 2005) was used to distinguish species A and species D from species B, species C and species E of the An. culicifacies complex.

Various studies carried out in the direction of biological behavior of members of An. culicifacies complex brought to the light various note-worthy differences. Members of the species complex are widely distributed and have specific distribution pattern (Subbarao, 1988). The distribution of the members of An. culicifacies complex is shown in figure 2. Sympatric species of An. culicifacies complex vary in their proportions in different seasons of the year (Subbarao et al., 1987a). Species A and B, and species B and C of this taxon were reported to have both pre-mating and post-mating barriers while, only a pre-mating barrier was observed between species A and species C (Subbarao et al., 1988c). Studies on host feeding preferences have revealed that species A is more anthropophagic than species B (Joshi et al., 1988). Further, species A is more susceptible to DDT and malathion than species B (Subbarao et al., 1988b, Raghavendra et al., 1992) and species C develops resistance to malathion at a faster rate than species B (Raghavendra et al., 1991).

Members of An. culicifacies complex are reported to have differential disease transmission potential. Vector incrimination studies using Immunoradiometric assays (IRMA) revealed that species A, species C and species D are vectors of both Plasmodium
vivax and Plasmodium falciparum (Subbarao et al., 1988c, 1992). Only two specimens of
species B were positive for P. vivax sporozoites; one in northern India (Subbarao et al.,
1988c) and other in southern India (Suguna et al., 1983). Similar studies carried out in
Pakistan (Mahmood et al., 1984), Arabia (Akoh et al., 1984) and Iran (Zaim et al., 1993)
also showed that species A is a vector of malaria. Entomological and epidemiological
studies carried out in eastern districts of Uttar Pradesh and northern Bihar, revealed that
malaria incidence was significantly low in areas where species B was prevalent
(Subbarao et al., 1988d, Tiwari et al., 1994). All these studies unequivocally established
that the role of species B as a vector of malaria in India is negligible, if any (Sharma,
1999).

Susceptibility studies carried out in laboratory using members of An. culicifacies
complex and P. vivax revealed that the species B was least susceptible to P. vivax
infection while species A was the most susceptible (Adak et al., 1999). The susceptibility
of species C to P. vivax was in between species A and species B. The differential
susceptibility of the members of An. culicifacies complex is not confined to the human
malaria parasites alone. Species A, B and C of the species complex when fed upon rodent
malaria parasite Plasmodium vinckei petteri or Plasmodium yoelii yoelii infected mice,
showed almost similar trends in terms of susceptibility as to P. vivax. Species B was least
susceptible to both the rodent malaria parasites and species A was the most susceptible
(Kaur et al., 2000). Different strains of species B collected from different geographical
localities of India were found to be differentially susceptible to P. vivax (Adak et al.,
2006).

2.6. Host Parasite Interaction

2.6.1. Sporogony

The part of the life cycle of malaria parasite in mosquito vector is termed as
sporogony (Figure 3). Sporogony is a complex process and involves six transition stages
of parasite viz., gametocytes, gametes, zygote, ookinete, oocyst and sporozoite, within
the mosquito (Grassi, 1900). Gametocyte positive blood reaches gut lumen where
exflagellation takes place (Mac Callum, 1897). Exflagellation stimulates the gametes to
Figure 3. Immune response of *Anopheles* against developing malaria parasite.

fuse to form zygote. Zygote develops into motile ookinete within the gut and approaches
gut epithelium (Freyvogel, 1966). Peritrophic matrix (PM), a chitinous matrix secreted by
insects in their gut, physically separates the food material and the midgut epithelium
(Waterhouse, 1953). Either after traversing peritrophic matrix or prior to its formation
ookinetes invade midgut epithelium. To cross the midgut, ookinetes first adhere and then
penetrate specialized epithelial cells termed as Ross cells (Shahabuddin and Pimenta,
1998). The ookinetes that egress midgut epithelial cell settle beneath the basal lamina for
5-7 days and are then transformed into oocysts (Terzakis et al., 1966, Sinden and Strong,
1978). The cytoplasm of mature oocyst divides to form tiny sporozoites. Thousands of
sporozoites are liberated into the hemolymph. Sporozoites from hemolymph reach
salivary glands and invade the tissue (Sterling et al., 1973). Sporozoites in the salivary
glands are injected into vertebrate host along with saliva during an infective bite.

2.6.2. Mosquito Immunity

Mosquitoes are exposed to a number of microorganisms throughout their life
cycle. As a process of evolution and for the sake of their existence, mosquitoes, like other
organisms have developed immune mechanisms to suppress and/or inhibit the
development of malaria parasite and other microorganisms (Dimopoulos et al., 1997).
The only known defense mechanism in anophelines is innate immunity.

2.6.2.1. Mosquito Immunity: First line of defense

Innate immunity of mosquitoes involves first as well as second line of defense
(Gillespie et al., 1997). First line of defense or external defense includes physical and
chemical barriers to the entry of microorganisms into the body. Physical barrier include
hard chitinous exo-skeleton and peritrophic matrix (PM) secreted outside the blood bolus
(Waterhouse, 1953). Secretion of digestive enzymes, especially proteases, into the gut
lumen poses a chemical barrier to the developing parasite (Gass and Yeates, 1979).
Digestive enzymes affect the malaria parasites in a stage specific manner. Immature
ookinetes are susceptible to protease digestion while mature forms are relatively resistant
(Gass and Yeates, 1979).
2.6.2.2. Mosquito Immunity: Second line of defense

An invader, which successfully breaches the physical and chemical barriers of the first line of defense, confronts the second line of defense of the mosquito. The malaria parasite that invades midgut epithelium after escaping digestive enzymes and peritrophic matrix is recognized as a non-self by the mosquitoes (Gillespie et al., 1997). Non-self recognition involves patterns of the surface molecules (e.g., peptidoglycans and lipopolysaccharide) on the invader by the pattern recognition receptors (PRRs) of the mosquito. Recognition of non-self triggers synthesis of various immune effector molecules in the mosquito. Innate immunity of mosquitoes comprises of both humoral and cellular factors (Figure 3). Humoral response includes melanotic encapsulation (Paskewitz et al., 1988), synthesis of anti-microbial peptides (Hoffmann and Hoffmann, 1990, Bulet et al., 1999) and synthesis of nitric oxide synthase (Luckhart et al., 1998), whereas cell-mediated immunity includes phagocytosis (Bayne, 1990). Besides all these, some other unknown mechanism is also involved that lyses the oocysts in mosquito gut epithelium (Vemick et al., 1995, Adak et al., 2006).

2.6.3. Molecular Immune Response

2.6.3.1. Immune response: Transcript level

During the last decade, tremendous progress has been made in the understanding of mosquito immune responses at transcript and protein level. A set of diverse immune genes is transcriptionally activated in epithelial cells upon microbial challenges. Serine protease (G13) (Dimopoulos et al., 1996), cecropin (Vizioli et al., 2000) and gambicin (Vizioli et al., 2001) genes were strongly induced in An. gambiae upon bacterial challenge. Many genes such as those encoding chitin-binding protein (ICHIT), nitric oxide synthase (NOS), defensin and Gram-negative bacteria-binding protein (GNBP) opsonin of An. gambiae are responsive to both bacteria and Plasmodium infection (Richman et al., 1996, 1997, Dimopoulos et al., 1997, 1998, Luckhart et al., 1998). The activation of these malaria-induced genes correlates with the passage of the parasites through the mosquito both temporally and spatially (Richman et al., 1997, Dimopoulos et al., 1997). Dimopoulos et al., (1998) used six immune markers viz., NOS, a serine
protease like gene \((ISPL5)\), galactose-binding lectins \((JGALE\ 20)\), \(ICHIT\), defensin and \(GNBP\), to map the immune response of malaria vector \(An.\ gambiae\) against \(P.\ berghei\). All the six genes were induced in coordination with the presence of malaria parasite.

The process of gene discovery was very slow until the advent of Expressed Sequence Tags (EST). In a pilot gene discovery project, 2,380 clone clusters were obtained from a hemocyte based cell line using EST. Among these, 38 showed significant similarities to the classes of known innate immunity genes including gene encoding putative serine proteases and serpins, pro-phenoloxidases (PPOs), cecropins, pattern recognition receptors and signaling proteins (Dimopoulos et al., 2000). Out of the 38 putative immune-related clones, transcripts of 18 clusters were immune induced, and one, with similarity to putative \(Drosophila\) scavenger receptor homologue, was repressed by exposure to heat-killed bacteria. (Dimopoulos et al., 2000).

The immune response posed by a mosquito is specific for a group of invaders depending upon the specific patterns on the cell surface of the invader. The response of \(An.\ gambiae\) to the malaria parasite extensively overlaps with but not identical to the response to bacteria (Dimopoulos et al., 2002). Most of the malaria-induced genes were also up-regulated by bacteria, for example, gene encoding PGRP-LB (Peptido-glycan-recognition protein) receptor, the Gram-negative binding protein (GNBP) opsonin, a fibrinogen domain (FBN) lectin, a thio-ester containing putative opsonin (TEP), the serine protease Sp14D, the CED-6-like phagocytic adaptor, and the lucine rich repeats (LRR) putative receptor. However, some malaria-inducible genes are down-regulated by bacteria and sterile injury. The latter group includes genes encoding isocitrate dehydrogenase, dsRNA binding RNase 3, and mitochondrial phosphate carrier.

The immune responses of mosquitoes differ from species to species or strain to strain of parasite and/or mosquito. The defensin gene was induced upon infection of \(P.\ berghei\) in \(An.\ gambiae\) (Richman et al., 1997) but not with the infection of \(P.\ gallinaceum\) in \(Ae.\ aegypti\) (Lowenberger et al., 1999). Even in the same strain of \(An.\ gambiae\), rodent malaria parasite \(P.\ berghei\) and human malaria parasite \(P.\ falciparum\) elicited differential immune responses (Tahar et al., 2002).
2.6.3.2. Immune response: Protein level

Most of the studies carried out in the field of mosquito immunity are at the transcription level, despite of the fact that various immune molecules, especially those having a pro-peptide were post-translationally modified upon immune challenge for example defensins (Richman et al., 1996), serine proteases (Muller et al., 1996), pro-phenoloxidases (Soderhall and Cerenius, 1998) and cecropins (Vizioli et al., 2000). A number of changes in the protein profile of salivary glands (Shandilya et al., 1999), hemolymph (Gakhar and Shandilya, 2000) and midgut (Gakhar and Shandilya, 2001) of *An. stephensi* were recorded after successive time intervals of *P. yoelii yoelii* challenge. Chun et al. (2000) studied the polypeptide profile in the hemolymph of refractory and susceptible strains of *An. gambiae* and found a refractory strain specific 35 kDa polypeptide. They observed a number of immune-inducible polypeptides and one of them upon sequencing showed homology to serine proteases. Similarly, Paskewitz and Shi (2005a) studied the hemolymph proteome of *An. gambiae*. Upon sterile injury, a set of seventeen proteins were induced in *An. gambiae*. These included two isoform of phosphoglycerate mutase (PGM), triose phosphate isomerase (TPI), an actin, two glutathione-S-transferases (GST S1-1, S1-2) and adenylate cyclase. Fourteen bacteria inducible proteins were not induced upon sterile injury. The transcript abundance of light and heavy chains of ferritin decreased in *An. gambiae* following bacterial infection. Recently, Shi and Paskewitz (2006) reviewed the importance of proteomics in insect immunity with different possible methodologies and their application. They suggested proteomic studies complemented with work on insect transcriptomes will provide new directions for further investigation of insect immunity.

2.6.4. Immune-Related Genes and Gene Families

In an important advancement in the field of malaria, genomes of all the three organisms constituting the malaria triangle i.e., human host (Venter et al., 2001), anopheline vector (Holt et al., 2002) and *Plasmodium* parasite (Gardner et al., 2002) were sequenced. Analysis of the *An. gambiae* genome sequence revealed 278 million base pairs and a strong evidence for about 14,000 protein-encoding transcripts (Holt et
al., 2002). A total of 18 families of *An. gambiae* were analyzed for which comparative evidence from *Drosophila* and other organisms suggested involvement of 242 genes in immune responses (Christophides et al., 2002). These immune-related gene families of *An. gambiae* showed a marked deficit of orthologs and excessive gene expansions in relation to *Drosophila* and other organisms. In contrast, the multifunctional Toll signal transduction pathway is substantially conserved, presumably because of counter-selection for developmental stability (Christophides et al., 2002).

### 2.7. *Plasmodium*-Refractory strains

The immune responses posed by anophelines against malaria parasites are highly potent. In some extreme cases, the sporogony of malaria parasites is terminated. Mosquito strains that are capable of completely blocking the sporogony of malaria parasite are termed as refractory strains. Several refractory lines are known to restrict the development of the parasite either by melanotically encapsulating all of them or by killing the ookinetes within the midgut epithelium (Vernick et al., 1995). For the first time a *Plasmodium*-refractory strain of *An. gambiae* was genetically selected for naturally occurring resistance in field population (Collins et al., 1986). The refractory strain conferred full refractoriness to *P. gallinaceum* and simian malaria parasites viz., *P. cynomolgi*, *P. inui* and *P. knowlesi* and was partial refractory to human and rodent malaria parasites. Feldmann and Pondurai (1989) selected refractory strains of *An. stephensi* against *P. falciparum* but the criterion for refractoriness is reduction of susceptibility to *P. falciparum* rather than completely blocking the sporogony. Recently, Adak et al. (2006) had identified a naturally occurring refractory strain of species B of *An. culicifacies* complex. The refractory strain confers 100% refractoriness to human malaria parasite *P. vivax* and partial refractoriness to *P. falciparum* (Adak et al., 2006).

Subsequent to the genetic selection of *Plasmodium* refractory strains of *An. gambiae* in 1986, various studies were initiated to investigate the genetic basis of refractoriness. Vernick et al. (1989) found that an incompletely recessive autosomal gene, the *Plasmodium* infectivity factor-Ceylon strain (*Pif-C*), is involved in conferring refractoriness to *P. cynomolgi*. In addition, the line was fixed for a semi-dominant
refractoriness allele at an unlinked autosomal locus, Pif-B (*Plasmodium* infectivity factor of B strain). Zheng *et al.* (1997) identified a major quantitative trait locus (QTL), *Plasmodium* encapsulation 1 (*Pen 1*) and two minor loci *Pen 2* and *Pen 3* in *An. gambiae* responsible for the encapsulation of *P. cynomolgi* B strain. *Pen 1* alone is responsible for ~54% of phenotypic variance in refractory phenotype and *Pen 2* contributes ~13% of the trait. Altogether the three loci accounts for ~70% of the variance of melanotic encapsulation of *P. cynomolgi* strain B. *Pen 3* maps in the general area where the pro-phenoloxidase gene is located. A diphenoloxidase gene-A2 is genetically linked to *Pen 1* of the refractory phenotype (Romans *et al.*, 1999). The *Pen 1* locus is highly polymorphic and the polymorphism was found to be the determinant of vectorial capacity in a vector population (Thomasova *et al.*, 2002). The melanotic encapsulation of *P. cynomolgi* Ceylon strain in *An. gambiae* L3-5 is controlled by *Pen 2* and *Pen 3* which were also involved in the encapsulation of *P. cynomolgi* strain B and a novel QTL that maps to chromosome 3R (Zheng *et al.*, 2003).

Melanotic encapsulation is a well-studied mechanism of insect immunity. In lower insects (dipterans) with less number of hemocytes, the melanotic encapsulation is acellular (Gotz, 1986) which involves laying a thick acellular capsule of melanin around the invader. Pattern recognition of an invader activates serine protease through a cascade of signaling in between the cells (Soderhall and Cerenius, 1998). Serine protease in turn activates pro-phenoloxidase (PPO) to form active phenoloxidase (PO). Latter oxidizes mono-and di-phenols, which subsequently forms melanin (Zhao *et al.*, 1995). The melanin, thus, formed polymerize to arrest the invader. Various serine proteases, characterized and cloned from mosquitoes seem to be involved in mosquito immunity i.e., in melanotic encapsulation and/or anti-microbial peptide synthesis (Gorman *et al.*, 2000). Six pro-phenoloxidase (PPO) genes (named PPO1-6) were characterized from cell line (4a-3b) of *An. gambiae* (Muller *et al.* 1999). Although none of the PPO genes was induced upon bacteria infection, PPO1 through PPO4 were up-regulated following blood meal.
2.8. Gene silencing in mosquito immunity

To precisely investigate the role of immune molecules, gene knockdown strategy using respective dsRNA is in use these days. Silencing of defensin gene in *An. gambiae* has no significant effect on the development and morphology of the *Plasmodium berghei* midgut stages (Blandin *et al.*, 2002). Similarly in *Ae. aegypti*, silencing of defensin A does not increase the susceptibility of the mosquito to bacteria (Bartholomay *et al.*, 2004). In both the genera i.e., *Anopheles* (Blandin *et al.*, 2002) as well as *Aedes* (Bartholomay *et al.*, 2004) silencing of defensin does not seem to affect mosquito survival.

The dsRNA mediated silencing of thio-ester containing opsonin (*TEP1*) completely abolished refractoriness in a genetically selected refractory strain of *An. gambiae* (Blandin *et al.*, 2004). Moreover, in susceptible mosquitoes this knockdown increased the number of developing parasites. The authors suggested that the *TEP1*-dependent parasite killing is followed by a *TEP1*-independent clearance of dead parasites by lysis and/ or melanization (Blandin *et al.*, 2004). Similar results were obtained with dsRNA-mediated knockdown of leucine rich immune protein (*LRIM1*). *LRIM1* causes a substantial majority of the ookinetes to be killed while crossing the midgut, before oocyst formation (Osta *et al.*, 2004a). These findings were in agreement with the observations that the parasites injected directly into the hemolymph of the refractory mosquito i.e., upon bypassing midgut, escaped melanization (Paskewitz and Shi, 2005b).

All the molecules synthesized by mosquito in response to invading parasite are not detrimental to the invader. During a long process of co-evolution parasite somehow learned to use the host molecules to evade the immune reaction mounted against it (Adini and Warburg, 1999). Genes encoding C-type lectins (*CTL*) belong to the group of immunity genes of *An. gambiae* that are diversified from their *Drosophila* homologue (Christophides *et al.*, 2002). *In vivo* silencing of *CTL* genes in *An. gambiae* showed that the *CTL4* and *CTLMA2* gene products protect the ookinetes against melanization (Osta *et al.*, 2004a). Functional knockouts of either of these *CTLs* resulted in massive melanization of the invading ookinetes. Apparently, the surviving parasites in gut use
CTL4 and CTLMA2 and potentially other protective molecules of mosquito origin to protect themselves against melanization (Osta et al., 2004a).

2.9. Alternate Splicing in Insect Immunity

The fine balance between the mosquito and parasite made the insect immunity very complex (Abraham et al., 2004, Srinivasan et al., 2004), perhaps, more complex than we understand. Recently, alternate splicing of immunoglobulin (Ig) superfamily receptor-Down syndrome cell adhesion molecule (Dscam), resulting in its more than 18,000 isoform, was reported in Drosophila (Watson et al., 2005). Theoretically, Dscam molecule of An. gambiae (AgDscam) can generate 31,920 alternatively spliced forms (Dong et al., 2006a). Challenges with different pathogens resulted in the production of AgDscam molecules with different adhesive characteristics and interaction specificities. In response to two different Plasmodium species viz., Plasmodium falciparum and Plasmodium berghei different AgDscam splice form repertoire were induced. dsRNA mediated silencing of AgDscam not only increased the susceptibility of the mosquito to experimental infections of bacteria and Plasmodium but also to the natural infection of various microbes (Dong et al., 2006a). Occurrence of alternate splicing in insects further enhances the complexity of insect immunity.
Gaps in Present Knowledge

Various groups around the globe are involved in investigating the malaria vector-parasite interaction for the past many years. In spite of the worldwide effort, our understanding of vector-parasite relationship is still limited.

- No study has been carried out regarding the immune response of any member of the *Anopheles culicifacies* complex to malaria parasites.

- It seems, immune responses of different sibling species of any malaria vector are not compared.

- The molecular basis of refractoriness in the refractory strain of *Anopheles culicifacies* is unknown.
Objectives

- To study and compare the immune responses of species A and B of *Anopheles culicifacies* complex to rodent malaria parasite *Plasmodium vinckei petteri* infection.

- To study and compare the immune responses of species A and B of *Anopheles culicifacies* complex to Gram-positive bacteria *Micrococcus luteus* infection.

- To study the role of gene encoding serine protease (*AcSP30*) and pro-phenoloxidase (*AcPP06A*) in refractory phenotype of the refractory mosquito.

- To compare the response of *AcSP30* and *AcPP06A* to *Plasmodium* challenge in refractory strain of *Anopheles culicifacies* with a susceptible strain of the species complex.