Vaccine development against Dengue and Shigellosis and implications for control of the two diseases in Brunei Darussalam

Lim Chun¹, Lakshmi Devi Telisinghe², Mohammad Moshadeque Hossain¹, Ranjan Ramasamy¹

I Institute of Medicine, Universiti Brunei Darussalam and ² Ministry of Health, Brunei Darussalam

Abstract

Dengue and shigellosis are common in the Asia-Pacific region. There are constraints on effective treatment of the two diseases that can pose a health risk to Brunei Darussalam. Vaccines against other flaviviral and enteric bacterial pathogens are currently available but not yet against the dengue virus or Shigella bacteria. The state of vaccine development against the two diseases and the prevalence of the diseases in Brunei Darussalam and neighbouring countries are reviewed. For dengue, live attenuated viral strains and chimaeric viruses based on a yellow fever virus backbone are in clinical trials. Shigellosis vaccines based on O antigen – protein conjugates, and O antigen incorporated into hydrophobic proteosomes, have undergone clinical trials. It is concluded that vaccines against the two diseases are not needed for routine or mass vaccination in the country, but that they will be useful in protecting travelers to endemic countries and in controlling potential epidemics.

1. Introduction

Dengue and shigellosis are two infectious diseases found in the Asia-Pacific region associated with high morbidity and mortality. Dengue is endemic in Brunei Darussalam and outbreaks occur from time to time in the country. Although shigellosis is not endemic in Brunei Darussalam, there is always a risk of infection spreading from neighboring countries to Brunei. There is no specific anti-viral drug available for the treatment of dengue infection. There are four serotypes of dengue viruses that circulate globally and this and other factors have restricted vaccine development. Shigella is gaining resistance to many antibiotics used in its treatment and several infectious species are prevalent worldwide. However vaccines against dengue and Shigellosis are now in clinical trials. This article examines the current state of vaccine development against the two pathogens and the potential use of such vaccines in Brunei Darussalam.

1.1 Dengue

Dengue is a global health problem accounting for 50-100 million cases of febrile illness annually, including more than 500,000 cases of dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS) with an approximate case fatality rate of 20%. The disease is endemic in more than 100 countries in different parts of the tropical developing world, placing more than two billion people at risk of infection [1].

Dengue is caused by an arbovirus and the virus belongs to the family Flaviviridae, genus Flavivirus. The dengue viruses consist of four antigenically distinct serotypes (DV1, DV2, DV3 and DV4). Infection with one serotype confers immunity for several years against that serotype but only short-lasting protection against other serotypes. Infection with one serotype may also increase the risk of developing DHF/DSS on subsequent infection with another serotype.

Dengue virus is transmitted to humans by the bite of infected female Aedes aegypti (the primary vector) and Ae. albopictus (a subsidiary vector) mosquitoes. The Aedes mosquito species have adapted well to human habitation and urbanisation. They often breed in stagnant water collections in domestic water storage containers, blocked drains, roof gutters, flower pots and rubbish deposits (Fig 1). Currently there is no specific antiviral drug to treat dengue infections leading to an urgent need to develop a vaccine.
Shigellosis is also of major health concern in many countries. There are approximately 164.7 million cases of shigellosis worldwide, of which 163.2 million occur in developing countries and 1.5 million in industrialized countries [2]. Each year approximately 1.1 million people die of shigellosis. There are also 580 000 cases of shigellosis reported among travelers from industrialized countries. Sixty-nine percent of all the infections and 61% of all deaths attributable to shigellosis involve children less than 5 years of age.

Shigellosis is caused by a group of bacteria belonging to the genus Shigella [3]. Shiga, a Japanese scientist, first discovered the causative agent over 100 years ago. Shigella was adopted as a genus in the 1950s and grouped into four species: Shigella dysenteriae, Shigella flexneri, Shigella boydii, and Shigella sonnei. S dysenteriae serotype 1 is responsible for deadly epidemics and S. boydii is restricted to the Indian subcontinent. S flexneri and S. sonnei are prevalent in developing and developed countries, respectively. S. flexneri, an enteroinvasive gram-negative bacterium, is responsible for the worldwide endemic form of bacillary dysentery [4].

Shigellosis is particularly prevalent in developing countries with poor sanitation [5]. The fecal-oral route is most common method of transmission of the disease. Ingestion of contaminated food or water, contact with a contaminated inorganic object and sexual contact are other modes of transmission. Vectors like the housefly can spread shigellosis by physically transporting infected faeces.

In recent decades, Shigella has gained resistance to the antibiotics that were initially effective in treating the infection. Shigella acquired resistance to sulfonamides in the 1940s, tetracycline and chloramphenicol in the 1950s, ampicillin in the 1970s and trimethoprim/ sulfamethaxazole in the late 1980s. A vaccine against shigellosis would therefore be extremely useful. The Lanzhou Vaccine Institute’s vaccine is the only available vaccine today but it is not licensed outside China [6].
2. Targets molecules for vaccine development.

2.1 Dengue

Dengue virus envelope surface projections are made up of dimers of the viral envelope (E) glycoprotein and membrane (M) protein [7]. The capsid (C) protein is the only other protein constituent in the virion. The E glycoprotein is responsible for attaching the virus to receptor on target cell membrane and fusing the virus envelope with the cell membrane. It also bears the virus neutralization epitopes. In native virions, the elongated three-domain molecule lies tangentially to the virus envelope in a head-to-tail homodimeric conformation. The E dimers are changed to stable target cell membrane-inserted homotrimers that realign themselves vertically to promote virus-cell fusion upon penetration of the virion into the target cell endosome.

The 10.5 kb-long genomic RNA is a single stranded mRNA which is translated into a precursor polypeptide. The individual viral proteins derive from this polypeptide 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 an RNA polymerase. In addition to the glycoprotein, only NS1 had 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. NS3 contains the largest number of T cell epitopes.

Various vaccines under development have focused on the dengue virus E protein for the following reasons:
- It binds host cell surface receptor
- It is a target of neutralizing antibodies
- It elicits the first antibody response with the longest lasting activity
- Several monoclonal antibodies specific to receptor binding domain of the E protein block virus adsorption and infectivity
- Purified E protein can induce neutralizing antibodies and protective immunity

Most vaccine designs also include the prM protein, implicated in the maintenance of the structural/antigenic integrity of the E protein.

2.2 Shigellosis

Natural and experimental exposure to Shigella antigens has been observed to induce clinical immunity [8]. For example, monkeys experimentally infected with S. flexneri 2a were unaffected when rechallenged with the same strain but became ill when rechallenged with S. sonnei or S. flexneri. Israeli soldiers, with pre-existing serum antilipopolysaccharide [LPS] antibodies, deployed to a field area were significantly less likely to become ill upon exposure to homologous Shigella serotype than seronegative soldiers. From this and other observations, immunity was found to be serotype-specific leading to the recognition that the O antigen of LPS as a crucial moiety to be included in a Shigella vaccine.

3. Vaccines under development against dengue and shigellosis

3.1 Dengue vaccines

Currently there is no vaccine against dengue approved for clinical use [9]. However there are two promising approaches to vaccines in the late stages of development viz. one involving the use of live attenuated dengue virus strains and the other using chimeric viruses. Other forms of experimental vaccines are also under development but these may be less immunogenic and therefore require more immunizations to be effective, which is a disadvantage for controlling epidemics. All dengue vaccines have to take into consideration that non-neutralising anti-viral antibodies may promote the development of DHS/DSS and that more than one serotype of dengue virus may be circulating in any one locality. Therefore for a vaccine to be effective it must elicit protection against all four strains, or at least all the strains occurring in a particular locality, and should not promote the subsequent development of DHS/DSS upon infection.

3.1.1 Live attenuated dengue virus strains

The first of these vaccines was developed at Mahidol University, Thailand [10]. The vaccines were made by serially culturing the wild type-virus in animal kidney cells.
Dengue and Shigella vaccines

63

DV1, 2 and 4 were grown in primary dog kidney (PDK) cells, and DV3 in African green monkey cells. The vaccine underwent laboratory studies and later clinical trials in Thailand in the mono and polyvalent forms.

The vaccine was licensed to Sanofi-Pasteur, France, for commercial development. In a phase 1 clinical trial, tetravalent neutralizing antibody seroconversion rates of 80-90% was achieved in children aged 3-14 years and antiviral activity remained stable for at least a year. DV3 was the most immunogenic. Various formulations of the tetravalent vaccine were tested in an attempt to obtain a similar immune response for each serotype. The Mahidol University developed vaccine strains have a lower infection, dissemination rates and transmissibility in Aedes aegypti than those of parent viruses [11]. Apart from this property, the passage in humans and mosquitoes did not change the characteristics of the vaccine strains. More recently, it has been reported that the Mahidol vaccine, while being immunogenic, has high reactogenicity in adults and children and that Sanofi-Pasteur have withdrawn their interest in the vaccine [12].

Another live, attenuated vaccine is being developed at the Walter Reed Army Institute of Research, USA in cooperation with GlaxoSmithKline (GSK) [9]. The vaccine is also made by serially culturing the wild type virus in PDK cells and a final passage in fetal lung cells of rhesus monkey. All four monovalent formulations induced neutralizing antibodies production and were well tolerated in humans. Tetravalent formulations prepared were evaluated in rhesus monkeys and were shown to cause seroconversion after two doses of the vaccine. Pilot studies in humans using three doses of the most promising vaccine combination managed to induce 90% neutralizing antibody formation to DV-1, 60% to DV-2, 3 and 25% to DV-4. A small-scale Phase 1 study involving children of age 6-9 years in Thailand produced acceptable immunogenicity and reactogenicity profiles. These attenuated virus strains were also poorly transmitted by mosquitoes, like the Mahidol strains, and are therefore considered unlikely to be transmitted under natural conditions [13].

3.1.2 Chimeric vaccines strains

Acambis, Cambridge, USA has applied the ChimeriVax system, which was originally developed to modify a Japanese Encephalitis vaccine, into a dengue vaccine [7]. The vaccine, Chimeri Vax-DV, was prepared by substituting the prM and E genes of an attenuated 17D strain of yellow fever virus with those from the dengue viruses. The chimeric viruses were shown to be attenuated, including after intracerebral injection of monkeys, and showed 92% efficacy at protecting monkeys from homologous DV challenge. The viruses did not show any replication in mosquitoes after blood virus mixtures oral feeding. A monovalent ChimeriVax-DV-2 vaccine formulation tested on 56 human volunteers in a Phase 1 clinical trial in USA showed 100% neutralizing responses to DV-2. In 2005, a Phase 1 trial of the tetravalent combination in collaboration with Sanofi-Pasteur showed seroconversion to all four dengue serotypes. Sanofi Pasteur has progressed this vaccine into Phase 2 trial.

Groups in three other institutions have used modified dengue viruses as the basis for a multivalent vaccine [14]. The National Institutes of Health, USA developed a DV4 mutant containing a 30bp deletion in the 3' non-coding region of the DNA as a genetic background for the construction of chimeric viruses. Phase 1 clinical trials of the mutant carried out in 20 adult volunteers exhibited only minor symptoms with 100% neutralizing antibody formation. The mutated virus was then used as the backbone for the construction viruses with DV1, 2 and 3 envelope glycoproteins by insertion of DV1, 2, 3 prM and E genes. Alternatively the deletion mutation was induced in DV1, 2 and 3.

The Centers for Disease Control, USA carried out a similar work by using an attenuated DV2 vaccine mutant (strain 16681, PDK-53) as a backbone to construct chimeric attenuated viruses by changing the prM and E genes with those from DV1, 3 and 4.

The US Food and Drug Administration has also created a chimeric vaccine by replacing 3 nucleotides in the terminal 3' stem structure of DV1 and inserting DV2-4 prM and E genes into the DV1 backbone. The candidate vaccines were found to be highly immunogenic in susceptible rhesus monkey.
3.1.3 DNA vaccines

A DNA vaccine expressing prM and E proteins of DV1 tested on *Aotus* monkeys showed that there was induction of a protective immune response [15]. More extensive pre-clinical and clinical trials are required before the DNA vaccine is acceptable for practical use as the general problem with DNA vaccines has been the requirement for multiple boosting immunisations to generate sufficient immunogenicity [16].

3.1.4 Inactivated and subunit vaccines

Successes in development of inactivated flavivirus vaccines against Japanese encephalitis and tick-borne encephalitis have triggered attempts to develop an inactivated dengue vaccine. DV2 grown in Vero cells was inactivated, purified, concentrated and tested in laboratory animals. Trials in monkeys have shown production of a protective level of antibodies [9].

Subunit vaccines have been developed by several researchers using the recombinant DNA techniques. In recombinant DNA techniques, specific genes encoding for protective antigens are cloned and expressed in other host cells including *E.coli*, yeast and insect cell systems. Recombinant E protein of DV2 produced using a baculovirus vector-insect cell system was able to induce neutralizing antibody production and provide partial protection to immunized monkeys [17].

A major drawback with inactivated or recombinant vaccines is the need for more effective adjuvants suitable for human use, to increase immunogenicity, thereby reducing the number of immunisations needed to achieve protection.

3.1.5 Vaccinia virus as vector for dengue vaccine

The Modified Vaccinia Ankara (MVA) vector with a restricted host range has been used for the construction of dengue recombinants. Trials whereby monkeys having repeatedly immunized with MVA recombinant expressing DV2 E protein were shown to produce virus neutralizing antibodies [18]. Construction of MVA recombinants expressing immunogenic E protein of other dengue virus serotypes is being planned. However pre-immunity to *Vaccinia* may limit its replication in immunized persons, as has been shown in trials with antigens from other pathogens, thereby similarly limiting efficacy against dengue in multiple immunizations. Pre-immunity to *Vaccinia* may be overcome by using a different viral vector, such as recombinant fowl pox virus, for boosting.

3.2 Shigella vaccines

The main approaches in *Shigella* vaccine development are the use of live attenuated bacteria vaccines and subunit vaccines [8]. The subunits vaccines consist of the conjugate vaccines, proteosome vaccines and the ribosomal vaccines.

3.2.1 Live attenuated oral vaccines

Two strategies to attenuate the bacteria are being used. The first approach is to create deletions in genes controlling metabolic processes, such as those involved in the biosynthesis of essential metabolites or transport of nutrients. The other approach is to mutate genes that encode specific virulence factors. These approaches are bedeviled by the fact that attenuated bacteria may cause serious infections in immunosuppressed and immunodeficient vaccinees in *Shigella*-endemic countries.

3.2.2 Parenteral conjugate vaccines

The National Institutes of Health has developed several vaccines by conjugating the O antigen of *S. sonnei*, *S. flexneri* or *S. dysenteriae* to *Pseudomonas aeruginosa* recombinant exoprotein A (rEPA). In one trial, the *S. sonnei* vaccine was shown to be immunogenic in 90%, and the *S. flexneri* vaccine in 73%, of adult Israelis. A phase 3 efficacy field trial involving Israeli soldiers showed that a single intramuscular injection of *S. sonneri*-rEPA conferred 74% efficacy against shigellosis [19]. A vaccine containing a several specific O antigens conjugated to a protein carrier may however be expensive for production.

3.2.3 Nasal proteosome vaccines

Another approach for developing the vaccine is by delivering *Shigella* LPS in proteosomes. LPS is non-covalently
complexed by hydrophobic interactions into proteosomes, or multimolecular vesicular structures, which are made from purified meningococcal outer membrane proteins. Proteosomes are also believed to generate mucosal adjuvanticity. A phase 1 trial of *S. flexneri* 2a LPS vaccine showed no efficacy against the primary endpoint (diarrhoea, dysentery, fever and early treatment) but apparently diminished the severity of illness [20].

3.2.4 Ribosomal vaccine

The International Vaccine Institute (IVI) in Korea has used a different approach to develop a *Shigella* vaccine. Researchers at IVI had discovered that ribosomes from the *Shigella* bacteria have specific O antigen non-covalently bound to their surface and free of the lipid A associated with O antigen in LPS. Preliminary results in mice have shown good antibody responses both in serum and faeces following parenteral injection with the purified ribosomes [6].

3.3 Dengue in Brunei and the region

Brunei Darussalam on the north-west part of Borneo Island is surrounded by the South China Sea to the north and the Malaysian State of Sarawak on all the other sides. Also located on the Borneo Island are the Malaysian State of Sabah and Indonesian State of Kalimantan.

Brunei consists of four districts: Brunei Muara, Tutong, Belait and Temburong. Brunei has an equatorial climate characterized by a consistent high temperature, high humidity and heavy rainfall. Rainfall varies from 2,500 mm on the coast to 7,500 mm in the interior annually. There is no specific wet season.

Figure 2 below shows the number of dengue cases notified in Brunei for the past 5 years23. Before this period (1995-2001), the annual number of reported cases was less than 10. The notified number of dengue cases has been on the rise since 2002. In the year 2003, there was a localised outbreak of dengue. The predominant infecting serotype is DV2 followed by DV1 as shown recently [22]. The Breteau Index, which estimates percentage of containers infested with Aedes larvae per number of inspected houses, has since remained low in the country. However this is not a definite indicator of the vector density.

![Figure 2. Total number of dengue cases reported in Brunei from year 2002 to 2006](image-url)
Figure 3. shows the distribution of dengue cases in the four districts of Brunei Darussalam [23]. The dengue cases reported were usually sporadic cases. Prior to 2005, there were no outbreaks in the Temburong district. However in 2005, there were an appreciable number of cases reported from this district.

![Figure 3. Dengue cases reported in four districts of Brunei from 2002 to 2006 [23].](image)

The outbreak of dengue which started in Kampong Ayer in 2003 mainly involved the Brunei Muara district where there was a definite local transmission. The disease spread to the neighbouring villages. The control of the outbreak was successfully carried out by adopting the following:

- Eradication and control of the *Aedes* larva, which was found breeding abundantly in and around houses of all the kampongs affected, by treating with the chemical larvicide Abate, and destroying all potential breeding places.

- Emergency spraying of insecticides carried out in all affected kampongs.

- The assistance of the penghulus and ketua kampungs and the community in enhancing the coverage of all control operations.

- Education of public on the nature of the disease and vector, and their positive involvement in control of vectors.

- Investigations of positive cases and serological testing of close contacts was used to assess the extent of the outbreak. The ability to rapidly report results by the State Laboratory in RIPAS was invaluable in this regard.

The outbreak in Brunei-Muara district was brought under control in two months through the efforts of the staff of the Environmental Health Division in the district, supported by the State Laboratory at RIPAS hospital. Following the 2003 outbreak, the Environmental Health Services continue to play a proactive role in the control of dengue in the country.

The following remain ongoing dengue control actions of the Environmental Health Services [Figure 4]:

- Source reduction

- Entomological survey of vector breeding and control of adults and larvae.

- Epidemiological surveillance with laboratory support

- Control of vectors by destroying breeding places by larviciding and adulticiding

- Involving the community in assisting Environmental Health services staff in controlling of vectors.

- Educating the community, including schoolchildren, on a healthy environment (‘healthy village’, sanitation of villages)
The incidence rate of dengue per 100,000 population in some of the countries in South East Asia is shown in Figure 5. Sarawak had 1799 cases of DF and 22 cases of DHF reported with an incidence rate of 78.7 cases per 100,000 population in the year 2006 (61.0 cases per 100,000 population in 2005) [24]. The incidence rate in 2006 is calculated based on the most updated 2005 population census available. In Sabah, 1,865 cases of DF and 36 cases of DHF were reported with an incidence rate of 64.8 cases per 100,000 population in year 2005 [25].
3.5 Shigellosis in Brunei and the region

In Brunei Darussalam, shigellosis is an uncommon but notifiable infectious disease under the Infectious Disease Order 2003 of Brunei. All health practitioners and laboratory personnel are obliged to report to the Ministry of Health upon identification of shigellosis cases. Shigellosis is classified as gastroenteritis or dysentery case. The Ministry of Health follows the guidelines of management of Shigellosis in the Control of Communicable Diseases Manual of the World Health Organization.

The high standard of living, with proper housing, good water supply and sanitation, strict commercial food safety regulations, and health screening of immigrant workers before entry are all factors that contribute to the better record of Brunei in limiting shigellosis compared to many Asian countries. However there is still a considerable risk of travelers carrying the disease into the country from the now frequent travel to endemic areas.

A recent study to obtain more accurate and current estimates of shigellosis in the Asian region has been carried out due to the belief that current estimates of shigellosis are under detected and inaccurate [5]. Three rural or semi rural sites (in China, Vietnam and Thailand) and three urban slum sites (in Bangladesh, Pakistan and Indonesia) were chosen. The study involved approximately 600,000 participants over a 1-3 year period. Sixty thousand cases of diarrhoea were detected and 5% were shigellosis.

The overall incidence of shigellosis was 2.1 cases per 1,000 residents per year in all ages and 13.2 cases per 1000 residents per year for children under 60 months old. The incidence of shigellosis increases after the age of 40 years old. During the study, several reasons possible for under detection of shigellosis were identified. Culture of stool samples is normally the diagnostic tool for *Shigella* detection in clinical settings. However evidence shows that a more sensitive detection method based on polymerase chain reaction (PCR) analysis detected *Shigella* DNA in one-third of culture-negative stool specimens. Another reason is that less than one-third of culture proven shigellosis cases presented with dysentery, which is a frequently used clinical case definition in government data collection. Shigellosis cases can present with other signs and symptoms such as fever, vomiting and others stated.

Shigellosis is endemic in Malaysian cities with a high prevalence of *S. flexneri*, and resistance to common antibiotics [27].
4. Discussion

No matter how advanced or effective is the existing treatment for infectious diseases, the ideal way to protect people from contracting the disease is by immunizing them with vaccines because this prevents morbidity and consequent mortality and saves on treatment costs. An ideal vaccine has to be free of adverse effects, easy to administer and provide long-term immunity. Such effective vaccines will greatly help control dengue and shigellosis in nearby Asian countries where the diseases are endemic.

Brunei Darussalam is surrounded by or close to countries where incidences of dengue and shigellosis are reportedly high and the diseases are endemic. This increases the risk of both diseases spreading to Brunei as there is constant and rapid movement of population to and fro across the border. The present modes of rapid transportation by air and land can introduce virulent forms of the diseases into Brunei through visitors or migrant workers entering Brunei and the return of Bruneian residents who contract the diseases abroad. DHF/DSS and antibiotic resistant strains of *Shigella* have not been reported in the recent past in Brunei. With urbanisation, dengue has found a foothold in Brunei and may cause outbreaks in the future, if not properly controlled.

Cost is another important factor. The Paediatric Dengue Vaccine Initiative estimates that a dose of dengue vaccine to the public sector will cost US$0.50 [28]. Assuming that the whole population of Brunei is vaccinated in the public sector, requiring one dose to provide lifelong immunity, mass vaccination will reduce the costs of treating patients with dengue in hospital, and vector control operations. It will also diminish economic loss due to reduced morbidity. However, additional manpower and equipment needed for vaccination, the costs of multiple doses of vaccine required to provide immunity, management of adverse events after vaccination, etc are other factors that have to be considered in the equation.

The two diseases are not a major threat to the public health at present. The strategies adopted by the Ministry of Health have therefore been very effective in controlling these two diseases in Brunei. However it is notable that the incidence rate of dengue in Brunei for the year 2006 is comparable to that in neighboring Southeast Asian countries. Although the available data indicates the total burden of these two diseases in Brunei is low, a continuous and comprehensive epidemiologic profile of these two diseases in the country is required.

Brunei has a national immunisation program for children [29], similar to that in neighbouring countries. Also available in Brunei are optional vaccines such as those for influenza and yellow fever. The efficacy of the current available experimental vaccines against dengue and shigellosis have yet to be perfected. Even if these vaccines were developed free from known adverse effects, there is always the possibility of unforeseen adverse effects, when additional immunizations to the current routine vaccinations are introduced.

Given the current burden of dengue and shigellosis, we may conclude that vaccines against these two pathogens are not needed for routine vaccination of the entire population of Brunei. However, increased travel between Brunei and neighboring countries and urbanization increase the risk of dengue and shigellosis outbreaks. Therefore if appropriately effective, safe and affordable vaccines become available, the possibility of implementing vaccinations to protect travelers may be considered. An appropriately polyvalent dengue vaccine may in particular be useful in helping to control localized outbreaks of the disease. Furthermore such effective vaccines may be stockpiled for use in preventing possible epidemics in neighboring countries from spreading to Brunei in the future. The use of vaccines in epidemics that spread across national boundaries will require close coordination among neighbouring countries.

5. Acknowledgements

We thank Dr Mohammad Hussein, Dr Pengiran Hishammuddin and Awg Kamaluddin bin Mohammad Yassin for valuable discussions, Hj Shamsul Bharine Hj Sabtu for compiling some of the data, and Haji Yaakub bin Ahmad for demonstrating the entomological survey in Kg. Ayer.
6. References


