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## 3. Vector-borne diseases

### 3.1. [Overview](#)

Numerous diseases are transmitted by insect vectors. Most are restricted to the tropics and are only seen in more temperate regions as imported diseases, because of the requirement for certain arthropod vectors like the Anopheles or Aedes mosquitoes.

Several vector-borne tropical diseases are protozoal infections, caused by protozoan parasites which replicate and multiply rapidly inside the host, invade the blood stream and lymphatic vessels or tissues, such as the agent of the African trypanosomiasis (sleeping sickness), *Trypanosoma brucei*, which is transmitted by the bite of a tsetse fly; the agent of the American trypanosomiasis (Chagas disease), *Trypanosoma cruzi*, which is transmitted by 'kissing bugs'; and the agents of leishmaniasis, which are transmitted through bites of phlebotomine sandflies. But by far the most important vector-borne protozoal infection is malaria, due to one of several *Plasmodium* parasites which are transmitted by the bite of any one of the 50 species of Anopheles mosquitoes. Malaria is one of the most important diseases in the world, killing children aged less than 5 years and exacting an enormous toll in lives, in medical costs and in days of labor lost in tropical regions of Africa, Asia, and Central and South Americas [1] [2]. There is hope, however, that a vaccine against malaria may be available within a few years [3].

A great many viruses also are transmitted by arthropod bites, mostly mosquitoes or ticks, and for that reason fall under the generic name of "arboviruses" (arthropod-borne viruses) (for a review see [4]. Most of these viruses belong to the Alphaviridae family, which includes the Eastern, Western and Venezuelan equine encephalitis viruses (EEEV, WEEV and VEEV, respectively), the O'nyong nyong virus and the Chikungunya virus (Chik); and to the Flaviviridae family, which includes the yellow fever virus (YFV), the four dengue fever viruses (DV), the Japanese encephalitis virus (JEV), the West Nile virus (WNV) and the tick-borne encephalitis virus (TBEV), not to mention the Murray Valley encephalitis, the Omsk haemorrhagic fever, the Kyasanur Forest disease and the Saint-Louis encephalitis (SLE) viruses. Several members of the Bunyaviridae family also are transmitted by mosquitoes, such as the Rift Valley fever virus (RVFV) and the California encephalitis virus, while others, such as the Crimean-Congo hemorrhagic fever (CCHF) virus, are transmitted by ticks. Several of these viruses are the agents of viral haemorrhagic fevers (Table1)1.

Not surprisingly, arboviruses can cause vast epidemics with impressive numbers of cases of illness or deaths. Dengue, the most prevalent mosquito-borne viral disease, is estimated to cause 100 million infections each year, 250 000-500 000 of which are the cause of severe illness [5]. Major epidemics of JE were reported from India and Nepal during the past few years [6]. The Rift Valley fever is endemic in Western and Eastern Africa where epidemic outbreaks in thousands of humans parallel epizootic outbreaks in sheep and cattle [7]. More than 265 000 people were infected during the recent Chik outbreak in Réunion, as well as 1 400 000 people in 2006 in India [8]. Japanese encephalitis accounts for up to 50 000 cases of encephalitis every year with case fatality rates of about 25%.

<b>Disease</b>	<b>Annual Incidence</b>	<b>Vector</b>	<b>Animal Reservoir</b>
Congo-Crimean HF (CCHF)	1000's	Ticks ( <i>Hyalomma</i> )	Hares, Crows, Cows, Ostriches
Dengue DHF/DSS	250 000-500 000	<i>Ae aegypti</i> <i>Ae albopictus</i>	None
Kyasanur Forest Disease (KFD)	100's	Ticks ( <i>Haemaphysalis</i> )	Monkeys, Rodents, Birds
Omsk Haemorrhagic Fever	100's	Ticks ( <i>Dermacentor</i> )	Field Mouse
Rift Valley Fever	10 000's	<i>Culex pipiens</i> , <i>Ae africanus</i> , <i>Anopheles</i> , etc	Sheep, Cattle, Camels
Yellow Fever (YF)	200 000 before vaccine introduction	<i>Ae aegypti</i> and others, <i>Haemagogus</i>	Monkeys

Table 1. Vector-borne viral haemorrhagic fevers (HF) [Ae: Aedes]

Other viruses that cause haemorrhagic fevers, such as the Hantavirus (Haemorrhagic fever with renal syndrome), Arenaviruses (Lassa virus, Junin virus), or filoviruses (Ebola, Marburg) are not vector-borne.

Significant epidemics of yellow fever have been found to occur almost every year in western Africa, with an estimated record 44 000 cases and 25 000 deaths in Nigeria in 1987-88. A 2001 YF outbreak in Abidjan, Côte d'Ivoire, required immunizing 2.6 million persons in 12 days. WHO estimates that there may be up to 200 000 cases of YF a year in western Africa, with 30 000 deaths, prompted GAVI and WHO to launch a major initiative to vaccinate more than 48 million people in 12 West African countries over the next 5 years [9], using the live attenuated 17D YF vaccine, which is mandatory for travelers to endemic areas of Africa and South America.

The epidemiology of arbovirus infection in man is influenced by the probability of contacts between the vector, the human population and, for many viruses, the amplifying vertebrate host, whether birds (most arboviruses that cause encephalitis), monkeys (YFV, KFV), horses (EEEV, WEEV, VEEV, WNV), sheep (RVF), pigs (JEV) or rodents, which serve as reservoir for the virus. Several arboviral infections are actually expanding geographically, as exemplified by the emergence of WNV in the Americas or JEV in Australasia, the spread of dengue, the reemergence of YFV in South America [10] [11] and the recent outbreak of Chik in northern Italy. Both yellow fever and dengue are transmitted between humans by *Aedes aegypti*, which are anthropophilic mosquitoes that breed in urban dwellings. Why is dengue virus much more widespread than yellow fever virus, which has never appeared in Asia, is not known. It may have to do with the fact that dengue occurs mainly in urban areas whereas outbreaks of yellow fever arise in remote areas. Moreover, dengue virus can be transmitted transovarially and sexually through mosquito populations [12].

Japanese encephalitis is widespread over South and South East Asia and Australasia, from Pakistan to the shores of Australia. The virus infects *Culex* spp mosquitoes that feed on birds, humans, pigs, horses, and breed in rice paddy fields. New irrigation projects that support agricultural development therefore increase the risk of disease outbreaks. As to West Nile virus, it attracted attention as a major pathogen after an unexpected outbreak of fever and encephalitis occurred in New York City in August

1999. Within a few weeks of its emergence on the American continent there were 62 confirmed cases and seven deaths among elderly people. The virus, which most likely had been introduced from Israel or Egypt [13] dispersed and spread throughout North America within the next five years.

In addition to protozoans and viruses, arthropods can also transmit bacteria, such as *Borrelia spirochaetes* responsible for Lyme disease and for louse-borne and tick-borne relapsing fevers, *Yersinia pestis*, the agent of plague, or *Bartonellas* and *Rickettsias* that are responsible for a variety of spotted fevers, including the Rocky Mountain spotted fever, typhus and Q fever.

This review will deal with Dengue, Japanese encephalitis (JE) and Malaria, which are the three most important mosquito-borne diseases in terms of morbidity and mortality and for which a vaccine is already available (JE) or will hopefully be available within the next five years; and with leishmaniasis, for which there unfortunately is no hope of a vaccine soon. Neither Tick-borne encephalitis (TBE), which is prevalent in an area that stretches from the West part of France up to Japan [14] nor Yellow fever, which is prevalent in most of West Africa and central South America will be discussed here, as efficient vaccines exist to fight them.

## **3.2. *Dengue***

### **3.2.1. *Introduction***

Dengue, an usually non-severe albeit debilitating viral fever ("breakbone disease"), is the most prevalent mosquito-borne viral disease in people. Dengue is endemic in most tropical and subtropical countries, many of which are heavily populated as well as a popular destination for tourists [15]. The disease is caused by the dengue virus (DV), of which four serologically different serotypes exist (DV-1, DV-2, DV-3 and DV-4), which actually are four viruses almost as genetically different from each other as JE, West Nile and SLE viruses are from one another [16]. Infection is believed to provide life-long immunity against reinfection by the same serotype, but not against the other serotypes. An individual could therefore experience a case of dengue-1 fever on one year, followed by a case of dengue-2 fever on the following year. Third infections however are very rare, and fourth infections have never been reported.

The dengue viruses are the only known arboviruses that have fully adapted to humans and do not need an animal reservoir, although dengue has been observed to also circulate in nonhuman primates. They are transmitted from human-to-human by the urban-dwelling *Aedes aegypti* mosquito, which has adapted to humans, laying their eggs in artificial containers in and around houses and feeding only on humans, and by *Ae albopictus* (the Asian tiger mosquito) and *Ae polynesiensis*. These latter mosquitoes feed on birds, reptiles, rats, cows, and humans. DV may be transmitted vertically from the female mosquito to her offspring. Most cases of transmission from human-to-human are through female mosquitoes which previously became infected when feeding on an infected person.

Dengue is primarily an epidemic disease of urban and peri-urban settings. Thus, in 1987, Thailand reported 175 000 cases and 1000 deaths; in 1996 Brazil, 180 000 cases; in 1998, countries in Latin America, the South-East Asian and the Western Pacific regions 1 300 000 cases and 3500 deaths. In 2001, almost 400 000 cases were notified in Brazil, which has known repeated epidemics and an increase in severe cases of dengue in adults since then [17]. Several large outbreaks also occurred in 2007 in Singapore, Cambodia, Malaysia, The Philippines and Vietnam, with more than 133 000 clinical cases reported and 850 deaths (182 in Cambodia alone).

### **3.2.2. Disease burden**

Some 2500 million (2.5 billion) people are estimated to currently be at risk of dengue in over 100 countries across the globe. It is estimated that between 50 - 100 million cases of Dengue fever, 500 000 cases of Dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) and more than 20 000 deaths from DHF/DSS occur each year [18]. Dengue has become one of the most important emerging disease problems among international travelers [15] and the second most frequent cause of hospitalization after malaria among travelers returning from the tropics [19] [20].

Dengue is a major cause of morbidity and mortality and a leading cause of hospitalization of children in many countries in tropical and subtropical areas of the world. The cost of illness to society is considerable. The past 25 years have seen the emergence and reemergence of epidemic dengue, with more frequent and larger epidemics associated with more severe diseases [21] [22], probably related to population growth, massive unplanned urbanization, modern transportations and the lack of effective mosquito control. Most primary infections cause a non fatal form of illness, but some individuals, mostly children, can experience a more severe form of the disease. Severe disease is referred to as dengue haemorrhagic fever (DHF), characterized by a transient increase in vascular permeability resulting in plasma leakage from blood vessels into tissues, oedema, especially in the chest and abdomen, as well as thrombocytopenia and bleeding seen as petechiae easily detected by the tourniquet test [23]. The patient can eventually go into hypovolaemic shock syndrome (DSS) and organ failure and die if not properly managed [24]. Children with severe dengue are particularly susceptible to DSS, the highest mortality being in infants, who show case fatality rates of up to 13% in hospital-admitted patients [5].

The risk of severe disease, which is estimated to occur in 250 000-500 000 patients a year, is associated with secondary infection by a different serotype, and may be due to antibody-dependent enhancement of macrophage infection by non-neutralizing but cross-reactive antibodies [24]. The increasing endemicity and co-circulation of several DV strains is therefore a leading contributor to the observed increased severity of dengue. Differences in strain virulence probably also are important [25]. In fact, the occurrence of DHF/DSS seems to actually depend on a combination of heterotypic antibody-dependent enhancement of macrophage infection [24] [26], viral load, strain virulence and host immune response [27] as well as to enhancement of dendritic cell infection [28]. DV enhancing antibody activity in plasma, as measured by in vitro assays, does not necessarily predict subsequent disease severity in secondary dengue virus infection [29] [30], as viral factors also contribute to disease severity. Thus, all DV-2 epidemics causing substantial DHF in the American region were associated with a South-East Asian genotype introduced to Cuba from Vietnam in 1981 and those of DV-3 with a virus from India or Sri Lanka [10].

The burden of severe disease remains proportionately much greater in Asian and Pacific countries than in the Americas. In a prospective study on 2114 school children in northern Thailand from 1998 to 2002, dengue accounted for 11% of all febrile illness cases, a burden of 3200 DALYs per million per year, in the same order as hepatitis B [31]. The study showed that dengue illness which does not require hospitalization accounted for half or more of DALYs lost to dengue and its cost was very substantive [32]. The infecting virus serotype also seems to be an important albeit variable determinant of DALYs lost, emphasizing the different contribution of the different serotypes to the disease burden.

### 3.2.3. Virology

DVs are members of the genus *Flavivirus* in the family *Flaviviridae*, which includes more than 70 related but distinct viruses, many of which are mosquito-borne, such as the yellow fever (YF) virus. Flaviviruses are enveloped, 40-50 nm-diameter viruses with an icosahedral capsid that protects the single-stranded, positive sense RNA genome. DV envelope surface projections are composed of dimers of the viral envelope (E) glycoprotein and of the membrane (M) protein, itself derived by furine-mediated cleavage from a prM precursor. The only other protein constituent in the virion is the capsid (C) protein. The E glycoprotein mediates virion attachment to receptor and fusion of the virus envelope with the target cell membrane and bears the virus neutralization epitopes. On native virions, the elongated three-domain E molecule lies tangentially to the virus envelope in a head-to-tail homodimeric conformation. Upon penetration of the virion into the target cell endosome, E dimers are converted to stable target cell membrane-inserted homotrimers that reorient themselves vertically to promote virus-cell fusion.

The 10.5 kb-long genomic RNA is a monocistronic mRNA which is translated into a precursor polyprotein from which the individual viral proteins eventually derive by cleavage, starting with the C, prM and E proteins followed by nonstructural proteins NS1 to NS5. NS3 is a protease and a helicase, whereas NS5 is the RNA polymerase in charge of viral RNA replication. In addition to the E glycoprotein, only one other viral protein, NS1, has been associated with a role in protective immunity. This glycoprotein is not present on the virion, but is found on the surface of infected cells. Immunization with NS1 has been shown to elicit protective immunity in animal models.

### 3.2.4. Vaccines

As there is no cross-protection between the 4 DV serotypes and because of fear of immune enhancement by heterotypic DV antibodies, only a tetravalent vaccine will be acceptable. Progress in DV vaccine development has been relatively slow, mainly because DV grows poorly in cell culture and because there is no reliable animal model for DHF. Also, tetravalent DV vaccines have often generated disappointing immunogenicity results as compared to monovalent vaccines, due to a phenomenon of interference between the 4 strains, the identity of the dominant serotype(s) depending on the nature and composition of the vaccine. The application of infectious clones technology to dengue vaccine development has greatly stimulated the development of candidate vaccines and the current pipeline of dengue vaccines is diverse and overall promising [33] [34] [35] [36].

#### 3.2.4.1. Live attenuated vaccines.

The initially favored strategy has been to attenuate DV strains by repeated passage of wt DV in cell culture in order to prepare a vaccine based on live attenuated virus strains. This was undertaken at Mahidol University in Bangkok, Thailand, using primary dog kidney cells, African green monkey cells and/or fetal rhesus monkey cell cultures. Live attenuated vaccines face the difficulty to define a correct balance between insufficient attenuation and over-attenuation of the candidate vaccine strains, the lack of correlation between in vitro markers of attenuation such as small plaque phenotype or thermosensitivity and in vivo attenuation, and the phenomenon of immunological interference between the four DV serotypes [37] [38]. The development of a tetravalent live attenuated vaccine was eventually taken over by Sanofi Pasteur [39] before being put on hold.

The Walter Reed US Army Institute of Research (WRAIR) succeeded in developing a tetravalent live attenuated vaccine by serial passage of all four DV strains in dog kidney cells, and tested various tetravalent formulations in adult volunteers, then in Thai children. [40] [41] [42]. The vaccine was

then licensed to GSK, which continued clinical trials [43] and is presently going through Phase II trials with the live attenuated tetravalent vaccine candidate.

The US National Institutes of Health (NIH) also have developed attenuated DV strains, using reverse genetics to create an attenuating 30-nucleotide deletion (?30) in the 3' untranslated region of the genome of the four DV strains [44] [45]. Two of the resulting virus strains, DV1-?30 and DV4-?30, were found to be attenuated in rhesus monkeys and safe and highly immunogenic at a dose of 10<sup>3</sup> PFU/vaccinee in Phase I/II clinical trials in human volunteers [46] [47]. However, this strategy did not yield suitable candidates for serotypes 2 and 3, which had to be generated by replacing the sequence coding for the M and E structural proteins in the attenuated DV4-?30 genome by the corresponding sequences from DV2 or DV3, thus yielding intertypic chimeric viruses DV2/4-?30 and DV3/4-?30, respectively [48]. The DV2/4-?30 virus strain was tested in humans and appeared safe and strongly immunogenic at the dose of 10<sup>3</sup> PFU/dose [49]. The replication of these chimeric viruses was also attenuated for *Ae aegypti*, indicating that they would likely manifest decreased transmissibility by mosquitoes [50]. The DV3/4-?30 virus was evaluated in clinical trials [51] but immunogenicity appeared to be weak. The 4 attenuated virus strains should eventually be combined together and tested as a tetravalent live attenuated candidate vaccine [52].

#### 3.2.4.2. Live chimeric virus vaccines

A homotypic chimeric virus approach also has been applied by the US Centers for Disease Control and Prevention to engineer DV2 chimeras by inserting the structural protein genes from DV1, DV3 and DV4 into an attenuated PDK53 DV2 genome that had been attenuated by replacing a portion of the DV terminal 3' stem and loop structure with that of West Nile virus [53] [54]. The clinical development of these DV2-based chimeras is being carried by Inviragen, in collaboration with the US CDC and Shantha Biotechnic.

Another approach, the engineering of heterotypic chimeric viruses, was initiated in the 1990s by Acambis, using the YFV 17D vaccine strain as the genetic background [55] and replacing the M and E structural protein-coding sequences in the YFV genome with those from either the four DV serotypes [56], or from JEV [57], or from WNV [58] [59]. The DEN-YF chimeras were developed by Acambis and licensed to Sanofi Pasteur. When injected to monkeys for safety and protective efficacy tests, the monkeys developed a brief viremia followed by a DV neutralizing antibody response and were protected against challenge with DV [60].

The chimeric viruses were tested and found to be safe and immunogenic in humans. A tetravalent combination of the four DEN-YF chimeras, ChimeriVax-Den vaccine [56] [61], was shown to induce a transient and low grade viremia in nonhuman primate and in human volunteers, followed by a solid immune response against the four serotypes with some strains showing dominant immunogenicity. A dose adjustment for the DV2 chimera resulted in a more balanced response. The chimeric viruses also were found to replicate and disseminate poorly in the body of mosquitoes, indicating that the risk of infection and transmission by mosquitoes in nature would be minimal. A Phase II trial is taking place in the USA and Latin America and a Phase IIb pediatric trial has been launched by Sanofi Pasteur in early 2009 in Thailand.

#### 3.2.4.3. Live recombinant, DNA and subunit vaccines

DV genes were inserted at the Naval Medical Research Center into a new, non-replicative adenovirus vector (Ad5) to engineer double recombinants expressing the prM and E sequences from both DV1

and DV2 and DV3 and DV4, respectively [62]. The pair of recombinants was tested in mice and shown to induce neutralizing antibodies to the four DV serotypes. It also was tested in macaques, in which it induced significant protection against challenge with all four DV serotypes [63].

The Naval Medical Research Center is pursuing a DNA-based vaccine approach, using a Biojector device for immunization. Evaluation of a Phase I study is ongoing.

Finally, a subunit dengue vaccine has been developed by Hawaii Biotech Inc using the truncated amino-terminal 80% of the E glycoprotein from each serotype plus the entire NS1 protein from DV2 [64] formulated in a proprietary adjuvant. The candidate vaccine elicited robust immune responses in nonhuman primates and clinical trials are envisaged in the near future. A novel dengue subunit vaccine candidate was developed using a consensus dengue virus envelope protein domain III (cEDIII). BALB/c mice immunized with the recombinant cEDIII in the presence of aluminium phosphate developed long-lasting neutralizing antibodies against all 4 serotypes of dengue virus [65]. Several groups are developing subunit vaccines based on domain III of the DV E protein, a strategy that is aimed at reducing the induction of crossreactive antibodies.

Another approach has used inactivated virus. A purified inactivated DV2 candidate vaccine has been tested and shown to elicit a good level of neutralizing antibody and protection in nonhuman primates [66].

The question of whether any of the candidate dengue fever vaccines will be ready by 2012 still is an open question, although it is the declared objective of both GSK and Sanofi Pasteur, both of which have presented timelines compatible with licensure by 2012.

### ***3.3. Japanese Encephalitis***

#### ***3.3.1. Introduction***

Japanese encephalitis (JE) is the most common cause of viral encephalitis in the Asian Pacific region. The virus exists in a transmission cycle between mosquitoes and pigs and/or water birds such as herons and egrets which are the main host reservoir. JE is therefore a mosquito-borne zoonotic viral infection, the reservoir of which is water birds and in which pigs play the role of amplifying host in rural areas. JE used to be mostly prevalent in countries with a temperate climate, including Japan, but data from tropical countries (Thailand, Cambodia, Indonesia) show that these zones also are favorable for JE transmission [16]. Indeed, JE can now be found from the extreme south-eastern part of Russia to the North of Australia and Papua New Guinea, and from Japan to the west of India. Some 50,000 cases of JE occur annually, with 25%-35% case fatality rates, and more than 30 % severe long-term disabilities in survivors [67].

Recent epidemics have led to increased demand for more effective, safe and affordable JE vaccines, leading to an extended registration and use of a live-attenuated vaccine that was previously largely confined to China. To meet increasing demand and to comply with GMP standards, a new plant for the vaccine is being built in China, which should become operational by 2010 and produce vaccines at GMP standards [68]. Together with the licensing of second generation JE vaccines, the global need for this vaccine should thus be met. It is anticipated that the mouse brain inactivated vaccine will be replaced by the less reactogenic and better controlled Vero cell-derived inactivated vaccine, which, together with the live attenuated SA14-2-14 vaccine and the live YF-JE chimeric vaccine hold great

promise for programmatic use in developing countries. Their accelerated clinical development and evaluation has been facilitated by the WHO.

### **3.3.2. Disease burden**

JE is endemic in parts of China and in Eastern, Southern and Southeastern Asia, and Papua New Guinea [10]. In the 1990s, JEV spread westward into Pakistan and eastwards into the western Indonesian archipelago, New Guinea and northern Australia [69]. The mechanism by which the virus reached the Torres straight in 2000, perhaps through a migratory bird, remains unknown [70]. JE is principally a disease of rural areas in which vector mosquitoes proliferate in close association with birds and pigs. The spread of the disease into non-endemic regions has been correlated with agricultural development and intensive rice cultivation and the breeding of pigs supported by irrigation programs. The disease is currently considered hyperendemic in northern India and southern Nepal, as well as in parts of central and southern India.

JE is the most important cause of acute encephalitis in eastern and southern Asia and carries with it a heavy burden of permanent neuropsychiatric sequelae. The figure of 50 000 cases of illness a year probably is an underestimate, because of inadequate surveillance and reporting and because most infections are asymptomatic, with a ratio of symptomatic to asymptomatic infections that can range from 1 in 25 to probably 1 in 250 infections [71]. In rural villages, exposure and infection occur at a very early age with half of all cases occurring in children less than 4 years of age. Typical incidence rates in those younger than 19 years range from 10 to 100 per 100 000 population per year [72]. Seroprevalence studies indicate nearly universal infection by early adulthood in those areas [73]. Transmission of JE is mostly seasonal in temperate areas, but year-round transmission is seen in Indonesia [74]. Large outbreaks of JE with clear summer seasonality [75] also are periodically reported on the Indian subcontinent, as illustrated in Uttar Pradesh, where 6097 suspected cases, including 1398 deaths, were reported between July 1 and November 10, 2005.

Needless to say, JE also poses risks to travelers and expatriates and to military personnel deployed overseas.

### **3.3.3. Virology**

The JE virus, JEV, belongs in the Flaviviridae family, where it forms a common serogroup with West Nile, Kunjin (a subtype of WNV), Murray Valley encephalitis and Saint Louis encephalitis (SLE) viruses. All members in the serogroup have avian vertebrate hosts and are vectored by *Culex* spp mosquitoes. JEV has also occasionally been recovered from *Aedes* spp mosquitoes. The virus is a 40-50 nm enveloped, positive single-stranded RNA virus, with an isometric 30 nm nucleocapsid core. The envelope is spiked with a mature membrane (M) protein and a glycosylated envelope (E) protein which is stabilized by disulfide bonds and comprises three domains (I, II and III) involved in antigenic properties, cell receptor binding and penetration of the virion into the host-cell. The 10 976 bases long single-stranded viral RNA encodes an uninterrupted open reading frame that is translated into a polyprotein precursor eventually processed into capsid (C) pre-M and E structural proteins and into seven non-structural proteins (NS1-NS5). All known JEV isolates, although comprising five genotypes, belong to a single serotype [76].

### 3.3.4. Vaccines

The control of JE is based essentially on three interventions: mosquito control, avoiding human exposure to mosquitoes and immunization. Mosquito control has been very difficult to achieve in rural settings and avoidance of exposure is difficult as *Culex* mosquitoes bite during day time. Immunization is the only effective method for sustainable control. Routine immunization of school-age children is currently in use in Korea, Japan, China, Thailand and Taiwan. The introduction of the JE vaccine into the Expanded Program of Immunization has helped curb the disease in countries like Thailand, Vietnam, Sri Lanka and China [77].

#### 3.3.4.1. Inactivated vaccines

Among the currently available vaccines is a formalin-inactivated vaccine derived from mouse brain-grown JEV strain Nakayama [55] [78], which still is produced by manufacturers in Korea, Thailand and Vietnam. The vaccine is relatively expensive, requires three doses on days 0, 7 and 30, followed by a booster at 1 year and thereafter at intervals of 3 years. The vaccine can often generate neurological adverse reactions. In addition to local and systemic side effects, individual cases of generalized urticaria and angioedema were reported in about 1 case per 1000 vaccinees after vaccination of travelers from western countries.

Another formalin-inactivated JE vaccine is prepared in China using the JEV P3 strain propagated in primary hamster kidney-cell cultures. The vaccine appears to be more immunogenic than that based on the Nakayama strain and can be integrated into the routine childhood immunization schedule but is not distributed outside of China. It is now largely being replaced by the live attenuated vaccine.

Several attempts are in progress to prepare inactivated JEV vaccines starting from virus grown in controlled cell line cultures. Several manufacturers are developing Vero cell-derived purified inactivated JE vaccines, either using the virulent Nakayama strain, as done by Japanese manufacturers, or starting from the attenuated SA14-14-2 JEV strain, as done by the Austrian biotech company Intercell. Phase I and Phase II clinical trials have shown that the vaccine was safe and immunogenic [79] and a Phase III trial was recently completed [80]. The Japanese vaccine candidates have been recently licensed in Japan, while the Intercell vaccine, Ixiaro, was licensed by the US FDA for adults. A two-dose rapid immunization schedule has been worked up for administration to travelers. Most people immunized with the Intercell vaccine developed protective neutralizing antibody levels that lasted for at least one year [81] [82] and the vaccine was well tolerated [83] [84]. The company pursues a separate clinical development for pediatric indication for endemic countries in a joint venture with Biological E, an Indian manufacturer. A large pediatric Phase IIb trial is currently taking place in endemic settings in India. Similarly, a Vero cell inactivated vaccine is now being produced in China by the Beijing Institute of Biological Products.

#### 3.3.4.2. Live attenuated vaccines

The live attenuated JE vaccine strain, SA14-14-2, which was obtained after 11 passages in weaning mice followed by 100 passages in primary hamster kidney cells, has been developed and used in China since 1988. The vaccine, which is produced by the Chengdu Institute of Biological Products in China, was licensed in recent years in several Asian countries and was extensively used from 2006 to 2008 in mass immunization campaigns in India. Although the product is not WHO prequalified at this time, much investment and efforts have been made to bring the production and quality control to international standards. The vaccine is produced on primary hamster kidney cells, lyophilized, and

administered to children at one year of age and again at two years, in annual spring campaigns [85]. Initial observational studies in southern China involving more than 200 000 children had demonstrated the vaccine safety, immunogenicity (99-100% seroconversion rate in nonimmune subjects) and protective efficacy over 5 years [78]. The short-term effectiveness of a single dose of SA14-2-14 was demonstrated in 2001 in a case control study on Nepalese children where an efficacy of 99.3% was reported [86]. A five year follow-up study found the single-dose efficacy was maintained at 96.2% [87]. Another five-year follow up study showed that neutralizing antibody persistence was close to 90% at 4 years and 64% at 5 years after a single-dose of the vaccine in adult volunteers [88]. Recent studies in the Philippines have demonstrated the safety and efficacy of the vaccine even when co-administered with measles vaccine at 9 months of age. Similar studies in Sri Lanka and Indonesia will help confirm these findings in other Asian settings [16].

Currently, more than 30 million doses of the live SA14-2-14 vaccine are distributed annually in southern and western China and exported to Nepal, India and Korea. Starting in May 2006, the SA14-2-14 live attenuated vaccine was used in India to vaccinate 9.3 million children in 11 districts scattered among 4 states where JE was considered as highly endemic. More than 500 adverse events were reported during the campaign, including 66 severe AE, of which 22 were fatal. These cases were reviewed by an expert committee which concluded that none of the deaths were attributable to the vaccine. The severe adverse events and critical press coverage nevertheless had a deep negative impact on vaccine acceptance in the rest of the country, highlighting the need for proper safety monitoring and case investigations.

#### 3.3.4.3. *Chimeric vaccines*

A promising approach for a future JE vaccine has been the construction of a YF-JE chimera based on the attenuated 17D YF virus genome, in which the YFV sequences encoding viral structural proteins prM and E were replaced by the corresponding prM and E sequences from JEV strain SA14-2-14. The resulting YF-JE chimeric virus, ChimeriVax-JETM, developed by Acambis and now licensed to Sanofi Pasteur, was grown on Vero cells and shown to elicit JEV neutralizing antibodies as well as protection against nasal and intracerebral virus challenge in rhesus monkeys [57] [89] [90]. The vaccine was tested in human adult volunteers in the USA, showing good safety and immunogenicity, with 94% of the vaccinees in the Phase II trial developing protective neutralizing antibody levels after a single dose [91]. The chimeric virus was shown not to replicate in mosquitoes which were fed the Chimerivax-JE vaccine [92], a further proof of attenuation. The vaccine has been undergoing Phase III clinical trials in the USA and Australia for adult indication, whereas a parallel pediatric development program has been launched in Thailand by SanofiPasteur.

#### 3.3.4.4. *Live recombinant JEV vaccines*

Replication-defective canarypox (ALVAC) and the highly attenuated vaccinia virus strain NYVAC were used as vectors to express the pr-M, E, NS1 and NS2a gene from JEV. The vaccine candidates were found to be well tolerated but their immunogenicity was too weak, especially in non-vaccinia immune volunteers, to warrant further development [93].

### 3.4. [Leishmaniasis](#)

#### 3.4.1. *Disease burden*

Leishmaniasis is caused by several species of flagellated protozoan parasites, *Leishmania* spp, that are transmitted by the bite of a female phlebotome sandfly. The disease is found in many areas of the

world, particularly in Africa, the Mediterranean Basin, South and Central Asia, the Middle East and Latin America. Several forms of the disease exist: cutaneous (CL), mucocutaneous (MCL) and visceral (VL, also called "Kala-azar"), which, after treatment, is often followed by a dermal manifestation known as post-kala-azar dermal leishmaniasis (PKDL). The typical lesion of CL is a chronic ulcer with intense lymphoid and monocytic infiltration and granuloma formation. VL is characterized by dissemination of the parasites through the reticuloendothelial system leading to pyrexia, wasting and hepatosplenomegaly.

For many years, the public health impact of leishmaniasis has been grossly underestimated, as a substantial number of cases were never recorded. WHO estimates the worldwide prevalence to be approximately 12-15 million cases, with annual mortality of about 60 000. The size of the population at risk is about 350 million [94]. About 1.5 million new cases are estimated to occur annually, but only 600 000 are officially declared. In addition, deadly epidemics of VL periodically flare up but go mostly unnoticed in spite of case-fatality rates as high as 10% or more. In the 1990s Sudan suffered a crisis with an excess mortality of 100 000 deaths among people at risk. There are an estimated 200 million population at risk on the Indian subcontinent, which reports 25 000 to 40 000 cases and 200-300 deaths every year [95]. A VL elimination program is currently being launched in India, Bangladesh and Nepal [96].

The expansion of leishmaniasis and the alarming rise in the number of cases is related to environmental changes such as deforestation, building of dams, new irrigation schemes and migration of non-immune people to endemic areas, which resulted in significant delay in the implementation of development programs in Saudi Arabia, Morocco, the Amazons and the tropical regions of the Andean countries. More recently, as a result of epidemiological changes, a sharp increase in the overlapping of HIV infection and visceral leishmaniasis has been observed, especially in intravenous drug users in southwestern Europe. The situation might worsen in Africa and Asia where the prevalence of HIV and Leishmania co-infections still is probably largely underestimated.

### **3.4. 2. Parasitology**

Leishmaniasis is a parasitic infection caused by the obligate, intracellular protozoan of the genus *Leishmania*, fifteen species of which can infect man. Main species in the Old World are: *L. major*, *L. tropica*, *L. donovani* and *L. infantum* and in the New World: *L. mexicana*, *L. brasiliensis*, *L. amazonensis* and *L. guyanensis*. The parasite has two life cycles, one in the alimentary canal of female phlebotome sandflies where it forms motile flagellated and elongated 'promastigotes' that are inoculated into the blood stream of a mammalian host when the female is having a blood meal; and a mostly intracellular cycle in the mammalian host where it matures inside macrophages, neutrophils and dendritic cells into tiny ovoid shape 'amastigotes' with a nucleus, a kinetoplast and a short flagellum [97]. The kinetoplast, which contains mitochondrial DNA, is a distinctive feature of both *Leishmanias* and *Trypanosomias*.

Recent studies have evidenced the existence of interspecific and intraspecific hybrids and recombinant genotypes among the natural populations of *Leishmanias* [98] [99], suggesting the formation of haploid gametes through meiosis and the generation of heterozygous progeny in the sandfly vector [100].

Cutaneous (CL) and mucocutaneous (MCL) leishmaniasis in Central and South America are caused by members of the *L. mexicana* and *L. braziliensis* species, whereas CL in South and Central Asia and the Middle East is caused by *L. tropica* and *L. major*. The majority of MCL cases occur in Bolivia, Brazil and Peru, whereas 90% of CL cases occur in Afghanistan, Iran, Saudi Arabia, Syria, Brazil and Peru. VL (kala-azar), the most lethal form of the disease, is caused by *L. donovani* in Sudan, India, Nepal,

Bangladesh and China, by *L. infantum* in North Africa and southern Europe, and by *L. chagasi* in Latin America. Transmission is most often zoonotic: the parasites (*Leishmania*) are transmitted from a wild animal reservoir (small rodents, dogs) by the bite of the female phlebotome sandfly. It also can be anthroponotic, the parasite being transmitted by the sandfly from an infected human host. In the Mediterranean Basin and Brazil, the main identified reservoir is the dog population, and the most effective control strategy has been the use of deltamethrin-impregnated dog collars [101] [102].

Cutaneous leishmaniasis usually leads to self-healing disease with life-long immunity against reinfection. Resolution is characterized by induction of IFN- $\gamma$  releasing CD4<sup>+</sup> T cells of the Th1 phenotype whereas failure to cure is associated with elevated levels of IL-4 and IL-10 and low levels of IFN- $\gamma$ , a typical Th2 profile [103] [104] [105]. It was demonstrated in experimental animal models that a dominant Th1 lymphocyte response (IL-2, IFN- $\gamma$ ) is associated with self-limited disease, whereas a dominant Th2 response (IL-4, IL-5) is linked to progressive disease.

### 3.4.3. Vaccines

Vector and reservoir controls may be useful under certain conditions but are not applicable in every epidemiological setting and require infrastructure and vigilance beyond the capability of many endemic countries. Vaccination, therefore, remains the best hope for control of all forms of the disease. Progress towards a vaccine however has remained very slow, even though evidence from animal studies demonstrate that protection can be achieved through immunization with purified proteins or DNA vaccines [106]. There is as yet no effective vaccine for prevention of any form of leishmaniasis.

#### 3.4.3.1. Leishmanization

For centuries, in some of the hyper-endemic areas of the Middle East, the pus of an active lesion was used to inoculate young children to protect them against future lesions on the exposed parts of the body, especially the face. The practice is known as leishmanization. Leishmanization has been practiced in several countries, including Uzbekistan, the only country where the measure is still in current use. *L. major* promastigotes grown in culture under GMP guidelines, rather than the exsudates from active lesions, have more recently been used for inoculation as a live vaccine. Genetically manipulated parasites with attenuated virulence or high sensitivity to chemotherapy might represent the ideal form of such a live vaccine [107].

#### 3.4.3.2. Inactivated whole-cell vaccines

In recent years most clinical trials of first generation vaccines in humans have evaluated the effect of three types of vaccines: a *L. amazonensis*-based vaccine derived from an earlier 5-valent vaccine (BIOBRAS, Brazil), a *L. mexicana*-based product (Instituto Biomedicina, Venezuela) and a *L. major*-based preparation (Razi Vaccine and Serum Research Institute, Iran). In addition, a trivalent preparation consisting of *L. braziliensis*, *L. guyanensis* and *L. amazonensis* antigens was evaluated in Ecuador [23]. Bacille Calmette-Guérin (BCG) was used as the adjuvant in some versions of the Venezuelan, Ecuadorian and Iranian candidate vaccines in an attempt to improve the vaccine's ability to induce cell mediated responses [108]. In trials of the combination of autoclaved *L. major* promastigotes with BCG as adjuvant by the Razi Institute in Iran against CL and in Sudan against VL, a limited efficacy was noted in converters to positive skin reaction to leishmania antigen (leishmanin) and unexpectedly in boys [109]. Alum-precipitated autoclaved *L. major* promastigotes plus BCG have demonstrated safety and substantial immunogenicity in Phase I and II studies in Sudan and in Iran

[110]. It is of note that treatments combining administration of antimonials and first generation leishmania vaccines in patients suffering from post-Kala-Azar dermal leishmaniasis (PKDL) have shown benefit to the patients, suggesting that even suboptimal leishmaniasis vaccines could have a role in a therapeutic setting.

#### 3.4.3.3. Subunit vaccines

Various subunit recombinant candidate vaccines also were tested in mice and provided some degree of protection against infection. These vaccines were based on a variety of Leishmania gene products including:

- the gp63 surface antigen, a glycoprotein with protease activity,
- lipophosphoglycan, a surface glycoconjugate;
- a 46 kD promastigote antigen derived from *L. amazonensis*;
- the Leishmania-activated C kinase (LACK)

Protection against *L. major* infection in mice was provided by DNA constructs encoding a number of such Leishmania antigens, including gp63 and LACK. A prime-boost vaccination regimen against experimental VL was tested in dogs with DNA/recombinant cysteine proteinases I and II and showed partial protection [111]. A prime boost DNA/ modified vaccinia virus Ankara (MVA) expressing leishmania trypanothione peroxidase (TRYP) was safe and immunogenic in dogs [112]. Further dog trials are eagerly awaited. There also is evidence that a 15 kD protein antigen derived from the salivary glands of the sandfly vector could be protective in mice when given as a vaccine [113]. The fact that the nucleotide sequence of the 8500 identified genes in the Leishmania (*L. major*) genome is now known [114] leads to hope that screening of new potential vaccine candidates will be accelerated [115].

A DNA vaccine encoding leishmania antigens LACK, TSA, LmST11 and CPa elicited only partial protection against hind footpad challenge in mice [116]. However, addition of Th1-driving adjuvants such as IL-12 or oligodeoxynucleotides (CpG) to the leishmanial antigens resulted in complete protection of susceptible mice against progressive disease, whereas no protection was observed in the absence of adjuvant [117]. The development of a chimeric vaccine made of the three recombinant leishmanial antigens LeIF, LmSTI-1 and TSA in the form of a fusion protein (Leish-111f) combined with monophosphoryl lipid A (MPL) in squalene oil as adjuvant was launched by a group at the Infectious Disease Research Institute in Seattle (WA) with the support of the Bill and Melinda Gates Foundation [118]. Phase I trials of the vaccine in healthy volunteers in the USA and initial efficacy testing as a therapeutic vaccine in patients in Latin America suggest safety and immunogenicity of the vaccine [94].

### 3.5. *Malaria*

#### 3.5.1. *Introduction*

Malaria is by far the most important tropical parasitic disease, killing two children aged less than 5 years every minute. Most of these deaths occur in sub-Saharan Africa [1] [119] [120]. It is estimated that around 900 000 people die each year from malaria and that more than 3 billion people are exposed to the risk of acquiring the disease worldwide, especially in sub-Saharan Africa, India and

South East Asia [121]. Deployment of an effective vaccine could save countless lives and improve the overall quality of life in the tropics and subtropics.

Malaria is caused by infection of red blood cells with protozoan parasites of the genus *Plasmodium*, which are transmitted by the bite of a feeding female mosquito of any one of the 50 species of *Anopheles* mosquitoes, of which the best known is *A. gambiae*. Four *Plasmodium* species infect humans, *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*, causing a spectrum of clinical disease ranging from moderate flu-like symptoms to severe malaria disease characterized by respiratory distress, coma, severe anaemia, hypoglycaemia, generalized convulsions and high mortality.

Uncomplicated malaria, with no evidence of vital organ dysfunction, has a relatively low case-fatality rate provided there is early case detection and appropriate management, including effective drug therapy. However, in areas of high disease burden and lack of prompt access to health care and accurate diagnostics, delay in treatment or use of ineffective drugs, uncomplicated malaria can rapidly progress to severe disease with case fatality rates in people receiving treatment of up to 15-20%. Untreated severe malaria is almost always fatal. Most at risk are those who have not acquired immunity to malaria, such as young children, travelers, and displaced persons. Malaria contributes significantly to anaemia in children living in endemic countries. In those who survive, profound sequelae may affect physical and mental development [2] [122] [123]. Malaria in pregnant women is a cause of adverse birth outcomes such as spontaneous abortion, stillbirth, premature delivery and low birth weight [124] as well as profound anaemia.

Malaria transmission was successfully reduced or eliminated between 1957 and 1972 from areas where it had occurred at low intensities in the Americas, Asia, Europe and Transcaucasia. This was achieved through vector control - DDT spraying - combined with improved access to treatment. In contrast, most of sub-Saharan Africa and some foci elsewhere continued to suffer high intensity malaria transmission. In recent years, malaria has reemerged in several areas after interruption of malaria control efforts that were not sustainable [125] and in the face of increasing drug and insecticide resistance. The combination of tools and methods to combat malaria now includes long-lasting insecticidal bed nets (LLIN) and artemisinin-based combination therapy (ACT), supported by indoor residual spraying of insecticide (IRS) and intermittent preventive treatment in pregnancy (IPTp) [126] [127].

### **3.5.2. Disease burden**

There were an estimated 247 million malaria cases among 3.3 billion people at risk in 2006, causing nearly a million deaths, mostly of children under 5 years of age. In 2008, 109 countries were endemic for malaria, 45 of which within the WHO African region. Provisional country-level estimates continue to be refined based on efforts to improve the global malaria endemicity map. As of January 2005, estimates of rates of total clinical malaria incidence indicated that around 59% of the world's clinical malaria cases occurred in Africa, around 38% in Asia and around 3% in the Americas. Over 80% of the deaths from malaria occurred in sub-Saharan Africa. In Mali alone, malaria kills close to 70 000 children under 5 years of age every year and contributes to substantial yearly economic losses. Malaria is estimated to be responsible for an estimated average annual reduction of 1.3% in economic growth for those countries with the highest burden [1] [128].

Parasite-vector-human transmission dynamics such as transmission potential of the different anopheline mosquitoes and climatic conditions greatly influence the variation in disease burden in different regions of the world. In addition, socioeconomic factors such as degree of poverty, quality of housing and access to health care and health education, as well as the existence of active malaria control programs providing access to malaria prevention and treatment measures also greatly affect the

disease burden variability. The most efficient malaria vector, *Anopheles gambiae*, occurs exclusively in Africa and also is one of the most difficult to control. Tropical areas of the world have the best combination of adequate rainfall, temperature and humidity allowing for breeding and survival of anophelines. A recent study shows that a substantial proportion of people originating from malaria endemic countries and not showing any sign of malaria harbored *Plasmodium* parasites in their blood and could potentially be a reservoir [129].

The pattern and intensity of malaria transmission determines the degree of protective immunity acquired by the residents of affected areas and the nature of the clinical disease profile. The majority of deaths in tropical Africa occur in areas of high transmission of *P falciparum* malaria. In much of sub-Saharan Africa, populations are continuously exposed to a fairly constant rate of malarial inoculations, and if the inoculation rates are high (annual entomological inoculation rate (EIR) >10 bites), then partial immunity to the clinical disease and to its severe manifestations is acquired early in childhood. In such areas, acute clinical disease is almost always confined to young children who have not yet acquired clinical immunity, and to pregnant women, whose immunity to malaria is temporarily impaired. In these 'stable' and high-transmission areas, adolescents and adults are partially immune and rarely suffer clinical disease, although they continue to harbour low blood-parasite densities. Immunity appears to be lost when individuals move out of the transmission zone, although the time course of this waning of immunity is not well documented.

In areas of low or highly seasonal *P falciparum* malaria transmission, where annual EIRs are usually <5 bites and may be <1, acquisition of immunity is slower. As a result, people of all ages may be at risk of suffering acute clinical malaria episodes, with a high risk of progression to life-threatening severe disease if not appropriately treated. This situation is seen in much of Asia and Latin America and the remaining parts of the world where malaria is endemic. In these areas, malaria epidemics may occur when inoculation rates increase rapidly, leading to a high incidence of malaria in all age groups.

The estimated costs of malaria in terms of strain on the health systems are enormous: in endemic countries, as many as 3 out of 10 hospital beds can be occupied by victims of the disease. A study in 2002 in Mozambique showed that 40% of outpatients and 60% of children admitted to hospitals suffered from malaria. The cost of malaria in sub-Saharan Africa is estimated to represent 1%-5% of GDP, a cost of about \$12 billion a year [130].

### **3.5.3. Parasitology**

The agents of human malaria are four species of *Plasmodium* protozoa: *P vivax*, *P falciparum*, *P ovale*, and *P malariae*. All are transmitted by *Anopheles* mosquitoes. The majority of malaria cases are caused by *P. falciparum* and *P. vivax*. [131]. Human cases of *P. knowlesi* malaria also occur in Malaysia, Thailand, Myanmar and parts of the Philippines as a zoonotic disease [132]. About 86% of all clinical malaria cases occur in Africa. Among the cases that occur outside the African Region, 80% are in India, Sudan, Myanmar, Bangladesh, Indonesia, Papua New Guinea and Pakistan. The second most common malaria parasite, *P vivax*, accounts for a higher proportion of the total malaria disease burden in Asia and in parts of the Americas, Europe and North Africa.

*Plasmodium* parasites have a complex life cycle that begins when an infected female mosquito injects sporozoites into a human when taking a blood meal [133] [134] [135]. The sporozoites invade the blood stream and within about 30 minutes migrate to the liver and invade hepatocytes. Sporozoites mature in the liver where they give rise to tens of thousands of merozoites over a period of 6-16 days. The sporozoite and liver stages are known together as the pre-erythrocytic stage of the life cycle. This initial phase is followed by the erythrocytic or asexual blood stage, when merozoites erupt into the blood stream, invade red blood cells (RBCs), multiply and mature over a few days and then are released by lysis of the infected RBCs. The RBC invasion-multiplication cycle repeats itself time after

time, giving rise to the classical malaria acute febrile episodes and rigors that occur every 48-72 hours due to the lysis of infected RBCs, which leads to the release of new mature merozoites in the blood. The third stage of the parasite is the sexual stage, when some merozoites differentiate into gametocytes, which, after being taken up by an anopheline mosquito, will sexually combine in the insect host to generate a zygote, from which new sporozoites will eventually emerge, ready to reinitiate the cycle.

Both the 23.3 megabase *P. falciparum* and the 26.8 megabase *P. vivax* genome sequences have been reported: they each carry more than 5200 genes distributed over 14 chromosomes [136] [137].

#### 3.5.4. Vaccines

Several lines of evidence suggest that a prophylactic malaria vaccine for humans is feasible. Firstly, naturally acquired immunity builds up during the first two decades of life in people living in malaria-endemic countries. This naturally acquired immunity is however partial and short-lived, and appears to depend on continuous antigenic stimulation, waning when antigen exposure ceases. Protection has been elicited by passive transfer of immunoglobulins from malaria-immune adults to malaria-naïve human volunteers. There also is experimental evidence that immunization of humans and animals with irradiated sporozoites results in partial or complete protection from an experimental infection with viable sporozoites [138] [139], which might pave the way to a genetically attenuated live sporozoite vaccine [140] [141] [142] [143].

However, multiple obstacles to the development of a malaria vaccine remain, that include the lack of general agreement on possible immune correlates of protection, the lack of predictive animal models and assays, and the multiple stages and antigenic diversity and variability of the parasite. Different antigens often are expressed at different stages of the parasite life cycle, and most show considerable polymorphism [144]. The genetic complexity of the parasite remains a significant challenge. Plasmodia have more than 5000 genes, and finding which ones code for appropriate candidate vaccine antigens may be a quagmire [145] [146].

Different vaccines are being developed that target the different stages of the parasite life cycle: pre-erythrocytic (sporozoite and liver stage), erythrocytic (blood stage) and sexual stages (for reviews, see [1] [2] [3] [147] [148] [149]). Pre-erythrocytic stage vaccine strategies aim to generate an antibody response that will prevent sporozoites from invading hepatocytes as well as to elicit a cell-mediated immune response that will inhibit intra-hepatic parasite development. This type of vaccine would be ideal for travelers because it would prevent infection and the advent of clinical disease. Erythrocytic stage vaccine strategies aim to elicit antibodies that will target merozoite antigens and/or antigens expressed on the surface of infected RBCs. These vaccines ideally should be able to induce antibody-mediated cellular toxicity and/or complement lysis, as well as T-cell responses that will inhibit the development of merozoites in RBCs. This type of vaccine is hypothesized to allow parasite densities to be controlled at levels which would minimise morbidity and would therefore be suitable for residents of endemic countries for morbidity reduction, but not for prevention of infection. Finally, vaccines targeting the sexual stage of the parasite aim not to prevent infection or disease but to prevent the transmission of the parasite to new hosts. Efficacious transmission blocking vaccines are thought to be highly desirable in pre elimination settings where interruption of transmission becomes a key aim of an immunization programme.

Almost all of the vaccines under development are directed at *P. falciparum*, which is responsible for the vast majority of severe malaria disease and deaths [150].

### 3.5.4.1. Pre-erythrocytic vaccines

The most advanced malaria vaccine candidate at this time is a pre-erythrocytic vaccine based on the circum-sporozoite protein (CSP) that is the predominant surface antigen of the sporozoites and is expressed early on infected hepatocytes. This vaccine, RTS,S/AS01, is made of recombinant chimeric virus-like particles (VLPs) produced in *Saccharomyces cerevisiae* combining the Hepatitis B surface antigen (HBsAg) with the C-terminus portion (aa 207-395) of *P. falciparum* CSP. The candidate vaccine has shown 40% protective efficacy in multiple clinical challenge studies conducted in partnership with the US Military Malaria Vaccine Program. A randomized controlled field efficacy trials in Gambian adults demonstrated 71% efficacy against time to infection for the first 9 weeks after vaccination, but low efficacy for the subsequent 6 weeks. Efficacy over the entire 15 week period was 34% and it has been proposed that heterogeneity of risk may partly or wholly account for the apparent waning seen in this study. The vaccine was found to be safe and well tolerated in adults in a hyperendemic region of western Kenya [151] as well as in 1-4 years-old children in Mozambique in whom it induced a strong antibody response to both the CSP and to HBsAg [152] and a TH1 CD4+ T cell response. The paediatric clinical development of RTS,S has occurred as a partnership between the PATH Malaria Vaccine Initiative and GSK.

A randomized phase IIb trial in 2022 Mozambiquan children aged 1 to 4 years showed 30% efficacy (95%CI 11-45%) over six months against time to first or only clinical malaria episode and 57% efficacy (95%CI 16-80%) against severe malaria [153]. Vaccine efficacy over an extended 18-month follow-up period was 35.3% against time to first or only episode of malaria and 48.6% against severe disease [154] [155]. In a study in Bagamoyo, Tanzania, 170 infants were immunized at 8, 12 and 16 weeks of age with RTS,S in co-administration with a DTPw/Hib vaccine. Seroconversion to CSP was 98.6%, and the efficacy of the RTS,S vaccine against first infection with *P. falciparum* at 6 month after vaccination was 65.2%, but GMT to diphtheria and tetanus vaccines were lower in co-administration with RTS,S [156].

All studies cited to date had used AS02 adjuvant, an oil-in-water emulsion with added MPL and QS21. Paediatric development has since shifted to AS01, which consists of a liposomal preparation with MPL and QS21. This adjuvant is more immunogenic for both IgG and CD4+ T cell responses. A randomised controlled study with about 850 children aged 5 to 17 months who were followed for 8 months in Kilifi, Kenya and Korogwe, Tanzania, showed an efficacy of 55% in terms of the rate of all episodes of malaria for RTS,S/AS01 [157]. The magnitude of the anti-CSP antibody responses in that study was substantially higher than in children that had received the RTS, S/AS02 vaccine previously. Whether the higher antibody titers associated with the use of AS01 will translate into a longer duration of protective efficacy for the RTS, S vaccine remains to be demonstrated.

Other clinical trials of the RTS,S vaccine are under way in combination with other candidate malaria vaccines [158] [159] [160]. A pivotal phase III trial design was planned to start in the second quarter of 2009 and is intended to enrol up to 16,000 children in 11 different sites in Burkina Faso, Ghana, Gabon, Malawi, Mozambique, Kenya and Tanzania, covering different transmission patterns.

Another CSP-based candidate vaccine was developed by Dictagen, Inc, in collaboration with the University of Lausanne, Switzerland, that contained a 102 aa synthetic peptide representing the C terminal portion of the CSP antigen formulated with Montanide ISA 720. The formulation was found to be safe in human volunteers and to elicit both an antibody response and a cellular immune response. The vaccine currently is in Phase IIa clinical trials.

The US Military Malaria Vaccine Program (USMMVP), in collaboration with Vical, Inc, developed a candidate DNA vaccine for malaria by mixing five plasmids that encoded five different *Plasmodium falciparum* antigens, including CSP, liver stage antigens 1 and 3 (LSA-1 and -3), exported protein 1 (EXP-1) and the sporozoite surface protein 2 (SSP-2, also known as thrombospondin-related adhesive protein, TRAP). The vaccine however only showed modest immunogenicity in nonhuman primates and elicited little protection against sporozoite challenge in human volunteers. More recently, USMMVP has conducted Phase I trials with Adenovirus 5 vectors that expressed CSP and the merozoite antigen AMA1. In addition, Crucell has been conducting a Phase I trial with adenovirus 35 recombinant for CSP in the USA in conjunction with NIAID.

The vaccine potential of *P. falciparum* LSA-3 was further investigated in nonhuman primates: both a DNA vaccine and a long LSA-3 synthetic peptide vaccine were able to elicit sterilizing immunity against sporozoite challenges in chimpanzees and *Aotus* monkeys, respectively [161] [162].

Another liver stage antigen, the TRAP antigen, was developed as a candidate vaccine by the Oxford University Malaria Vaccine Clinical Trials Group, which conducted studies of a DNA, a fowlpoxvirus (FPV) and a MVA-based vaccines expressing the TRAP antigen. The recombinant FPV and MVA vaccines were tested independently or in prime-boost combinations with or without the DNA vaccine and found to be well tolerated and highly immunogenic in human volunteers in the UK in terms of induction of specific CD4+ T cells [163] [164]. Trials in Gambia and in Kenya (DNA/MVA prime-boost and FPV/MVA prime-boost) failed however to demonstrate protective efficacy of these approaches [165].

Live recombinant vaccines expressing CSP determinants also were developed by Oxford University using a chimpanzee Adenovirus (AdCh63), MVA or FPV as vectors. These vaccines are in early clinical development. The first clinical trials of AdCh63/MVA ME-TRAP were begun in 2008 with a challenge trial planned for 2009. The prime-boost combination of an Ad35-CSP vaccine with the RTS,S/AS01 vaccine was found to be very immunogenic and to elicit robust protection against challenge in rhesus macaques [165]. Results were less clear-cut for prime-boost immunization with RTS,S and the MVA-CSP recombinant vaccine, whose combination only elicited a modest 33% protection against sporozoite challenge in volunteers [166].

#### 3.5.4.2. Erythrocytic vaccines

The choice of clinical case definitions and end-points in efficacy trials of erythrocytic vaccines, which aim to decrease morbidity by reducing parasitemia, still remains a difficult issue [167] [168]. Various groups, focusing on different merozoite antigens and different regions of the proteins, and using different antigen expression systems and formulations are developing a variety of erythrocytic stage malaria vaccine candidates. Results of preliminary efficacy trials involving some of these candidates should be made available over the next couple of years.

Blood stage vaccine candidates currently in early clinical trials are the merozoite surface proteins 1 (MSP-1), 2 (MSP-2), and 3 (MSP-3), apical membrane antigen 1 (AMA-1), the glutamate-rich protein (GLURP) and the serine repeat antigen protein (SERA) [169]. Anti-MSP-1 antibodies have been reported to strongly correlate with reduced risk of clinical malaria in Ghanaian children [170]. The two allelic forms of the C-terminal 42 kD region of MSP-1 were formulated with Alhydrogel and used to immunize 60 malaria naïve individuals, the large majority of whom developed anti-MSP-1 antibodies. However, these antibodies only showed minimal growth inhibition of *P. falciparum* in vitro [171]. A candidate vaccine containing MSP-1 adjuvanted with AS02, which had shown promising protective

efficacy in nonhuman primate models, also failed to induce protection or to decrease parasitemia in 1-4 years old children in Kenya in spite of good immunogenicity results [172].

The Combination B vaccine, which was developed in collaboration between the Walter and Eliza Hall Research Institute in Australia, the Swiss Tropical Institute in Lausanne and the Papua New Guinea Institute for Medical Research, combined the merozoite surface proteins MSP-1 and MSP-2 with the ring-stage infected erythrocyte surface antigen RESA and induced a 62% reduction in parasitemia in a Phase II trial on 5-9 years old children in Papua New Guinea [173], but this effect was restricted to parasites expressing one of the two allelic forms of MSP-2 [174]. A bi-allele MSP2 vaccine is now under development and a phase I trial of this vaccine recently took place in Australia.

Another promising vaccine in clinical development is an MSP-3 -based vaccine. The vaccine was tested in Phase I trials [175] [176] and found to elicit antibodies able to block multiplication of *P. falciparum* in red blood cells in vitro in a monocyte-dependent manner, a property of natural cytophilic IgG1 and IgG3 antibodies from malaria-immune African adults [177]. Passive transfer of the vaccinated volunteers' sera into *P. falciparum*-infected humanized SCID mice reduced or abrogated parasitemia [178]. The levels of cytophilic IgG3 antibodies against conserved regions of MSP-3 and the 24 kD glutamate-rich protein (GLURP) both significantly correlated with protection against clinical *P. falciparum* malaria in naturally exposed individuals in an area of hyperendemicity in Myanmar [179]. A GLURP-based candidate vaccine formulated with either alum or montanide ISA 720 similarly elicited dose-dependent cellular and humoral immune responses with high levels of cytophilic IgG1 antibodies that inhibited *P. falciparum* growth in vitro in the presence of monocytes [180]. The MSP-3-based vaccine is currently in Phase II clinical trials in Mali. A MSP3-GLURP fusion protein termed GMZ2 has progressed to the stage of phase I trials in sub-Saharan Africa with plans to presently progress to Phase 2 trials.

Another antigen of interest that was tested in Phase I trials is the AMA-1 antigen, which is known to be expressed at both the hepatic and erythrocytic stages. The AMA-1 vaccine which was developed by the Walter Reed Army Institute of Research using AS02 as the adjuvant induced a robust humoral immune response that lasted for more than one year. Field trials of that formulation have been initiated in Mali [181]. Phase I trials of an alhydrogel AMA-1 formulation were also conducted in Mali: the vaccine was found to be well tolerated [182] [183]. AMA-1 was also expressed in combination with MSP-1 as a fusion protein (PfCP2.9) that was formulated with montanide ISA 720. The resulting candidate vaccine was tested in Phase I trials in Chinese volunteers [184] [185] and was found to be well tolerated and to elicit high anti-PfCP2.9 antibody titers. The resulting antibodies, however, failed to show much effect on the growth of the parasite in vitro. The ISA720 formulation has been found to introduce several challenges for clinical vaccine development and is not now recommended for use by IVR.

Despite encouraging progress, the lack of immune correlates of protection together with the high polymorphism of many of the erythrocytic stage antigens constitute major obstacles to the development of vaccines that target the blood stage of the parasite cycle. In contrast with pre-erythrocytic stage vaccine candidates, erythrocytic vaccine candidates lack an appropriate human artificial challenge model and have had to rely on natural transmission in the field to provide a proof-of-concept of their efficacy. Their development is therefore slower and necessitates major commitment, intensive collaboration as well as high-level coordination supported by adequate funding. A recent meeting stressed the potential of the use of optimised clinical challenge models for screening blood stage vaccines in the future.

#### *3.5.4.3. Transmission-blocking vaccines*

Transmission-blocking vaccines aim to prevent onward transmission to humans by targeting the sexual stages of *P. falciparum* and blocking sexual mating so as to prevent the development of sporozoites in *Anopheles* mosquitoes. Antibodies against gametocytes could act directly in humans, or at a later stage in mosquitoes. This approach has the advantage of having robust in vitro assays that could be used to demonstrate proof-of-concept, as well as a relatively clear effector immune response. Several candidates are in clinical development, including vaccines that target the Pfs25 or Pvs25 and Pvs 28 surface proteins, but the ISA51 formulation of these vaccines turned out to be unacceptably reactogenic [186]. Also, a major challenge for this vaccine approach is proving true field efficacy [187] [188].

#### *3.5.4.4. Pregnancy malaria vaccines*

In Africa, where an estimated 50 million women become pregnant each year, maternal malaria causes untold numbers of abortions, stillbirths and over 10 000 maternal deaths. In addition, malaria infection causes more than 200 000 low-birthweight babies to die within their first year of life. The possibility of developing a placental malaria vaccine by targeting Plasmodium antigens expressed on the surface of infected erythrocytes that attach to placental proteins is the subject of promising R&D efforts [124].

#### *3.5.4.5. P. vivax vaccines*

If overall malaria disease burden continues to decrease then it is likely that *P. vivax* control will become a high priority within the malaria community. *P. vivax* disease already accounts for a rising proportion of cases in co-endemic areas. The WHO has taken a leading role in providing guidance on the *P. vivax* vaccine R&D agenda. Phase 1 trials of Pvs25 and *P. vivax* CSP-based peptide vaccines have been the only *P. vivax* clinical trials in recent years. A potentially promising approach based on *P. vivax* Duffy Binding Protein is under development by a group in India.

#### **3.5.5. Concluding remarks**

Given the complexity of the parasite and the slow development of naturally acquired immunity, it is increasingly thought by many experts that an effective malaria vaccine may have to contain antigens from multiple stages of the parasite. Several groups are working on such combination approaches for which numerous challenges have to be overcome including possible competition between the different antigens, compatibility of antigen presentation systems, and cost and complexity of evaluating incremental improvements.

International efforts to combat malaria have been scaled up in recent years, including among its many actors: the WHO, UNICEF, UNDP, the World Bank, the NIH in the USA, the Wellcome Trust in the UK, USAID, the European Malaria Vaccine Initiative (EMVI), the Malaria Vaccine Initiative (MVI) of PATH, The European Commission, EDCTP, and the Bill and Melinda Gates Foundation. The Roll Back Malaria Partnership plays a key role in coordinating malaria control strategy <http://rbm.who.int/aboutus.html> [189] [190].

It has been estimated that the cost for supporting the minimal set of malaria interventions required to effectively control malaria is around US\$ 3.2 billion per year for the 82 countries with the highest

burden of disease (US\$ 1.9 billion for Africa alone). Increased commitment and financial support, through programmes such as the Global Fund to Fight AIDS, Tuberculosis and Malaria which disbursed more than US\$ 200 million in 2003-2004 to 28 countries, will be needed to support control strategies in an effective and sustainable way. The next 5 years is likely for the first time to witness the submission of a malaria vaccine for possible registration (or "positive scientific opinion" - the EMEA article 58 equivalent). The malaria community will then need to consider the role of a partially efficacious pre-erythrocytic malaria vaccine as an addition to the current complement of malaria control measures. Generation of appropriate clinical trial data to allow assessment of public health impact of vaccination in the context of existing control measures will be a crucial component of this process.