Leishmaniases are parasitic diseases with an important clinical and epidemiological diversity. Its burden remains important: 88 countries, 350 million people at risk, 500,000 new cases of VL per year, 1–1.5 million for CL and DALYs: 2.4 million (Desjeux, 2004b). Most of the burden is concentrated in few countries which allows clear geographic priorities. It is still an important public health problem due to not only environmental risk factors such as massive migrations, urbanisation, deforestation, new irrigation schemes, but also to individual risk factors: HIV, malnutrition and genetic. Leishmaniasis is part of those diseases which still requires improved control tools. Consequently WHO/TDR research for leishmaniasis has been more and more focusing on the development of new tools such as diagnostic tests, drugs and vaccines (Desjeux, 2004a; Desjeux, 2004b).

The disease manifestation is determined by both the species of *Leishmania* and the host immune system, although certain species are associated with specific clinical conditions. For example, visceral leishmaniasis (VL) usually results from infection with either *L. donovani* or *L. infantum* (Vanloubbeecck and Jones, 2004). Although chemotherapeutic treatments for the leishmaniases exist, the drugs are costly, limited and toxic (Murray *et al*., 2005). Furthermore, available treatments are threatened by drug resistance (Croft *et al*., 2006). Even with treatment, various disease forms can cause lifelong disfigurement and scarring. Thus, as with all infectious diseases, prevention of leishmaniasis is superior to a cure. A prophylactic vaccination would prove to be the most effective strategy to control infection and spreading of this group of diseases (Rodriguez-Cortes *et al*., 2007). However, despite substantial efforts spent in developing a vaccine, there is currently no licensed vaccine against human leishmaniasis (Reithinger *et al*., 2007).

Human infection with *Leishmania* results in diverse clinical and immunopathological situations. The capacity of the parasites to cause this wide range of disease manifestations depends upon their ability to evade the immune defense mechanisms by performing a well-tuned orchestra of host parasite interactions inside the
macrophages. The symptoms of VL vary between individuals and according to geographical foci. However, some of the common symptoms include high undulating fever often with two or even three peaks in 24 hours and drenching sweats which can easily be misdiagnosed as malaria, chills, rigors, weight loss, fatigue, poor appetite, cough, insomnia, abdominal pain, joint pain, anorexia, epistaxis and diarrhoea. Clinical signs include splenomegaly, hepatomegaly and lymphadenopathy (Hashim and Elhassan, 1994). The immunological features include circulating immune complexes, elevated levels of IgG, IgM, IgE and IgG subclasses, decreased responsiveness to the lipopolysaccharide required for induction of interleukin-1 (IL-1) production, immunosuppression and a low cell mediated immunity (CMI) in terms of delayed type hypersensitivity (DTH) (Fagarasan and Hongo, 2000; Carvalho et al., 1981).

The incubation period is highly variable; the disease can appear anytime between ten days to over one year. Even longer incubation periods have been documented (WHO, 1997). The duration of the disease can be 1-20 weeks, in endemic areas of Western Sudan the illness usually lasts for about 12-16 weeks with an average of about 6 weeks (Hashim and Elhassan, 1994). VL can be complicated by serious secondary bacterial infections such as pneumonia, dysentery and pulmonary tuberculosis, which often contribute to the high fatality rate of VL patients. Other complications though rare include haemolytic anaemia, acute renal damage and severe mucosal haemorrhage (WHO, 1991).

*Leishmania* specific antibodies, disseminated by the immune system of the host cannot approach intracellular phases of the parasite in hepatocytes, splenocytes and bone marrow cells (Garg et al., 2005). Hence protective immune response in experimental leishmaniasis has been shown to be related to the presence of cell mediated immunity and positive delayed type hypersensitive (DTH) response to leishmanial antigens in the skin. Studies described that cell mediated immunity is associated with activation of Th1 cells producing IFN-γ, IL-2 and TNF-α (Pinelli et al., 1995). Interferon gamma (IFN-γ) and IL-2 induce nitric oxide-dependent parasite killing by infected macrophages (Ravindran and Ali, 2004). On the other hand, active disease is characterized by a marked humoral response which in turn is associated with expansion of Th2 cells (Reis et al., 2006). These cells secrete anti-inflammatory cytokines IL-4, IL-10 and IL-5. It has been shown that the Th1 cytokine, IFN-γ promotes immunoglobulin switching from IgM to the IgG2a isotype.
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At the same time, IL-4 triggers switch from IgM to IgG1 and IgE. Indeed, IgG2a and IgG1 kinetics indirectly reflect the Th1/Th2 responses. The relative production of these isotypes can thus be used as a marker for the induction of Th1-like and Th2-like immune responses, respectively. In particular, there are several reports introducing vaccines for leishmaniasis in murine model which demonstrate the correlation of Th response and IgG isotypes (Shimizu et al., 2002; Iborra et al., 2005; Lange et al., 2004). Also, IL-5, a Th2-derived cytokine, co-operates with IL-4 in inducing production of IgG1 and IgE antibodies (Purkerson and Isakson, 1992; Cocks et al., 1993; De Kruyff et al., 1990). Susceptibility and resistance to Leishmania infection in the mouse model have also been demonstrated to be associated with the emergence of a unique subset of T cells, namely the T regulatory (Treg) cells. CD4⁺CD25⁺ T cells produce IL-10 upon in vitro stimulation with parasite antigens. IL-10 is a potent inhibitor of IFN-γ production which in turn is a key cytokine to control leishmaniasis (Belkaid, 2003).

There is general agreement that the establishment of a protective anti-leishmanial immune response requires the presentation of appropriate antigens by antigen-presenting cells, the induction and expansion of Th1 lymphocytes and the activation of macrophages for efficient killing of parasites. In the early days of vaccine development, when live or killed whole parasites were considered, identification of the antigens which induce the appropriate immune responses leading to host protection was a difficult task. Then, it became possible to identify and characterize relevant protein antigens and, most importantly, to produce them in unlimited amounts with the help of gene cloning and monoclonal antibodies. Thereafter, a number of leishmanial antigens have been exploited in an attempt to produce a vaccine against leishmaniasis. Whole killed Leishmania antigen had been used in both prophylactic and therapeutic vaccines and the latter may have a particular role in drug resistant cases. Theoretically, the killed vaccines from cultured Leishmania parasite should be cost effective in endemic countries. However, their registration is hampered by the lack of standardization. Extensive vaccination trials in Brazil and Ecuador have shown that a cocktail of five killed Leishmania stocks or a single strain of L. amazonensis induces significant protection from natural infection (Bertholet et al. 2005). Phase III trials with a vaccine formulation of killed Leishmania organism mixed with a low concentration of BCG as an adjuvant have also yielded
promising results. Preliminary studies using autoclaved L. major mixed with BCG have been successful in preventing infection with L. donovani (Bertholet et al., 2006). Therapeutic vaccines with killed parasite have also demonstrated their good safety profile with encouraging results, despite their poor prophylactic outcomes, in South America and Sudan. Autoclaved and heat-killed antigens of L. donovani were also tested in our laboratory and were found to induce cellular and humoral responses in experimental VL (Nagill et al., 2009).

Vaccination remains the best hope for control of all forms of the disease, and the development of a safe, effective and affordable antileishmanial vaccine is a critical global public-health priority. Although a great number of antigens have been tested in experimental models for protection against the disease, no effective vaccine against leishmaniasis is yet available. Significant antigenic diversity and digenetic life cycle hampers the development of vaccine. Various methods have been used previously in an attempt to isolate and select relevant Leishmania antigens for vaccine development, some of which are T cell cloning, screening antigen pools and screening cDNA libraries (Rafati et al., 2001). There are a number of Leishmania antigens that might act as vaccine candidates. Gp63 a surface expressed glycoprotein or leishmanolyisin is delivered by a plethora of immunization regimens, however, promising findings from animal models were overshadowed by mostly negative T cell responses in humans (Russo et al., 1991). Parasite Surface Antigen 2 (PSA-2) or gp46 is another vaccine candidate that belongs to a gene family present in all Leishmania species except L. braziliensis (McMahon-Pratt et al., 1992). Immunization with the native polypeptides derived from promastigotes protected mice against infection (Kedzierski et al., 2004) but vaccination with a recombinant protein derived from either promastigotes or amastigotes protein did not protect mice against infection (Sjolander et al., 1998a). Similarly, DNA vaccination conferred protection in mice when used as either prophylactic (Sjolander et al., 1998b) or therapeutic vaccines (Handman et al., 2000). Another extensively tested antigen is the Leishmania homologue for receptors of activated C kinase (LACK) that is expressed throughout leishmanial life cycle (Mougneau et al., 1995). Immunization with LACK appears to promote the expansion of IL-4 secreting T cells skewing the response towards detrimental Th2 responses (Launois et al., 1997). To date, LACK has been found to show
protection mainly in the *L. major* model but it failed to protect against VL (Melby et al., 2001b). Other antigens from different *Leishmania* species that had been tested in animal models include amastigote cysteine proteases (CP) (Rafati et al., 2005), cysteine proteinase A2, amastigote membrane proteins P4 and P8 (Soong et al., 1995), kinetoplastid membrane protein-11 (KMP-11) (Basu et al., 2005), *Leishmania chagasi* recombinant antigen (LCR1) (Streit et al., 2000), hydrophilic acylated surface protein B1 (HASPB1) (Stage et al., 2000), leishmanial antigen ORFF (Tewary et al., 2005), acidic ribosomal protein P0 (Iborra et al., 2003), paraflagellar rod protein 2 (PRP-2) (Saravia et al., 2005), NH36, a main component of the fucose-mannose ligand (Aguilar-Be et al., 2005) and proteophosphoglycan (PPG) (Samant et al., 2009). In addition, molecules such as ATP synthase alpha chain, beta-tubulin and heat shock 70-related protein 1 precursor have been recently identified as novel vaccine candidates (Bhowmick and Ali, 2008).

Hsps also have been found to have a variety of important and surprising roles in immunity (Sargent et al., 1989). They are known to induce release of cytokines by different immune cells (Multhoff et al., 1999; Asea et al., 2000). Hsp70 is a major target of immune responses to a wide variety of pathogens including bacteria, fungi, helminths (worms) and protozoan parasites (Young and Elliot, 1989; Kaufmann, 1990; Young et al., 1990; Young, 1990). Hsp83 has also been found to show immunogenicity, for example *Leishmania infantum* Hsp83 when fused to *Toxoplasma gondii* Rop2 protein elicited strong cellular response in an adjuvant-free vaccination system. Mixture of two proteins produced a Th1/Th2 mixed response (Echeverria et al., 2006). The immunostimulatory properties of these two antigens encouraged us to evaluate their immunoprophylactic potential in murine leishmaniasis

Data obtained using different strains of inbred mice indicate that genetic variation in the host has a major influence in determining the outcome of infection. Part of this variation probably reflects differences in the ability of the host to respond to individual antigens. A high responder to one antigen may be a low responder to another antigen. Accordingly, a vaccine to be used in an outbred population, such as humans, will probably require several different antigens to guarantee a satisfactory overall response by most if not all of the population. Therefore, the development of polyvalent vaccines incorporating many antigens is necessary to ensure that all individuals in the population
including low responders to some antigens will get the benefit of the vaccine. It is now clear that many antigens may combine to elicit protective immune responses. A number of studies have been directed towards the use of multicomponent vaccines against leishmaniasis (Li et al., 1999; Wunderlich et al., 2000). However only one vaccine, Leish-111f, has reached the clinical trials (Coler and Reed, 2005). It is a single polyprotein composed of three molecules; the L. major homologue of eukaryotic thiol-specific antioxidant (TSA), the L. major stress-inducible protein-1 (LmSTI1) and the L. braziliensis elongation and initiation factor (LeIF) (Skeiky et al., 2002). Initial vaccination trials in mice demonstrated that Leish-111f was able to protect mice against cutaneous leishmaniasis by L. major and L. amazonensis (Skeiky et al., 2002). It was also demonstrated that the Leish-111f vaccine could induce partial protection against VL in animal models (Coler et al., 2007), however, it failed to protect dogs against the disease in Phase III trial (Gradoni et al., 2005). Leish-110f is a slightly improved version of the original construct and is tested in dogs as a therapeutic vaccine in combination with chemotherapy. It led to reduced number of deaths and higher survival probability (Miret et al., 2008). Human Phase I and II clinical trials (safety and immunogenicity) of Leish-111f have been completed over the past few years in Brazil, Peru, Columbia and India (Kedzierski, 2010). Although the antigens mentioned above have shown good protection against leishmaniasis in various animal models, no effective vaccine is yet available that could provide complete protection.

Purified protein antigens and peptides, the preferred subunit vaccine candidates, are poor immunogens. Administration of these immunogens usually induces immunological tolerance unless injected in combination with an adjuvant (Gupta et al., 1993). Adjuvants can be used to improve the immune response to vaccine antigens in several different ways: adjuvants can increase the immunogenicity of weak antigens; enhance the speed and duration of the immune response; modulate antibody avidity, specificity, isotype or subclass distribution; stimulate cell mediated immunity (CMI), enhance immune responses in immunologically immature, or senescent individuals; or decrease the dose of antigen in the vaccine and reduce costs. It is widely recognized that many vaccines will require the simultaneous administration of adjuvants to enhance immunogenicity and efficacy. In addition, immunity induced by vaccines often
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necessitates specific enhancement of a polarized immune response, e.g. Th1 versus Th2, and this would require adjuvants possessing specialized mode of actions such as TLR ligation (Pashine et al., 2005). Therefore research has been focused on the identification of adjuvants that stimulate cellular immunity and those that act through Toll-like receptor 4 (TLR4) have shown particular promise (Coler and Reed 2005; Cluff et al., 2005). Among the adjuvants approved for use in humans, alum has had the greatest clinical use in past (Vogel and Powell, 1995). The ability of bacteria to be taken up by dendritic cells, coupled with the ability to express foreign genes in bacteria, has made them attractive delivery vehicles for vaccines. Such a vaccine could exploit attenuated bacteria such as BCG (Convit et al., 1987; Michel et al., 2006). Several other types of particulate adjuvants or delivery systems have been tested for use with Leishmania vaccines; they include liposomes, montanide ISA 720, CPG oligodeoxynucleotides (CPG ODN), glucan, saponins, freund's adjuvants and monophosphoryl lipid A (MPLA) (Skeiky et al., 2002; Coler et al., 2007; Santos et al., 2002; Walker et al., 1999). MPLA is the only adjuvant that had been approved by Food and Drug Administration of USA for human use. It is a detoxified form of lipid A derived from the lipopolysaccharide of Salmonella minnesota R595. The adjuvant activity of MPL is attributed primarily to its ability to activate antigen presenting cells and induce cytokine cascades (Ulrich and Myers, 1995). Several studies have demonstrated the ability of MPL to activate monocytes and macrophages (Ribi et al., 1984 and Masihi et al., 1986). Presumably through the activation of these cells, vaccine antigens are more readily phagocytised, processed, and presented. It has also been reported that, MPL-SE formulated with rLeish-11f elicits protective immunity against L. major infection (Skeiky et al., 2002). Autoclaved L. donovani (ALD) along with BCG was found to be protective against experimental VL (Srivastava et al., 2003). Autoclaved L. major plus BCG has been found to be protective against L. donovani langur monkeys (Dube et al., 1998). Recently, studies in our lab also demonstrated protective potential of ALD along with 78kDa in experimental leishmaniasis (Nagill and Kaur, 2010). These studies inspired us to formulate vaccines in combination with MPLA and ALD.

Leishmaniasis are a diagnostic challenge because of the broad spectrum of clinical manifestations. However, differential diagnosis is important because diseases of
other etiologies with a clinical spectrum similar to that of the leishmaniases (e.g., leprosy, skin cancers, and tuberculosis for CL and malaria and schistosomiasis for VL) are often present in areas of endemicity. Also, clinical disease severity is mainly due to the infecting *Leishmania* species, and there is growing evidence that the therapeutic response is species and, perhaps, even strain specific (Singh and Sivakumar, 2003; Zijlstra et al., 2000)

Parasitological diagnosis remains the gold standard in leishmaniasis diagnosis because of its high specificity (Zijlstra et al., 1992). This is typically undertaken by microscopic examination of giemsa stained slides of the relevant tissue to visualise the parasite. Specimens examined are usually skin biopsies or aspirates from bone marrow or spleen. In fact, these methods have many drawbacks like spleen hemorrhage and sometimes record high rates of false negative results (Sundar and Rai, 2002). All these factors necessitate the development and application of reliable serological tests. Various serological tests include IFAT, ELISA and CIEP for diagnosis of VL. The sensitivity of ELISA (99.5%) and IFAT (99.04%) is more than that of CIEP (96.6%) while specificity of these two tests is lesser than CIEP (Mittal et al., 1991). The basis for IFAT is detection of antibodies which are demonstrated in very early stage of infection and are undetectable after six to nine month of cure (Palma and Gutierry, 1991). However, the test is unsuitable for field conditions (Singh and Sivakumar, 2003).

Direct Agglutination Test (DAT) is a simple, sensitive and specific test in this category (Singh and Sivakumar, 2003) but is unable to distinguish between active disease and past infection. Since total *Leishmania* promastigotes are used as antigen in routine assay, false positive reactions are frequent due to cross reaction with the sera from other diseases. Therefore, an antigen that determines less cross reactivity is the best suggested for serodiagnosis of leishmaniasis (Sundar and Rai, 2002).

ELISA is a valuable, rapid, sensitive and commonly used serodiagnostic tool for almost all infectious diseases including leishmaniasis. The sensitivity and specificity of ELISA can be improved by the use of defined antigens (Blaxter et
al., 1988). Several recombinant antigens like rGBP from *L. donovani*, rORFF from *L. infantum*, rgp63, rK9, rK26 and rK39 from *L. chagasi* (Montoya et al., 1997; Martin et al., 1998; Jensen et al., 1999; Rajasekariah et al., 2001; Raj et al., 1999) have been developed and tested. Of these, the rK39 antigen is found to be highly sensitive and predictive of onset of disease manifestation in VL patients but the method is costly. Several heat shock proteins from *Leishmania* belonging to the 60, 70, 83, and 90 families also have been tested for the serodiagnosis of leishmaniasis. *L. major* Hsp60 was tested with cutaneous leishmaniasis sera (Rey-Ladino et al., 1997). *L. braziliensis* Hsp83 and Hsp70 with cutaneous, mucocutaneous and diffuse cutaneous leishmaniasis sera (Skeiky et al., 1997), and *L. donovani* Hsp70 (MacFarlene et al., 1990) and Hsp83 of *L. infantum* with canine VL sera (Angel et al., 1996). These studies inspired us to explore diagnostic potential of Hsp70 and Hsp83 in murine VL.

The antigen detection techniques are ideal methods to diagnose the disease because they indicate the stage of active disease (Kohanteb et al., 1987). However, detection of antigen in the patient’s serum is complicated by the presence of high level of antibodies, circulating immune complexes, serum amyloid, rheumatoid factor and autoantibodies; all of which may mask immunologically important antigenic determinants or competitively inhibit the binding of free antigen. Till date no satisfactory antigen detection system is currently available. A new latex agglutination test (KATEX) for the detection of low molecular weight leishmanial antigens in the urine of patients with VL has also been developed. The results obtained with KATEX using samples collected from patients of different foci of VL indicate that, the test works well regardless of the geographical origin of samples. The test had 100% specificity but the sensitivity is low to moderate (48-87%) (Sundar et al., 2007; Alam et al., 2008).

Molecular methods to diagnose the disease are capable of detecting nucleic acids unique to the parasite and therefore are capable to address the limitations of conventional methods. A variety of nucleic acid detection methods targeting both DNA and RNA have been developed. PCR is proved to be a highly sensitive and specific technique amongst these methods. Different DNA sequences in the genome of *Leishmania* like ITS region, gp63 locus, telomeric sequences, and sequence targets in rRNA genes such as 18s rRNA and SSU-rRNA and both conserved and variable regions in kinetoplast DNA (kDNA)
minicircles are being used by various workers (Attar et al., 2001; Santos-Gomes et al., 2000; El Tai et al., 2001; Pizzuto et al., 2001; Wortman et al., 2001). Although the serological tests have been evaluated with various degrees of sensitivity and specificity, a universal method of field diagnosis to replace the gold standard of kala-azar diagnosis by histological demonstration of parasites from the splenic or bone marrow aspirate is still lacking (Singh and Sivakumar, 2003; Kubar and Fragaki, 2005; Sundar and Rai, 2002).

Considering the above studies, the present study was designed to evaluate the immunodiagnostic and immunoprophylactic potential of three vaccine formulated by the cocktail of two heat shock proteins (Hsp70 and Hsp83) in combination with adjuvants ALD and MPLA against experimental murine visceral leishmaniasis.