Evaluation of first generation vaccines against human leishmaniasis and the implication of Leishmanin Skin Test (LST) response in disease prevalence.

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This thesis addresses some of the questions about effectiveness of candidate vaccines against leishmaniasis, the epidemiology of leishmaniasis infection and immune response to the antigen. This study is in fact the continuation of the work done by previous investigators to identify a safe, efficacious, inexpensive vaccine that could be easily produced in/by endemic countries.

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This study brings together the results of several leishmaniasis vaccine clinical trials over a number of years; from 1980's to 2000's. I wish to thank the investigators of all the trials that were used in this combined analysis for their valuable work towards a vaccine against leishmaniasis.

Dedicated to my parents for their love and support

SUMMARY

This study reports the detailed analysis of the data from ten different clinical trials of first generation prophylactic vaccines (FGV) against leishmaniasis. With the exception of one trial, clinical trials of leishmaniasis FGV's have failed to show efficacy. However, some trials have indicated reduced infection rates in the subset of participants whose leishmanin skin test (LST) had converted after vaccination. Additionally boys were observed to be protected more than girls by the vaccine in one trial.

Objectives

One objective of this study was to re-assess the effectiveness of FGV's in providing protection. This was done both, overall, with all vaccines and trials included and separately, in more homogeneous subsets of clinical trials. The justification for this re-assessment was the potential ability of the combined analysis to bypass the trial-specific limitations of individual studies and bring about the advantages of a larger sample size. Efficacy was also evaluated in different demographic groups identified by age, gender and endemic/non-endemic origin of participants. In addition to efficacy, immunological response (indicated by LST) to leishmanial antigen introduced by vaccination or naturally was evaluated in different demographic groups. LST reaction is an indication of delayed-type hypersensitivity (DTH) and has been used by investigators to assess exposure. Another objective of this study was to assess the merit of LST as a marker of infection and/or immunity in endemic and non-endemic populations.

Findings

Vaccine efficacy:

Vaccine efficacy was re-evaluated by meta-analysis. Results were consistent with the conclusion in most clinical trials that FGV's were not efficacious - *L. amazonensis* vaccines in South America were possibly the only exception. Restricting the analysis to more homogeneous subsets of trials (similar vaccine, same causative parasite, etc) did not change this overall conclusion. Furthermore, overall no evidence of protection associated with LST conversion after vaccination in the vaccine arm was observed. Additionally, different demographic groups were not different in their infection rates after vaccination.

Other factors associated with lower incidence:

In ALM+BCG (Autoclaved *L. major* + Bacille Calmette-Guerin) vaccine trials in Iran, it was observed that immunological reactivity (LST>0) 80 days after vaccination was associated with reduced incidence if LST measurement one year after study injection were LST≥5. This observation, was independent of the study treatment (i.e., no difference observed between the ALM+BCG and BCG alone arms). This could indicate a level of BCG related protection.

Reduced infection rate associated with study participation (again, regardless of treatment) was observed in ALM+BCG trials in all participants of endemic origin

with previous natural exposure (screening LST≥5). This confirms the protective effect of endemic exposure associated with LST conversion.

Under endemic conditions, trials conducted with non-endemic participants showed higher rates of infection than those with endemic participants; another observation suggesting the effect of endemic immunity.

Exposure and LST response:

By design, two of the trials used in this analysis enrolled volunteers with LST>0 at screening. This allowed studying natural exposure in demographic groups. Inhabitants of an endemic focus were observed to have a different LST profile (more frequent with smaller induration) from residents of a newly endemic focus.

LST as a marker of immunity:

LST measurement changes as a result of exposure to leishmanial antigen. However, LST does not provide a reliable marker of immunity independently of the information about factors that gave rise to it. For example, LST converts in a far greater percent of vaccine arm participants compared to BCG participants. However, this difference is not associated with better protection. Additionally, LST is subject to significant variability from measurement to measurement. For example, in Borkhar, 38% of participants with LST>0 at screening, had LST=0 eighty days after vaccination. This could constitute a source of misclassification of previous exposure. Also, LST reflects immune system stimulation due to different factors: natural exposure, vaccination and even BCG could affect the LST response. Therefore, unless the reasons for a reactive LST are known, LST cannot be used as a marker of immunity.

Thesis organization

This thesis is organized into 8 Chapters:

CHAPTER 1 - Background
CHAPTER 2 - Research objectives and methods
CHAPTER 3 - Efficacy of killed whole-parasite vaccines in prevention of leishmaniasis - a meta-analysis
CHAPTER 4 - Prophylactic efficacy of whole-parasite killed vaccines in demographic subgroups
CHAPTER 5 - Immunological response (measured by LST) in demographic subgroups to the leishmanial antigen introduced by vaccine or natural exposure
CHAPTER 6 - LST response as a correlate of immunity
CHAPTER 7 - Discussion
CHAPTER 8 - Conclusions and recommendations for further research

ZUSAMMENFASSUNG

Diese Studie berichtet über die detaillierte Datenanalyse von 10 verschiedenen klinischen Studien der prophylaktischen Impfstoffe der ersten Generation (Englisch: „first generation prophylactic vaccines“ FGV) gegen Leishmaniose. Mit Ausnahme von einem Versuch haben klinische Studien von Leishmaniose FGVs bisher keine Wirksamkeit gezeigt. Jedoch haben einige Versuche verringerte Infektionsraten in einer Untergruppe der Versuchsteilnehmer gezeigt, deren Leishmaniose Hauttest (Englisch: „leishmanin skin test“ LST) sich nach der Schutzimpfung umgewandelt hatte. Des Weiteren wurde in einer Studie beobachtet, dass Jungen besser von dem Impfstoff geschützt werden, als Mädchen.

Zielsetzung

Eine Zielsetzung dieser Studie war es, neu einzuschätzen, inwieweit FGV einen wirksamen Impfschutz herstellen können. Dieses wurde in zwei Gruppen untersucht: insgesamt mit allen Impfstoffen und allen Versuchen zusammen, und separat mit homogeneren Untergruppen klinischer Studien. Die Rechtfertigung für diese Studie war die Möglichkeit der kombinierten Analyse, die Versuchs-spezifischen Beschränkungen der einzelnen Studien überbrückt sowie die Vorteile einer größeren Stichprobe. Die Wirksamkeit wurde in den verschiedenen demographischen Gruppen ausgewertet, die durch Alter, Geschlecht und endemischen/nicht-endemischen Ursprung der Teilnehmer bestimmt wurden. Zusätzlich zur Wirksamkeit wurde die immunologische Reaktion zum Leishmaniosis Antigen (angezeigt durch LST), das durch Schutzimpfung oder natürlich eingeführt wurde, in den verschiedene demographischen Gruppen ausgewertet. Die LST-Reaktion ist ein Indiz für eine verzögerte Überempfindlichkeit (Englisch: „delayed-type hypersensitivity” DTH) und wird in Studien verwendet, um eine Exposition festzustellen.

Eine weitere Zielsetzung dieser Studie war, den Benefit von LST als Markierung der Infektion und/oder der Immunität in den endemischen und nicht-endemischen Gruppen zu evaluieren.

Ergebnisse

Wirksamkeit des Impfstoffes:

Die Wirksamkeit des Impfstoffes wurde durch eine Meta-Analyse neu bewertet. Das Ergebnis der Meta-Analyse deckt sich mit den Schlussfolgerungen der meisten klinischen Studien, die FGVs als nicht wirkungsvoll bewerten. Die wahrscheinlich einzige Ausnahme bilden die L. Amazonensis-Impfstoffe in Südamerika. Die Beschränkung der Analyse auf homogenere Untergruppen der Studien (ähnlicher Impfstoff, der gleiche kausale Parasit, usw.) erbrachte keine Änderung der Schlussfolgerung. Darüber hinaus wurde in der Impfgruppe beobachtet, dass die Umwandlung des LST-Tests nach Impfung nicht mit einem Impfschutz assoziiert ist. Zusätzlich unterschieden sich die verschiedenen demographischen Gruppen nicht in ihren Infektionsraten nach der Schutzimpfung.

Andere Faktoren, die mit geringer Inzidenz assoziiert sind:

Bei Versuchen mit dem ALM+BCG (Autoklavierten *L. major* + Bacille Calmette-Guerin)-Impfstoff im Iran wurde beobachtet, dass die immunologische Reaktion (LST> 0) 80 Tage nach der Impfung mit einer verringerten Inzidenz verbunden war, wenn der LST ein Jahr nach der Impfung LST≥5 war. Dieses Ergebnis war unabhängig von der Art der Impfung (d.h., kein Unterschied zwischen den ALM+BCG und nur-BCG Gruppen). Dieses könnte ein gewisses Mass eines BCG-bezogenen Schutzes anzeigen.

Die verringerte Infektionsrate, die mit Studienteilnahme verbunden ist (wiederum unabhängig von der Behandlung) wurde in den ALM+BCG- Versuchen in allen Teilnehmern der endemischen Gruppe mit vorangegangener natürlicher Exposition beobachtet (LST≥5). Dieses bestätigt den schützenden Effekt der endemischen Exposition verbunden mit LST-Umwandlung.

Unter endemischen Bedingungen zeigten die Versuche, die mit nicht-endemischen Teilnehmern durchgeführt wurden eine höhere Infektionsrate, als die mit endemischen Teilnehmern; eine weitere Beobachtung, die auf den Effekt der endemischen Immunität hinweist.

Exposition und LST-Antwort:

Zwei der Studien, die in dieser Analyse untersucht wurden, hatten Freiwillige mit einem LST>0 eingeschlossen. Dieses ermöglichte die Untersuchung natürlicher Exposition in den demographischen Gruppen. Es wurde beobachtet, dass Bewohner einer endemischen Region ein anderes LST-Profil aufwiesen (häufiger mit geringerer Verhärtung), als Bewohner eines neu entstandenen endemischen Gebietes.

LST als Immunitäts-Marker:

Die Messung von LST ändert sich als Resultat von einer Belastung durch das Leishmaniose-Antigen. Jedoch liefert LST keine zuverlässige Markierung der Immunität unabhängig der Informationen über Faktoren, die sie verursachten. Zum Beispiel wandelt LST sich zu einem weit größeren Prozentsatz in den Impf-Gruppen um, als BCG-Gruppen. Jedoch ist dieser Unterschied nicht mit besserem Schutz verbunden. Darüber hinaus ist der LST-Test sehr variabel: Zum Beispiel hatten in Borkhar 38% der Teilnehmern mit einem LST>0 bei den Vortests, einen LST von 0 achtzig Tage nach der Impfung. Dieses könnte eine Quelle für Fehlklassifizierung der vorhergehenden Expositiondarstellen. Ebenso spiegelt der LST die Immunsystemanregung aufgrund verschiedener Faktoren wider: natürliche Exposition, Impfung, und sogar BCG konnten die LST-Antwort beeinflussen. Folglich eignet sich der LST nicht als Immunitäts-Marker; es sei denn, die Gründe für einen reagierenden LST sind hinreichend bekannt.

Gliederung

Diese Arbeit ist in acht Kapitel gegliedert:

KAPITEL 1 - Hintergrund

KAPITEL 2 - Forschungszielsetzungen und -methoden
KAPITEL 3 – Die Wirksamkeit von abgetöteten Voll-Parasit-Impfstoffen in der Prävention der Leishmaniose - eine Meta-Analyse
KAPITEL 4 – Die prophylaktische Wirksamkeit des Voll-Parasit-Impfstoffes in den demographischen Untergruppen
KAPITEL 5 – Immun-Reaktion (gemessen durch LST) in den demographischen Untergruppen zum Leishmaniose-Antigen durch Impfstoffe oder natürliche Exposition
KAPITEL 6 - LST-Antwort als Korrelat der Immunität
KAPITEL 7 - Diskussion
KAPITEL 8 - Zusammenfassungen und Empfehlungen für weitere Forschung

ABBREVIATIONS

ACL	1. Anthroponotic cutaneous leishmaniasis 2. American cutaneous leishmaniasis (by various species)
ALM	Autoclaved *Leishmania major*
Alum-ALM	ALM precipitated in aluminium hydroxide (a more immunogenic, formulation of ALM)
AVL	Anthroponotic visceral leishmaniasis
BCG	Bacille Calmette-Guerin, at 1/10 of the normal dose used as an adjuvant in ALM+BCG *Leishmania* vaccine
CL	Cutaneous leishmaniasis
DCL	Diffuse cutaneous leishmaniasis
DTH	Delayed type hypersensitivity
Efficacy	The percentage reduction in the incidence of leishmaniasis in vaccinated individuals compared to the control group
End point	The outcome of interest, could be determined by change in status (appearance of a lesion), time to the change of status (time to appearance of the lesion), or the severity of symptoms (e.g., size or number of lesions).
FGV	First generation vaccines including ALM+BCG, alum-ALM+BCG and other vaccine candidates
GCP	Good clinical practice (international guidelines for clinical trials)
GLP	Good laboratory practice (international guidelines for laboratory production)
GMP	Good manufacturing practice (international guidelines for manufacturing pharmaceuticals)
IFN-γ	Interferon γ
IL-2, IL-4, etc	Interleukin 2, interleukin 4, etc. cytokines secreted by cells of the immune system
LR	Leishmaniasis recidivans
LST	Leishmanin Skin Test used to evaluate immune response to leishmanial antigens. This procedure includes intradermal inoculation of 0.1 mL of leishmanin (containing 5-20 µg of *Leishmania* protein from killed whole promastigotes), the result will be evaluated by measuring induration induced at the site of inoculation in 48-72 hours. Normally (but arbitrarily) an induration of 5 mm or larger is considered as a positive response.
LST conversion	Changes from negative LST to positive
LZ	Leishmanization. Using live *Leishmania major* to cause artificial cutaneous leishmaniasis. This method is proposed to be used as live challenge in efficacy trials of candidate vaccines for

	leishmaniasis.
MCL	Mucosal or mucocutaneous leishmaniasis
MST	Montenegro skin test (alternate designation for LST, frequently used by Latin American investigators)
PKDL	Post kala azar dermal leishmaniasis
TFN-α	Tumour necrosis factor-α
VL	Visceral leishmaniasis (kala azar)
ZCL	Zoonotic cutaneous leishmaniasis
ZVL	Zoonotic visceral leishmaniasis

CHAPTER 1

BACKGROUND[1]

1.1 Etiology of leishmaniasis

Leishmaniasis is a vector-borne disease caused by several species of protozoan parasites of the genus *Leishmania* (table 1.1). Genus *Leishmania* includes the two subgenus *Leishmania* (in the Old World) and *Viannia* (in the New World). Leishmanial infection has diverse clinical manifestations, including cutaneous (CL), mucocutaneous (MCL), diffuse cutaneous (DCL), visceral (VL or kala-azar), post kala-azar dermal leishmaniasis (PKDL) and recidivans (LR) (WHO Expert Committee on the Control of the Leishmaniases, 1990).

Table 1.1 - Main species of *leishmania* with pathogenicity in human
(SOURCE: http://www.bio.tuebingen.mpg.de/membio/staff/thilg.html Citation: April 2006)

Kingdom	Protista						
Subkingdom	Protozoa						
Phylum	Sarcomastigophora						
Subphylum	Mastigophora						
Class	Zoomastigophora						
Order	Kinetoplastida						
Suborder	Trypanosomatina						
Family	Trypanosomatidae						
Genus	*Leishmania*						
Section	Peripylaria		Suprapylaria				
	L. braziliensis complex: *L. braziliensis* *L. panamensis* *L. guyanensis*	*L. peruviana*	*L. donovani* complex: *L. donovani* *L. infantum* *L. chagasi*	*L. major*	*L. tropica*	*L. aethiopica*	*L. mexicana* complex: *L. mexicana* *L. amazonensis* *L. pifanoi* *L. garnhami* *L. venezuelensis*
Host	mammals, including humans						
Disease pattern in humans	CL (Pian Bois), MCL (Espundia)	CL (Uta)	VL (Kala Azar), CL, PKDL	CL (oriental sore)	CL (orien-tal sore), VL	CL, DCL	CL (Chiclero's ulcer), DCL

[1] This chapter has been in part published in the Vaccine (see Annex)

1.2 Transmission:

Leishmaniasis is transmitted via the bite of the female phlebotomine sandfly. There are 30 proven species of sandflies of the genus *Papatasi* or *Lutzumyia* acting as the vector in the transmission of the disease. Table 1.2 depicts some of the main pathogenic species. Leishmaniasis has both, zoonotic (reservoir in animals) and anthroponotic (reservoir in humans) forms. Many mammalian species constitute the reservoir and/or the host for the disease including dogs, rodents and humans.

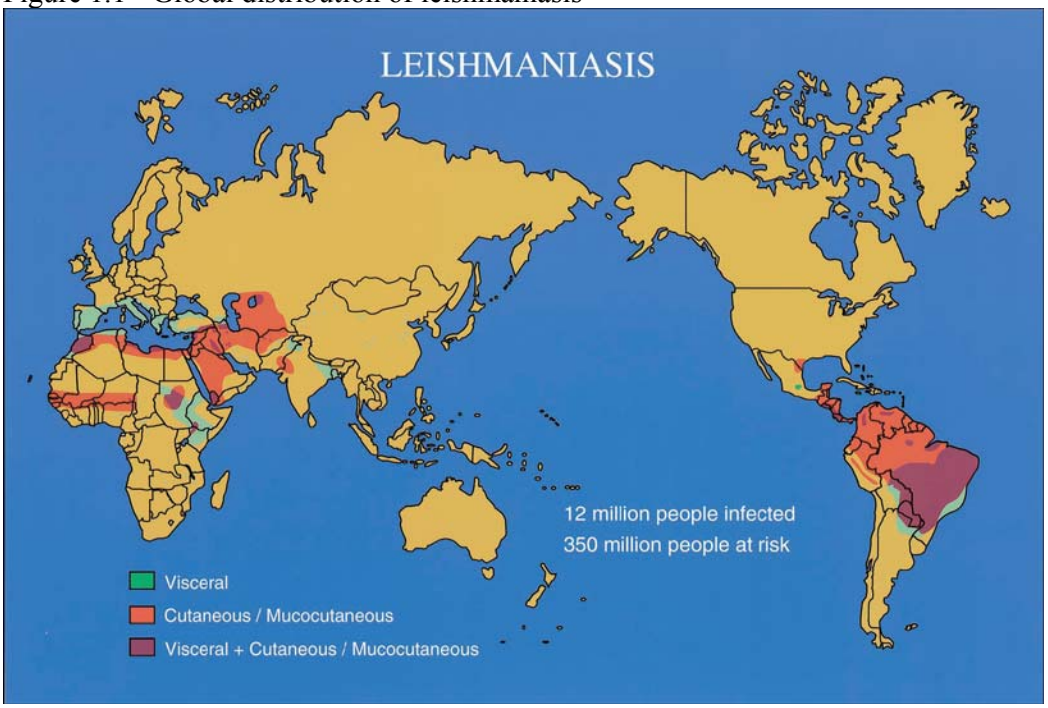
Table 1.2 - Proven and suspected sandfly vectors in the Old World

Parasite	**Clinical association**	**Geographical distributions**	**Proven or suspected vectors**
L. donovani	AVL; PKDL	China	*P. alexandri*
		Indian subcontinent	*P. argentipes*
		East Africa	*P. martini*
		East Africa	*P. orientalis*
L. infantum	ZVL; ZCL	Southern Europe	*P. ariasi*
		Southern Europe; Eastern Mediterranean	*P. perniciosus*
		Eastern Mediterranean	*P. langeroni*
		China	*P. chinensis*
		China; Eastern Medit	*P. major*
L. major	ZCL	Africa, Middle East, South-west Asia	*P. papatasi*
		Africa	*P. dubosqi*
L. tropica	ACL; LR	Africa, Middle East, South-west Asia	*P. sergenti*
		Kenya	*P. saevus*
L. aethiopica	CL; MCL; DCL	East Africa	*P. longipes*
		East Africa	*P. pedifer*

a. AVL, anthroponotic visceral leishmaniasis; PKDL, post kala-azar dermal leishmaniasis; ZVL, zoonotic visceral leishmaniasis; ZCL, zoonotic cutaneous leishmaniasis; ACL, anthroponotic cutaneous leishmaniasis; LR, leishmaniasis recidivans; MCL, mucocutaneous leishmaniasis; DCL, diffuse cutaneous leishmaniasis.

1.3 Geographical distribution and global burden:

Figure 1.1 - Global distribution of leishmaniasis



Source: (Handman, 2001)

Leishmaniasis is a public health problem in 88 countries (figure 1.1), including 72 developing countries, with 80% of their population earning incomes less than $2 daily)(Davies et al., 2003;WHO, 2006). Most cases of leishmaniasis occur in Asia, Africa and Latin America. The following statistics are provided by the World Health Organization (WHO, 2006):

- 90% of all visceral leishmaniasis cases occur in Bangladesh, Brazil, India, Nepal and Sudan;
- 90% of mucocutaneous leishmaniasis occurs in Bolivia, Brazil and Peru;
- 90% of cutaneous leishmaniasis cases occur in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria

Its burden is higher among the poor, with anaemia and malnutrition being among the major risk factors for death (Collin et al., 2004). The estimated global prevalence of all forms of the disease is 12 million, with 1.5 to 2 million added cases annually of CL (with average duration of few months to one year) and 500,000 of VL (with average duration of several months to more than one year) (WHO, 2002;WHO, 2006). Due to increases in urbanization and other risk factors, different forms of leishmaniasis have wider presence and more severe prevalence world wide than ever before (WHO, 2002). Recent epidemics have caused hundreds of thousands of deaths and immeasurable morbidity and economic consequences. In 1977 in Bihar, India there were 100,000 cases of kala-azar with the death rate in Vaishali district of 28.7% of affected cases (Thakur, 1984). In Sudan (Western Upper Nile) in 1993, 100,000

people died over a period of 5 years from an area with less than one million population (Desjeux, 1996). It took five years to control the epidemic.

The transmission cycle for natural leishmanial infection requires the presence of the mammalian reservoir and the sandfly vector. All factors contributing to the spread of the reservoir and vector also contributes to the spread of leishmaniasis. The epidemiology of leishmaniasis infection is affected by naive human populations migration to endemic areas. Examples include outbreaks of cutaneous disease caused by *Leishmania tropica* in Afghan refugees, visceral disease in Sudanese refugees, and cutaneous disease caused by *L. major* in American forces in Iraq (Berman, 2005;Weina et al., 2004). Additionally, human interventions in previously non-endemic areas (e.g., construction of dams) could trigger the outbreak of leishmaniasis in the non-immune local population (Neouimine, 1996). New foci could also be created by introduction of the parasite into a susceptible and previously non-endemic area and could lead to outbreak in the non-immune residents (Neouimine, 1996).

There are important interactions between *Leishmania* and HIV infections. HIV infection in leishmaniasis endemic areas has lead to a higher incidence of leishmaniasis (Puig and Pradinaud, 2003). Compared to immune competent persons, AIDS patients in endemic VL foci have 100-1000 times the risk of developing clinical VL. Also, VL infection accelerates the development of HIV into AIDS (Desjeux and Alvar, 2003). Another factor contributing to the higher incidence of leishmaniasis in HIV endemic areas is the higher rate of sandfly infection after feeding on the blood of immunodeficient persons. Infectivity seems to be proportional to the severity of infected immunodeficiency (Molina et al., 2003). In addition, in HIV positive individuals, normally non-pathogenic strains of *leishmania* and even lower trypanosomatids could cause infection (Chicharro and Alvar, 2003;Molina et al., 2003) .

1.4 Clinical forms

Despite their similarities, pathogenic species of *Leishmania* cause different forms of the disease (CL, MCL, DFL, VL, PKDL - table 1.3). Furthermore, possibly due to the interactions between the vector, the parasite, the host and the environment, basic clinical manifestations within the different forms of the disease vary by endemic region (Berman, 2005;WHO Expert Committee on the Control of the Leishmaniases, 1990).

1.4.1 Cutaneous Leishmaniasis (CL)

Infection with any of several species can produce cutaneous leishmaniasis. According to the causative parasite species, CL can be classified into Old World and New World CL. The former is primarily due to *L. major* (known as rural or zoonotic CL -ZCL*)*, *L. tropica* (urban or anthroponotic - ACL), and *L. (L) aethiopica* but also due to *L. infantum* and *L. donovani*. New world CL is caused by *L. mexicana*, *L. (L) amazonenesis*, *L. braziliensis*, *L. (V) panamensis*, *L. (V) peruviana*, and *L. (V) guyanensis* and also *L. chagasi* (Murray et al., 2005;WHO Expert Committee on the Control of the Leishmaniases, 1990).

CL due to *L. major* tends to heal spontaneously and without systemic complication or dissemination to other sites. Individuals with history of CL are usually protected against future infection (Khamesipour et al., 2005;WHO, 2002;WHO/TDR, 2002). CL due to other species may lead to other complications (such as mucosal involvement in *L. tropica* or *L. aethiopica* infection) (WHO Expert Committee on the Control of the Leishmaniases, 1990;Yaghoobi and Hoghooghi-Rad, 2001). In infection with *L. major* and *L. tropica*, an erythematous papule at the site of the sandfly bite is normally the initial stage of the lesion later developing into a painless nodule which typically ulcerates in 1-3 months(Dowlati, 1996;Murray et al., 2005). The lesion duration in ZCL cases is usually less than a year and in ACL (*L. tropica*) up to 2 years. Cases with lesion duration longer than one year in ZCL or 2 years in ACL are considered chronic (Dowlati, 1996;Murray et al., 2005). Compared to CL due to *L. major*, infections due to *L. tropica* and *L. aethiopica* takes longer to heal(Dowlati, 1996;WHO Expert Committee on the Control of the Leishmaniases, 1990). Leishmaniasis recidivans (LR) is the rare, lupoid chronic form caused by the recurrence of *L. Tropica* up to 40 years after the initial infection (Marovich et al., 2001). Although the localized form is far more common, American CL caused by the New World species could range from a single, self healing lesion to multiple, slowly progressive nodules characteristic of diffuse CL(Barral et al., 1995;Machado et al., 2002).

HIV-associated cutaneous leishmaniasis has been relatively infrequent but this will probably change in the future (Murray et al., 2005). HIV+ cases infected with CL seem to experience more frequent recurrences and longer treatment periods (Couppie et al., 2004). The success of antiretroviral drug distribution in countries such as Brazil has had a positive impact on the incidence of leishmaniasis among HIV patients (Rabello et al., 2003). American CL is also associated with the destructive MCL form.

1.4.2 Mucocutaneous (Mucosal) Leishmaniasis (MCL):

MCL is a potentially life threatening, degenerative inflammatory form of leishmaniasis of the nasal and oral mucous membranes, extending to the pharynx. The appearance of the disease resembles leprosy and it is associated with the same stigma. Diagnosis and treatment is difficult (Evans, 1993). In the Old World MCL is rarely seen and is usually due to *L. tropica* and *L. aethiopica* (Kharfi et al., 2003;WHO Expert Committee on the Control of the Leishmaniases, 1990). However, cases associated with other species have also been reported (el-Hassan and Zijlstra, 2001;Guddo et al., 2005;Yaghoobi and Hoghooghi-Rad, 2001). In Sudan, MCL is rarely observed as an aftermath of VL (unlike PKDL)(el-Hassan and Zijlstra, 2001).

MCL in the New World is primarily due to *L. braziliensis*, *L. panamensis* and *L. guyanensis* (Weigle and Saravia, 1996;WHO Expert Committee on the Control of the Leishmaniases, 1990). About 1-10% of CL patients in the New World develop mucosal manifestation within 1-5 years of healing (Murray et al., 2005). CL in the New World is associated with the risk of developing mucosal infection if the treatment is delayed due to the causing parasite genus not being rapidly identified (Peyron-Raison et al., 1996).

Mucosal leishmaniasis begins with erythema and ulcerations at the nares, proceeding to nasal septum perforation and destructive inflammatory lesions. The latter can obstruct the pharynx or larynx and produce remarkable disfigurement (Murray et al., 2005).

1.4.3 Diffuse Cutaneous Leishmaniasis (DCL)

DCL is a rare form of the disease caused by various species. In the Old World the primary causative agent is *L. aethiopica*.and in the New World the *L. mexicana* complex and specifically *L. amazonensis* (Silveira et al., 2005;WHO Expert Committee on the Control of the Leishmaniases, 1990). In DCL patients, the absence of delayed type hypersensitivity (DTH) is associated with widespread plaques, papules or multiple nodules especially on the face or the limbs and could resemble leprosy. DTH normally is observed after cure. The disease does not heal spontaneously and tends to recur (Silveira et al., 2005;WHO Expert Committee on the Control of the Leishmaniases, 1990).

1.4.4 Visceral Leishmaniasis (VL)/Kala Azar:

VL is primarily caused by members of the *L. donovani* complex (*L. donovan*i in Sudan and India, *L. infantum* in other parts of the Old World and *L. chagasi* in the New World). Some cases of VL in humans and dogs are due to *L. tropica* (Alborzi et al., 2006;Lemrani et al., 2002;Mohebali et al., 2005;Sacks et al., 1995). Because of its associated complications and severity, VL has more significant public health consequences than other forms of leishmaniasis. Children are especially susceptible (Murray et al., 2005;WHO Expert Committee on the Control of the Leishmaniases, 1990). The spleen, the liver, the mucosa of the small intestine, the bone marrow, lymph nodes and other lymphoid tissues are heavily parasitized (WHO Expert Committee on the Control of the Leishmaniases, 1990). Symptoms include fever, weight loss, splenomegaly, hepatomegaly, lymphadenopathy, cachexia, nausea and it is typically fatal if not appropriately treated (Seaman et al., 1996;Zijlstra and el-Hassan, 2001b).

1.4.5 Post Kala Azar Dermal Leishmaniasis (PKDL):

PKDL is the dermal complication developed in VL patients after clinical cure and is characterized by a macular, maculopapular, and nodular rash which normally starts around the mouth. The rash is typically the only complication in PKDL. Chronic PKDL is the source of considerable morbidity. PKDL is mainly seen in Sudan and India (about 50% of the Sudanese and 5-10% of Indian VL patients) and is restricted to infection with *L. donovani* (Musa et al., 2002;Zijlstra et al., 2003;Zijlstra and el-Hassan, 2001a). PKDL follows within 6 months of VL cure in Sudan and 2 years or longer in India. While the Indian PKDL needs treatment, the Sudanese form tends to self heal within 1 year post onset. Chronic cases, however, need treatment and are hard to cure (Musa et al., 2002;Zijlstra and el-Hassan, 2001a).

PKDL is believed to play a role in the transmission of kala azar by providing the human reservoir in the skin of the PKDL patient despite recovery from the symptoms of the visceral disease (Zijlstra et al., 2003).

Table 1.3 - Clinical manifestations of leishmanial infection

Characteristic:	Disease Form			
	Mucocutaneous	**Cutaneous**	**Diffuse**	**Visceral**
Lesions	Ulcerative destruction of the nasal septum	Single, occasionally a small number, ulcerated lesions with elevated borders and necrotic centre	Non-ulcerated nodules spread through different areas of the body	Internal Organs
Histopathology and parasite numbers	Granulomatous reactions with very few parasites	Chronic inflammatory responses with moderate number of parasites	Monotonous macrophagic infiltration with abundant parasites	Marked macrophagic proliferation with heavy parasitism in the haematopoietic organs
Anti-*Leishmania* antibody levels	Low	Low	Moderate to high	high
Anti-*Leishmania* CMI (in vitro and in vivo tests)	Strongly positive	Positive	Negative	Negative

1.5 Current control methods

Given the significance and the public health burden, effective control of leishmaniasis is an important item on the public health agenda of many endemic countries. Due to the complexity and diversity of the epidemiology, transmission and pathology of leishmaniasis, no single diagnosis, treatment or control approach could offer the complete solution. HIV co-infection is making the situation more complex by changing the epidemiology of the disease and presenting new problems in diagnosis and case management (Sinha et al., 2005). Moreover, control methods, even when potentially viable, are seldom used (Guerin et al., 2002). Overall, current methods of control and case treatment have been unable to provide a solution in areas of high burden (Ahluwalia et al., 2003;Dantas-Torres and Brandao-Filho, 2006;WHO/TDR, 2004).

Currently, three general categories of control methods can be identified: vector control, reservoir control and case identification/therapy. Of these methods, vector control through insecticides is considered as the most effective (de Oliveira and de Araujo, 2003;Dye, 1996;Thakur and Kumar, 1992). However, sustainable vector and reservoir control methods require infrastructure resources beyond the means of most endemic countries. Dispersed communities, hard to reach areas, vast desert areas,

difficulties of identifying and treating/eliminating infected dogs or wild animals in forests or deserts and existence of human reservoirs in anthroponotic infection are among many challenges in the way of such control methods. Sustainability of vector and reservoir control efforts is an essential requirement for their success. In developing country settings, the enormity of the problem and the cost and labour associated with such measures makes them difficult to implement (Dantas-Torres and Brandao-Filho, 2006). As an example, the eradication efforts and disinfection in Bukhara region next to Kisil-Kum desert (Uzbekistan) over a 15 year period did not lead to a significant reduction in rates of infection. The vast desert with high prevalence of infection among rodents does not allow such measures to be effective (Gafurov, 1999). Once such efforts are discontinued due to financial, logistic or health concerns, re-emergence of vector colonies would be the likely outcome. To cite an example, it is possible that after the DDT spraying in India was stopped (1958-1970), newly emerging sandfly colonies used PKDL cases as the reservoir to start the epidemic in Bihar in early 1970's (Thakur and Kumar, 1992).

Among other factors contributing to the problem is that vector and reservoir control measures are developed under laboratory conditions that are different from natural settings in a variety of ways. For example sandfly reaction to fabrics treated with repellents and insecticides under laboratory conditions could be different from that in the wild. One reason for such a difference could be that infected sand flies have a more aggressive biting behaviour and probe more frequently than uninfected sand flies. Such factors could compromise the efficacy of impregnated uniforms (Asilian et al., 2003;Croft et al., 2006a). Additionally, the use of chemically treated fabrics could cause skin irritation and absorption into the systemic circulation (Asilian et al., 2003;Schreck et al., 1984;Schreck et al., 1986).

For the past 60 years, the first line treatment for leishmaniasis has consisted of long courses of pentavalent antimonials. Despite their toxicity, these drugs constitute the most practical and economically feasible chemotherapy currently available. Unfortunately, resistance to antimonials is becoming a major and growing problem. It is estimated that 60% of VL patients in Bihar, India do not respond to antimonials due to drug resistance (Bryceson, 2001;Croft et al., 2006b;Dube et al., 2005). Resistance to antimonial drugs has also been observed in VL caused by *L. donovani* in the Sudan (Khalil et al., 1998) and in ACL caused by *L. tropica* in Iran (Hadighi et al., 2006), which may be pointing at the possibility of a similar problem in Afghanistan which is the world's largest focus of CL (Reithinger and Coleman, 2007). Other, recently discovered drugs such as amphotericin B, miltefosine, paromomycin, are expensive and unavailable to most patients in developing countries (Croft and Coombs, 2003).

Diagnostic tests constitute another aspect of case management. Unfortunately, there are shortcomings in the sensitivity and specificity of currently available tests. In addition, they are not available to all patients in rural areas (WHO/TDR, 2004).

For many infectious diseases, reducing susceptibility through vaccination is a highly effective method of control (Dye, 1996;Guerin et al., 2002). However, vaccination is not currently an option for leishmaniasis control since a prophylactic vaccine against leishmaniasis is yet to be developed (Khamesipour et al., 2006;Modabber and Reed, 2004).

Overall, current control methods present significant challenges including toxicity, cost, practicality, and sustainability. Any one of these obstacles in developing country settings could render these measures impractical. These serious challenges and the growing prevalence of drug resistant organisms underscore the need for an effective prophylactic vaccine reagent as a viable alternative strategy for control (Handman, 2001;Khamesipour et al., 2006;Webb et al., 1998).

1.6 Immunology

Studying host response to the parasite has been facilitated by using animal models, particularly inbred strains of mice. After inoculation with *L. major*, leishmaniasis-prone BALB/c mice develop uncontrolled, extensive lesions which eventually cause death. Other strains, such as C57BL/6, CBA/J, C3H and B10D2, on the other hand, develop a small self healing lesion and an effective immune response shortly after infection (Campos-Neto, 2005;Handman et al., 1979;Webb et al., 1998). Other strains of mice have different levels of susceptibility to various species of *Leishmania*. These differences are attributable to the differences in immunological response to the infection by each strain. Since *Leishmania* is an intracellular protozoa, the relevant immune response is T cell-mediated. The susceptibility in BALB/c mice is linked to the predominant development of Th2 subset of CD4+ cells which leads to an antibody oriented response. In the resistant mice, on the other hand, response is the development of Th1 subset of CD4+ cells(Campos-Neto, 2005;Handman et al., 1979;Webb et al., 1998). Stripping otherwise resistant mice of their T cells makes them susceptible to infection, while injection of these mice with T cells from recovered mice restores resistance (Mitchell et al., 1980).

In humans, most of the evidence suggesting the association of Th1 with cure and immunity is applicable to CL. In contrast, in chronic CL or ML cases, a combination of Th1 and Th2 response is observed. In VL, a different pattern of cytokine profile occurs (Modabber and Reed, 2004).

Cell-mediated immunity is facilitated by different proteins, known as cytokines, that are secreted by T lymphocytes, especially CD4+ T cells, after activation by antigens displayed on the MHC (major histocompatibility complex) of APC's (antigen presenting cells). In innate immune response cytokine production is mainly done by macrophages, while in adaptive immunity, they are mainly secreted by T cells.

Activated CD4+ T cells, start the production of cytokines and at the same time become able to respond and bind to cytokines. Interleukin-2 (IL-2) is among the first cytokines to be produced by CD4+ T cells within 1-2 hours after activation. IL-2 plays an important role in clonal expansion of both CD4+ and CD8+ T cells, within one or two days after activation. Both CD4+ and CD8+ cells differentiate into effector cells and memory cells. CD4+ T cells can differentiate into subsets of effector cells known as Th1 and Th2 cells (type 1 and type 2 helper T cells). Th1 cells stimulate phagocyte-dependant immunity, which is the beneficial response in the context of intracellular infection. Th2 cells, on the other and, stimulate phagocyte-independent, eosinophil- and IgE-dependent immunity which can exacerbate the disease. Differentiation of naive helper T cells into Th1 rather than Th2 is believed to be facilitated by IL-12 produced by parasite-activated macrophages and dendritic cells.

In the absence of IL-12, the T cells themselves (and perhaps other cells) produce IL-4 which stimulates differentiation into the Th2 subset and IgE production. Therefore, IL-12 is an essential pretext of Th1 response(Abbas and Lichtman, 2004).

Th1 and Th2 subsets of CD4+ T cells secrete different cytokines which lead to their distinct actions (table 1.4).

Table 1.4 - Cytokines in relation to Th1 and Th2 subsets of CD4+ cells

Cytokine	Th1	Th2
IL-2	++	-
IFN-γ	++	-
TNF-β	++	-
G-CSF	++	+
TNF-α	++	++
IL-3	++	++
IL-4	-	++
IL-5	-	++
IL-6	-	++
IL-10	-	++

IFN-γ: Interferon-γ, IL: Interleukin, GM-CSF: granulocyte macrophage-colony stimulating factor, TNF: tumor necrosis factor α or β.
Source: (Cabrera, 1994)

These cytokines are mutually inhibitory. For example, IL-10 and IL-4 down regulate Th1 differentiation, while IFN-γ down regulates Th2 expansion. IL-10 inhibits IFN-γ and promotes the persistence of the parasites in skin lesion. IL-4 also inhibits INF-γ production and its disruption enables BALB/c mice to resist infection (Belkaid et al., 2001;Cabrera, 1994;Campos-Neto, 2005;Kopf et al., 1996;Mosmann and Moore, 1991)

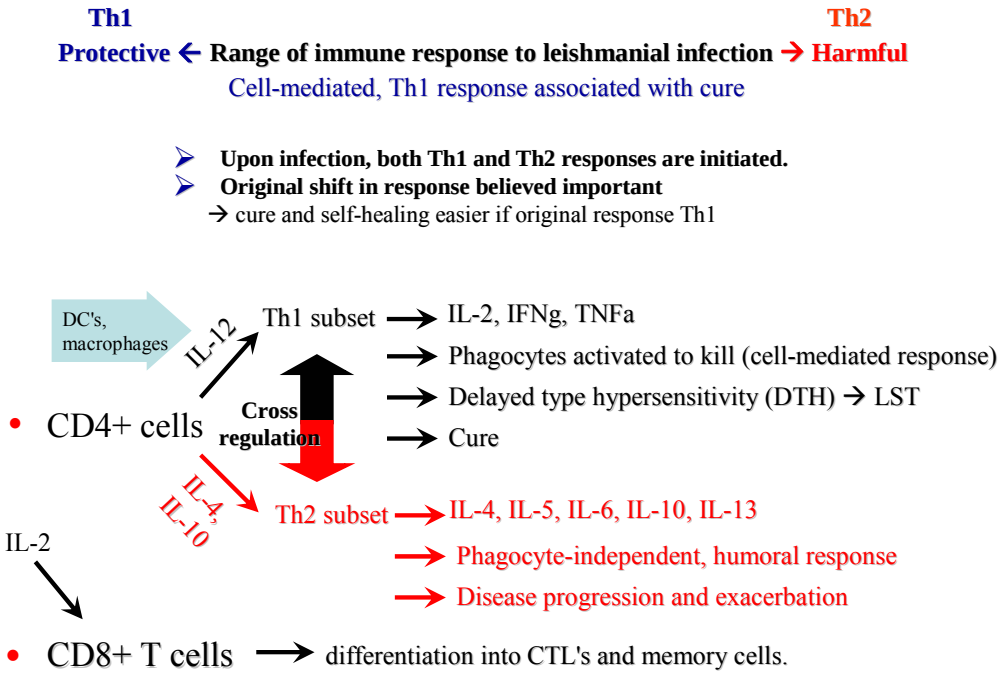
The most important cytokine produced by Th1 cells is IFN-γ (interferon-γ), a potent activator of macrophages. Macrophage activation for effective killing of ingested parasites is the differentiating aspect of cell mediated response to intracellular infection and is conducted in a nitric oxide-dependent manner (Abbas and Lichtman, 2004;Vanloubbeeck and Jones, 2004b)**.** It should be noted that early supply of IFN-γ is mainly by NK (natural killer) cells (Cabrera, 1994). It is important to emphasize that immunity is not associated with an exclusive Th1 differentiation, but rather the predominance of a Th1 response (Erb et al., 1996;Vanloubbeeck and Jones, 2004a;Vanloubbeeck and Jones, 2004b). Factors that drive CD4+ differentiation into the Th1 subset relate host genetics, parasite factors and the role of the vector and include(Cabrera, 1994;Vanloubbeeck and Jones, 2004a;Vanloubbeeck and Jones, 2004b):

1. Type of Antigen presenting cell (APC)
2. Endogenous cytokine levels
3. Nature of the antigen recognized

In addition to CD4+ and their differentiation into Th1, activation of CD8+ cells has been shown to play an important role in protection, both after primary infection and during a memory response (Vanloubbeeck and Jones 2004).

Both CD4+ and CD8+ stimulate macrophage killing. CD8+ cells play another, more direct role in parasite eradication. Naive CD8+ cells differentiate into CTL's (cytolitic T lymphocytes) that kill infected host phagocytes, thus eliminating the parasite. Both CD4+ and CD8+ recognize, and are stimulated by, the MHCs (major histocomatibility complexes) displayed by antigen presenting cells (APCs) such as macrophages and dendritic cells. Peptides associated with class I MHC molecules (from parasites that escape into the cytoplasm from the vesicle formed inside the macrophage) are recognized by CD8+ T cells while peptides associated with class II MHC molecules (obtained within the vesicles) are recognized by CD4+ T cells. Expansion of CD8+ cells is also dependent on help from CD4+ cells through IL-2 action (Abbas and Lichtman, 2004;Vanloubbeeck and Jones, 2004a;Vanloubbeeck and Jones, 2004b). Immunology of Leishmaniasis is summarized in figure 1.2.

Figure 1.2 - Immunology of leishmaniasis



As mentioned earlier, in humans, the direct and clear association between immunity or cure and Th1 response applies to CL where predominant Th1 response and elevated levels of IFN-γ producing cells has been observed in healing lesions. In contrast, in chronic CL or ML cases, a combination of Th1 and Th2 response coupled with abundant IL-4 is observed. In VL, on the other hand, both IFN-γ and IL-4 are

raised during the disease, but they decline after the disease (Modabber and Reed, 2004). Table 1.5 demonstrates the immunology in different forms of leishmaniasis.

Table 1.5 - Main Immunological features of leishmanial infection in man

Species	disease	Primary Immune response DTH	Antibody	Parasite in Lesion	Self cure
L. tropica	CL	present	variable	present	yes
	LR	strong	variable	scanty	no
L. major	CL	present	present	present	rapid
L. aethiopica	CL	weak	variable	present	slow
	DCL	absent	variable	abundant	no
L. donovani	VL	absent	abundant	abundant	rare
	PKDL	variable	variable	variable	variable
	CL	present	variable	present	yes
L. braziliensis	CL	present	present	present	yes
	MCL	present	present	scanty	no
L. mexicana	CL	present	variable	present	yes
	DCL	absent	variable	abundant	no

Source: (WHO Expert Committee on the Control of the Leishmaniases, 1990)

1.6.1 Delayed-type hypersensitivity and skin test for leishmaniasis

Cell mediated immune response includes recruitment of T cells that recognize the specific antigen into the infection site and activation of macrophages that have phagocytosed and display that specific antigen. This type of response involves production of substances that are toxic to microbes and, if released into the extracellular space, to normal tissues. The resulting tissue injury is known as hypersensitivity. The antigen-specific nature of this process leads to a similar response every time the immune system encounters the same antigen (Abbas and Lichtman, 2004).

The same reaction could be induced by injecting the specific antigen into the skin of a previously exposed (infected or immunized) individual. This reaction is called delayed-type hypersensitivity (DTH). The term "delayed" signifies the length of time (24 to 48 hours after challenge with the antigen) for circulating effector T cells to accumulate at the site (Abbas and Lichtman, 2004). DTH is used as a diagnostic test in different diseases, e.g., Tuberculosis (Purified Protein Derivative - PPD) and leishmaniasis (Leishmanin Skin Test - LST). LST is widely used as a diagnostic tool for leishmaniasis (Handman, 2001;Khalil et al., 2000a;Khamesipour et al., 2006;Reed, 1996)).

1.6.2 Leishmanin skin test (LST) and its application in vaccine clinical trials

The widely used diagnostic test for present or past infection with *Leishmania* species is the Leishmanin Skin Test (LST), know as Montenegro Skin Test (MST) in the New World. LST is essentially an indicator of DTH due to exposure to leishmanial antigens and is used in detecting CL, MCL and asymptomatic VL. However, it is negative in acute VL patients(Reed, 1996;Saran et al., 1991). LST has been

classically used in epidemiological studies for detection of exposure to Leishmanial antigen (Reed, 1996) and is frequently used in vaccine clinical trials to assess immune response to the vaccine.

LST consists of intradermal injection of 0.1 ml of leishmanin. Leishmanin is produced slightly differently by different laboratories (e.g., 6 x 10^6 promastigotes/ml of Phosphate Buffered Saline (PBS) containing 0.01% of thimerosal, as produced by Pasteur Institute, Iran: http://www.pasteur.ac.ir/researchDepartment/Immunology/new_page_1.htm). Results are measured in 48 to 72 hours. The emergence of a skin reaction to the agent is indicative of delayed-type hypersensitivity (DTH) due to previous exposure to the antigen. By convention, an induration ≥ 5 millimetres (measured by the ballpoint pen method (Sokal, 1975) is considered to be a positive result, while a smaller induration is considered as negative. Skin test conversion signifies an increase to ≥ 5 (or another pre-determined value) due to infection or vaccination.

A 98% sensitivity has been reported for LST in CL patients after 6 weeks from the onset of lesion (Manzur and ul Bari, 2006). However, it is important to note that different preparations of leishmanin, and even leishmanin produced by different laboratories, have different sensitivity (Akuffo et al., 1995;Solano-Gallego et al., 2001).

LST with leishmanin prepared from *L. major* promastigotes seems to be sensitive in detecting exposure even when the cause of the infection is a species other than *L. major* (Ben Alaya Bouafif et al., 2001;Ben Salah et al., 2005). As such, LST conversion is routinely observed in a percentage of the control arm participants in clinical trials (for example in (Bahar et al., 1996;Momeni et al., 1999;Sharifi et al., 1998).

In Leishmaniasis vaccine trials, LST plays an important role as the method of detection of previous exposure. Studies of vaccines for prophylaxis normally require participants to be LST negative. This allows identifying: 1) previous exposure and asymptomatic infection and 2) the effect of the vaccine in inducing an immune response (by converting LST). In several efficacy trials there has been an important finding regarding LST results in the vaccinated arm: even if efficacy is not observed for the entire vaccinated arm, significantly lower incidence is usually observed in the subset of vaccinated participants with LST conversion post-vaccination; i.e., those whose immune system responds to the treatment (Antunes et al., 1986;Khalil et al., 2000a;Momeni et al., 1999). It has been suggested that this apparent protection is due to the vaccine. However, in the trial in Esfahan by Momeni et al, the significantly lower incidence was observed in LST converted participants, regardless of their treatment assignment.

1.7 Vaccine development

1.7.1 Introduction

Given the enormity of the public health burden in several developing countries and the problems with control and treatment as discussed earlier, any method of

prevention would be of great value. A prophylactic vaccine could constitute a reasonable and practical approach to prevention and control, if the price could be kept at levels affordable to the at-risk population in developing countries. Unfortunately, the time and the cost required for the development of a vaccine and the low potential for a sizeable return on investment makes leishmaniasis vaccine development an unattractive investment for drug manufacturing companies (Khamesipour et al., 2005;Modabber and Reed, 2004). A feasible approach to leishmaniasis vaccine production would be to create facilities with GMP capabilities in developing countries and to seek local governments' support for purchasing and distribution. Philanthropic support for the transfer of technology and production under GMP conditions is needed as most donor agencies for medical research are not interested in development (Modabber and Reed, 2004). Fortunately, there is awareness and commitment on the part of foundations such as the Bill and Melinda Gates and international and non-governmental organizations to address this significant health problem.

Development of a vaccine against leishmaniasis has been the focus of many investigators around the world. During the past several decades, many vaccine candidates have been developed and some have entered the clinical development stage. However, to-date, no prophylactic vaccine for widespread use against leishmaniasis is available. Despite the discovery of many antigens which showed immunogenicity in animals, some of which have been shown to be safe and immunogenic in humans (Handman, 2001;Khalil et al., 2000a;Khamesipour et al., 2006), prophylactic efficacy for killed or sub-unit vaccines remains to be shown in a reproducible manner. The only efficacious prophylaxis against leishmaniasis to-date is infection with live, virulent *Leishmania* parasites, known as leishmanization (LZ).

Although an efficacious vaccine for leishmaniasis has not yet been identified, the existence of a natural model of protection (i.e., LZ) and the accumulating evidence of the effectiveness of immuno-chemotherapy in leishmaniasis patients (discussed later in this chapter) support the idea that developing an efficacious prophylactic vaccine is feasible.

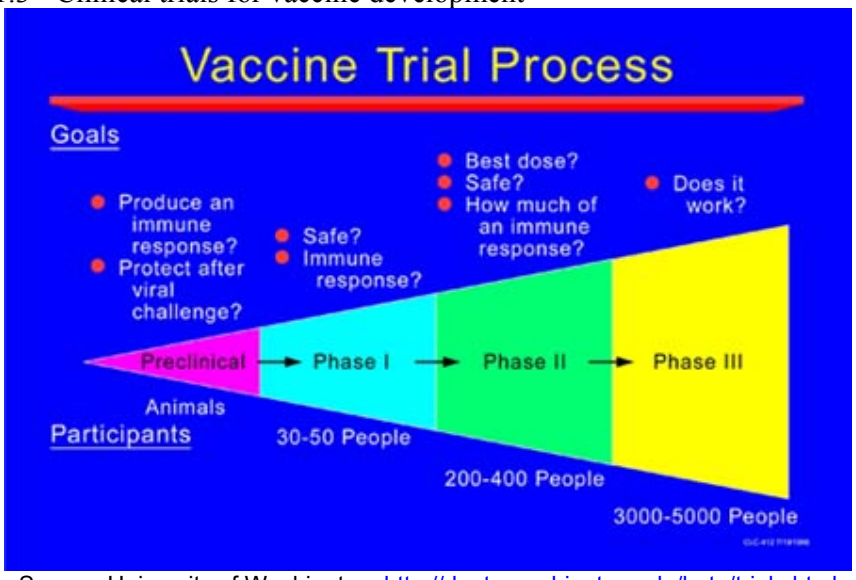
1.7.2 Development process

Many challenges lie in the way of vaccine development. Since vaccines are destined for use in healthy individuals and frequently young children, it is critical to ensure the safety of the product. Efficacy is important not only from a medical standpoint but also from the standpoint of ethics. Requirements of Good laboratory practices (GLP), Good Manufacturing Practices (GMP) and Good Clinical Practices (GCP) guarantee the reliability and reproducibility of the development and manufacturing process, clinical evaluation of the product and the evaluation of the vaccine's safety and efficacy. Quality control (QC) and quality assurance (QA) safeguards and requirements must all be met (Khamesipour et al., 2006).

After showing promising results in the discovery stage, a candidate antigen would have to prove safe and immunogenic in preclinical studies and only then enter the clinical trial stage, provided that technical and financial support are available. All phases of clinical trials have a focus on safety of the product. For "first in human" studies (phase 1), the primary assessment is of safety. Successful phase 1 studies are

followed by evaluation of safety and immunogenicity in larger numbers of subjects (phase 2). Phase 3 trials are also heavily oriented towards the assessment of efficacy. Hence, Phase 3 trials involve many more participants (in the thousands) than phase 1 (dozens) and phase 2 (hundreds) trials. The process of clinical trial from pre-clinical to phase 3 is demonstrated in figure 1.3. Phase 4 or post marketing trials aim to detect any safety problems after the vaccine is released and also to evaluate effectiveness under normal conditions of use. Normally Phase 3 trials for vaccines take several years to complete, depending on the incidence rate of the disease. A more rpid method to assess efficacy is to challenge study participants with live parasite after a pre-specified length of time post-vaccination. A challenge system, such as LZ, could bring about significant time and cost saving in the development process. Steps in clinical development of vaccines are displayed in the following figure.

Figure 1.3 - Clinical trials for vaccine development



Source: University of Washington, http://depts.washington.edu/hptu/trials.html

1.7.3 Live virulent parasite (Leishmanization)

Leishmanial infection, particularly CL, is associated with subsequent protection against future infection (Khamesipour et al., 2006). The strength of the immune response to infection seems to be directly correlated with the rate of protection in patients (Ben Salah et al., 2005). Historically, it has been known that leishmanial infection induces long lasting protection against future infection (Khamesipour et al., 2005). This knowledge in the endemic foci of *Leishmania* in South West Asia led to the practice of inoculation of at-risk individuals, usually children, with the pus from live lesion of a patient. However, in modern days, this is done with cultured parasite and is known as leishmanization (LZ) (Modabber and Reed, 2004). In Uzbekistan the first LZ campaign began in 1967 and lasted until 1970, modelled after a similar campaign in Turkmenistan. The standard preparation, registered in Uzbekistan for prophylaxis, consists of a mixture of live and killed promastigotes (Gafurov, 1999) and is the only prophylactic live vaccine in current use(Khamesipour et al., 2006).

LZ was used in Iran (1980's) and Israel (1970's) as a prophylaxis against leishmaniasis. It is not currently practiced in either country (Jaffe and Greenblatt, 1991;Khamesipour et al., 2006). In Israel, LZ program was discontinued because of loss of infectivity of the parasite due to repeated sub-culturing as well as immunosuppression that resulted in reduced responsiveness to diphtheria, pertussis and tetanus (DPT) vaccine in children following LZ (Jaffe and Greenblatt, 1991;Khamesipour et al., 2006). In Iran, in the preliminary experiment by Nadim et al, LZ achieved 80% efficacy (Nadim et al., 1983;Nadim et al., 1997). After this initial trial, the practice was used to immunize nearly 2 million individuals in Iran who were at risk of leishmaniasis after moving into endemic areas as a result of the Iran-Iraq war. Despite its effectiveness, the LZ campaign in Iran was stopped in 1986 and is currently not used as a method of vaccination in Iran and Israel due to a small percentage of cases with complications such as protracted and non-healing lesions (Nadim et al., 1997).

Development and registration of live, attenuated or drug-sensitive parasites (through culture, chemical, radiation or genetic manipulation) could lead to a significant improvement in LZ and enable its standardization, either as a vaccine or a challenge in clinical trials. However, questions such as parasite reversion to its original pathogenic state, consequences of its release into the environment should be addressed (Handman, 2001).

In addition to its potential as a prophylactic vaccine (after standardization), LZ (even in its current unstandardized form) could be used, and is currently used in Iran, as a tool in vaccine development; i.e., as challenge in vaccine efficacy trials in hyper endemic foci (where trial participants are in very high risk of natural infection). LZ challenge could provide a number of advantages (Khamesipour et al., 2005):

- Reduce the length of follow-up time
- Reduce the number of volunteers needed in a trial
- Therefore, reduce the trial cost
- Provide a consistent/standardized challenge to make better comparison between the vaccine and the control arm, without unaccounted variation due to differences resulting from natural infection
- Since efficacy vaccine trials take place in endemic foci, LZ would bring about a second advantage: conferring protection in study volunteers who are at great risk of natural infection

LZ with virulent, non-attenuated parasites also has certain shortcomings such as its differences with natural infection and variability inherent in production of the parasites which makes LZ difficult to standardize.

The safety and protective ability of LZ was observed in a clinical trial in Iran (Khamesipour et al., 2005).

1.7.4 Whole parasite vaccines

This general class of vaccines against leishmaniasis consists of vaccines made with whole killed parasites. As a result, standardization is a problem in the way of registration of these vaccines. In recent years the three killed parasite vaccines

evaluated in most human studies have been: *L. amazonensis* (BIOBRAS, Brazil), *L. mexicana* (Instituto Biomedicina, Venezuela) and *L. major* (Razi Vaccine and Serum Institute, Iran)(Modabber, 2000). In addition, a trivalent preparation consisting of *L. brazilensis*, *L. guyanensis* and *L. amazonensis* antigens was evaluated in Ecuador (Armijos et al., 1998). The adjuvant used in the Venezuelan, Ecuadorian and Iranian preparations was BCG.

1.7.4.1 Killed whole parasite (first generation) prophylactic vaccines, New World

First generation vaccines have been the subject of experiments in Latin America since early 20th century. The two main vaccines evaluated in the New World have been the pentavalent preparation in Brazil by Mayrink et al, which was later simplified to a monovalent *L. amazonensis* vaccine with no adjuvant. Originally, this vaccine was produced using merthiolate for parasite inactivation. The autoclaved preparation was also evaluated with similar immunogenicity results to the merthiolate preparation (Modabber, 2000;Velez et al., 1997). The other vaccine produced in Venezuela has been autoclaved *L. mexicana* with BCG adjuvant, used in immunotherapy of patients. Prophylactic studies of this vaccine apparently faced difficulties due to less-than-expected incidence (Modabber, 2000). Additionally, a trivalent preparation consisting of *L. brazilensis*, *L. guyanensis* and *L. amazonensis* antigens was evaluated in Ecuador (Armijos et al., 1998)

Brazil:

Brazilian investigators conducted trials of different preparations of killed parasites as early as 1939 by Sales-Gomes followed by Pessoa and colleagues in 1940's who used a polyvalent vaccine of 18 strains of *Leishmania* (Genaro et al., 1996).

These efforts were later followed in the 1970's by Mayrink and colleagues in Brazil who developed a pentavalent vaccine which, after 2 intramuscular injections (one week apart), was able to convert the MST (Montenegro skin test) results in 78.4% of vaccinated volunteers within three months, with no major side effects. Despite the fairly large sample size (614 vaccinated vs 974 control) the efficacy of the vaccine could not be assessed since no cases occurred in the study area after vaccination (Genaro et al., 1996;Mayrink et al., 1978;Mayrink et al., 1979).

Suggestion of protection associated with vaccination was given by another trial of 2 injections of the same vaccine by Mayrink et al (Mayrink et al., 1985) in a clinical trial with 216 volunteers in the vaccine arm and 266 in the control arm. Two years after vaccination the numbers of followed-up volunteers had dropped to 203 and 179 in the vaccine and control arms. Despite the reduced sample size, statistically significant results ($P < 0.01$) were observed between the vaccine and control arms with respect to the number of cases (1.7% vs 8.9%, respectively). However, the trial was not a double blind controlled trial(Genaro et al., 1996).

In 1981 and 1983 two randomized, double blind, controlled trials of the pentavalent vaccine were conducted by Mayrink, Antunes, and colleagues with Brazilian army personnel. As in previous trials, 2 intramuscular injections, one week apart were administered. The vaccine concentration was somewhat different (Antunes et al.,

1986). In the 1981 trial, 667 and 645 volunteers were included in the vaccine and placebo arms, respectively. LST conversion was 35%, considerably lower than in previous trials. This was attributed to the immunosuppression effect of the routine army yellow fever vaccination about 60 days prior to the study injection. Trial results did not show a significant difference between the vaccine and the placebo arms, despite some complications due to difference in incidence rate in the two army training groups in the study. In the 1983 trial, 658 and 616 army recruits were randomly assigned to the vaccine and placebo arms, respectively. LST conversion rate was 68% (more than that in the1981 trial, possibly because study started 120 days after routine yellow fever vaccination). Again, trial results were not significant.

However, importantly, 1981 results in group 1 of trainees (who spent a longer time in the jungle and experienced greater incidence of the disease) did show significantly lower incidence in those whose LST results had converted (induration ≥ 5) after vaccination (compared to either both placebos or non-LST converted vaccines). The reason for the overall lack of statistical significance seems to be the lower-than-expected incidence in the Group 2 in 1981 and the entire 1983 participants. Sample size calculations in both these studies were based on an expected incidence of 10%. Although more participants were registered in the study than the calculated sample size of 580.

Despite promising results, because of the difficulties of producing and standardizing the 5-strain Leishvacin® presented difficulties in preparation and registration. Additionally, subsequent studies demonstrated the immunogenicity of a single-strain *L. amazonensis* antigen vaccine. To address the development of a vaccine against CL, two Vaccine Advisory Groups with the participation of representatives of national and international organizations interested in developing an anti-leishmaniasis vaccine were organized by World Health Organization in Washington DC, USA and Belo Horizonte, MG, Brazil in February and September 1991, . It was suggested that studies with the vaccine should continue, using only one strain, the *L. amazonensis* (IFLA/BR/67/PH8). This strain was chosen because its antigens induced high stimulation indexes for lymphocytes from vaccinated volunteers it is easy to grow in non-cellular media, it is internationally known and it is taxonomically well defined" (Genaro et al., 1996;Mayrink et al., 2002).

It was later observed that *L. amazonensis* promastigotes have similar immunogenicity in mice as the 5-strain Leishvacin (Mayrink et al., 2002). Another study confirmed the immunogenicity of 2 and 3 doses of various concentrations of the monovalent vaccine in human volunteers (De Luca et al., 2001).

Colombia:

Immunogenicty and safety of three doses of the monovalent *L. amazonensis* vaccine was confirmed in Colombia in a phase 2 and a phase 3 randomized, double blind clinical trials. The phase 2 trial in 296 army volunteers was conducted (intradermally) with BCG as adjuvant and also intramuscularly without BCG. The vaccine with BCG was only administered twice (despite the protocol requiring 3 doses), due to the BCG-induced lesions that were unacceptable to volunteers. The intramuscular administration proved to be safe and immunogenic and as a result the phase 3 was conducted (Velez et al., 2000).

The phase 3 trial was conducted in LST negative (<3 mm LST induration) army volunteers. Participants in the vaccine arm (n = 1295) and the placebo arm (n = 1302) were followed for 12 months. The vaccine was safe and immunogenic but did not provide protection against CL (Velez et al., 2005). Participants in this study were not LST tested after vaccination. Therefore, it is not possible to compare incidence in those whose LST results converted after vaccination with those who did not.

Ecuador:

The only leishmaniasis vaccine clinical trial in which prophylaxis efficacy was observed (regardless of LST conversion after vaccination) was conducted in Ecuador (Armijos et al., 1998). In this study, safety, immunogenicity and efficacy of 2 intradermal doses of a locally prepared vaccine were assessed against 2 doses of BCG alone (n = 438 vaccine arm vs n = 406 controls). The vaccine consisted of *L. braziliensis*, *L. guyanensis* and *L. amazonensis* promastigotes originally collected from the lesions of patients living in the study area, mixed with BCG. After 12 months of follow up, the vaccine was shown to be safe and the protective efficacy of the vaccine was evaluated at 72.9%, with 2.1% incidence of CL in the vaccine arm vs 7.6% in the control arm (Armijos et al., 1998). In this study, similar to some of Mayrink's works, the incidence in the control arm was less than the expected 13-15% (Armijos et al., 1995a;Armijos et al., 1995b). However, the sample size calculation had been based on 7.5% incidence, rather than 14%.

The differentiating aspects of this trial are 1) the origin of the vaccine being from locally obtained parasites, and 2) the average age of subjects being around 5.5 years (5.4±3.9 vaccines and 5.7±3.9 controls).

After the original 12 months of follow up, these subjects were followed up, in a separate study, for another 4 years. Results indicated that although the protective efficacy was still significant between the 13th and the 18th months, it was not so after the 19th month of follow-up (Armijos et al., 2003). The incidence in each 6-month period and the total incidence from the 19th to the 60th month were not significantly different between the vaccine and the control arm subjects who were followed-up.

However, examining the table provided in the article by Armijos, et al, suggests that the loss of statistical significance in the difference between arms after the 19th month was not due to an increase in the number of cases in the vaccine arm, but rather a reduction in the number of new cases in the control group. In addition, the gradual reduction in the number of subjects followed seems to be another factor contributing to differences that are not significant (number of subjects were calculated for each 6-month follow-up period based on incidence values reported in the article). It could, therefore, be argued that this study is inconclusive with respect to the erosion of the vaccine-derived protection after 18 months of vaccination.

The other vaccine trial conducted by Armijos et al, compared 2 doses of vaccine (n = 750) against placebo (n = 756). The vaccine consisted of autoclaved *L. amazonensis* mixed with BCG. Although the vaccine was safe and immunogenic (significantly more LST conversion in the vaccine arm), results indicated no protection associated with vaccination; not even in the LST-converted subgroup (Armijos et al., 2004). In

this trial, the average age of participants was 11.2±10.5 in the vaccine arm and 10.5±10.1 in the placebo arm; on average nearly twice as old as those in the earlier study. Authors mention the possibility of the parasite killing method (heat), and the geographic origin of the parasite or the parasite species used in the vaccine as possible factors contributing to its ineffectiveness. As in some of the other studies, the observed incidence in the placebo arm was less than expected. This must have lead to a reduction in statistical power and contributed to inconclusive results.

1.7.4.2 Killed whole parasite (first generation) prophylactic vaccines, Old World

After the LZ program in Iran in the 1980's, a national vaccine development program was planned and the production of the killed *Leishmania* vaccine was started at the Razi Vaccine and Serum Institute, Hessarak, Iran, using the same organism as used in the LZ program(Khamesipour et al., 2006;Modabber, 2000). Phase 1 and 2 clinical studies of safety and immunogenicity of different doses of inactivated *L. major* promastigotes with or without a low dose of BCG (as adjuvant) were conducted in non-endemic areas in Iran. The potential advantage of BCG adjuvant was that it could facilitate introducing leishmaniasis vaccination into the national vaccination programs, which already included BCG vaccination. These early results demonstrated the ability of a low dose of BCG in enhancing the immune response to the antigen (Bahar et al., 1996;Dowlati et al., 1996). It should be noted that in the study by Bahar et al, vaccines produced by two methods of parasite inactivation (thimerosal treatment vs autoclaving) were used with similar results. Since autoclaving provided a less complicated and less expensive method of preparation, it was recommended by the authors as the preferred method of vaccine preparation for the future efficacy trials (Bahar et al., 1996). The autoclaved *L. major* (ALM) preparation mixed with BCG adjuvant (10% the dose normally used in vaccination against TB) was used in several field trials in Iran and Sudan. The results of some of these studies in the late 1990's (multiple injections in Bam, Iran, multiple injections in Zavareh, Iran and multiple injections in Borkhar, Iran) have not yet been published. This preparation was later replaced by an improved formulation involving precipitation of the ALM in aluminium hydroxide (alum), known as alum-ALM. Alum-ALM mixed with BCG showed significantly higher ability to convert the LST results in vaccinees.

Iran:

Esfahan single injection (Esf1): Momeni et al conducted a randomized, double-blind, controlled field efficacy trial of ALM+BCG against ZCL due to *L. major*. One injection of the vaccine (n = 1188) was compared with one injection of BCG alone (n =1122). Study volunteers were from healthy, LST-negative (LST=0), residents of an airbase in an endemic part of Isfahan, Iran. Volunteers' age ranged from 5 to 72 years. The distinguishing aspect of the sampled population was that they had moved into the airbase from throughout Iran, including non-endemic areas (Momeni et al., 1999). Study duration was originally planned to be 1 year, but after interim analysis, another year of follow up was recommended. Post-vaccination LST was conducted on day 80 and again in one year.

The annual incidence rate in the area during the years prior to the study had been consistently around 4-6% in the native community. Study results indicated higher

annual incidence rate (about 10% in the controls, for those with LST results obtained). Authors indicate higher LST conversion compared to other studies conducted in non-endemic or low endemic areas in Iran. They attribute the higher LST conversion in the vaccine arm and the conversion observed in the control arm to natural exposure. It is possible that the higher response reflect the non-endemic origin of participants.

Although the two years of follow-up did not produce significant results to support vaccine efficacy overall, infection rates in the LST converted individuals (in the vaccine and the control arm combined) were lower than in those whose LST had not converted (7.3% vs 11.3 in the vaccine arm and 3.4% vs 10% in the control arm). This is consistent with earlier discussed findings in Brazil (Antunes, Mayrink et al. 1986). The difference is that in Brazil the significant difference was between LST converted vaccine participants and all others, while in Esfahan the difference was between participants whose LST converted and those who did not, regardless of BCG or vaccine treatment. This may reflect the fact that in Brazil the control treatment was not BCG. Another outcome of this study was the lower disease severity in vaccinated children less than 14 years old. A subsequent study of the immunogenicity data from this trial did not substantiate a significant correlation between proliferation response (IFNγ production) to ALM and the magnitude of LST response (Mahmoodi et al., 2003). Authors compared several different subgroups of the study but did not compare the vaccinated volunteers whose LST had converted with other groups. Authors raise the possibility that BCG may not be an ideal control arm treatment since it could induce a similar immune response to the ALM+BCG.

Bam single injection (Bam1): Randomized, double-blind, BCG controlled clinical trial in Bam, Kerman, assessed the efficacy of ALM+BCG in protection against the anthroponotic CL (ACL) caused by *L. tropica* in LST-negative (LST = 0) school children 5 to16 years old (Sharifi et al., 1998). One injection of the vaccine (n = 1839) was compared with one injection of BCG alone (n = 1798). Post vaccination LST was conducted on day 80 and again after 1 year.

Previous studies had indicated a 2% annual incidence rate. Two years of follow-up uncovered infection rates that were not significantly different between the vaccine and the control arms (2.8% and 3.3%, respectively). The LST conversion was significantly greater in the vaccine arm (16.5% vs 3.6% on day 80 after vaccination), but overall, less than that observed in Isfahan, possibly due to the lower incidence rate in Bam compared to Esfahan. Protection in the LST converted subgroups was not reported. Protection was observed in vaccinated boys, but not girls. Also, it was noticed that through the 6th month of follow up more cases were identified in the vaccine arm, while fewer were identified thereafter. This observation may point at the longer time it takes for the *L. tropica* to lead to clinical infection

Other studies were conducted in Iran with multiple doses of ALM+BCG. These studies were carried out in Bam, province of Kerman and in Borkhar and Zavareh in the province of Esfahan. Results have not been published, but will be used in the analysis in the present investigation. Briefly, they include the following trials

Bam 3-injections (Bam3): Sharifi, et al conducted a study in LST = 0 school age children with 3, one month apart injections of ALM+BCG. BCG was used as the

control treatment. Two post vaccination LST measurements (day 80 and 1 year) were taken.

Borkhar 3-injection (Bor3): Khamesipour et al conducted a 3-injection trial in school age children in Borkhar, Esfahan province. The two main distinguishing aspects of this trial was 1) Inclusion criteria allowed any initial LST values accepted into the trial and 2) although the first 2 injections of the vaccine were one month apart, the third injection was one year after the first. Several LST measurements were taken throughout the trial.

Zavareh 3-injection (Zav3): Khamesipour et al also conducted a 3-injection trial in Zavareh, in the province of Esfahan. Participants' age ranged from 5 to 59. Vaccine injections were one month apart. In this study only the pre-vaccination LST was conducted.

Additionally, a clinical trial assessing the safety, immunogenicity and effectiveness of alum-ALM (alum-precipitated ALM an improved formulation of ALM) mixed with BCG followed by LZ challenge was conducted in a hyper-endemic area of Esfahan. This study (unpublished) did show the safety of alum-ALM+BCG but was inconclusive about the vaccine efficacy against challenge due to problems with the LZ reagent. This trial with an improved study design is currently underway in Iran.

Sudan:

Safety and immunogenicity of ALM+BCG was assessed in endemic and non-endemic areas of Sudan (Khalil et al., 2000b;Satti et al., 2001). Subsequently, a double-blind randomized field efficacy trial of two injections of ALM+BCG (n = 1155) vs BCG alone (n = 1151) was conducted in the late 1990's in Sudan in an area endemic for VL due to *L. donovani* (Khalil et al., 2000a). LST-negative (LST = 0) volunteers between the ages of 1 and 65 years were admitted to the study. Post vaccination LST conversion was significantly higher in the vaccine arm (30% vs 7% after 42 days of vaccination). However, after 2 years of follow up, the rate of infection in the two study arms were not significantly different (11.5% in the vaccine arm and 12.3% in the control arm). Although the overall vaccine efficacy was not significant, individuals whose LST converted after vaccination had a significantly lower incidence of VL than non-responders (7.2% vs 12.7%), regardless of the BCG or vaccine treatment. These results are consistent with findings in studies in Esfahan and Brazil, discussed earlier (with the difference that the control treatment in Brazil was not BCG).

In addition to the studies of ALM+BCG, safety and immunogenicity of different doses (10, 100, 200 and 400 µg of leishmanial protein) of alum-ALM+BCG was assessed in Khartoum (Kamil et al., 2003). LST conversion 42 days after vaccination was observed in all volunteers in the 10 µg, 100 µg and 400 µg doses but only in one of five volunteers in the 200 µg group. All doses were safe with minimal, local side effect (Kamil et al., 2003). Additionally, an extended phase 2 study (Khalil et al., 2006) confirmed safety and immunogenicty of alum-ALM+BCG compared to vaccine diluent as the control treatment in 544 leishmanin non-reactive children younger than 15 years old. The four cases observed in this study were all in the control arm.

Although not an efficacy trial, this evidence may suggest lower incidence associated with vaccination.

1.7.4.3 BCG as adjuvant in first generation vaccine trials:

Bacille Calmette-Guérin (BCG) has been used in several of the formulations of first generation leishmania vaccines. In the New World, the vaccines tested in Brazil did not contain adjuvant. However, Armijos used BCG (about 1:2 dose of what is normally used for vaccination) in both his trials; with the locally produced trivalent vaccine and with the Brazilian Leishvacin. In immunotherapy, variable doses of BCG were used depending on the PPD results of each volunteer, per Convit's protocol (Cabrera, 1994). In the Old World different doses of BCG were tested in phase one trials (Dowlati et al., 1996) and subsequently 1/10 of the normal dose (in TB vaccination) was used in all leishmaniasis vaccine trials (Alimohammadian et al., 2002;Bahar et al., 1996).

The use of BCG has both advantages and disadvantages. Injection of BCG mixed with the antigen significantly increases the LST response to the vaccine; i.e., it enhances the desirable Th1 response. Additionally, it has been observed that LST conversion due to vaccination is associated with reduced infection (Alimohammadian et al., 2002). Therefore, BCG inclusion as an adjuvant could help the immunogenicity and protection due to the vaccination. However, BCG, even without the antigen can induce short-term LST conversion. Therefore, from the standpoint of assessing the efficacy of the vaccine, BCG is not a good candidate for use in the control arm efficacy trials. (Alimohammadian et al., 2002;Armijos et al., 1998).

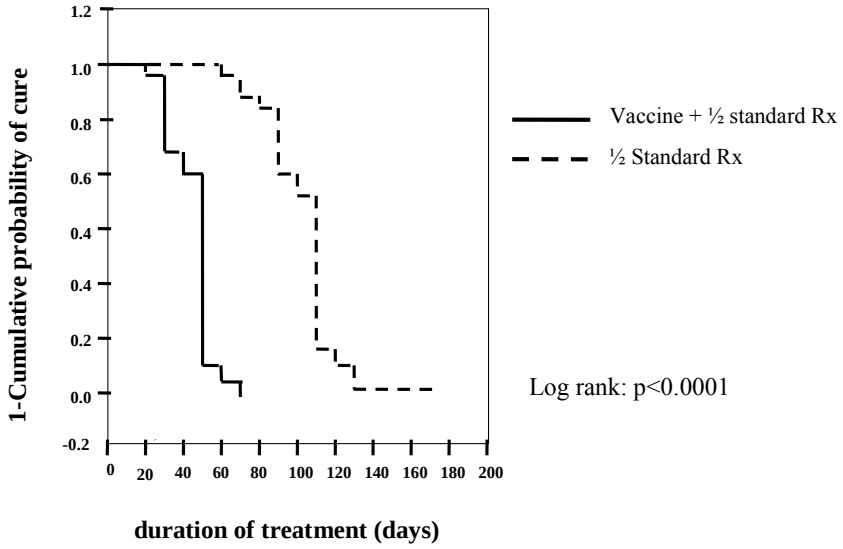
1.7.4.4 Killed whole parasite (first generation) therapeutic vaccines

Although prophylactic efficacy has not been demonstrated for any of leishmaniasis candidate vaccines, several vaccine candidates have been shown to be efficacious in treatment of different forms of leishmaniasis when used in combination with chemotherapy. In fact, some candidate vaccines have shown efficacy when used alone.

As early as 1939, Sales-Gomes tested the effect of killed *Leishmania* in patients in Brazil. He noticed a decrease in the size of lesions associated with this therapy (Genaro et al., 1996). Mayrink in Brazil and Convit in Venezuela experimented with different vaccine formulations beginning in the 1980's.

In Brazil, Mikhalick and Myrink compared different vaccination protocols with a pentavalent vaccine (Leishvacin) and observed that immunotherapy could be a viable alternative to drug therapy in patients who can not tolerate Glucantime (Genaro et al., 1996). Machado-Pinto and Mayrink compared the monovalent *L. amazonensis* vaccine plus half does of the standard antimonial treatment against half dose of standard antimonial treatment alone. Results suggested much faster cure in 100% of patients in the immunochemotherapy arm (compared to 8.2% in the chemotherapy arm - figure 1.4) and fewer side effects (Machado-Pinto et al., 2002).

Figure 1.4 - Immuno-chemotherapy vs chemotherapy in Brazil, after Machado Pinto et al., 2002



In Venezuela, Convit compared 3 injections of a combination of *L. Mexicana* promastigotes and BCG without chemotherapy vs standard antimonial regimen in 94 patients with localized CL. Cure rate in both study arms were 94%, but with far fewer side effects in the vaccine arm (Convit et al., 1987).

Subsequent studies by Convit and colleagues supported the therapeutic efficacy and safety of vaccine therapy of patients with various forms of leishmaniasis, including cases with severe ML and DCL (Convit, 1996;Convit et al., 2003;Convit et al., 2004). Figures 1.5 and 1.6 depict the results of Convit's 1996 investigation.

Figure 1.5 - Side effects of Chemotherapy, immunotherapy and BCG alone, after Convit 1996

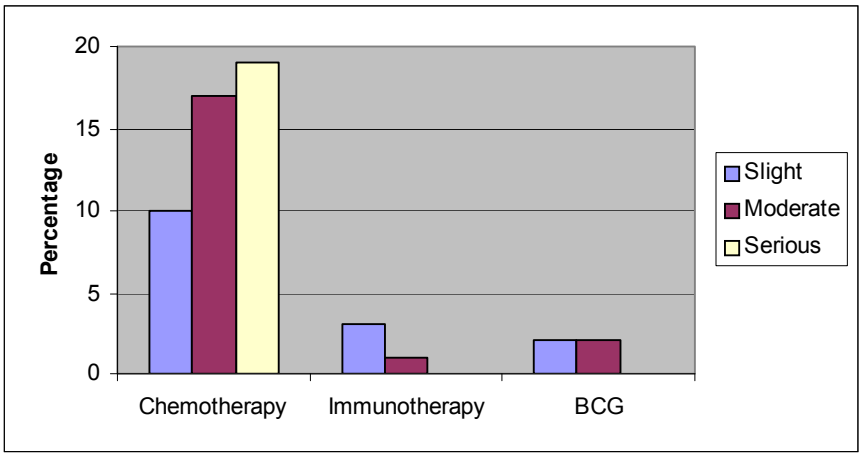
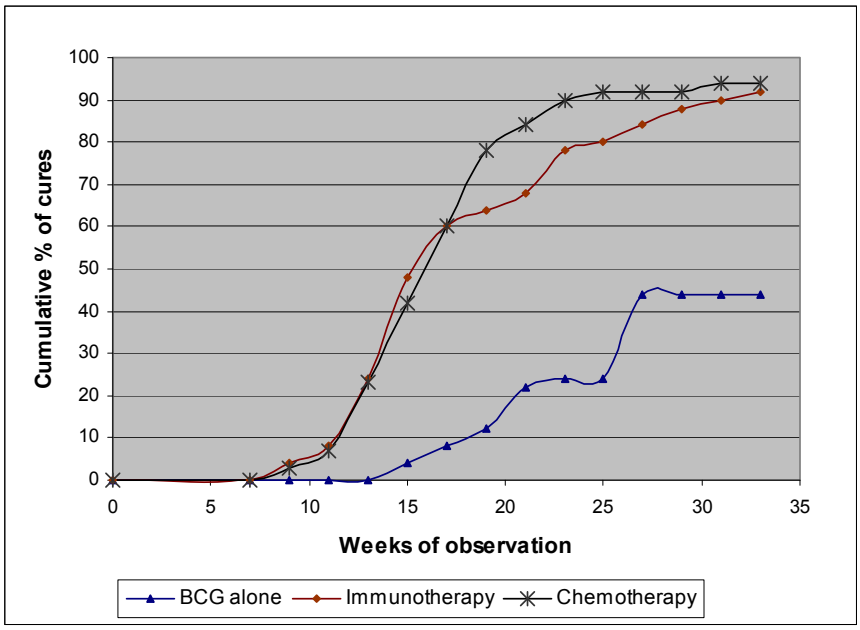
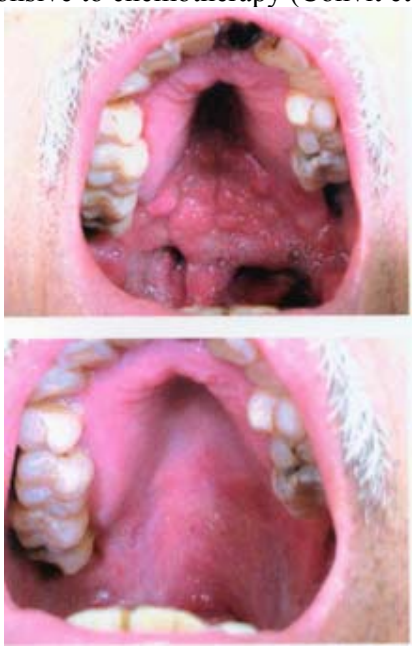


Figure 1.6 - Healing of localized leishmaniasis by chemotherapy, immunotherapy and BCG alone, after Convit 1996



The following figure shows a patient before and after immunotherapy in Convit et al, 2004.

Figure 1.7 - Mucosal leishmaniasis before and 6 months after immunotherapy in a patient non-responsive to chemotherapy (Convit et al., 2004)



Immunochemotherapy in *Leishmania*/HIV co-infection cases has had promising results (Genaro et al., 1996). The latest therapeutic assessment by Mayrink et al (Mayrink et al., 2006) supports these earlier findings by Convit, Mayrink and their colleagues in Venezuela and Brazil. Results demonstrate the effectiveness of immunotherapy with or without standard pentavalent antimonial treatment. The effect in combination therapy was shorter length of therapy along with >98% cure. In immunotherapy alone, the same level of cure was achieved in slightly longer length of time.

Recently in Sudan two trials suggested the ability of immunotherapy with whole-parasite, alum-precipitated autoclaved *L. major* promastigotes combined with BCG to induce quicker healing in chronic PKDL patients as compared to standard treatment with antimonial drugs (Pentostam) (Musa et al., 2008). The fact that even suboptimal leishmaniasis vaccines could be effective in the therapeutic setting is encouraging support for the pursuit of a prophylactic vaccine (WHO/IVR, 2006).

1.7.5 Modified antigen vaccines

Modified antigen vaccines include second generation vaccines (sub-unit, purified protein, DNA, etc) and vaccines made with modified live parasites.

1.7.5.1 Second generation vaccines

Second generation vaccines encompass a number of different antigens and delivery systems and include purified protein, DNA's or otherwise manipulated (genetically, chemically, etc) antigens with the goal to develop a well characterized vaccine with well defined effect on the immune system. The common advantage of second generation vaccines is that they are well defined and easily standardized. This is a major advantage and renders these vaccines better candidates for production and registration. The development of well defined vaccine candidates against leishmaniasis has been the result of understanding the immunological mechanism that mediate protection as well as genome sequencing of *L. major* (Khamesipour et al., 2006). , subunit vaccines (purified, recombinant proteins), recombinant bacteria or viruses with leishmanial antigens, DNA vaccines, synthetic peptides (introducing the antigen protein's peptide rather than the entire protein) and non-protein antigens (parasite surface glycol-lipids such as lipophosphoglycan (LPG) (Handman, 2001;Khamesipour et al., 2006;Modabber, 1995).

Currently, the only second generation vaccine in clinical development is Leish-111f with MPL-SE which is the fusion of three leishmanial antigens: LmSTI1 (*L. major* Stress Inducible protein), TSA (Thiol-Specific Antioxidant) and LeIF (*Leishmania* elongation and initiation factor). Leish-111f in its current formulation is injected after mixing with MPL-SE (monophosphoryl lipid A in squalene emulsifier) as adjuvant (Coler and Reed, 2005;Modabber and Reed, 2004). A phase 1 trial in the US showed the safety, immunogenicity of the vaccine and therapeutic trials in Latin America have been conducted (Coler and Reed, 2005;Ghalib and Modabber, 2007). In dogs, however, multiple doses of Leish-111f did not protect against *L. infantum* infection (Gradoni et al., 2005)

Another approach has been based on the immune response to the sandfly salivary proteins inoculated into the host during a blood meal (Belkaid et al., 2000). It has been observed that prior exposure of mice to the bite of uninfected sandflies is associated with reduced rate of infection (Kamhawi et al., 2000). In endemic regions, many individuals are exposed to the bites of Leishmania-free phlebotomines before being bitten by an infected sand fly (Kamhawi et al., 2000). In recent years there have been efforts for identifying salivary proteins that could lead to protection in humans (Handman, 2001;Valenzuela et al., 2001).

1.7.5.2 Modified live parasite vaccines

Modified live parasite vaccines consist of live parasites that are chemically or genetically attenuated or manipulated (drug sensitized, suicide genes added, etc) to be infectious but not pathogenic (Handman, 2001). Attenuated parasites are taken into the macrophage in the same way as the virulent organisms and persist long enough to induce an immune response (Kedzierski et al., 2006). Persistence of the antigenic effect within the immune system is the main advantage of live attenuated parasites and could lead to a long lasting protection, much like live virulent parasites (Scott, 2005;Selvapandiyan et al., 2006) methods of attenuation included long term culture, γ-irradiation, temperature sensitive mutations or random mutations induced by chemical agents(Selvapandiyan et al., 2006). A major problem with these methods the possibility of reversion to the virulent state. Such reversion can occur spontaneously in healthy individuals or under conditions of weak host immune system(Selvapandiyan et al., 2006).

These challenges can be met with newly developed live attenuated strains because they (i) possess genetically defined mutations; (ii) persist in the host without being virulent; (iii) have less chance of reversion to the virulent phenotype because of irreversible genetic modifications; (iv) are amenable to further genetic manipulation if a candidate vaccine causes adverse reactions; (v) can be produced in large quantities in well defined conditions suitable for human vaccination; and (vi) can be tested along with new adjuvants or parasite antigens to enhance protective immune response or in combination with antileishmanial drugs to reduce pathogenesis, if needed(Selvapandiyan et al., 2006).

1.7.6 Vaccines against canine leishmaniasis

In parallel to vaccine development for humans, dog vaccines have been a focus of investigators. Dogs play a major role as a reservoir in transmission in human communities (Gavgani et al., 2002). Prevention in dogs could have significant effect on transmission reduction and incidence in humans within the same endemic focus (Gavgani et al., 2002;Saraiva et al., 2006). A number of different dog vaccines with good results have been under investigation (Gradoni, 2001;Rafati et al., 2005).

1.7.7 Overview

As suggested by the foregoing review, until now laboratory and clinical experiments have identified several vaccine candidates with the potential ability to produce the beneficial immune response against leishmanial infection. Furthermore, those candidate vaccines that have undergone clinical trials, whether for prophylaxis or therapy, have been shown to be safe, without untoward systemic consequences, generally tolerable and immunogenic in humans. Complications tend to be local and mostly associated with the use of adjuvant, particularly BCG which induces a lesion and leaves a scar. The following points are central when considering the feasibility of an efficacious prophylactic vaccine against leishmaniasis:

1. The natural model for protection against leishmaniasis exists: cured cases tend to be immune to new infection. This has been also demonstrated by LZ experiences.
2. From a biological and immunological standpoint, a leishmaniasis vaccine seems to be feasible because the antigenic profile of the parasite in various stages of its life cycle (extra- and intra-cellular forms) undergoes little variation (Modabber and Reed, 2004). In addition, the antigenic variation across different species of *Leishmania* is not extensive and cross protection in mice and monkeys has been observed(Vanloubbeeck and Jones, 2004a;Vanloubbeeck and Jones, 2004b).
3. There is only one target host cell for the parasite (the macrophage).
4. In all clinical trials in healthy volunteers, the candidate vaccines have proven to be not only safe, but immunogenic.
5. The therapeutic efficacy of various vaccine candidates has been demonstrated in several studies. This indicates the ability of these vaccines in producing an effective Th1 immune response.
6. In several trials where LST was performed to measure immune response to vaccination, lower rates of infection were observed in the subset of participants whose LST converted after vaccination.
7. An affordable and efficacious leishmaniasis vaccine could be major addition to the armament for the control of the disease.

Therefore, developing a vaccine against leishmaniasis is desirable and feasible. Yet, long-term prophylactic efficacy in humans has not been clearly, consistently and reproducibly demonstrated. Even vaccine candidates with therapeutic efficacy and immunogenicity in healthy subjects have not been shown to have prophylactic efficacy. Improving the structure of these vaccines, by introducing new adjuvants or antigens, could potentially improve their efficacy (e.g., adding alum to ALM+BCG, as discussed earlier). Another contributing factor to the failure of clinical trials in demonstrating prophylactic efficacy could be the limitations and conditions imposed by the design and conduct of phase 3 vaccine trials that could adversely affect the outcome. This subject will be discussed later, since one of the objectives for this thesis is to address some of these limitations.

1.8 Rationale for the thesis

Given the problems with existing methods of control, an affordable prophylactic vaccine would be a major step forward in the control of leishmaniasis. First generation vaccines have the advantage that they can be produced relatively simply at low cost within endemic countries. With one exception, results of clinical trials of first generation vaccines do not support their efficacy. However, their safety and immunogenicity, as important requirements, have been demonstrated. Additionally, a lower disease incidence has been repeatedly observed in individuals who responded to vaccination (LST converted). Given the need for a cost effective method of prevention and observations that support at least partial effectiveness of the FGVs, it seems the idea of FGV efficacy can not be rejected without thorough analysis of the existing body of clinical trial data. Therefore, in addition to continuing vaccine discovery and development efforts, it would be important to reassess the existing body of clinical trial data to further examine hypotheses related to prophylactic efficacy. Such reanalysis seems even more necessary in light of the mostly unavoidable problems and shortcomings in the design and conduct of clinical trials.

1.8.1 Potential problems with individual field efficacy trials

Field efficacy trials for prophylactic vaccines are subject to certain conditions and limitations that could adversely affect their outcome. The design of field efficacy vaccine trials should allow the vaccine to demonstrate its true ability to reduce the rate of infection in those who are vaccinated. For this to happen, a desirable statistical power (ability to detect a real difference) should be achieved. Parameters that affect power calculation are

a) disease incidence,
b) expected vaccine efficacy,
c) sample size

For a given sample size, lower incidence or lower efficacy could adversely affect power.

Certain aspects of vaccine efficacy trials can reduce the effective power of the study to less than its nominal value. Problems arising from the design and conduct of efficacy trials with the ability to affect study power include:

Problem 1: Expected incidence of leishmaniasis could vary dramatically due to known or unknown reasons. Examples of reduced incidence can be found in several of Mayrink's trials in Brazil (discussed).

Problem 2: Vaccine efficacy trials must be done in endemic foci, so that infection can be observed and compared between study arms. Any travelling out of the endemic foci by study participants (in either study arm) would reduce the effective incidence.

Problem 3: Unlike drug trials, vaccine efficacy trials normally take a long time to allow study participants enough exposure and the expected incidence rate to take effect. After several years, many of the original study participants could be lost to follow-up due to a variety of reasons, leading to a reduction of effective sample size.

Problems 5: In endemic foci a subset of the population are immune due to either

- genetic make up (Nylen et al., 2006;Ramirez and Guevara, 1997),
- previous exposure to the bite of the uninfected vector which would not cause infection but could generate a certain level of immunity (Kamhawi et al., 2000) without affecting LST.

Therefore, a certain number of those who would have otherwise been protected by the vaccine in the vaccine arm only, are protected due to other reasons. More importantly, they have immune counterparts in the placebo arm since randomization guarantees that individuals with these types of undetected immunity are assigned to both study arms with equal probability. Thus, some of the vaccine's potential for inducing immunity unilaterally in the vaccine arm would not be realized and would be wiped out by similar immunity in the control arm. Study power for detecting vaccine efficacy will be reduced proportionately to the percent of such individuals in both arms.

Problems 6: In addition to the above, LST performance is subject to considerable variation due to a variety of other reasons. This variability in performance could allow unintended inclusion of individuals who are immune due to previous infection in the study. This, too, would allow inclusion of already immune participants in both trial arms.

These and a number of other problems and inconsistencies between individual trials could have adverse effect on the study power and the ability to draw a correct conclusion. Furthermore, they can not be prevented by randomization.

1.8.2. Analyses in thesis

Combining and re-analysing clinical trial data would allow testing of new hypotheses in addition to re-examination of original hypotheses. It can bring about the advantages of a greater sample size and the ability to observe efficacy in various sub-groups (demographics, etc), identify new risk factors and shed light on ways to improve upon existing vaccine candidates or methods of assessment (e.g., trial designs). This approach would also enable new hypotheses that could not be originally tested due to the requirement of adherence to randomization and intention to treat principle in the original trial analysis.

Results could provide a different and enhanced view of the existing information and could lead to conclusions beyond what has already been made in individual trials, or confirm that new statistically significant results cannot be arrived at despite the greater power afforded by larger sample size.

The re-examination of the data will enable re-evaluation of efficacy of FGV's for prophylaxis against leishmaniasis in different settings: various vaccine types, different causative parasites and the use of adjuvants. A meta analytic approach will be used for this re-evaluation. Efficacy should also be examined in demographic subgroups

via meta-analysis to study differences in response to vaccination due to demographics. Furthermore, the immunological response in various subgroups to vaccination and natural exposure should be assessed. Studying these differences could shed light on immunological distinctions between endemic and non-endemic populations. Finally, since LST is widely used in epidemiological and clinical studies, its merits to provide a marker of immunity should be assessed. A variety of analytical techniques will be used to address these objectives.

CHAPTER 2

RESEARCH OBJECTIVES AND METHODS

2.1 Goal and objectives

The overall goal of the present study is to re-analyse the existing data of clinical trials of first generation leishmaniasis vaccine candidates with the view to re-examine the efficacy and immunogenicity (DTH response) of the most promising vaccines and to reassess LST as a correlate of immunity. We will investigate whether the information from all these trials combined supports the feasibility of a first generation leishmaniasis vaccine and could shed additional light on the following specific objectives:

Objective 1: Re-evaluate the efficacy of FGV's for prophylaxis against leishmaniasis.

Ho1: None of the FGV's for which there are trial data has the ability to confer protection in the population.

A. Efficacy of all vaccines overall: This will indicate the ability of leishmanial antigens, overall, in producing immunity and immune response, regardless of the species used in the vaccine.
B. Efficacy of different vaccine candidates prepared by various methods (parasite killed by heat, merthiolate, etc): *L. major* (against *L. major*, *L. tropica* and *L. donovoni*), *L. amazonensis* (against ACL), and re-examination of 3-antigen cocktail of *L. amazonensis*, *L. Guayanensis* and *L. Brasiliensis* (against ACL).
C. Contribution of adjuvant (comparison of vaccines with or without adjuvant(BCG or alum)

Ho2: The studied FGV's are unable to confer immunity even in specific subgroups

D. Comparison of efficacy in the subgroups identified by demographic and risk behaviour: age, gender, occupation, native origin (endemic vs non-endemic area native).

Objective 2: Assess immunological response as a result of vaccination and natural exposure.

Ho: Inhabitants of endemic and non-endemic areas and various demographic groups do not differ in their immunological response to leishmanial antigens (introduced as a vaccine or natural exposure).

A. Comparison of immunological response to leishmanial antigens (measured by LST) between various demographic groups (age and gender) and between inhabitants of endemic foci and those originally from non-endemic areas. This includes assessment and comparison of :
   - response in the vaccine arm (as a result of vaccination + natural exposure)

- response in the control arm (as a result of natural exposure)

Objective 3: LST as a marker of immunity

Ho: LST does not constitute a surrogate marker of immunity (overall and in endemic area natives)

A. Ability of LST to indicate the underlying immunological response to leishmanial antigens.
B. Relationship between LST response and actual protection. This objective is to verify the suggestion by some studies that FGV's can confer immunity in LST converters.
C. Comparison of this relationship between subgroups identified by demographic and risk behaviour: age, gender, occupation, native origin.

2.2 Methods

2.2.1 Data

The data from the following leishmaniasis vaccine trials in Iran, Sudan and South America were used in this research (some from published articles and some provided by the principal investigators). These trials constitute all randomized, blinded, controlled efficacy trials of leishmaniasis vaccines with the exception of Armijos' second trial in Ecuador (Armijos et al., 2004) (which did not reach conclusive results due to significantly lower rates of leishmaniasis than expected).

A. Zav3: Phase 3 trial of 3 injections of ALM+BCG against *L. major* in Zavareh, Iran (Khamesipour, personal communication, not published)
B. Bor3: Phase 3 trial of 3 injections of ALM+BCG against *L. major* in Borkhar, Iran (Khamesipour, personal communication, not published)
C. Bam3: Phase 3 trial of 3 injections of ALM+BCG against *L. tropica* in Bam, Iran (Sharifi, personal communication, not published)
D. Bam1: Phase 3 trial of single injection of ALM+BCG against *L. tropica* in Bam, Iran (Sharifi et al., 1998)
E. Esf1: Phase 3 trial of a single injection of ALM+BCG against *L. major* in Esfahan, Iran (Momeni et al., 1999). The study population included many who had moved from non-endemic areas to the trial site
F. Sudan 2: Phase 3 trial of 2 injections of ALM+BCG against *L. donovani* in Sudan (Khalil et al., 2000a)
G. Brazil 1981 and 1983: Efficacy trials of 2 injections of a 5-strain vaccine (composed of brasiliensis and mexicana complexes, including L. *guyanensis* and *L. amazonensis*) in Amazonas, Brazil (Antunes et al., 1986). Exposure limited to military missions.
H. Colombia3: Phase 3 trial of 3 injections of *L. amazonensis* vaccine in Colombia (Velez et al., 2005) Exposure limited to military missions.
I. Ecuador2: Efficacy trial of 2 injections of a 3-strain vaccine (*L. guyanensis*, *L. brasiliensis*, *L. amazonensis*)+ BCG, in Ecuador (Armijos et al., 1998)

Individual level data were available and were used to calculate relative risk for trials A-E above, while published information was used for F-I.

2.2.2 Analysis

Examination of structure and distribution of the data obtained from above studies to ensure their suitability and compatibility.

Analysis of the data to address the stated objectives, using appropriate statistical methods -- including conventional techniques (graphical analysis, cross tabulation, regression and tests of statistical significance) and meta analytic techniques for combined analysis of different trials. If the original participant-level data for a given study were not available the analysis was based on published trial results (this was the case for South American and Sudan trials).

The data used in this study represent trials that are different with respect to:

1. Parasite species used in the vaccine (*L. amazonensis* in South American vaccines, *L. major* in Iran and Sudan trials),
2. Parasite geographical origin; e.g., local parasites used in Equador vaccine
3. Antigen preparation (merthiolate vs heat killed),
4. Same antigen but different vaccine dose; e.g., single, double or triple injection of ALM+BCG in various trials,
5. Disease causing parasite and its epidemiology; e.g., *L. tropica* in Bam.
6. Study population; e.g., indigenous to an endemic area, age composition,

2.2.2.1 Analysis methodology

This study is a re-examination of previous efficacy vaccine trials and relies on conventional analytic techniques as well as meta-analysis as a technique to ascertain underlying relationships not observable in individual trials. Reviews provide an important tool for access to the results of multiple investigations. Systematic reviews are believed by many to allow more objective appraisal of previous research(Egger et al., 2005a). In addition to confirming previous results, systematic re-evaluation of studies could identify new findings and generate new research questions to be addressed in the future. Randomized, controlled clinical trials of the prophylactic efficacy of leishmaniasis vaccine candidates have been conducted since the 1970's. The results of these studies vary from trial to trial, owing to the particular circumstances, disease epidemiology and immunology, the vaccine candidate, etc. Meta-analysis would enable studying the results of these studies in concert, thus minimizing the "noise" due to the specific limitations of individual trials and helping to resolve if there are conflicting findings in some studies.

<u>Internal validity and sources of bias in clinical trials and the present investigation:</u>

Internal validity implies that differences between groups in a clinical trial are attributable to the treatment of interest, plus some random error (Juni and Egger, 2005). In conducting systematic reviews and meta analyses it is important to ensure that the design and implementation of the original studies were appropriately done to maximize internal validity and minimize selection bias, bias due to outcome assessment and attrition bias(Egger et al., 2005b;Juni and Egger, 2005).

By design, and in contrast to observational studies, double blind randomized controlled clinical trials, when well conducted, have an important safeguard against bias (Egger et al., 2005b).

The trials used in the present investigation have all been randomized, double blind controlled studies. Therefore, appropriate measures for minimizing bias have been taken in their design and conduct. In addition, most of these trials were done with collaboration and monitoring of WHO/TDR for adherence to protocol and measures of GCP (good clinical practice). Data management, progress reports and monitoring reports by TDR monitors were retrieved from WHO archives and studied to ensure trials were done appropriately according to the approved protocol.

The study in Zavareh (unpublished) was not done with formal sponsorship of TDR. However, TDR staff in charge of leishmaniasis were informed of the progress of the trial and maintained involvement. The conduct and procedures of this trial were discussed with the Center for Training and Research in Skin Diseases and Leprosy, Medical University of Tehran, Iran and the staff involved in performing and data management of the trial. The 1981 and 1983 studies by Mayrink were done independently of TDR. However they are accepted as landmark trials of leishmaniasis vaccines and generally accepted as having been appropriately designed and conducted. The second Armijos trial, also conducted with TDR involvement, suffered a lower than expected incidence rate which rendered the results inconclusive (since the power was calculated based on a much higher incidence rate). This trial was, therefore, not used in the present analysis.

Statistical approaches and considerations in meta-analysis:

Note - In the following explanation of meta-analysis models and effect estimation, reference will be made to a usual 2 by 2 table such as the following:

Study i	Event	No event	Group size
Vaccine	ai	bi	n1i
Control	ci	di	n2i

where i identifies clinical trial. In the present study, as in the original trials, "effect" is presented as relative risk (RR), where $RR = (a_i/n_{1i}) / (c_i/n_{2i})$.

* * *

Two general approaches to processing data for meta-analysis include random effect and fixed effect models.

1) Fixed effect model**:** The fixed effect model assumes that individual studies measure a single (hence, "fixed"), true effect, θ, which will be estimated by the meta-analysis pooled effect. Consequently, the differences between effects in individual trials should be due to random errors of measurement -- rather than a real difference in what they measure. Therefore, it is expected that variation in the measurement of the effect in different studies be comparable and reflect only chance. If variation between and within different studies are comparable and not widely different (i.e.,

studies are homogeneous), the fixed effect assumption is expected to hold. As a result, in calculating the common, or pooled, effect, the average of the statistic of interest (e.g., relative risk) is calculated by simply weighting individual study effects according to their trial size (i.e., weighting by the relative quantity of information provided by the trial). These weights can be calculated using a variety of methods, including inverse variance (I-V) and Mantel Haenszel (M-H) methods (Deeks et al., 2005).

Pooled effect estimation in the fixed effect model (inverse variance and Mantel-Haenszel methods):

The inverse variance method uses the inverse of the standard error (SE) of the effect measured in a study "i". With RR being the effect of interest where

$\theta_i = Ln(RR_i) = Ln((a_i/n_{1i})/(c_i/n_{2i}))$

SE of the logarithm of RR_i (or $LnRR_i$) is obtained by

$SE(Ln\ RR_i) = \sqrt{(1/a_i)+(1/c_i)-(1/n_{1i})- (1/n_{2i})}$

The weight calculated for individual effects is

$\omega_i = 1 / SE(\theta_i)^2$

Thus, since n_{1i} and n_{2i} in the denominator of SE transfer to the numerator of the weight, weighting by ω_i is equivalent of weighting individual effects by their respective sample size.

The pooled effect size is given by

$Pooled\ \theta = \sum \omega_i\ \theta_i / \sum \omega_i$

With SE given by

$SE\ (pooled\ \theta) = \sqrt{1/ \sum \omega_i}$

The heterogeneity statistic is given by

$Q = \sum \omega_i\ (\theta_i - pooled\ \theta)^2$

Mantel-Haenszel Method is another fixed effect method of estimation. In this method, similar to the inverse variance method, for each study, the effect size from each trial θ_i is given weight and the pooled effect is estimated as:

$Pooled\ \theta = \sum \omega_i\ \theta_i / \sum \omega_i$

Unlike inverse variance method, relative effect measures are combined in their natural scale, although their standard errors and confidence intervals are computed on the log scale. Weights for RR's are calculated as:

$\omega_i = c_i n_{1i}/N_i$

Standard error of log RR is given by:

$SE(\ln(RR) = \sqrt{P}/(R*S)$

Where

$P = \sum ((n_{1i}\, n_{2i})(a_i + c_i)-(a_i\, c_i\, N_i) / N^2_i); R = \sum ((a_i\, n_{2i}) / N_i); S = \sum ((c_i\, n_{1i}) / N_i)$

2) Random effect model: This model assumes the "true" effect to be not fixed, but an overall average effect with a normal distribution. Each individual study is assumed to measure a point in this distribution. Therefore, individual studies could vary due to where they target in this distribution. Consequently, compared to the fixed effect model, more heterogeneity between various studies is expected. Therefore, a greater variation than attributable to chance (i.e., greater variation between studies compared to the variation within studies) would indicate heterogeneity and the appropriateness of a random effect model. Due to the heterogeneity and the assumption that individual studies may not measure exactly the same thing, less emphasis (compared to the fixed effect model) is placed on size alone in weighting individual study results. This will place more emphasis on the differences between studies. A common method for calculation of the pooled effect in random effect analysis is the DerSimonian-Laird method as described below (Deeks et al., 2005).

Pooled effect estimation in the random effect model:

The random effect model assumes a normal distribution for the treatment effect with mean θ and variance τ^2. The DerSimonian-Laird estimate of τ^2 is as follows

$\tau^2 = (Q-(k-1)) / (\sum \omega_i - (\sum \omega_i^2 / \sum \omega_i)')$

Where Q is the heterogeneity statistic, with τ^2 set to zero if $Q<k-1$. The ω_i are calculated as in the inverse variance method, described above. As in the fixed effect model, the natural logarithm of the effect size (if the effect size is RR or OR) is used.

Individual study weights are given by

$\omega_i = 1/ (SE(\theta_i)^2 + \tau^2)$

As can be seen the main difference between the DerSimonian-Laired and the inverse variance methods is in the inclusion of τ^2 in the former. If τ^2 is zero, the two models would be equivalent. The pooled effect is given by

Pooled $\theta = \sum \omega'_i \theta_i / \sum \omega'_i$

with its standard error being

SE (pooled θ) $= 1/ \sum \omega'_i$

A measure of Heterogeneity in meta-analysis is I-squared that is based on Cochrane's Q. I-squared measures the amount of heterogeneity not explainable by chance (i.e, variablilty between studies) (Huedo-Medina et al., 2006). Values of I-squared range from 0% to 100%.

2.2.3 Thesis structure

This study is organized in the following chapters:

CHAPTER 1 - Background
CHAPTER 2 - Research objectives and methods
CHAPTER 3 - Efficacy of killed whole-parasite vaccines in prevention of leishmaniasis - a meta-analysis
CHAPTER 4 - Prophylactic efficacy of whole-parasite killed vaccines in demographic subgroups
CHAPTER 5 - Immunological response (measured by LST) in demographic subgroups to the leishmanial antigen introduced by vaccine or natural exposure
CHAPTER 6 - LST response as a correlate of immunity
CHAPTER 7 - Discussion
CHAPTER 8 - Conclusions and recommendations for further research

CHAPTER 3

EFFICACY OF KILLED WHOLE-PARASITE VACCINES IN PREVENTION OF LEISHMANIASIS - A META-ANALYSIS[2]

Most trials of leishmaniasis vaccines have demonstrated no prophylactic efficacy when comparing the vaccine and the control arms. However, the trial by Armijos et al (Armijos et al., 1998) suggested efficacy when comparing the two arms of the randomized trial of a locally produced tri-valent vaccine. In addition, reduction in infection rate in a subset of the randomized sample (in the vaccine arm) has been demonstrated in several trials. Studies by Antunes/Mayrink et al, Momeni et al and Khalil et al (Antunes et al., 1986;Khalil et al., 2000a;Momeni et al., 1999) have shown reduced incidence in the subset of participants whose LST results converted as a result of vaccination. Additionally, Sharifi (Sharifi et al., 1998) showed a significant protection in boys, but not in girls. On the therapeutic side, several trials have demonstrated the efficacy of these vaccines for treatment of leishmaniasis patients.

The objective of this analysis is to examine the results of all of these trials combined so that the effect of any possible specific adverse conditions or limitations associated with individual trials could be minimized. This objective will be addressed by first examining the data from all previous efficacy trials and continuing with the analysis of more homogenous subsets of trials to study efficacy in groups that are homogeneous with respect to a given factor (such as the parasite species used in the vaccine). The following 3 sets of analysis will be undertaken:

A. Efficacy of all vaccines overall: This will indicate the ability of leishmanial antigens, overall, in producing immunity and immune response, regardless of the species used in the vaccine.
B. Efficacy of different vaccine candidates prepared by various methods (parasite killed by heat, merthiolate, etc): L. major (against *L. major, L. tropica* and *L. donovoni*), *L. amazonensis* (against ACL), and re-examination of 3-antigen cocktail of *L. amazonensis*, *L. Guayanensis* and *L. Brasiliensis* (against ACL).
C. Contribution of adjuvant (comparison of vaccines with or without adjuvant(BCG or alum)

3.1 Description and comparison of trials

Tables 3.1a and 3.1b provide a summary description and comparison of various trials used in this study.

[2] This chapter has been submitted in part for publication in th Vaccine (see Annex)

Table 3.1a - Leishmaniasis first generation vaccine trials

Author	**Sharifi**	**Momeni**	**Sharifi**	**Khamesi-pour**	**Khamesi-pour**	**Khalil**	**Antunes, Mayrink**	**Antunes, Mayrink**	**Velez**	**Armijos**	**Armijos**
Year Published	**1998**	**1998**	**N/P**	**N/P**	**N/P**	**2000**	**1986**	**1986**	**2005**	**1998**	**2004**
Study designation	Bam1	Esf1	Bam3	Bor3	Zav3	Sudan2	Brazil 1981-2	Brazil 1983-2	Colombia3	Ecuador2	N/A
Background											
Country, Area	Iran, Bam	Iran, Esfahan	Iran, Bam	Iran, Borkhar	Iran, Zavareh	Sudan, Gedaref	Brazil, Amazonas	Brazil, Amazonas	Colombia	Ecuador	Ecuador
Year(s) study conducted	1994-1997	1994-1997	1997-2000	1997-2000	1997-2000	1997-1999	1981	1983	2001-2003	1995-6?	2002?
Targetted parasite causing local disease	*L. tropica*	*L. major*	*L. tropica*	*L. major*	*L. major*	*L. donovani*			*L. pnamensis, L. braziliensis*	*L. panamensis, L. basiliensis, L.amazonensis*	*L. panamensis, L. basiliensis, L.amazonensis*
Expected annual incidence in controls	2%	5%	2%	6%	6% ?	9%	10%-25%	10%-25%	5%	7.5%	3%
Number of volunteers screened	12156	4712	6524	5869 [a]	2053	5093	?	?	3018	?	4164
Trial Design											
Number of volunteers accepted and randomized	3637	2453	4217	2191	2008	2306	1312	1274	2597 [b]	1042	1995
N in vaccine arm (original, received complete vaccination schedule ---if known)	1839	1256, 1118	2149, 2082	1107, 964	945	1155	667	658	1302, 1252	552, 438	1009, 750
N in control arm (original, final)	1798	1197, 1122	2068, 2008	1084, 956	1063	1151	645	616	1295, 1251	487, 406	986, 756
originally planned (nominal) power	80%	80%	90%	90%	90%	90%	90%	90%	80%	90%	90%
Hypothesized vaccine effectiveness (expected %reduction in annual incidence)	50%	50%	50%	50%	50%	50%	50%	50%	50%	50%	50%
LST requirement for inclusion (LST=0 mm, LST<5 mm , etc)	LST=0 mm	LST=0 mm	LST=0 mm	LST≥ 0 mm	LST≥ 0 mm	LST=0 mm	LST negative	LST negative	LST<3 mm	LST< 5 mm	LST< 5 mm
Length of time post vaccination for LST testing	80 days, 1 yr	80 days, 1 yr	80 days, 1 yr	80 days, 1yr, 1yr+80 days	Not done	42 days, 1 yr	40-45 days	40-45 days	Not done	1 month	2 months
Number of vaccine injections	1	1	3	3	3	2	2	2	3	2	2
Time between vaccine injections	single dose	single dose	30 days	75 days, 1 yr	30 dqys	28 days	7 days	7 days	20 days	30 days	56 days
Dose of *Leishmania* antigen injected (each injection)	1 mg ALM	1 mg ALM	1 mg ALM	1 mg ALM	1 mg ALM	1 mg ALM	240 ug Nitrogen	240 ug Nitrogen	11.11 mg protein/mL	72 mill promast.	240 ug Nitrogen
Injection method	ID (0.1 mL)	ID (0.1 mL)	ID (0.1 mL)	ID (0.1 mL)	ID (0.1 mL)	ID (0.1 mL)	IM (1 mL)	IM 1(mL)	IM (1 mL)	ID (0.1 mL)	ID (0.1 mL)
Control treatment	BCG	BCG	BCG	BCG	BCG	BCG	PBS + merthiolate	PBS+ mrthiolate	Saline (ph=7.4)	BCG	BCG
Duration of follow up (months)	12+12	12+12		36	24	24	12	12	12	12+48	12+14
Type of case detection during follow up (A=active, I=inactive)	A/I	A/I	A/I	A/I	A/I	A/I	A	A	A	A	A

[a] 2671 of 5869 were qualified for randomization but 492 participated in phase I/II trial.

[b] Recruitment was done in 3 groups: Sept-Nov/01 (989), Jan-March/)2 (1131), Jul-Aug/02 (477)

Table 3.1a (continued) - Leishmaniasis first generation vaccine trials

Author	**Sharifi**	**Momeni**	**Sharifi**	**Khamesi-pour**	**Khamesi-pour**	**Khalil**	**Antunes, Mayrink**	**Antunes, Mayrink**	**Velez**	**Armijos**
Year Published	**1998**	**1998**	**N/P**	**N/P**	**N/P**	**2000**	**1986**	**1986**	**2005**	**1998**
Product Profile										
Parasite species in vaccine	*L. major*	*L. major*	*L. major*	*L. major*	*L. major*	*L. major*	*5 Leishmania strains* [g]	*5 Leishmania strains* [g]	*L. amazonensis*	*L. braziliensis, L. guyanensis, L. amazonensis*
Parasite killing method	Heat	Heat	Heat	Heat	Heat	Heat	Merthiolate	Merthiolate	Merthiolate	Phenol
Antigen origin	Iran	Iran	Iran	Iran	Iran	Iran	Brazil	Brazil	Brazil	Ecuador
Adjuvant	BCG	BCG	BCG	BCG	BCG	BCG	None	None	None	BCG
Vaccine Antigen manufacturer	Razi, Iran	Razi, Iran	Razi, Iran	Razi, Iran	Razi, Iran	Razi, Iran	Local	Local	Biobras	Local
Adjuvant (BCG) manufacturer	Pasteur, Iran	Pasteur, Iran	Pasteur, Iran	Pasteur, Iran	Pasteur, Iran	Pasteur, Iran	N/A	N/A	N/A	Tokyo
BCG concentration/injection	1/10 normal dose	1/10 normal dose	1/10 normal dose	1/10 normal dose	1/10 normal dose	1/10 normal dose	N/A	N/A	N/A	1/2 normal dose (500,000 organisms)
Leishmanin manufacturer	Pasteur, Iran	Pasteur, Iran	Pasteur, Iran	Pasteur, Iran	Pasteur, Iran	Pasteur, Iran	?	?	Colombian Nat'l Inst. of Health	Local
Leishmanin composition	*L. major*	*L. major*	*L. major*	*L. major*	*L. major*	*L. major*	?	?	*L. amazonensis, L. panamensis*	same as vaccine
Findings										
Yr 1: Observed incidence in controls	2.17% [c]	N/A [d]	3.77%	1.75%	1.70%	N/A [f]	11.07% (gr 1), 1.40% (gr 2)	1.30%	6.80%	7.60%
Yr 1: Observed incidence in vaccine arm	2.01% [c]	N/A [d]	3.16%	1.36%	1.40%	N/A [f]	8.70% (gr 1), 1.16% (gr 2)	0.61%	7.76%	2.10%
Yr 1: Efficacy (= 1-(% case vaccine arm/%case control arm)	7.4%	N/A	16.2%	22.3%	17.6%	N/A	21.4% (gr 1), 17.1% (gr 2)	53.1%	-14.1%	72.4%
Yr 2: Observed incidence in controls	1.2% [c]	N/A [d]	1.76%	4.04% [e]	10.70%	N/A [f]	N/A	N/A	N/A	9.19% h
Yr 2: Observed incidence in vaccine arm	0.83% [c]	N/A [d]	1.68%	4.67% [e]	11.40%	N/A [f]	N/A	N/A	N/A	4.41% h
Yr 2: Efficacy	30.8%	N/A	4.5%	-15.6%	-6.5%		N/A	N/A	N/A	52.0%
Volunteers endemic origin	Low endemic	Mixed (army base)	Low endemic	Endemic	New endemic	different villages	Mixed (army concripts)	Mixed (army conscripts)	Mixed (army)	Endemic
Age (range, mean)	6-15, 9.1	5-72, 18.2	6-12, 7.41	6-13, 8.45	5-59, 19.12	1-65, 6.9	18.6	18.6	>18, 19.8	approx mean 5.6
Sex (% male)	50.60%	47.40%	50.80%	50.70%	46.80%	45.70%	100%	100%	100%	44.16%
Protection observed in the overall sample	No	No	No	No	No	No	No	No	No	Yes
Protection in those with converted LST after vaccination	No	Yes	No	No	Not done	Yes	Yes	No	Not done	N/A
LST conversion rate (per protocol) 40-80 days post vaccination (vaccine arm, Controle arm)	16.5%, 3.2%	36.2%, 7.9%	18.2%, 2.0%	29.9%, 6.1% (Received 2 doses year 1)	Not done	30%, 7%	33%	70%	Not done	85.1%, 20.1%

(Footnotes on the next page)

Bor3, Zav3, Bam3 findings are ITT. Other trials from published information.

[c] Cumulative 2-year incidence: Vacc=2.8%, BCG=3.3%, overall efficacy=15%

[d] Annual rate not reported. Cumulative 2-year incidence: vacc=18.0%, BCG=18.5%, overall efficacy=3%

[e] Third year incidence in Bor3: 6.64% (Control), 6.44% (Vaccine)

[f] Annual rate not reported. Cumulative 2-year incidence: vacc=11.5%, BCG=12.3%. Overall efficacy=6%

[g] Brasiliensis and mexicana complexes

[h] Reported by Armijos 2003

3.1.1 Demographic composition

The demographic make up of trials used in this analysis are presented below:

Table 3.1b - Age and sex distribution in clinical trials used in the meta-analysis

Study	Bam1		Esf1		Bam3		Bor3		Zav3		Sudan2		Brazil1981 - 2		Brazil 1983 - 2		Colombia3		Ecuador2	
Trial arm	**V ***	**C ***	**V**	**C**	**V**	**C**	**V**	**C**	**V**	**C**	**V**	**C**	**V**	**C**	**V**	**C**	**V**	**C**	**V**	**C**
N	1838	1795	1190	1124	2149	2068	1107	1084	941	1055	1155	1151	667	644	658	616	1302	1295	438	406
Age (yrs)																				
Minimum	6	6	5	5	6	6	6	6	5	5	<3	<3								
Maximun	15	15	67	72	12	12	13	13	59	59										
Mean	9.1	9.1	18.0	18.8	7.4	7.4	8.2	8.7	19.0	19.2	6.5	7.2	18.6	18.6	18.6	18.6	19.8	19.8	5.4	5.7
P value (Kruskal-Wallis H)	0.822		0.118		0.132		0.000		0.666		0.010		--		--		--		0.271	
SEX																				
% Female	47.1	51.8	52.7	52.6	48.8	49.6	46.3	52.4	50.2	48.9	53.3	55.3	0.0	0.0	0.0	0.0	0.0	0.0	57.5	54.3
P value (Fisher Exact)	0.003		0.496		0.302		0.002		0.323		0.599		N/A		N/A		N/A		0.349	

* V=vaccine arm, C=control arm

Note: The Kruskal-Wallis test and associated probabilites test the equality of mean for age

Trials in Brazil and Colombia (4 out of 11 trials), were done strictly in male soldiers, leaving the Ecuador trial as the only South American study with female+male participants. Other trials include a mix of gender and age.

3.1.2 Vaccine immunogenicity (DTH) in various trials

LST measured 42 to 80 days post-vaccination (depending on the trial) in vaccine and control arms in various trials are displayed in the table 3.2. LST induration is an inexact indication of the intensity of immune response (DTH) to the antigen--i.e., the ability of the vaccine to produce a cell mediated immune response. LST induration equal to or greater than 5 mm is generally accepted as positive response and an indication of significant immunogenicity.

The table indicates that LST conversion is more frequently observed in the vaccine arms of trials. It is also observed in control arms in a small percentage of participants as a result of BCG injection (if BCG is used as placebo) or possibly natural exposure. As can be seen, post vaccination LST was not conducted in all trials.

It should be pointed out that the Brazil study in 1981 by Mayrink, Antunes et al (results reported along with 1983 trial results by Antunes et al. 1986), was conducted in two separate cohorts. These cohorts were different with respect to observed incidence, duration of exposure, previous vaccination history, etc. In the present analysis, these two cohorts are treated as two separate studies (identified as Brazil 1981A-2 and Brazil 1981B-2) due to the differences in trial conditions and participants' vaccination history (Antunes et al., 1986). This is reflected in the following table.

Table 3.2 - LST conversion 42-80 days post-vaccination in initially LST negative individuals

Post vaccination LST	Trial arm	N	% LST ≥5 mm
Bam1	V *	1807	16.5
	C *	1761	3.3
Esf1	V	1168	36.2
	C	1104	7.9
Bam3	V	1980	18.2
	C	1935	2
Bor3	V	608	29.9
	C	538	6.1
Zav3	V	772	--
	C	901	--
Sudan2	V	1919	30
	C	1005	7
Brazil1981A-2	V	311	33
	C	--	--
Brazil1981B-2	V	338	37
	C	--	--
Brazil 1983-2	V	611	68
	C	--	--
Colombia3	V	--	--
	C	--	--
Ecuador2	V	--	--
	C	--	--

* V = vaccine arm, C = control arm

-- = LST not measured

Note: In most of the listed trials an exclusion criterion of LST>0 mm or, at least, LST negative (e.g., LST>3 in Colombia) was used. However, in Bor3 and Zav3, LST was not an exclusion criteria and volunteers with any values of LST were enrolled in order to compare the vaccine effect in those with or without previous exposure. To address the current objective of overall vaccine efficacy, participants in Bor3 and Zav3 with LST>0 mm were excluded for consistency with other trials and to be able to isolate the effect of the vaccine from that of previous natural exposure.

3.2 Efficacy of all vaccines overall

This analysis combines previous trials, regardless of vaccine formulation (e.g., the species of the parasite used as the antigen, the use of adjuvants, etc), or the epidemiology of the local disease (species of the parasite causing the disease, incidence rate, etc). As mentioned, Zav3 and Bor3 participants with pre-vaccination LST>0 mm were excluded from this analysis (12.2% of Zav3 and 40% of Bor3). Trial results are summarized in table 3.3.

Table 3.3 - Results of efficacy clinical trials over the entire follow-up period

Study	Follow-up (months)	Vaccine Total N	Vaccine Cases	Control Total N	Control Cases	% Case (vaccine)	% Case (control)	Vaccine efficacy
Bam1	24	1838	52	1795	60	2.83	3.34	15%
Esf1	24	1190	214	1124	208	17.98	18.51	3%
Bam3	24	2082	81	2008	93	3.89	4.63	16%
Bor3	36*	604	64	561	63	10.60	11.23	6%
Zav3	24	742	102	868	109	13.75	12.56	-9%
Sudan2	24	1155	133	1151	141	11.52	12.25	6%
Brazil 1981A-2	12	322	28	289	32	8.70	11.07	21%
Brazil 1981B-2	12	345	4	356	5	1.16	1.40	17%
Brazil 1983-2	12	658	4	616	8	0.61	1.30	53%
Colombia3	12	1302	101	1295	88	7.76	6.80	-14%
Ecuador2	12	333	7	316	24	2.10	7.60	72%

* Although there were 3 years follow up, only cases from years 2 and 3 are included in this analysis because vaccination was completed end of yr 1.

More details about the conduct of these trials are available in tables 3.1a and 3.1b, presented earlier (table 3.1a).

3.2.1 Meta-analysis results

The hypothesis to be tested:

H0: RR(in pre-vaccination LST=0 participants, all trials combined) = 1
H1: RR(in pre-vaccination LST=0 participants, all trials combined) ≠ 1

To test this hypothesis, the "metan" program in Stata 9 was used to calculate relative risk estimates and corresponding 95% confidence intervals. The inverse variance (I-V) and the Mantel-Haenszel (M-H) methods were used separately to fit the fixed effect model and the DerSimonian-Laird (D+L) method to fit the random effect model. The

RR values, associated confidence intervals and the weights assigned by each estimation method are presented in the following table.

Table 3.4 - Relative risks, 95% confidence intervals and meta-analysis weights

					Weight (%)		
Study	**N**	**RR**	**95% Conf. Int**		**I-V**	**M-H**	**D+L**
Bam1	3633	0.846	0.587	1.22	6.32	7.26	8.33
Esf1	2314	0.972	0.818	1.155	28.37	25.59	19.66
Bam3	4090	0.84	0.628	1.124	9.94	11.32	11.41
Bor3	1165	0.944	0.679	1.31	7.84	7.81	9.72
Zav3	1610	1.095	0.851	1.408	13.34	12.02	13.66
Sudan2	2306	0.94	0.753	1.174	17.08	16.89	15.63
Brazil 1981A-2	611	0.785	0.485	1.271	3.64	4.03	5.39
Brazil 1981B-2	701	0.826	0.224	3.049	0.49	0.59	0.86
Brazil 1983-2	1274	0.468	0.142	1.547	0.59	0.99	1.02
Colombia3	2597	1.142	0.867	1.503	11.15	10.55	12.27
Ecuador2	649	0.277	0.121	0.633	1.23	2.95	2.05
					100	100	100

The weight assigned to the Ecuador trial varies substantially (by about 100%) from the IV method to the D+L. This reflects the tendency of the fixed effect model to give smaller weights to smaller trials.

The heterogeneity statistics estimated by the three methods are very similar, as indicated by the following table. The p values associated with the heterogeneity chi square statistics in all methods are >0.05. These results suggest that the heterogeneity among the trials is minimal and results are consistent with a fixed model. Comparing the RR's (table 3.4) from the Old World trials with those of the New World trials shows that most of the heterogeneity is due to the latter group. However as the weights in table 3.4 indicate, there is very little difference in the estimation of the pooled RR by the three methods.

Table 3.5 - Heterogeneity statistics in the 3 methods of meta-analysis

	Heterogeneity			
Method	**Chi square**	**d.f.**	**p**	**I-squared**
I-V	14.59	10	0.148	31.50%
M-H	14.62	10	0.146	31.60%
D+L	14.62	10	0.146	31.60%

I-square is a measure of heterogeneity not explainable by chance, ranging from 0% to 100%

Pooled RR estimates and the 95% CI estimated by the 3 methods (table 3.6) are very similar regardless of the model used and lead to similar conclusions. Overall, the hypothesis of RR=1 (i.e., no efficacy) can not be rejected.

Table 3.6 - Meta-analysis pooled estimates of RR and confidence intervals

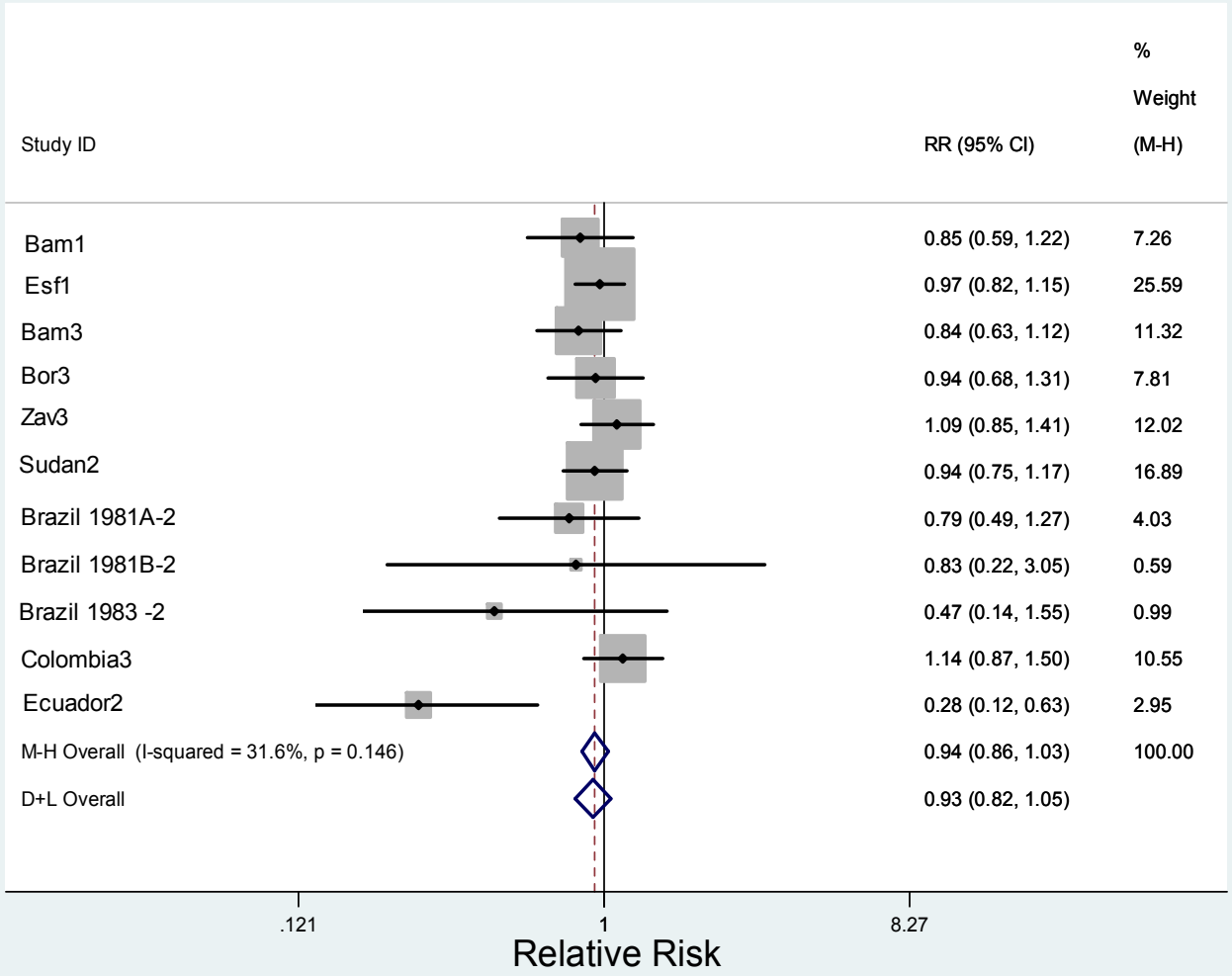
Method	Pooled RR	95% Conf. Int		Test of RR=1 z value	P
I-V	0.947	0.864	1.038	1.16	0.246
M-H	0.939	0.857	1.029	1.34	0.179
D+L	0.928	0.821	1.049	1.2	0.231

Therefore, the hypothesis that the overall effect is not different from 1, i.e.,

H0: RR(in pre-vaccination LST=0 participants, all trials combined) = 1 cannot be rejected.

The "forest plot" in figure 3.1 presents a graphical comparison of the results of these trials and the pooled effect. Gray square boxes represent the relative size of each trial with the centre dot and the line in the centre of each square representing the RR and its 95% CI. The overall RR is depicted by two blank diamond boxes, representing the M-H and the D+L estimates.

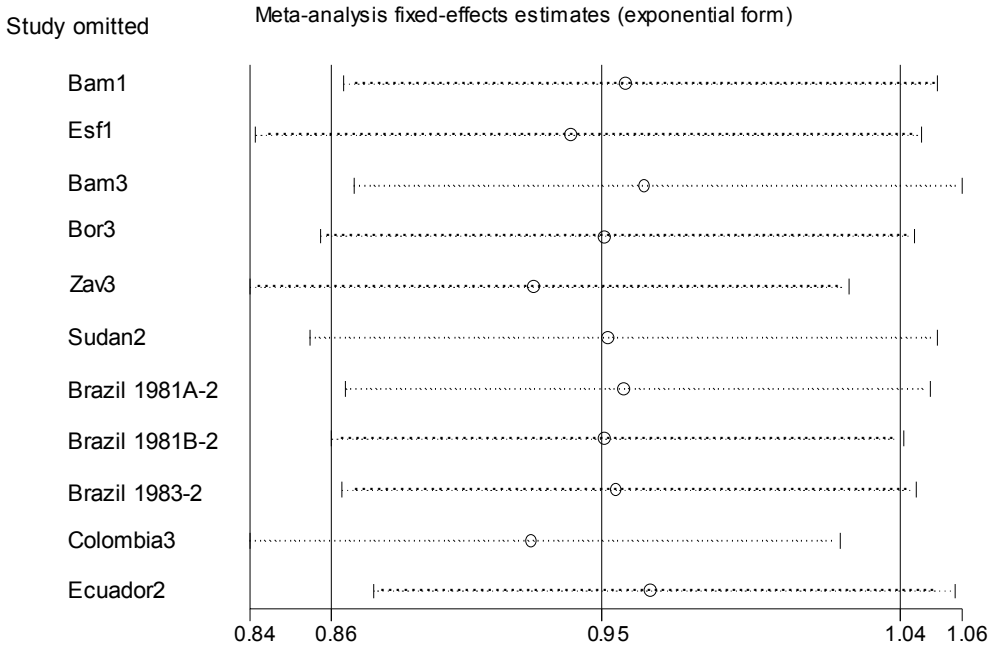
Figure 3.1 - Forest plot of vaccine efficacy measure in different leishmaniasis vaccine trials



The forest plot summarizes the information in the previous tables. While Old World trials are clustered around the vertical line of RR=1 (i.e., homogeneous but with minimal efficacy), South American trials tend to be scattered on the left of that line, suggesting more heterogeneity but also more efficacious results. The Ecuador trial, the only trial with significant results, is located in the far left of the forest plot. Despite their lower individual RR values, these trials have limited impact on the pooled RR due to their smaller sample sizes. Other statistics, such as the heterogeneity statistics and the pooled RR (with 95% CI) are also depicted on the graph.

A graphical display of the influence of individual trials on the pooled RR is presented in the following figure. This graph shows the values of the pooled RR, when studies are omitted one at a time. The reference line in the centre is the overall, pooled RR. Thus, the pooled RR when Esf1, Zav3 or Colombia3 trials are omitted tends to the left of the overall estimate. In other words, these trials have relatively strong influence on bringing the overall RR closer to 1. Both Bam trials, Brazil1981A-2 and Ecuador2 have a favourable influence on the pooled estimate.

Figure 3.2 - Influence of individual trials on pooled RR

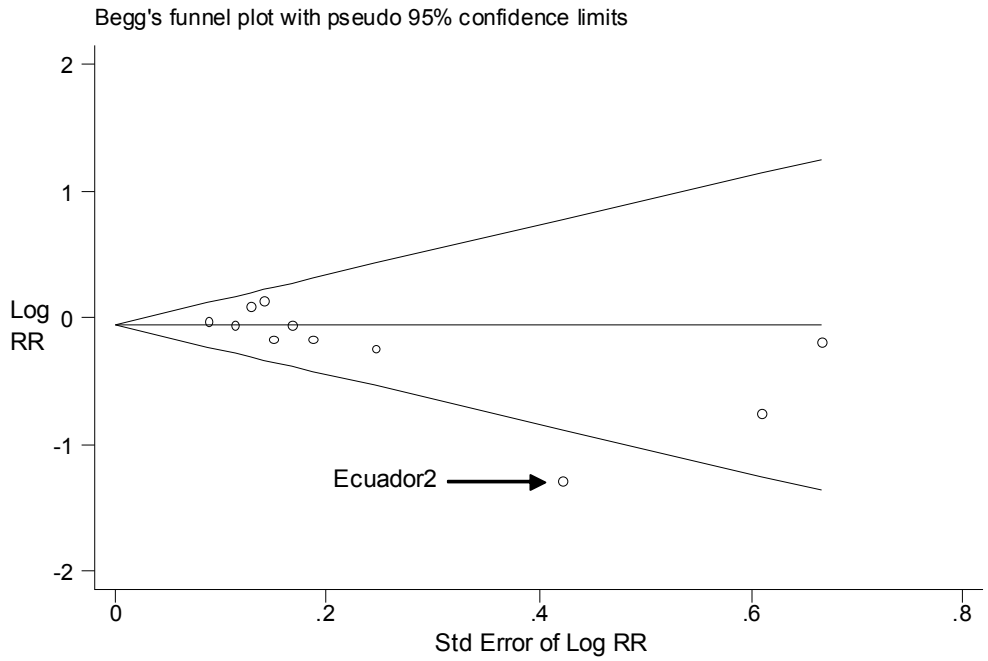


The above graph and the funnel plot in figure 3.3 suggests the RR from the Ecuador trial to be somewhat out of range for its given study size and variability. Additionally figure 3.3 Additionally, the downward trend of all points in figure 3.3 may indicate a small bias (better vaccine effect in smaller studies).

A statistical analogue of a funnel plot is Egger's test which is a regression-based approach to assessing bias (symmetry of the plot). It consists of a weighted regression of the effect size on standard error: $RR = b_0 + b_1 s$ where weights are inversely proportional to the variance of the effect. However, simulations by (Sterne et al., 2005) have demonstrated low sensitivity for this test when the number of trials

is less than 20 or the bias is not substantial. Therefore, the results of Egger's test are not reported here.

Figure 3.3 - Funnel plot of leishmaniasis vaccine trials



3.3 Efficacy related to different vaccine formulations and causative parasite

In further analysis of these vaccine trials it should be considered that they were conducted to test the efficacy of different vaccines based on different antigens, against different causative agents and with or without BCG. It is reasonable allow for these sources of difference by studying groups of more similar trials with a common vaccine formulation and/or epidemiological setting.

Two sets of analyses will be conducted in groups of trials that are similar with respect to antigen/vaccine formulation and causative parasite.

3.3.1 Subgroup analysis

3.3.1.1 Analysis based on vaccine formulation:

The candidate vaccines that were in trial in the Old World and the New World were different on a number of characteristics. Chemical inactivation of the parasite was the common method in vaccine preparation in the New World trials. In contrast, those in the New World were all heat killed (see the trial comparison table). Another distinction between the Old World and the New World vaccines was the parasites species used as the antigen in the vaccine. *L. amazonensis* was the common parasite used in all South American vaccines (in Ecuador it also included *L. braziliensis* and *L. guyanensis*) usually without adjuvant (except in Ecuador), while *L. major* plus BCG constituted the Old World vaccines. Consequently, differences in the results of the Old World and the New World trials could be due to one or more of such factors that are in effect simultaneously. Due to this confounding, it is not practical to separately

assess the effect of parasite species and the method of inactivation. But it is possible to study the two effects in combination. In this analysis, the symbols NW (New World) and OW (Old World) will be used to identify the two groups of trials.

As an exploratory step, a regression analysis was conducted to investigate whether the effect size in the OW vaccines is significantly different from that in the NW (metareg command in Stata's metan program). The regression model was set up as: LnRR= f(vaccine formulation), i.e., natural log of RR regressed on a binary variable indicating Old World and New World. Results, presented below, suggest no significant difference in RR due to vaccine formulation.

Table 3.7 - Regression results: LnRR= f(vaccine formulation)

	Coef.	Std. Err.	z	p	95% Conf. Int.	
Vacc formulation	0.04	0.12	0.34	0.73	-0.20	0.29
Constant term	-0.09	0.11	-0.79	0.43	-0.31	0.13

At the next step, OW and NW trials were meta analysed separately to investigate RR in each set of trials.

3.3.1.1.1 New world (NW) trials:

The hypothesis to be tested is formulated as:

H0: RR(in pre-vaccination LST=0 participants, NW trials combined) = 1
H1: RR(in pre-vaccination LST=0 participants, NW trials combined) ≠ 1

The following table present the results of NW trials:

Table 3.8 - South American trial results and meta-analysis weights

Study	N	RR	95% Conf. Int.		Weight (%)	
					I-V	D+L
Brazil 1981A-2	611	0.785	0.485	1.271	21.28	26.77
Brazil 1981B-2	701	0.826	0.224	3.049	2.89	10.85
Brazil 1983-2	1274	0.468	0.142	1.547	3.46	12.22
Colombia3	2597	1.142	0.867	1.503	65.16	31.71
Ecuador2	649	0.277	0.121	0.633	7.21	18.45

The heterogeneity observed in the results of these trials is as follows:

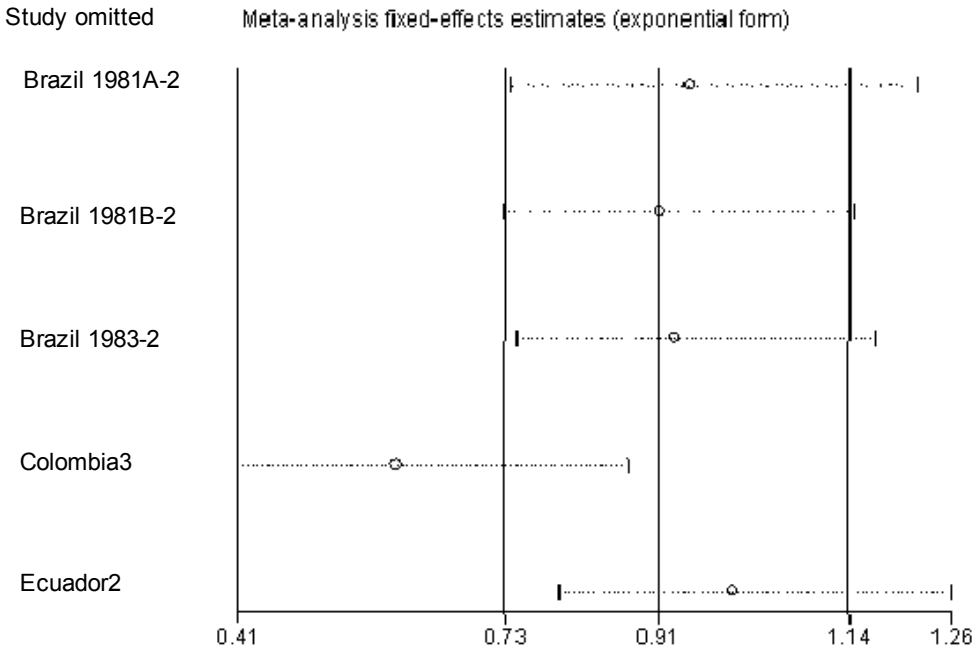
Table 3.9 - Heterogeneity in South American trials

Method	Heterogeneity Chi sq	p	I - squared
I-V	12.12	0.017	0.67
D+L	12.18	0.016	0.672

These statistics point at the significant heterogeneity in NW trials. This heterogeneity stems primarily from the Colombian trial with the smallest effect and largest sample

size. This heterogeneity is demonstrated by the following table. As a result of its sample size, in the meta-analysis of these trials, the Colombia trial sways other trial results in its own direction.

Figure 3.4 - Influence by Colombia trial



Due to the sizeable difference between Colombia and other trials' results, the overall RR is not statistically significant based on the random effect model (which is the appropriate model, given the large heterogeneity) or even the fixed effect model (table 3.10). Therefore, the hypothesis that the RR in all NW trials is not different from 1 can not be rejected.

Table 3.10 - Meta-analysis results for South American trials (all trials)

	Pooled			Test of RR=1	
Method	**RR**	**95% Conf. Int.**		**z**	**p**
I-V pooled RR	0.914	0.732	1.142	0.79	0.43
D+L pooled RR	0.688	0.41	1.156	0.141	0.158

Excluding the Colombia trial reduces the heterogeneity to non-significant levels and leads to statistically significant pooled RR based on the trials in Ecuador and Brazil, as follows:

Table 3.11 - Meta-analysis results for South American trials (Colombia excluded)

	Pooled			Test of RR=1	
Method	**RR**	**95% Conf. Int.**		**z**	**p**
I-V	0.604	0.414	0.88	2.63	0.009
M-H	0.576	0.398	0.834	2.93	0.003
D+L	0.551	0.315	0.966	2.06	0.037

As indicated by the above results of three estimation models, exclusion of the large trial in Colombia renders the combined result of other trials in South America significant. This approach is directed by the heterogeneity stemming from the Colombia trial. However, it should be kept in mind that this trial was a large, well managed, internationally sponsored trial and its exclusion could introduce an unknown bias. Furthermore, it could be argued that in most circumstances, by exclusion of trials with effect values in the opposite direction of other trials, one could finally reach a subset with statistically significant combined effect; an unjustifiable approach. One trial-specific situation that could provide an explanation for the difference between Colombia and other trials may be the possibility of ineffectiveness *L. amazonensis* in creating cross immunity with *L. panamensis*, an important causative species in Colombia. There is some evidence epidemiologically that those infected with *L. amazonensis* are still susceptible to infection by *L. panamensis* (R. Killick-Kendrick, personal communication). Another explanation may be that in the Colombia trial, by design, volunteers with LST<3 mm at the time of screening were enrolled. To the extent that such reactive LST (LST>0) is linked to endemic immunity, the study power would be compromised and efficacy estimate biased downward. This extent is unknown and the individual level data to identify the number of LST reactive participants was not available for verification. Therefore, at the current level of information, this analysis provides some basis for further exploration of the difference between Colombia and other trials.

3.3.1.1.2 Old world (OW) Trials: The OW trial set was studied to see if a strong homogeneity and/or influence of a particular trial is a possible source of overall low efficacy. No evidence of significant heterogeneity or influence was observed, with the M-H heterogeneity chi square of 2.36 (p= 0.797). H-M Pooled RR = 0.951 (p=0.330). Therefore, no further breakdown of the OW trials into more homogeneous sets seems to change the results.

3.3.1.2 Analysis based on causative *Leishmania* species:

The causative parasite between South America trials (*L. braziliensis, L. Mexicana or L. Peruviana* complexes), Iran trials (*L. major or L. tropica*) and the Sudan trial (*L. donovani*) are distinct. Multiple parasite species could cause the disease in each location in South American trial sites and there is not enough information to separate the trials into homogeneous subsets with respect to the causative parasite. Therefore, the NW set can not be analysed. The OW trials, on the other hand provide a better opportunity for this analysis since there are multiple trials with the same parasite species causing the disease: two trials in Bam, where the majority of cases are due to *L. tropica*, and three in Esfahan, Borkhar and Zavareh, where the disease is due to *L. major*. Since there is only one trial of *L. donovani* in Sudan, it can not be further analysed.

Tables 3.12 and 3.13 display the results of pooling of Bam and Esfahan/trials: The heterogeneity chi square for the both sets of analysis have p>0.05.

Table 3.12 - Bam1 and Bam3 trials

Method	Pooled RR	95% Conf. Int.		Test of RR=1 z	p
M-H pooled RR	0.843	0.671	1.058	1.47	0.141

Table 3.13 - Esf1, Bor3, Zav3 trials

Method	Pooled RR	95% Conf. Int.		Test of RR=1 z	p
M-H pooled RR	0.999	0.877	1.139	0.01	0.993

From the above analyses, it is concluded that the breakdown of the data into sets with common causative parasite does not improve efficacy results, although the RR in Bam trials seems to be slightly better.

Consistent results are reached by a regression analysis of lnRR regressed on a binary variable with 0 for *L. major* and 1 for *L. tropica*, as follows (table 3.14):

Table 3.14 - Regression results of Bam1, Bam3, Bor3, Esf1, Zav3 trials

	Coef.	Std. Err.	z	p	95% Conf. Int.	
parasite	-0.17	0.13	-1.27	0.20	-0.43	0.09
constant	0.00	0.07	-0.02	0.99	-0.13	0.13

3.4 Contribution of adjuvant (analysis based on adjuvant)

The role of BCG in induction of the Th1 immune response is well known and its presence in leishmaniasis vaccines is believed to play a role in the vaccine immunogenicity (Alimohammadian et al., 2002). Although BCG led to LST conversion in a percentage of trial participants, the conversion was generally not long lasting. The down side of using BCG as an adjuvant is the requirement to use it as the control treatment if double blinding is desired. Double blinding with BCG and the effect of BCG in LST conversion and (possibly) a certain level of resulting protection could make it difficult to see the relationship between LST conversion and vaccine induced immunity (Armijos et al., 1998). To address the question of the role of BCG in protection, trials with and without BCG adjuvant will be compared for induced protection.

Table 3.15 depicts the results of regressing the LnRR on a binary variable named "adjuvant" with 0 for trials without BCG and 1 for those with it. A significant model coefficient would indicate a relationship beyond chance:

Table 3.15 - Meta regression results of Bam1, Bam3, Bor3, Esf1, Zav3 trials

	Coef.	Std. Err.	z	p	95% Conf. Int.	
adjuvant	-0.07	0.13	-0.53	0.59	-0.32	0.18
constant	0.00	0.12	0.03	0.98	-0.23	0.23

As the table demonstrates, neither the model slope nor the constant are statistically significant ($p > 0.05$). Therefore the hypothesis of "no relationship" cannot be rejected.

3.5 Summary: As discussed in Chapter 1, with the exception of the trial in Ecuador no other efficacy trials had shown significant protection attributable to any of the leishmaniasis vaccines, when comparing the entirety of the two randomized trial arms. The forgoing analysis combines the results of all efficacy trials to go beyond limitations of individual trials. Potentially, many problems and limitations could adversely affect the results of individual trials. Problems such as lower-than-expected incidence, misclassification of exposed/protected people as unexposed and occasional problems with the conduct of a trial could have a negative impact on a given trial's statistical power. Other problems such as the length of the required follow-up period could also affect the final sample size and power. Combining the results of trials in a meta-analysis framework and simultaneously analysing them could help bypass problems with individual trials.

All studies were first combined for an overall analysis without accounting for differences in their vaccine antigen, use of adjuvant, causative parasite, etc. Next more homogeneous trials (based on the vaccine type and causative parasite species) were grouped and analysed separately. The efficacy results of the overall analysis of all trials combined were not different from the results of individual studies; i.e., no efficacy. Similarly, grouping trials by their antigenic content (separately for *L. major* and *L. amazonensis* vaccines) did not suggest significant protection. However, when considering *L. amazonensis* vaccine trials (i.e., all those in South America), the exclusion of the largest clinical trial, a TDR-sponsored study conducted in Colombia, and leaving the Ecuador and the Brazil trials in the analysis, yielded significant efficacy results. This approach could be disputed since it ignores the information from the largest trial in South America. Additionally, it could be viewed as an arbitrary exclusion of a trial. The argument in favour of this approach is that, the Colombia trial could act as the centre of gravity to all trials, drawing their results in its own direction by imposing possible trial specific limitations. This, coupled with the possible ineffectiveness of *L. amazonensis* in producing cross immunity against *L. panamensis* provide a justification for excluding the Colombia trial. Additionally, the Colombia trial included a number of participants with pre-vaccination LST between 0 and 3 mm. If any protection could arise as a result of the pre-vaccination LST>0, then this would have had an effect on the study power. In light of this observation, it may be worthwhile to further explore the Colombia trial results and possibly the *L. amazonensis* potential for vaccine development.

Grouping trials by other factors such as the causative parasite (*L. tropica* or *L. major*) or inclusion of BCG as adjuvant did not lead to significant efficacy results.

CHAPTER 4

INCIDENCE AND PROPHYLACTIC EFFICACY OF KILLED WHOLE-PARASITE VACCINES IN DEMOGRAPHIC SUBGROUPS

It is generally believed that in the context of leishmanial infection, immunity is correlated with previous/repeated exposure. This is also seen in other diseases such as malaria where, in endemic areas, a form of acquired immunity develops with continuous/repeated exposure. Demographic characteristics, primarily age and gender, are associated with behaviour that could augment the risk of exposure and/or infection. Playing and working outdoors, sleeping habits, coverage of the body and so on, are all age/gender related behaviour and can affect risk in an endemic area. Additionally, age is directly related to the length of exposure and accumulated likelihood of infection or immunity. Genetic differences between males and females could also have an impact on response to the leishmanial antigen. Consequently, it is important to investigate vaccine efficacy separately in demographically homogeneous sub-groups. The demographic variables of primary importance which have been measured in all leishmaniasis vaccine trials are age and gender. Other variables such as school and grade, also collected in some studies, highly correlate with these two. The focus of this analysis is assessing efficacy in various groups of age and gender.

The studies in Colombia and Brazil included only male participants of around 18-20 years old (soldiers) and did not present age/gender variety. The Sudan trial data were not available for individual participant level analysis and consequently was not used in the analyses in this chapter. Because participant-level data were available for trials conducted in Iran, including Bam1, Bam3, Bor3, Esf1 and Zav3, these trials were used to address the objectives of this and subsequent chapters. Additionally, these trials provided a better mix of age and gender. The added advantage was that they all evaluated the efficacy of the same vaccine (ALM+BCG) against BCG.

The overall hypothesis is formulated as:

H0: The studied FGV's are not able to confer protection even in specific subgroups
H1: The studied FGV's lead to reduction in incidence in certain sub-groups

This hypothesis will be tested by assessing vaccine (ALM+BCG) efficacy in the subgroups identified by demographic variables age, gender and endemic/non-endemic origin of participants.

Three of the trials used in this analysis (Bam1, Bam2, Bor3) included school age participants, 6-13 or 15 years. Zav3 and Esf1 included participants as young as 5 or as old as 59 or 72 years. Tables 4.01 and 4.02 show the distribution of age and gender in these trials combined.

Table 4.01 - Age distribution in Iran trials combined

Age category	Frequency	%
1 = (5-6 yrs)	1420	10.3
2 = (7 yrs)	2711	19.6
3 = (8 yrs)	2797	20.2
4 = (9-10 yrs)	3142	22.7
5 = (11-15 yrs)	2169	15.7
6 = (16-25 yrs)	517	3.7
7 = (> 26 yrs)	1069	7.7
Total	13825	100

Table 4.02 - Gender in Iran trials combined

Gender	Frequency	%
F	6898	49.9
M	6937	50.1
Total	13835	100

Although all trials were conducted in *Leishmania* endemic areas, they did not all include participants from endemic origins, as explained below.

Esf1: Esfahan is an *L. major* endemic area. However, participants in the trial consisted not of native residents but of army families in an airbase. Their origins, therefore, were from diverse areas, many coming from non-endemic areas.

Zav3: Participants were Zavareh residents. Zavareh is a new *L. major* endemic focus (or possibly the site of recent epidemics). Its endemicity started with an epidemic, after which cases have been identified on a regular basis. Therefore, residents are essentially from a non-endemic background, though living in a newly endemic area.

Bor3: Borkhar is an established *L. major* focus and participants were native area residents.

Bam1, Bam2: Bam is an established endemic focus of *L. tropica*. Trial participants were native area residents.

The distribution of the endemic/non-endemic origin of participants for the combined sample is as follows:

Table 4.03 - Endemic origin in Iran trials combined

Endemic Origin	Frequency	%
No	3992	28.9
Yes	9843	71.1
Total	1385	100

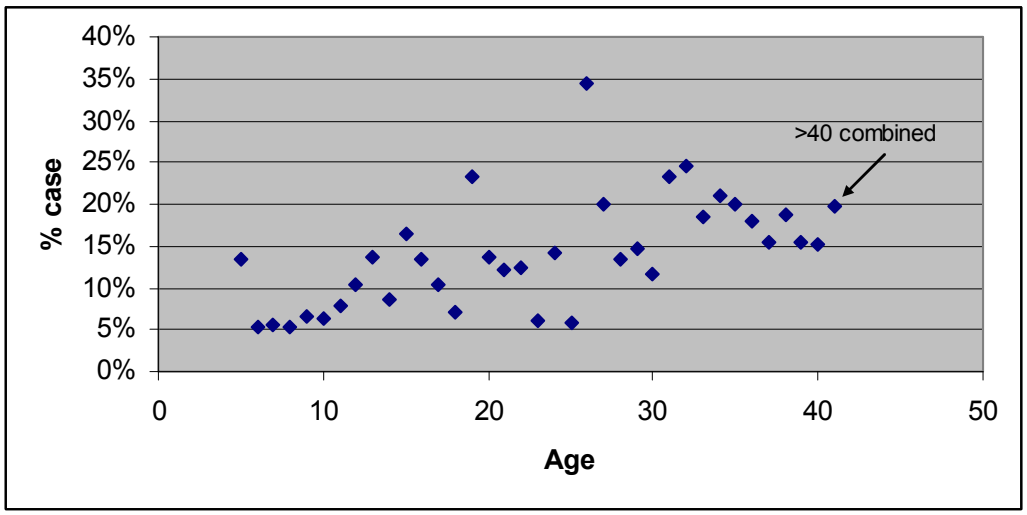
4.1 Age, incidence and vaccine efficacy

It would be helpful to explore the age-incidence relationship before addressing the age-efficacy question.

* * *

To study the effect of age on incidence data from all trials are combined in Figure 4.E (below). However, the apparent increased incidence with age may be spurious. Combining the data in this form is misleading since Zav3 and Esf1, the only trials with participants older than 16, were also the trials with highest incidence. Incidence in higher ages would only reflect the overall high incidence in these two trials. For this reason, combining all data, would lead to the significant, but biased, relation between efficacy and age (with significance level < 0.000).

Figure 4.E - Spurious relationship: % Case by age of participants



The appropriate method for this analysis would be comparing age-incidence distribution within the different trials. For estimating the age-related vaccine efficacy a meta analytic approach, similar to the overall efficacy analysis described in Chapter 3 could be used.

* * *

In studying the age/incidence distribution, it is important to note that two factors work against detecting the effect of age on incidence:

- Exposure (and resulting endemic immunity) increases with time/age and is manifested by pre-vaccination LST>0 (as will be indicated in the next chapter). As a result, eliminating LST>0 volunteers would minimize or remove from the trial sample the effect of age on exposure, endemic immunity and consequently incidence (in participants of endemic background). By design, LST>0 volunteers were not enrolled in Bam1, Bam3 and Esf1. In this chapter, to be able to see the effect of age on incidence, participants with pre-vaccination LST>0 in Bor3 and Zav3 have been included in most analyses.
- Similarly, limiting age to less than 15 years (Bam1, Bam3, Bor3) would take away from the effect of age on incidence since older individuals would be the ones with longer exposure and higher endemic immunity.

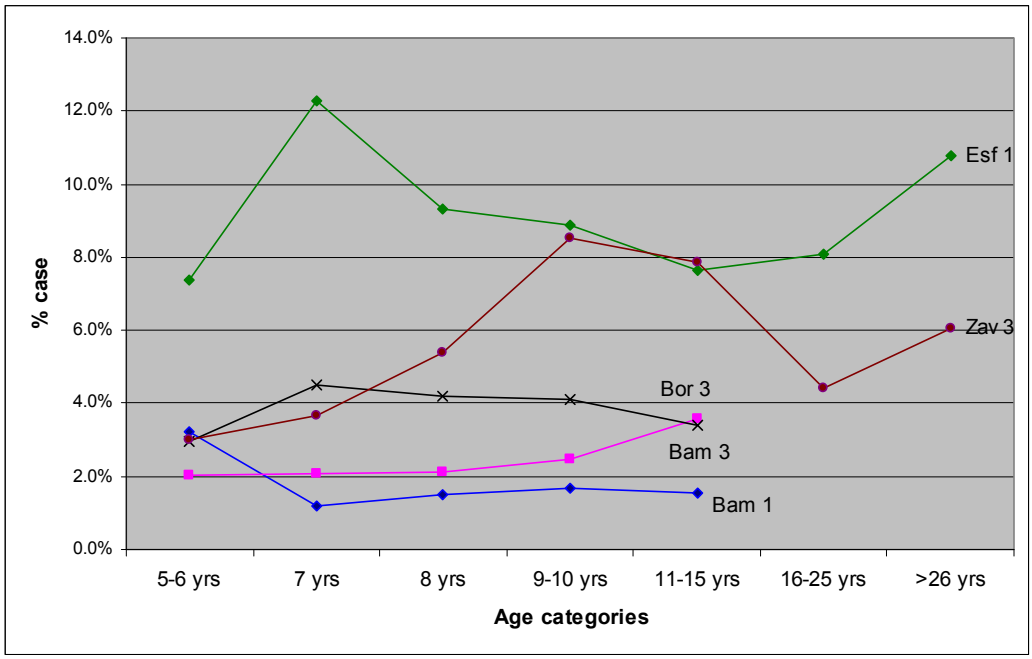
Figure 4.1 demonstrates the age/incidence distribution in the 5 trials. The age/incidence distribution in Bam1, Bam3 and Bor3 trials is not significant; possibly

partially due to age restriction in these trials. Statistically significant distributions are observed in Zav3 (chi sq=16.66, p=0.011) and Esf1 (chi sq=14.73, p=0.022), the two trials with participants' age ranging from 5 to 59 or 72. These significant values show the dependence of incidence on age, but trend tests do not suggest a significant linear downward trend in these trials. A downward age/incidence relationship would be consistent with exposure-induced immunity in endemic populations. In Zav3, where exclusion criteria did not impose a limitation on age or LST status of participants, lack of a significant downward trend confirms that participants do not have the age/incidence characteristics expected of endemic populations.

Despite the difference in the endemic origin of participants and average incidence, incidence distribution by age in Esf1 resembles Bor3 within their common age range. In Bor3, age/incidence distribution is not significant (p=0.067), but the downward trend in incidence with age can be recognized after age 7 (see the following graph=). Trends in Bam trials are not significant and possibly consistent with the exclusion of pre-vaccination LST>0.

Overall incidence in Esf1 and Zav3 were the highest of all trials. This is attributable to participants' non-endemic origin (*leishmania*-naive).

Figure 4.1 - Average annual incidence and age distribution in the 5 trials in Iran



Trend tests not significant for any of the trials

To further explore the relationship between age and infection, logistic regression was used to test whether age had a direct effect on incidence within each trial. In table 4.1 the OR (odds ratios) of incidence by age are displayed. The OR indicates for a one year increase in age the change in log odds (logarithm of the probability of being case divided by the probability of not being case). A larger OR indicates generally larger incidence at higher ages. OR is not significant in Bor3, even when including those with pre-vaccination LST>0. This is partially an effect of the age limitation to 13 in

this trial which restricts the effect of age on immunity and consequently incidence. Zav3 results are not significant and this is not as surprising since participants were not from endemic origin and therefore endemic immunity (a direct downward trend with age) was not expected. When considering different trials separately, the odds ratio of incidence/age is significant only in Esf1. This relationship though significant is not very strong and, as figure 4.1 indicates, is not linear, as will be discussed later.

Table 4.1 - Odds ratio for the effect of age on incidence in separate trials

OR of incidence by age in:	N	OR	Std. Err.	P>|z|	95% Conf. Int.	
Bam1	3633	0.988	0.060	0.85	0.877	1.114
Bam3	4087	1.073	0.083	0.36	0.922	1.248
Bor3 (all pre-vac LST)	2123	0.989	0.042	0.80	0.989	0.042
Bor3 (pre-vac LST=0)	1275	1.000	0.057	1.00	0.894	1.118
Esf1	2314	1.009	0.004	0.04	1.001	1.017
Zav3 (all pre-vac LST)	1659	0.997	0.007	0.61	0.984	1.009
Zav3 (pre-vac LST=0)	1454	1.000	0.007	0.95	0.987	1.014

To observe the age/incidence distribution in trials with endemic and non-endemic participants, these trials were combined as shown in table 4.2. Results did not show a significant difference in age-based incidence in either group:

Table 4.2 - Odds ratio for the effect of age on incidence in endemic and non-endemic areas (pre-vaccination LST=0)

OR of incidence by age in:	N	OR	Std. Err.	P> |z|	95% conf. Int.	
Non-endemic	3768	1.007	0.004	0.07	1.00	1.01
Endemic	8995	0.982	0.032	0.57	0.922	1.046

Breaking these groups further down to separate the effect of vaccine from BCG within endemic/non-endemic participants (table 4.3) did not yield significant results:

Table 4.3 - Odds ratio for the effect of age on incidence in trials arms in endemic and non-endemic participants (pre-vaccination LST=0)

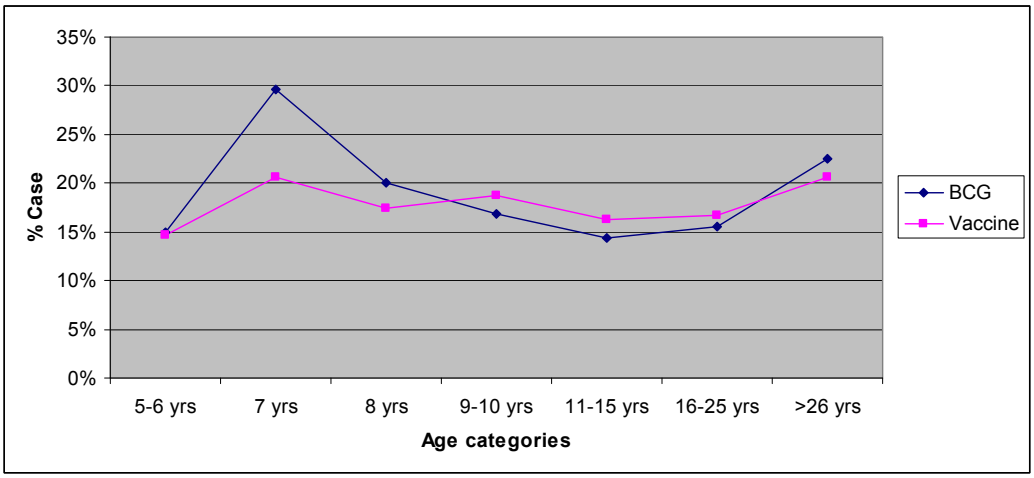
OR of incidence by age in:	N	OR	Std. Err.	P> |z|	95% Conf. Int.	
Control/non-endemic	1909	1.009	0.005	0.077	0.999	1.019
Control/endemic	4419	0.984	0.044	0.726	0.902	1.075
Vaccine/non-endemic	1859	1.004	0.005	0.406	0.994	1.014
Vaccine/endemic	4576	0.977	0.045	0.612	0.892	1.070

The age/incidence relationship in Esf1was further studied by breaking down the Esf1 data into vaccine and control arms. Resulting odds ratio in the control arm is marginally significant (table 4.4) and does not demonstrate a strong linear relationship (as demonstrated in figure 4.2 below with trend test p=0.318 for BCG, p=0.229 for vaccine). Moreover, the same relationship is not observed in Zav3, the other trial with non-endemic participants. Therefore, it is likely that the marginally significant OR is due to chance.

Table 4.4 - Esf1 odds ratio for the effect of age on incidence in trial arms

OR of incidence by age in:	N	OR	Std. Err.	P> |z|	95% Conf. Int.	
control arm	1124	1.012	0.006	0.042	1.000	1.024
vaccine arm	1190	1.005	0.006	0.373	0.994	1.018

Figure 4.2 - Incidence in trial arms in Esf1



* * *

Before proceeding with the meta-analysis, it is noteworthy that in the Bam3 trial, the number of cases in participants 9 years or older is significantly smaller in the vaccine arm than the control arm, as demonstrated in table 4.5. Since the difference in efficacy between the age groups were not significant and the same observation was not made in the other Bam trial data, or any other trials, this finding will not be further considered.

Table 4.5 - Bam3: significant vaccine arm protection in >9 yrs participants

Bam 3-inj

		Vaccine			
% case in age group		0	1	Total	Fisher Exact
< 9 yrs		0.04	0.04	0.04	p = 0.368
	N =	1723	1844	3567	
9 yrs or older		0.07	0.03	0.05	p = 0.036
	N =	282	238	520	
Total		0.05	0.04	0.04	
	N =	2005	2082	4087	

* * *

4.1.1 Meta-analysis to investigate age/efficacy relationship

4.1.1.1 Estimating the pooled RR for all age categories simultaneously

If due to any age-related reasons (such as immunological differences between ages), vaccine effectiveness in different age groups varies, this relationship could be explored by examining efficacy in various age groups. Furthermore, if all relationship between age and exposure/immunity are not removed by excluding LST>0, then some interrelationship between age and vaccine effectiveness could be present, if the vaccine is in fact efficacious. The hypothesis to be tested via meta-analysis is:

H0: Overall RR(estimated based on all age- and trial-specific RR's) = 1
H1: Overall RR(estimated based on all age- and trial-specific RR's) ≠ 1

This analysis initially estimates RR's separately for each age category within each trial. Next, a meta analytic approach is taken to estimate the pooled RR for all of these estimates using a fixed or random effect model. In this way, age-specific RR's are first estimated within the homogeneity of a given age group in a given trial. These group-specific RR's would represent the pure effect within a homogeneous age/trial group, without being averaged down by other age/trial groups. The pooled RR would then be based on these individually more reliable estimates.

Additionally, this approach allows studying whether RR's in a given age category are systematically different from those in other age categories. This can be visually examined in the graphs in the following pages.

Tables 4.6 and 4.7 and figure 4.3 demonstrate the results of the age/trial meta-analysis estimation of overall RR. As discussed in the previous chapter, large variability in individual RR's would lead to significant heterogeneity in the model. Therefore, if variation in RR's due to age and/or trial were substantial, significant heterogeneity and between-group variation, indicated by a significant τ^2, would be expected.

Table 4.6 - Meta-analysis results for age-specific/trial specific RR's

	Pooled			Test of RR=1	
Method	RR	95% Conf. Int.		z	p
M-H pooled RR	0.957	0.855	1.072	0.76	0.45
D+L pooled RR	0.960	0.851	1.084	0.65	0.513

Table 4.7 - Heterogeneity statistics for meta-analysis of age/trial RR's

	Heterogeneity Chi		
Method	sq	p	I - squared
D+L or M-H	29.93	0.367	0.64

As indicated by the above tables, heterogeneity and its associated chi square statistic are not statistically significant (τ^2 estimate is 0.007 and its associated heterogeneity chi sq.=29.93, p=0.367). This lack of significant heterogeneity suggests that RR within age/trial groups do not vary greatly; i.e., age/trial combination is not a

significant determinant of RR. This does not provide support for age specific vaccine effect.

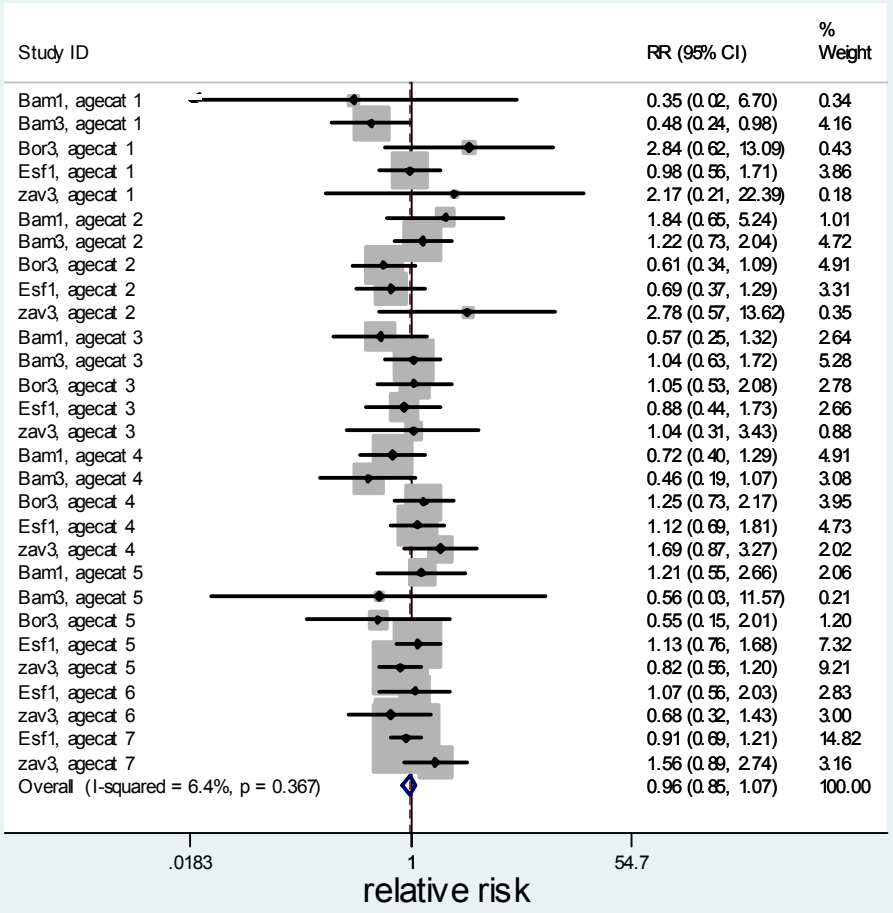
The forest plot in the next page (figure 4.3) indicates the deviations of individual RR's from the pooled estimate (represented by the dashed centre line). In this graph, RR's have been sorted by age-category. Thus, all age-based RR's for various trials are displayed one after another. In case of meaningful/systematic differences between age groups RR's for the same age category would cluster around the same value on the same side of the overall estimate line. Such clustering is not observed in the graph. Therefore, the visual examination of forest plots does not support the notion that there is a relationship between vaccine efficacy and age.

Age categories are defined as in previous figures (e.g., figure 4.1 and 4.2) as follows:

Age category	Age range (yrs)
1	5-6
2	7
3	8
4	9-10
5	11-15
6	16-25
7	>26

Grouping age into these categories was based on assumed behavioural differences between them coupled with large enough sample size in each group. For example, the 5-6 years category was believed to be different enough from the 7 years category in that 7 years of age is the official age for entering elementary school. Therefore, it is conceivable that 7 year olds are different in their exposure patterns from 5-6 year olds. Similarly, 7 and 8 year olds could be assumed to have some systematic differences in their behaviour since at that young age one year of difference could translate into measurable difference sin behaviour and exposure. Additionally, enough sample size was available for separating the two to observe possible differences. The same logic was behind separating 9-10 years and then 11-15 years categories. The 15-25 year group was separated from 25+ years mainly so that a young adults could be distinguished from all older ages.

Figure 4.3 - Forest plot of the age-specific RR's in all trial, sorted by trial within the same age



Results of diagnostic measures including are presented below and do not show any biasing effect or significant influence by any of the age/trial combinations on the overall estimate. The following 2 graphs indicate these results.

Figure 4.4 - Funnel plot: checking for out of range RR values

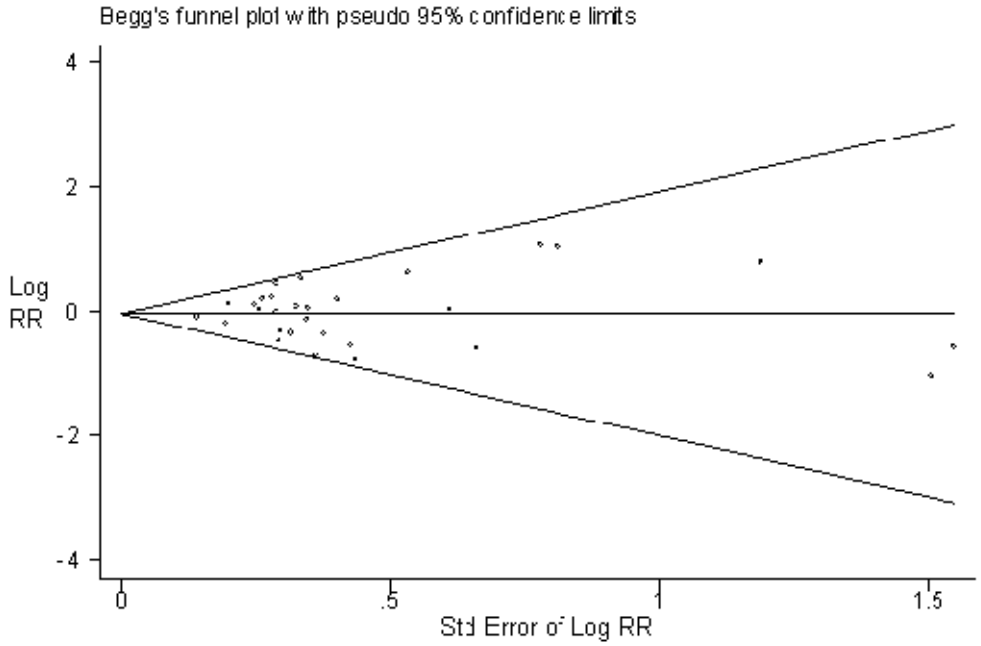
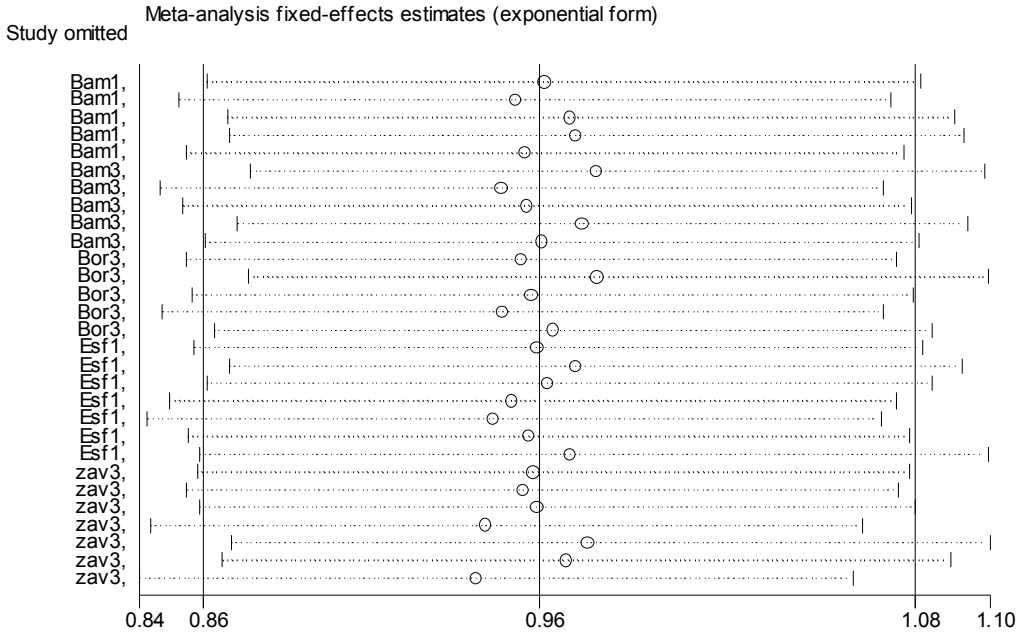


Figure 4.5 - Influence of individual age categories on the pooled RR



Overall, the meta-analysis of all age-specific effects in all trials does not lead to the rejection of the null hypothesis of RR=1. Furthermore, visual inspection of forest plots does not suggest systematic differences between age groups.

4.1.1.2 Estimating RR within each age category separately

With the above approach of estimating the pooled RR over all age categories failing to show a significant age/efficacy relationship, an alternative approach of estimating vaccine efficacy within each age group separately was used. The difference between age group RR's would then show the effect of age. The hypothesis tested was:

H0: Age-specific $RR_{(\text{estimated based on age-specific RR's in all trials})} = 1$
H1: Age-specific $RR_{(\text{estimated based on age-specific RR's in all trials})} \neq 1$

To estimate the vaccine efficacy within each age category, a series of meta analyses were conducted, estimating pooled RR's separately for each age category. Individual, age-specific RR's are the same those as used in the previous analysis. What contrasts this analysis is that RR's for the seven age categories are separately estimated (pooled over the 5 trials). Estimation is done within groups that are homogeneous with respect to age, without allowing the effect of the other age-groups to average down the results. Assuming that the RR is systematically and significantly different between some of the age groups, this would allow observing the pure effect within each age category. In this way, it would become clear if the vaccine were more effective in a certain group but not in others. Results are presented below.

5-6 years old: The RR in Bam3 is significant (as indicated by the 95% confidence interval that does not contain 1), while in other trials it is not significant. In Zav3 and Bor3, in fact, the direction of the effect is unexpected (higher incidence in the vaccine arm). However, these reverse RR's have minimal impact on the overall RR due to their small weights which reflects their relatively few participants in this age group (table 4.8).

Table 4.8 - Meta-analysis results in the 5-6 year old category

Age= 5-6 years

Study	RR	95% Conf. Int.		% Weight
Bam1	0.35	0.02	6.70	3.76
Bam3	0.48	0.24	0.98	46.37
Bor3	2.84	0.62	13.09	4.84
Esf1	0.98	0.56	1.72	43.03
zav3	2.17	0.21	22.39	1.99
M-H pooled RR	0.84	0.56	1.25	100.00

Test of RR= 1 : z= 0.87 p = 0.384
Heterogeneity chi-squared = 6.03 (d.f. = 4) p = 0.197
I-squared (variation in RR attributable to heterogeneity) = 33.7%

7 years old category: The RR in none of the trials is statistically significant. Similarly, the overall RR is also not significant. The direction of the RR in both Bam trials is opposite expectation but not significant (table 4.9).

Table 4.9 - Meta-analysis results in the 7 year old category

Age= 7 years

Study	RR	95% Conf. Int.		% Weight
Bam1	1.84	0.65	5.24	7.06
Bam3	1.22	0.73	2.04	33.01
Bor3	0.61	0.34	1.09	34.33
Esf1	0.70	0.37	1.29	23.14
zav3	2.78	0.57	13.62	2.46
M-H pooled RR	0.97	0.72	1.31	100.00

Test of RR= 1 : z= 0.19 p = 0.846

Heterogeneity chi-squared = 7.46 (d.f. = 4) p = 0.113

I-squared (variation in RR attributable to heterogeneity) = 46.4%

8 years old: Similar to the 7 years old category, all trial RR's and the overall RR are not statistically significant, showing no vaccine effect (table 4.10).

Table 4.10 - Meta-analysis results in the 8 year old category

Age= 8 years

Study	RR	95% Conf. Int.		% Weight
Bam1	0.57	0.25	1.32	18.54
Bam3	1.04	0.63	1.72	37.08
Bor3	1.05	0.53	2.08	19.54
Esf1	0.88	0.44	1.73	18.68
zav3	1.04	0.31	3.43	6.16
M-H pooled RR	0.92	0.68	1.26	100.00

Test of RR= 1 : z= 0.51 p = 0.612

Heterogeneity chi-squared = 1.66 (d.f. = 4) p = 0.798

I-squared (variation in RR attributable to heterogeneity) = 0.0%

9-10 years old category: also shows no significant effect by the vaccine in any of the individual trials or overall (table 4.11).

Table 4.11 - Meta-analysis results in the 9-10 year old category

Age= 9-10 years

Study	RR	95% Conf. Int.		% Weight
Bam1	0.72	0.40	1.29	26.29
Bam3	0.46	0.19	1.07	16.46
Bor3	1.25	0.73	2.17	21.15
Esf1	1.12	0.69	1.81	25.31
zav3	1.69	0.87	3.27	10.79
M-H pooled RR	0.99	0.76	1.29	100.00

Test of RR= 1 : z= 0.05 p = 0.959

Heterogeneity chi-squared = 7.77 (d.f. = 4) p = 0.100

I-squared (variation in RR attributable to heterogeneity) = 48.5%

11-15 years old: Similar results to younger age groups are seen in the 11-15 year olds (table 4.12).

Table 4.12 - Meta-analysis results in the 11-15 year old category

Age= 11-15 years

Study	RR	95% Conf. Int.		% Weight
Bam1	1.21	0.55	2.66	10.28
Bam3	0.56	0.03	11.57	1.04
Bor3	0.55	0.15	2.02	6.02
Esf1	1.13	0.76	1.68	36.58
zav3	0.82	0.56	1.20	46.07
M-H pooled RR	0.96	0.74	1.23	100.00

Test of RR= 1 : z= 0.36 p = 0.721

Heterogeneity chi-squared = 2.48 (d.f. = 4) p = 0.648

I-squared (variation in RR attributable to heterogeneity) = 0.0%

16-25 years old: Esf1 and Zav3 were the only trials with volunteers older than school age. The RR in Esf1 and Zav3 in the 16-25 years group are in the opposite direction, leading to an overall RR that is not significant (table 4.13).

Table 4.13 - Meta-analysis results in the 16-25 year old category

Age= 16-25 years

Study	RR	95% Conf. Int.		% Weight
Esf1	1.07	0.57	2.03	48.56
zav3	0.68	0.33	1.43	51.44
M-H pooled RR	0.87	0.54	1.41	100.00

Test of RR= 1 : z= 0.56 p = 0.573

Heterogeneity chi-squared = 0.83 (d.f. = 1) p = 0.363

I-squared (variation in RR attributable to heterogeneity) = 0.0%

25 years and older: Similar to the previous age group, the RR's in the two trials are in reverse direction (table 4.14). Moreover, their direction is reverse of that in the RR in the same trial in the 16-25 year category. This is not a problem since these RR's are not statistically significant. The overall RR is, again, not significant.

Table 4.14 - Meta-analysis results in the >25 year old category

Age> 25 years

Study	RR	95% Conf. Int.		% Weight
Esf1	0.91	0.69	1.21	82.43
zav3	1.56	0.89	2.74	17.57
M-H pooled RR	1.03	0.80	1.32	100.00

Test of RR= 1 : z= 0.21 p = 0.834

Heterogeneity chi-squared = 2.75 (d.f. = 1) p = 0.098

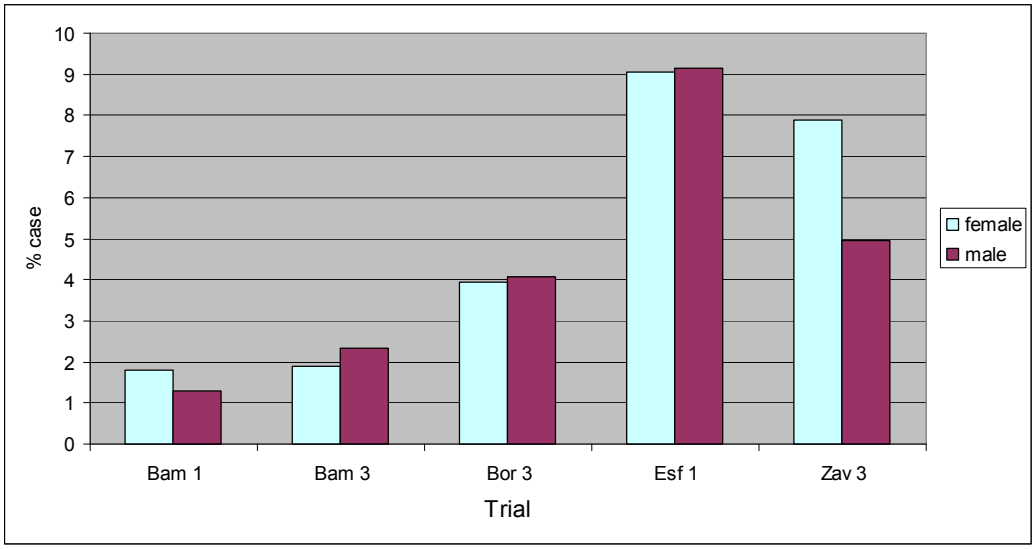
I-squared (variation in RR attributable to heterogeneity) = 63.6%

The only significant RR in this analysis is that in the 5-6 year olds in Bam3 -- which is therefore, more likely the product of chance. The pooled RR in none of the age groups is significantly different from 1; indicating that the vaccine efficacy (1-RR) is not significantly different from 0, even when considering the effect in homogeneous age groups separately. These results do not contradict the findings in the previous analysis where the pooled RR for all age groups and all trials was not significant. In summary, the data do not support the notion that vaccine efficacy is age related.

4.2 Gender, incidence and vaccine efficacy

Overall, in all trials, there are 8.11% cases among males compared to 8.92% among females (Fisher Exact p=0.088) without any significant difference in distribution in endemic/non-endemic participants or *L. major*/*L. tropica* trials. The following graph shows the distribution of infection by gender within each trial:

Figure 4.6 - Average annual incidence in male/female participants by trial (study arms combined)



Zav3 is the only trial with a statistically significant difference ($p < 0.05$) in % cases among males and females.

4.2.1 Vaccine efficacy in males and females

Trial by trial efficacy results in males and females are given in the following tables. The only significant relationship is the significantly lower incidence in boys in the Bam1 trial ($p = 0.006$). This is not observed in girls in the same trial. . Furthermore, The same magnitude of difference was not observed in any other trial. In Zav3, where male participants experienced significantly lower overall incidence compared to women ($p < 0.05$), the difference was not significant between study arms. Male and female incidence rates in various trials are depicted in tables 4.15a and 4.15b.

Table 4.15a - Percent infection in male/female participants in vaccine trials (endemic) in participants with pre-vaccination LST=0

	Bam 1		Bam 3		Bor 3	
SEX	**C**	**V**	**C**	**V**	**C**	**V**
F	3.01%	4.16%	4.53%	3.04%	12.33%	9.67%
	929	865	994	1019	300	319
M	3.70%	1.64%	4.75%	4.70%	10.66%	11.63%
	866	973	1011	1063	269	387
Total	3.34%	2.83%	4.64%	3.89%	11.47%	10.82%
	1795	1838	2005	2082	569	706

Table 4.15b - Percent infection in male/female participants in vaccine trials (non-endemic) in participants with pre-vaccination LST=0

	Esf 1		Zav 3	
SEX	C	V	C	V
F	18.10%	18.18%	14.29%	15.99%
	591	627	385	344
M	18.95%	17.76%	10.59%	10.98%
	533	563	406	328
Total	18.51%	17.98%	12.39%	13.54%
	1124	1190	791	672

For all trials combined, the overall efficacy results for male and female participants are not statistically significant (table 4.16).

Table 4.16 - Percent infection in male/female participants (all trials combined) in participants with pre-vaccination LST=0

	Overall			
SEX	C	V	Total	P
F	8.50%	8.39%	8.45%	0.87
	3199	3124	6323	
M	8.23%	7.45%	7.83%	0.26
	3135	3314	6449	
Total	8.37%	7.91%	8.13%	
	6334	6438	12772	

Regressing infection rate on a binary variable (0= control, 1=vaccine), separately in males and females shows similar results, i.e., the hypothesis of no difference in protection between males and females cannot be rejected (Table 4.17).

Table 4.17 - Vaccine odds ratio by gender in participants with pre-vaccination LST=0

	Odds ratio	p	95% conf. Int.	
Female	0.98	0.854	0.83	1.17
Male	0.90	0.262	0.75	1.08

4.2.1.1 Meta-analysis to investigate gender/efficacy relationship

To address the effect of gender on efficacy, the following hypothesis will be tested:

H0: $RR_{\text{(estimated based on sex- and trial-specific RR's)}} = 1$
H1: $RR_{\text{(estimated based on sex- and trial-specific RR's)}} \neq 1$

A meta analytic approach was adopted to test the hypothesis. This approach is similar to the one used in assessing efficacy in age groups. In this analysis, RR's for male and female participants were first estimated separately within each trial. The pooled RR was then estimated based on these gender-based RR's. Results are presented in table 4.18 and figure 4.7.

Table 4.18 - Gender-based analysis of vaccine RR in vaccine trials (0 = F, 1 = M)

Study	RR	95% Conf. Int.		% Weight
Bam1-SEX= 0	1.381	0.85	2.243	4.96
Bam1-SEX= 1	0.445	0.246	0.805	6.22
Bam3-SEX= 0	0.672	0.429	1.053	8.37
Bam3-SEX= 1	0.991	0.673	1.458	9.04
Bor3-SEX= 0	0.78	0.479	1.268	6.44
Bor3-SEX= 1	1.169	0.761	1.795	6.63
Esf1-SEX= 0	1.004	0.791	1.275	20.23
Esf1-SEX= 1	0.937	0.73	1.203	19.06
zav3-SEX= 0	1.086	0.793	1.487	11.52
zav3-SEX= 1	1.007	0.668	1.518	7.54
M-H pooled RR	0.952	0.851	1.066	100

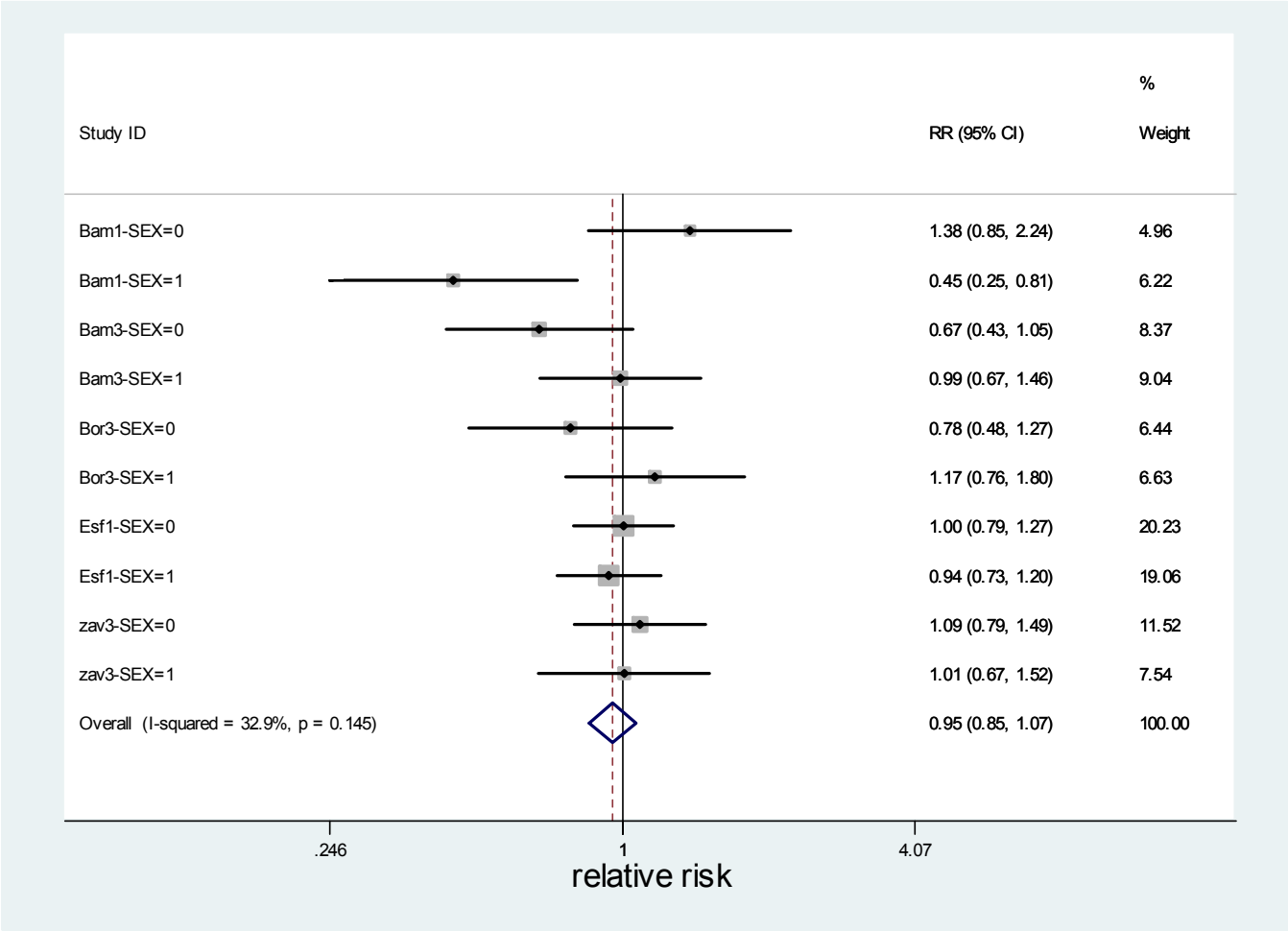
Test of RR= 1 : z= 0.85 p = 0.397

Heterogeneity chi-squared = 13.41 (d.f. = 9) p = 0.145

I-squared (variation in RR attributable to heterogeneity) = 32.9%

As table 4.18 indicates, the heterogeneity calculated via the Mantel-Haenszel method is not significant (p= 0.145), implying the appropriateness of the fixed effect model. The random effect model was nevertheless estimated for comparison with the above. However, regardless of the method of estimation, the overall effect is not statistically significant. As indicated, the only significant RR is in boys in Bam1. No other gender/trial group shows a significant effect. Furthermore, the overall RR is not significant. In light of these meta analytic results, a correlation between gender and incidence cannot be ascertained. These results are summarized in the forest plot in figure 4.7.

Figure 4.7 - Forest plot of gender-based meta-analysis

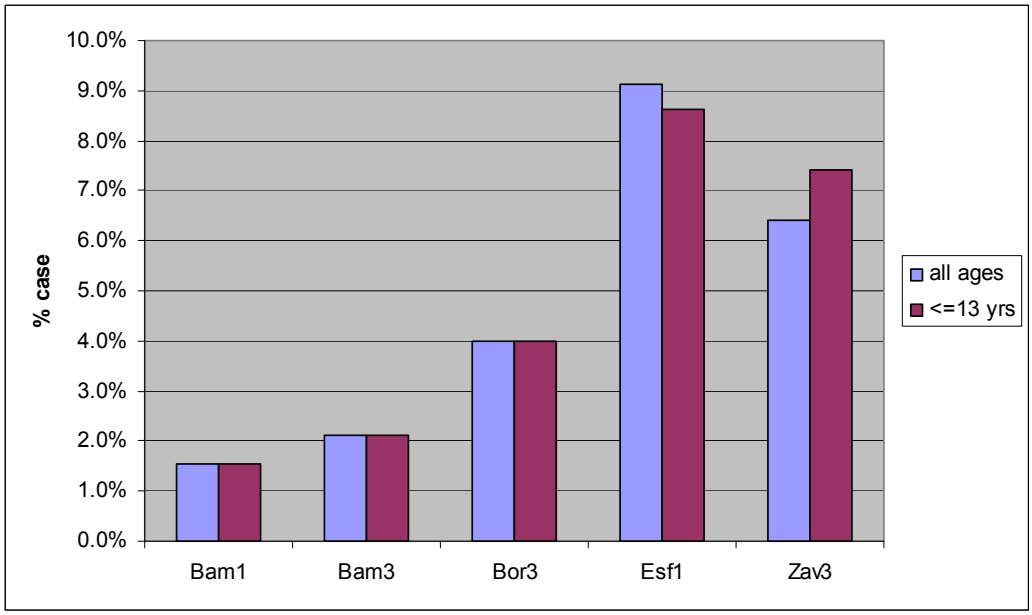


4.3 Endemic origin, incidence and efficacy

Residents of endemic areas are routinely exposed to sandfly bites. Although exposure to the infected sandfly bite leads to some level of infection, a large percentage of exposed individuals never develop clinical symptoms. Recovery from clinical disease normally is synonymous with immunity. Even in individuals who do not develop clinical infection, the constant exposure, which resembles a prime/boost vaccination method, is normally associated with LST reactivity and increased chances of immunity. Such immunity is expected to increase with exposure and age - since more frequent exposure for a longer period of time should increase the immunization effect (Davies and Mazloumi Gavgani, 1999). Due to this mechanism, one would expect lower infection rates in populations from endemic origin compared to those from non-endemic origin residing in endemic foci. Although endemic immunity that is manifested by LST can be identified in both arms, if it is not associated with LST>0 (for example in people who are genetically non-responsive to the antigen), then it cannot be identified. In such cases, the LST=0 inclusion criteria would be less effective and leave some exposed individuals in the study. By extension, if the vaccine is in fact efficacious, one could observe lower vaccine-attributable protection in clinical trials with endemic participants, since endemically immune participants would be present in both study arms with equal probability leaving fewer participants to be protected by the vaccine in the vaccine arm and more individuals already protected without the vaccine in the control arm. Therefore, to the extent that endemic immunity exists in both arms, vaccine-induced protection will be underestimated since those who would otherwise be protected only in the vaccine arm by vaccination are now already immune and present in both arms; thus reducing the statistical power of the study through reducing the difference in protection observed between study arms (since a percentaqge of those already protected in the control arm by endemic immunity would otherwise succumb to infection during the follow up).

As described earlier, two of the Iran trials, Zav3 and Esf1 were trials in endemic (or high incidence) areas with participants of non-endemic origin. Comparison of the much higher incidence in these trials with relatively lower incidence in Bam1, Bam3 and Bor3 (with local endemic area residents as trial participants) points partially at the difference due to the endemic make-up of study subjects (as displayed in figure 4.8 and table 4.19). Although total incidence is not significantly different between Zav3 and Esf1 ($p=0.626$), it is different between Bor3 and Zav3 ($p<0.001$).

Figure 4.8 - Average annual incidence in trials with participants of endemic/non-endemic origin by age



As expected, incidence in the non-endemic participants in Esf1 and Zav3 exceeds that among endemic participants -- even after excluding all ages >15 in Esf1 and Bor3 to make them consistent with other trials. This observation is consistent with the large number of *Leishmania*-naive participants in non-endemic populations who are expected to experience higher disease rates upon introduction to endemic conditions. The lower incidence in endemic participants in Bam1 and Bam3 reflects the epidemiology of the local ACL disease but potentially is also partially driven by the endemic immunity among the endemic participants. The lower rate in Bor3, however, probably reflects site differences as well as endemic immunity which would not be present in Esf1 and Zav3.

Table 4.19 compares incidence in vaccine and control arms in endemic and non-endemic participants. It is clear that despite the higher incidence in the latter, response to the vaccine is not significantly different between the two.

Table 4.19 - Incidence in vaccine and control arms, endemic and non-endemic

	Treatment			
	C	**V**	**Total**	**P**
Non-endemic	15.83%	16.11%	15.97%	0.42
	2022	1961	3983	
Endemic	5.75%	5.25%	5.50%	0.27
	4855	4988	9843	
Total	8.71%	8.32%	8.51%	
	6877	6949	13826	

Odds ratios (vaccine/control) for endemic and non-endemic participants are given in the table 4.20 and suggest no vaccine efficacy in either of groups of endemic origin.

Table 4.20 - Odds ratios of vaccine to control, endemic and non-endemic

	N	OR	Std. Err.	P>|z|	95% conf. Int.	
Non-endemic	3983	1.022	0.088	0.80	0.86	1.21
Endemic	9843	0.909	0.080	0.28	0.764	1.081

The result of this study suggests that there is no difference in vaccine efficacy between the endemic and the non-endemic trials. Neither group of trials shows protection in the vaccine arm. Despite the higher incidence and the favourable conditions in the Esf1 and Zav3 trials for detecting protection due to vaccine, vaccine efficacy cannot be observed.

4.4 Summary: Immunity to leishmaniasis could be related to genetic and demographic characteristics. Demographic characteristics, including age and gender, correlate with risk behaviour and exposure patterns. Exposure could lead to two outcomes: clinical or sub-clinical infection; both of which increase the likelihood of immunity to future infection. There are two ways in which age can influence incidence and immunity: 1) dissimilar risk behaviour in different age groups which leads to higher exposure in certain age categories, 2) cumulative chance of exposure by age.

The present analysis did not confirm a strong relationship between incidence with age or gender. Moreover, it did not suggest efficacy for the ALM+BCG vaccine in any of the demographic groups.

Since demographics were expected to be related to incidence and immunity in endemic foci, lack of such relationship in the data was unexpected. However, consideration of the data collection procedures sheds some light on this question. When demographic variables are used as exclusion criteria, to the extent that these variables are correlated with immunity and incidence the natural relationship between incidence and demographics may be concealed. In the clinical trials analysed in the present study, one of the factors with potential impact on observing age/incidence relationship was age limitation of 6-13 or 15 years in Bam1, Bam3 and Bor3. It has been argued that endemic immunity settles in at an early age, perhaps by 5 or 6 years. Whether this is the case or if immunity prevalence continues to increase with age, the 6-15 age limitation in these trials could have compromised the effect of age.

Another factor in the way of observing the effect of age and other demographic variables on incidence was exclusion of pre-vaccination LST>0 volunteers in Bam1, Bam3 and Esf1. Endemic immunity is expected to be associated with LST>0. At the same time, prevalence of LST>0 and endemic immunity increases with age. Excluding volunteers with LST>0 may have reduced the effect of age on immunity and consequently on incidence in these clinical trial samples. Three of the trials used for the forgoing analysis (Bam1, Bam3, Esf1) used LST>0 as an exclusion criterion. Zav3 is the only trial where neither age nor LST limitations were used as exclusion criteria. However, Zav3 participants were chiefly not from endemic background and therefore are unlikely to show the effect of age on endemic immunity and incidence. Together these factors seem to be the explanation for lack of a strong relationship between demographics and incidence.

In this analysis, vaccine efficacy could not be confirmed in any of the demographic groups, when considering all data combined. Although significantly greater vaccine related protection was observed in boys in Bam1, this observation could not be confirmed in other trials.

In summary, the results presented in this chapter do not in general support the notion that demographic characteristics affect vaccine efficacy significantly: none of the demographic factors discussed were found to directly and strongly correlate with vaccine efficacy across all trials and demographic groups, although greater vaccine-related protection in a subgroup in one trial was observed. These findings are subject to limitations in the age range of trial participants. Certain effects may be observable only in very high or very low values of a variable such as age. In this regard, the Esf1 trial provided the ideal combination of endemic foci + *Leishmania*-naive participants + a wide age range. Perhaps this was the reason why Esf1 was the only Iranian trial that showed lower incidence among vaccination-sensitized participants (those with converted LST, 80 days after vaccination, in both arms combined, as discussed in Chapter 1).

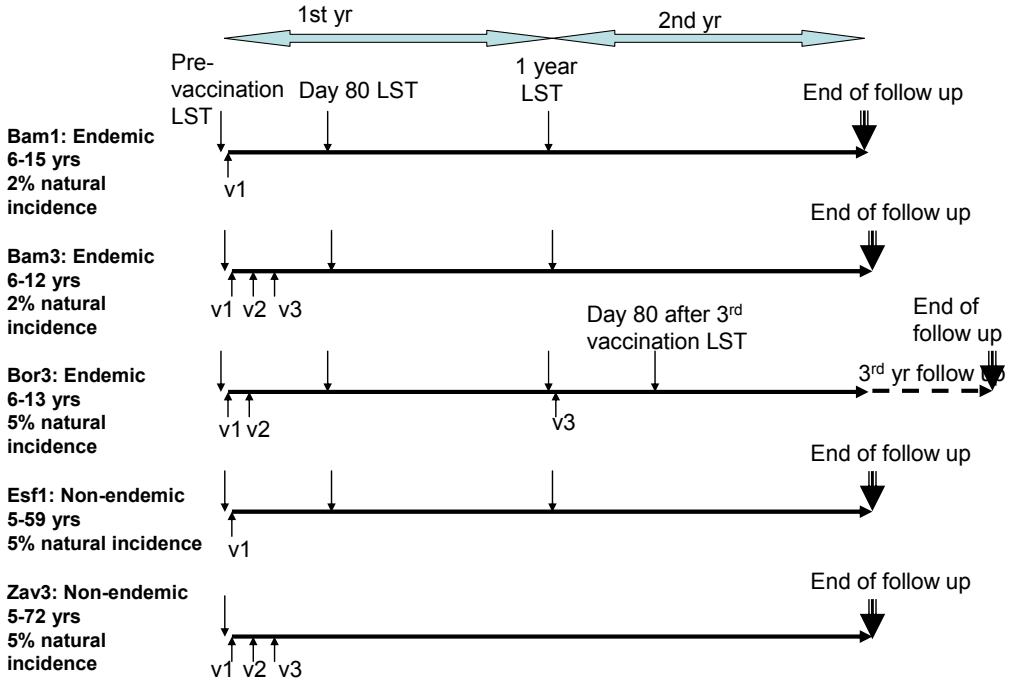
CHAPTER 5

IMMUNOLOGICAL RESPONSE (MEASURED BY LST) IN DEMOGRAPHIC SUBGROUPS TO THE LEISHMANIAL ANTIGEN INTRODUCED BY VACCINE OR NATURAL EXPOSURE

In this chapter the immune response to the antigen (as measured by LST), the development of immune response over time (from before vaccination to the end of one year follow-up) and the effects of demographic factors, gender, age and endemic origin are assessed.

The vaccine trials used in this analysis are the 5 trials in Iran which provide participant-level data. All of these trials, with the exception of Zav3, provide LST measurements 80 days and one year post-injection. Zav3 and Bor3 provide LST measurement prior to the vaccine injection. Esf1 and Zav3 include participants of non-endemic origin (i.e., originally from non-endemic areas) while others include chiefly participants originally from the endemic trial site. Figure 5.1 summarizes these trial design elements:

Figure 5.1 - Timing of vaccination and LST in Iran trials



A non-zero leishmanin skin test could indicate cellular response due to exposure to the leishmanial antigen naturally or via immunization. To a limited degree, it could also indicate exposure to other immunomodulating agents such as BCG. However, it is also possible that a certain percentage of people in endemic foci are non-responsive to the antigen. LST in such people is expected to stay negative.

The dogma maintains that previous infection or repeated exposure, common in endemic foci, is detectable via LST≥5 and is associated with immunity (endemic immunity). In other words, LST≥5 (generally accepted as "positive"), if not indicative of a developing infection, would be associated with protection (Ben Salah et al., 2005). Pre-vaccination LST could provide some information about existing levels of immunity in the study sample.

However, partially because of this reason, normally pre-vaccination LST> 0mm (or in some trials LST>5mm) is used as an exclusion criterion in leishmaniasis vaccine trials. This has the desirable consequence of allowing the assessment of the vaccine effect in *Leishmania*-naive individuals without confounding by the potentially protective effect of previous exposure. However, this disallows estimating the vaccine effectiveness in those with previous exposure; who constitute a large part of the populations in endemic areas.

As indicated, in Bor3 and Zav3, by design, LST>0 was not an exclusion criterion and volunteers with LST>0 (but no disease history) were admitted. This allowed a more representative cross section of endemic residents with respect to exposure and enabled assessing the correlation between pre-vaccination LST>0 and: disease incidence (thus, testing the dogma), post-vaccination immunological response and vaccine efficacy. On the negative side, this introduced potentially immune individuals to both arms of these trials, thus reducing statistical power.

Pre- and post-vaccination LST measurements indicate the degree of stimulation in the immune response at various points in time and could be linked to vaccine efficacy. LST measurements over time are inter-related; immune response at one point in time is probably not independent of it at an earlier time. Therefore, it is important to study different LST readings, their inter-relationship and their link to vaccination. More over, LST and immune response should be studied in connection to potentially risk-related characteristics such as demographics and endemic origin.

The overall hypothesis of this chapter is**:**

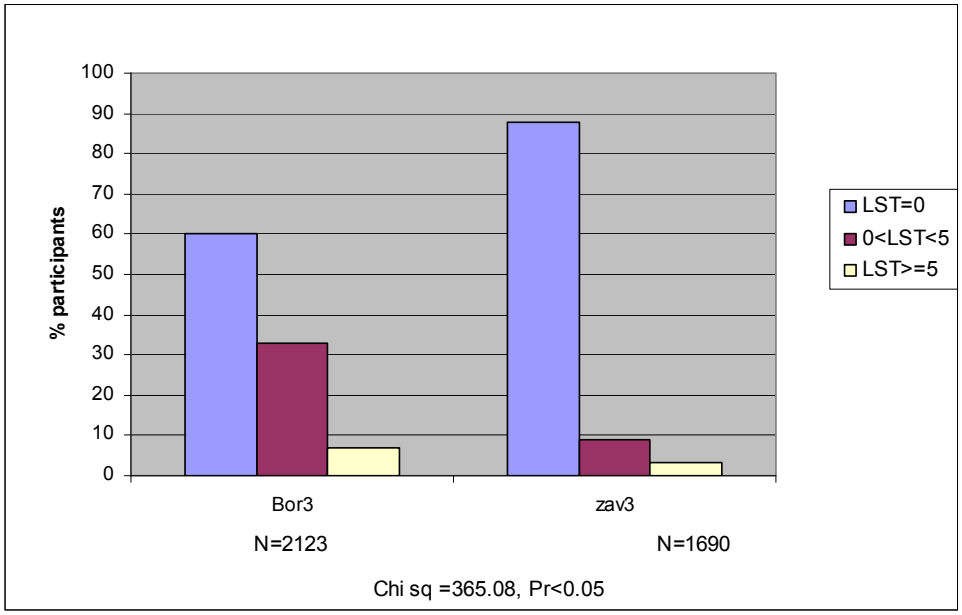
H0: Inhabitants of endemic and non-endemic areas and various demographic groups do not differ in their immunological response (measured by LST) to leishmanial antigens introduced as a vaccine or natural exposure.
H1: Demographic differences are associated with different immunologic responses in inhabitants of endemic and non-endemic areas.

5.1 Overall observations: LST results, before and after vaccination

5.1.1 Pre-vaccination LST

Pre-vaccination LST in Bor3 and Zav3 are presented below. Compared to Zav3, a significantly higher percentage of LST>0 was observed in Bor3, suggesting greater prevalence of exposure in the already well established endemic area of Borkhar compared to the newly endemic area of Zavareh. This is depicted in the graph below.

Figure 5.2 - Pre-vaccination LST distribution in Bor3 and Zav3



By contrast, the size of the LST reaction in those with LST>0 is greater in Zav3 than in Bor3 (right side of the table below), suggesting that in Zav3 with participants of non-endemic origin the lower prevalence is accompanied with more acute reaction.

Table 5.1 - Pre-vaccination LST induration in Bor3 and Zav3

	Overall sample			LST>0 only		
Study	**Obs**	**Mean**	**Std. Dev.**	**Obs**	**Mean**	**Std. Dev.**
Bor3	2123	1.09	2.07	848	2.72	2.51
Zav3	1690	0.48	1.71	210	3.91	3.22
	t= 9.664 p= 0.00			t= -5.765 p= 0.00		

It should be noted that the prevalence of LST>0 in either of these areas is smaller than that observed in other endemic areas previously studied(Sassi et al., 1999).

5.1.2 Post-vaccination LST

The following graphs and tables depict LST results 80 days and one year after vaccination in participants with pre-vaccination LST=0. Findings are discussed in the sections 5.1.2.1 and 5.1.2.2. Zav3 is excluded because there was no post vaccination LST measurement in that trial.

Figure 5.3 - Day 80 LST in vaccine and control arms (pre-vaccination LST=0)

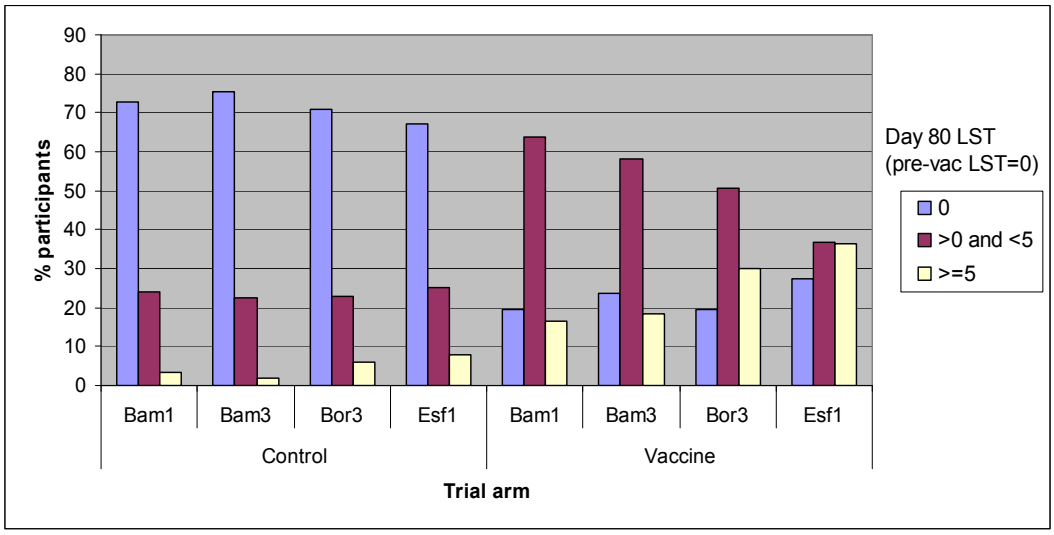


Figure 5.4 - One-year LST in vaccine and control arms (pre-vaccination LST=0)

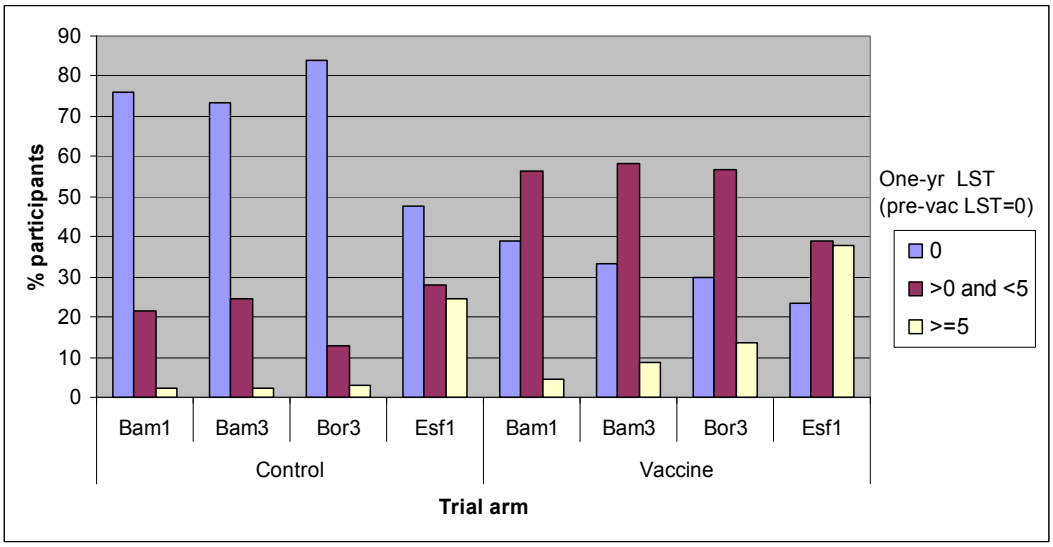


Table 5.2 - Mean LST induration on day 80 and one year after vaccination --- Pre-vaccination LST=0

		Day 80 LST		One-year LST	
Study		BCG	Vaccine	BCG	Vaccine
Bam1	mean	0.70	2.73	0.52	1.45
	std. dev.	1.46	1.94	1.49	1.64
	N	1761	1807	1654	1681
Bam3	mean	0.50	2.49	0.76	2.29
	std. dev.	1.12	2.09	1.51	1.99
	N	1932	1980	1855	1899
Bor3	mean	0.90	3.33	0.59	2.53
	std. dev.	2.19	2.73	1.73	2.21
	N	538	608	516	542
Esf1	mean	1.05	3.43	2.71	4.08
	std. dev.	2.03	2.98	3.86	3.84
	N	1104	1168	888	922

All paired comparisons between same-arm day 80 and one-year LST are significant at 0.000; except Bor3 in BCG arm at p=0.0314

Table 5.3 - Mean LST induration (if LST>0 in each evaluation) on day 80 and one year after vaccination --- pre-vaccination LST=0

		Day 80 LST		One-year LST		P	
Study		BCG	Vaccine	BCG	Vaccine	BCG	Vaccine
Bam1	mean	2.58	b 3.39	2.18	2.38		
	std. dev.	1.73	1.56	2.38	1.48		
	% LST> 0	27.2%	80.4%	24.0%	61.0%	0.020	0.000
	N (LST> 0)	479	1452	396	1024		
Bam3	mean	2.03	b 3.27	c 2.85	d 3.42		
	std. dev.	1.41	1.79	1.58	1.44		
	% LST> 0	24.5%	76.3%	26.8%	66.9%	0.105	0.000
	N (LST> 0)	473	1511	497	1271		
Bor3	mean	a 3.08	4.14	c 3.70	d 3.61		
	std. dev.	3.12	2.43	2.71	1.75		
	% LST> 0	29.1%	80.4%	15.9%	70.1%	0.000	0.000
	N (LST> 0)	157	489	82	380		
Esf1	mean	a 3.19	4.71	5.17	5.33		
	std. dev.	2.38	2.48	3.97	3.55		
	% LST> 0	32.9%	72.8%	52.5%	76.7%	0.000	0.042
	N (LST> 0)	363	850	466	707		

1. Day 80 LST: All differences between vaccine and BCG arms within the same trial/same evaluation are significant at p<0.001.
2. One-year LST: Differences between arms in Bam1 and Bam3 are significant at p<0.0001; in Bor3 at 0.33; in Esf1 at p=0.0549.
3. All pairs of differences between trials are significant at 0.00 or lower except those indicated a: p=0.951, b: p=0.061, c: p=0.095, d: p=0.506

5.1.2.1 LST 80 days after vaccination

As expected, LST response is significantly larger in the vaccine arm than the BCG arm in all trials; in both size and frequency. A certain level of LST response is

observed in BCG-alone arms of all trials, possibly due to the BCG-stimulated Th1 response and DTH.

A common pattern of LST response is not shared by trials with the same number of vaccine doses (Bor3 and Bam3 vs Esf1 and Bam1). Of note is Bam3 trial where after 3 doses of BCG or vaccine, the mean day 80 LST tends to be lower than in Bam1 or Bor3.

However, trial site (which encompasses the effect of endemic origin, incidence and causative species) seems to be a distinguishing factor: in both treatment arms, conversion to LST≥5 is more frequent in Esf1 and Bor3 (*L. major* endemic areas with 5-6% annual disease incidence) compared to Bam1 and Bam3 (*L. tropica* endemic area with 2% annual incidence).

Although Esf1 participants are largely from non-endemic origin, their intensity of LST response 80 days after vaccination is somewhat but not substantially greater than that in Bor3. However, in the course of the follow up, LST response becomes more frequent and stronger in both arms (particularly BCG) in Esf1, whereas they drop in Bor3.

5.1.2.2 LST one year after vaccination

As observed in other studies (Alimohammadian et al., 2002) some erosion in the vaccine-induced immune response is manifested by an increase in the number of participants with LST=0 in the vaccine arms in Bam1, Bam3 and Bor3 trials. At the same time, natural exposure during study follow-up were not able to maintain day 80 LST levels in endemic participants (or these endemic participants did not respond strongly to such exposure). By contrast, in Esf1 with non-endemic participants, the frequency of LST>0 and the average LST size in both arms increased from day 80 to the one year evaluation (particularly in the BCG arm).

In contrast to Bam1, LST response after the 3 doses in Bam3 trial did not decrease; rather, LST reactivity and magnitude increased particularly in the BCG arm. Despite the drop in the LST response in Bam1 and Bor3 trials, a certain level of LST response is maintained, particularly in Bor3.

In Esf1, unlike the other trials, an increase is observed in the percentage of one-year LST>0 and its magnitude compared to day 80 - this increase is observed in both arms, but it is substantial in the BCG arm. This could be attributable to the non-endemic background of participants and their resulting sensitivity to the natural exposure during the trial follow-up.

The greater increase in the frequency of one year LST>0 in the BCG compared to vaccine arm in Esf1 may indicate a natural limit for the number of people who can become LST reactive. This observation cannot be verified in Zav3 since post vaccination LST was not measured. However, examining the change in LST from day 80 to one year after vaccination in all other trials reveals the following:

- The change is very little in the BCG arm of Bam1 and Bam3 trials with endemic participants (stays at about 25%). Contrary to Bam trials, in Bor3, the endemic

area with a high annual incidence, the percent of LST>0 in the BCG arm almost halves (29.1% to 15.9% - see previous table). The change is in the opposite direction (increase significantly) in the BCG arm in Esf1 with non-endemic participants (table below).

- A point of maximum (a ceiling) may exist for the percent of individuals who become LST reactive. In the vaccine arm of trials by day 80 between 72-80% became LST>0. Over the next several months, a point of equilibrium was reached as the effect of the vaccine eroded and at the same time participants received the infected bites of sandflies. While in endemic participants the percent of LST>0 diminished during the follow up (from 78.7% to 65%), in non-endemic participants it grew (from 72.8% to 76.7%).

Table 5.4 - LST reactivity in endemic and non-endemic participants in vaccine and BCG arms: before, 80 days after and one year after vaccination

	Pre-vac	Day 80			One-year		
	Total	BCG	Vaccine	Total	BCG	Vaccine	Total
Non-endemic (% LST> 0)	12.4	32.9	72.8	53.4	52.5	76.7	64.8
N	1690	1104	1168	2272	888	922	1810
Endemic (% LST> 0)	39.9	27.6	78.7	53.6	25.2	65.4	45.4
N	2123	4630	4775	9405	4377	4437	8814
Total (% LST> 0)	27.7	28.7	77.6	53.5	29.8	67.3	48.7
N	3796	5734	5943	11677	5265	5359	10624

Note: Pre-vac LST observed only in Zav3 (non-endemic) and Bor3 (endemic)

5.1.3 Pre- and post-vaccination LST (in Bor3)

Bor3 trial provides the only opportunity to study the relationship between pre- and post-vaccination LST. Figures 5.5 and 5.6 display changes in pre-vaccination LST after 80 days and one year of vaccination, respectively.

Figure 5.5 - Day 80 LST in Bor3

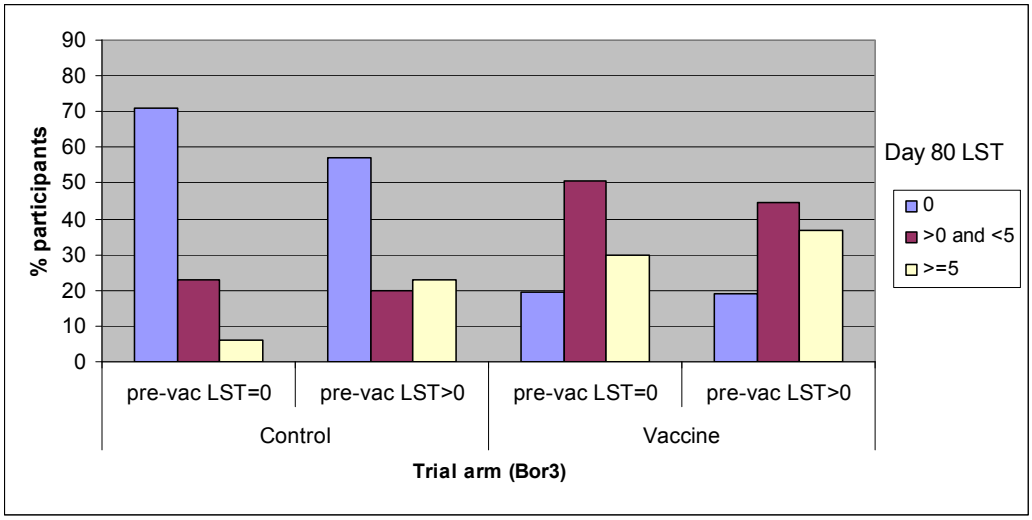
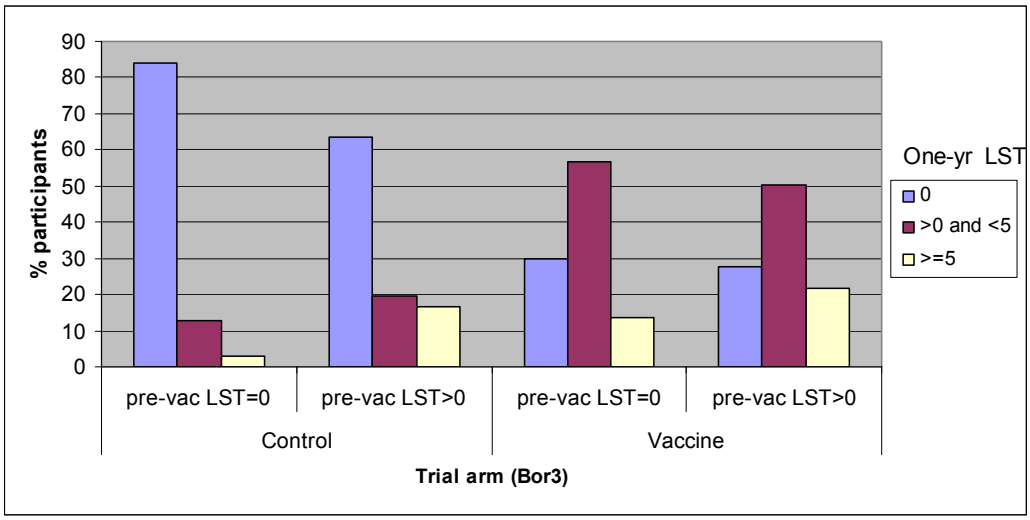


Figure 5.6 - One-year LST in Bor3



Tables 5.5 and 5.6 present changes in LST over the study period in Bor3. Among those with pre-vaccination LST<5, more of the vaccinated compared to BCG participants maintained their LST>0 status 80 days after vaccination. Vaccination led to one year LST reactivity even if day 80 LST values were non-reactive. As table 5.6 indicates, vaccination caused a sharper jump in LST in individuals with pre-vaccination LST=0 compared to LST>0. An important observation in table 5.5 is that there is a notable percentage of participants whose LST measurement changed from non-zero at screening to zero on day 80. Over one third (300/779=38.5%) of Borkhar participants with pre-vaccination LST had LST=0 on day 80.

Table 5.5 - Mean values of one-year LST associated with LST on day 80 and pre-vaccination in Bor3 for vaccine and BCG arms

		BCG				Vaccine			
		Day 80 LST categories				Day 80 LST categories			
Pre-vac LST		0	1	2	Total	0	1	2	Total
0	mean LST 1yr	0.33	0.74	4.17	0.62	1.72	2.52	3.17	2.56
	std. dev.	1.15	1.69	4.50	1.82	1.63	2.23	2.40	2.23
	N	322	103	23	448	99	250	151	500
	% of total	71.9%	23.0%	5.1%		19.8%	50.0%	30.2%	
> 0and< 5	mean LST 1yr	0.56	1.02	4.83	1.13	2.26	2.44	3.24	2.65
	std. dev.	1.73	1.64	4.37	2.51	1.81	2.16	3.17	2.48
	N	176	62	29	267	50	111	72	233
	% of total	65.9%	23.2%	10.9%		21.5%	47.6%	30.9%	
≥5	mean LST 1yr	0.33	3.33	7.22	6.29	3.00	2.86	5.98	4.72
	std. dev.	0.82	1.26	4.20	4.47	3.55	2.04	4.19	3.89
	N	6	3	48	57	7	14	30	51
	% of total	10.5%	5.3%	84.2%		13.7%	27.5%	58.8%	
Total	mean LST 1yr	0.41	0.89	5.83	1.22	1.95	2.51	3.52	2.72
	std. dev.	1.38	1.70	4.49	2.77	1.82	2.20	3.02	2.50
	N	504	168	100	772	156	375	253	784
	% of total	65.3%	21.8%	13.0%		19.9%	47.8%	32.3%	

LST categories are 0: 0mm, 1: >0mm up to 5mm, 2: LST≥5mm

Table 5.6 - Mean LST induration 80 days and one year post vaccination in pre-vaccination LST reactive participants in BCG or vaccine arm in Bor3

		Day 80		One-year	
Pre-vac LSt		**BCG**	**Vaccine**	**BCG**	**Vaccine**
LST= 0	mean	0.90	3.33	0.59	2.53
	std. dev.	2.19	2.73	2.99	2.21
	N	538	608	516	542
LST> 0	mean	2.73	4.15	2.08	3.08
	std. dev.	4.60	4.00	12.77	3.00
	N	399	380	352	315

Differences between LST=0 and LST>0 are significant in BCG arm in both evaluations ($p<0.0005$); in the vaccine arm: day 80 $p= 0.03$, one-year $p=0.10$.

The following regression models summarize the effect of earlier LST reaction and vaccination on day 80 LST measurement. The first model (table 5.7a) estimates the effect of prevaccination LST and the vaccine (vs BCG alone) on day 80 LST. The variable named Vaccine in the model is a dichotomous variable with 1 = vaccine and 0 = BCG. This model does not include an interaction term and tests the effect of the two independent variables Vaccine and Pre-vac LST independently of one another. Results indicate a significant direct relationship between both of these variables and the magnitude of day 80 LST. The beta values suggest that the pre-vaccination is the more important driver of day 80 LST.

Table 5.7a - Regressing day 80 LST on pre-vaccination LST and vaccine

Dependant: Day 80 LST	**Coef.**	**Std. Err.**	**t**	**P> |t|**	**Beta**
Pre-vac LST	0.78	0.03	23.76	0.000	0.46
Vaccine	2.02	0.14	14.73	0.000	0.28
CONST	0.79	0.11	7.54	0.000	.
R-squared	0.286				
Adjusted R-sq	0.285				
P (F test)	< 0.001				

To see the effect of prevaccination LST>0 in the two study arms separately, the second model (table 5.7a1) also includes an interaction defined as the product of Vaccine and Pre-vac LST variables (multi-collinearity was not a problem in the model, with the multiple correlation between the interaction term and its components around 0.5). For the BCG arm, the value of the interaction term is 0. In the vaccine arm, when the value of pre-vaccination LST>0 the interaction term is non-zero (and equals the pre-vaccination LST measurement).

Table 5.7a1 - Regressing day 80 LST on pre-vaccination LST, vaccine and their interaction

Dependant: Day 80 LST	Coef.	Std. Err.	t	P> |t|	Beta
Pre-vac LST	1.05	0.05	22.68	0.000	0.61
Vaccine	2.60	0.15	17.00	0.000	0.36
Interaction (Vaccine by Pre-vac LST)	-0.52	0.06	-8.06	0.000	-0.23
CONST	0.49	0.11	4.47	0.000	
R-squared	0.309				
Adjusted R-sq	0.308				
P (F test)	< 0.001				

In table 5.7a1, the significant, negative coefficient for the interaction term suggests that in the vaccine arm, non-zero pre-vaccination LST is associated with relatively smaller day 80 LST as compared to the BCG arm. This can also be verified via table 5.6. Since a non-zero prevaccination LST is necessary for the interaction term to also be non-zero, this observation may also indicate the role of previous exposure in keeping day 80 LST at moderate levels despite vaccination.

Table 5.8 presnts a similar model to that discussed above but has the one-year LST value as the dependent variable. This model estimates the effect of previous LST measurement (both prevaccination LST and day 80 LST) as well as vaccination in the two study arms. Results are similar to those seen for day 80 LST discussed above with a negative coefficient for the interaction term. Overall, the model indicates a greater association between pre-vaccination LST than day 80 LST with one-year LST measurement.

Table 5.8 - Regressing one-year LST on day 80 LST, pre-vaccination LST, Vaccine and their interaction

Dependant: One-year LST	Coef.	Std. Err.	t	P> |t|	Beta
Day 80 LST	0.30	0.03	8.56	0.000	0.23
Pre-vac LST	0.33	0.02	17.44	0.000	0.42
Vaccine	1.14	0.14	8.19	0.000	0.21
Interaction (Vaccine by Pre-vac LST)	-0.63	0.19	-3.38	0.001	-0.09
CONST	0.33	0.09	3.81	0.000	
R-squared	0.368				
Adjusted R-sq	0.366				
P (F test)	< 0.001				

From above data presentations (tables 5.5 through 5.8), it is clear that LST reaction at any stage is related to both, previous immune response and vaccination status. Primarily, however, natural exposure (boosted by BCG or vaccine) plays a major role in a lasting (one year) LST response. These observations are summarized below:

Strong immune response to natural exposure, manifested by pre-vaccination LST≥5, influences post-vaccination LST results: in the BCG arm, both 80 days and one year after vaccination, LST response is more frequent and stronger if pre-vaccination LST≥5. Vaccination also plays a significant role in increasing LST.

Based on table 5.5, larger pre-vaccination LST in the BCG arm leads to larger day 80 LST. In the vaccine arm this relationship is more moderate. This is demonstrated in tables 5.7a1 and 5.8, where the negative interaction term suggests vaccination in participants with reactive pre-vaccination LST leads to lower day 80 and one-year LST results than in those with pre-vaccination LST=0.

It should be noted that of those with pre-vaccination LST>0 nearly 19% in the vaccine arm and over 57% in the control arm reverted to LST=0 by day 80 (64% and 28% respectively reverted in one year). This demonstrates the volatility inherent in LST and its implication for vaccine clinical trials: had the volunteers been screened 80 days later, 38.5% of those with pre-vaccination LST>0 would have been accepted into the trial as having LST=0, and assumed unexposed, a source of misclassification.

5.1.3.1 Estimation of the number of non-responsive participants in the sample

The design of the Bor3 study which includes a number of LST tests including pre-vaccination, allows a rough estimate of the prevalence of lack of sensitivity/responsiveness to the leishmanial antigen in Bor3. The subset of the sample who received the vaccine, whose LST stayed at 0 from pre-vaccination until after the final dose of the vaccine (4 LST evaluations overall), and who did not develop the clinical disease would include non-responders; i.e., the percentage of such individuals would be the upper limit for the vaccine arm prevalence of those whose immunology does not respond to the parasite. A total of 28 participants with these qualifications could be identified. This suggests 3.72% (95% CI: 2.37%-5.07%) of all vaccinated trial participants with complete LST profile (753 individuals) potentially lack sensitivity to the antigen. Furthermore, if they indeed lack sensitivity to the antigen, they could be potentially immune.

This estimate has an upward bias due to: a) during the follow up some of these participants may not have been exposed to the infected sandfly bite in the first year; some such individuals could have responded to natural exposure had there been another LST test at the end of the 3 year follow up, and b) LST imperfect sensitivity and (for example, the BCG within the vaccine mix may have not been effective, leading to LST's inability to identify exposure).

5.2 LST results in demographic groups

5.2.1 Pre-vaccination LST

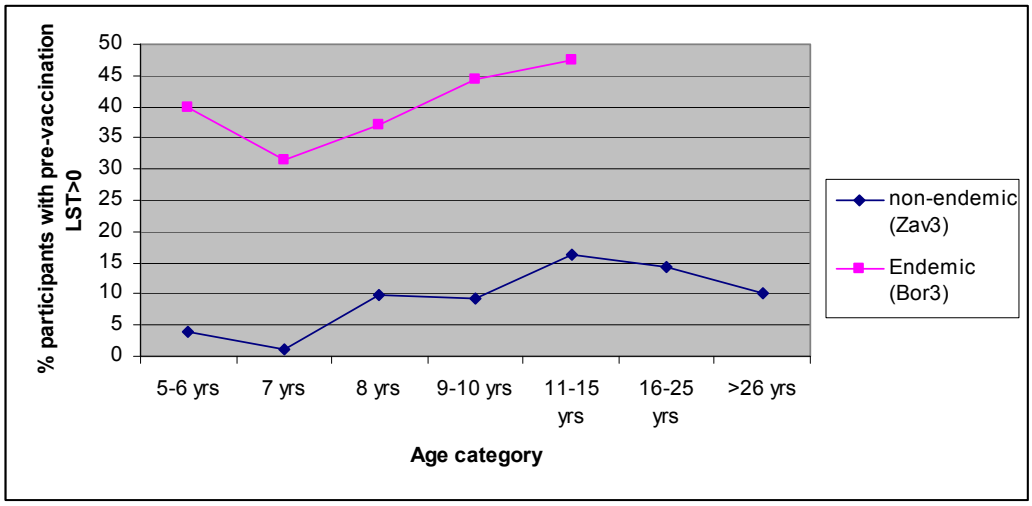
As indicated earlier, the naturally acquired pre-vaccination LST>0 is more prevalent but with generally smaller induration in Bor3. LST prevalence and induration (among those with LST>0) in both trials is age dependent. This is indicated in the following table and figure 5.7. In ages up to 13 years, the relationship is equally significant in both trials.

Table 5.9 - Mean values of pre-vaccination LST (including all LST values) in Bor3 and Zav3 and ANOVA* results

	Bor3			Zav3		
Age categories	**Mean**	**Std. Dev.**	**Freq.**	**Mean**	**Std. Dev.**	**Freq.**
5-6 yrs	0.86	1.60	236	0.20	0.99	50
7 yrs	0.87	1.93	445	0.04	0.41	94
8 yrs	1.02	2.01	461	0.34	1.19	93
9-10 yrs	1.23	2.21	764	0.32	1.06	170
11-15 yrs	1.41	2.35	217	0.60	1.71	592
16-25 yrs	--	--	--	0.57	1.75	321
> 26 yrs	--	--	--	0.52	2.28	360
Total	1.09	2.07	2123	0.49	1.72	1680

ANOVA results: Bor3: F=4.36, Pr>F=0.001; Zav3: F=2.21, Pr>F=0.040

Figure 5.7 - Age-based pre-vaccination LST>0 in endemic and non-endemic participants (by design, Endemic participants were limited to school age children)



(Trend test in Bor3: Z=4.36, p<0.001; in Zav3: Z=2.33, p=0.020)

The relationship between age and prevalence of naturally developed LST up to the 11-15 yr age group shows age-dependence in both Bor3 and Zav3. However, when considering age groups above 11-15 years (figure 5.7) this relationship is non-linear in Zav3. This is a deviation from the expected linear LST/age relationship in endemic foci, where longer exposure leads to more prevalent LST>0 in older residents.

The regression results in table 5.10 (based on participants with pre-vaccination LST>0 only) demonstrate the age-dependence of pre-vaccination LST induration in Bor3 (endemic) and Zav3 (non-endemic). The coefficient for participants' endemic origin is negative; indicating significantly smaller average LST induration in endemic participants in Bor3. Age is a statistically significant predictor, although the 0.049 R squared indicates that other factors than age are stronger determinants of LST induration.

Table 5.10 - Regression results of pre-vaccination LST on age in Bor3 and Zav3
LST=f (age+endemic) -- including only those with pre-vaccination LST>0

	Coef.	Std. Err.	t	P> |t|	Beta
age	0.07	0.02	4.74	0.00	0.18
endemic	-0.48	0.26	-1.86	0.06	-0.07
CONST	2.55	0.34	7.47	0.00 .	

Adjusted R squared=0.049

The relationship between pre-vaccination LST and gender in Bor3 and Zav3 is presented in table 5.11. In Bor3 a significantly higher percent of girls (45.4% vs 34.7% boys) had non-zero pre-vaccination LST. By contrast, in Zav3, no significant relationship between gender and pre-vaccination LST could be observed.

Table 5.11 - Distribution of pre-vaccination LST by Gender

Study		F	M
Bor3	N	1042	1081
	% with LST= 0	54.6	65.3
	% with LST> 0	45.4	34.7
Zav3	N	831	859
	% with LST= 0	88.0	87.2
	% with LST> 0	12.0	12.8

Fisher's exact: Bor3 p<0.001, Zav3 p=0.342

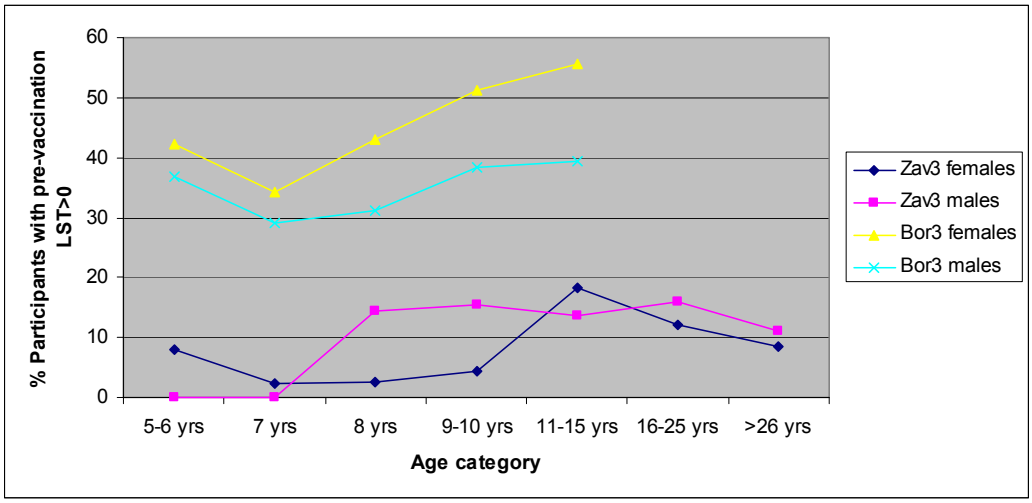
Although the percentage of pre-vaccination LST>0 is greater in females in Bor3, the average LST induration in both trials is larger in males (the significant difference in Zav3 holds true even when age is limited to <15, p=0.0001). This is indicated in the table 5.12. On average, pre-vaccination LST size in males in both trials (all ages) is significantly higher than in females.

Table 5.12 - Mean values of pre-vaccination LST in males and females with pre-vaccination LST>0 in Bor3 and Zav3

	Bor3					Zav3				
	Obs	Mean	Std. dev.	t	p> t	Obs	Mean	Std. dev.	t	p> t
Female	473	2.48	2.20	-3.11	-0.002	100	3.10	2.60	-3.71	0.000
Male	375	3.02	2.83			110	4.69	3.52		

To see the interaction between age and gender, graph 5.8 below breaks the age/LST relationship further down by gender. Trend tests and tests of LST mean differences between male and female participants (presented below) confirm age-LST relationship in both males and females in Bor3.

Figure 5.8 - Age-based pre-vaccination LST>0 in endemic (Bor3) and non-endemic (Zav3), male and female participants



Trend test in Bor3: females: Z=3.82, p<0.001, males: Z=1.84, p=0.066 (Z=2.10, p=0.036 if mean LST is used instead of %LST>0); no significant trend in Zav3 (females: p=0.135, males: p=0.073)

In Bor3, pre-vaccination LST is linearly age-related in males and females, as indicated in table 5.13. The same relationship in Zav3 (marginally significant at p =0.049) does not show gender-dependence. The initial high LST in Bor3 may indicate disease while the subsequent drop and gradual rise may indicate LST due to building up of immunity.

Table 5.13 - Male female comparison on mean pre-vaccination LST in Bor3 and Zav3

	Bor3		Zav3	
Age categories	**Male**	**Female**	**Male**	**Female**
5-6 yrs	0.83	0.91	0.40	0.00
7 yrs	0.89	0.84	0.09	0.00
8 yrs	1.11	0.93	0.22	0.43
9-10 yrs	1.25	1.22	0.12	0.55
11-15 yrs	1.55	1.27	0.48	0.74
16-25 yrs	--	--	0.39	0.67
> 26 yrs	--	--	0.39	0.60

In Zav3, the difference between males and females is mainly in ages 8-10 and, assuming this reflects a real difference, it may be due to greater exposure in 8-10 year old boys in this newly endemic area (girls may be more covered or spend less time outdoors particularly during peak hours of sandfly activity). However, as stated, other than this difference there is no consistent trend with age.

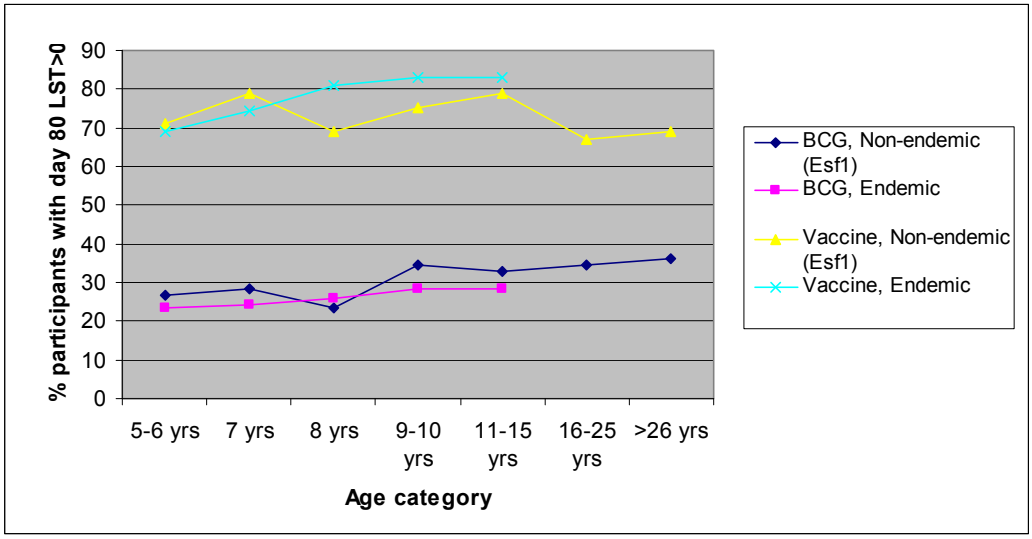
Overall, these findings suggest that in an established endemic area, as expected, most people eventually are exposed (i.e., increased chance of exposure with age), regardless of gender and, as a result, pre-vaccination LST is age-related. On the other

hand, in a newly endemic area pre-vaccination LST may be more related to the differences in exposure for male and female participants, in childhood (ages 8-10) without a continuous trend with age.

5.2.2 Day 80 LST

Trend tests for day 80 LST over age categories are significant for all except vaccine arm, non-endemic participants. Nonetheless, these relationships are not very strong or consistent (figure 5.9) and do not seem to have immediate epidemiological explanation. The finding for the Esf1 BCG arm is consistent with the finding in Chapter 4 (table 4.4) that in the BCG arm in Esf1 there is a positive but borderline significant relationship between age and incidence. The increased percentage of LST>0 in higher ages in the BCG arm in Esf1, therefore, may show developing infection due to natural exposure. This is, however, unlikely since the study start was selected so that the 80 days after vaccination would fall within the non-transmission period.

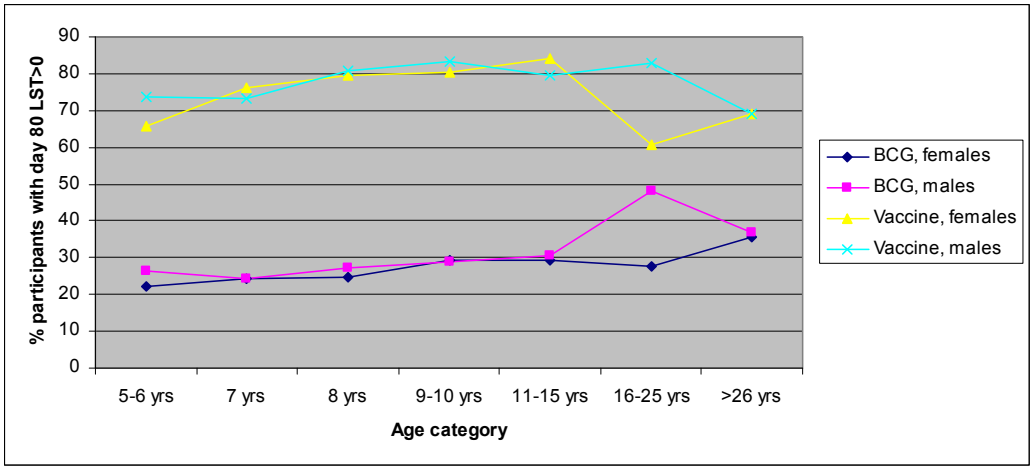
Figure 5.9 - Age-based day 80 LST>0 in participants from endemic or non-endemic origin (Endemic participants were limited to school age children-- all pre-vac LST=0)



Trend test for age: Non-endemic BCG: z = 2.29, p=0.022, vaccine: z = -1.05; p=0.292; Endemic BCG: z = 2.74, p=0.006, vaccine: z = 7.28, p<0.001

Graph 5.10 shows the relationship between gender and LST. The only interaction between gender and age is the difference observed between male and female participants in the 16-25 year age category, both in the vaccine and the BCG trial arms. The only immediate explanation is that local customs and risk behaviours promote such differences.

Figure 5.10 - Age-based day80 LST>0 in male and female participants in vaccine and control arms (endemic participants were limited to school age children-- all pre-vac LST=0)



Trend test for age: Females BCG: z = 3.86 p< 0.001, vaccine: z = 1.40, p=0.161; Males BCG: z = 3.63, p<0.001, vaccine: z = 1.65, p=0.099

In Bor3 male participants in the BCG arm with pre-vaccination LST>0 have a significantly greater tendency to maintain their LST levels after 80 days and after one year (table 5.14). This is probably independent of further natural exposure in these male participants since by day 80 the re-exposure to the sandfly bites has presumably not yet started (study start date was chosen so that day 80 would be before the start of the transmission season).

Table 5.14 - Post-vaccination LST in control arm participants with pre-vaccination LST>0 in Bor3

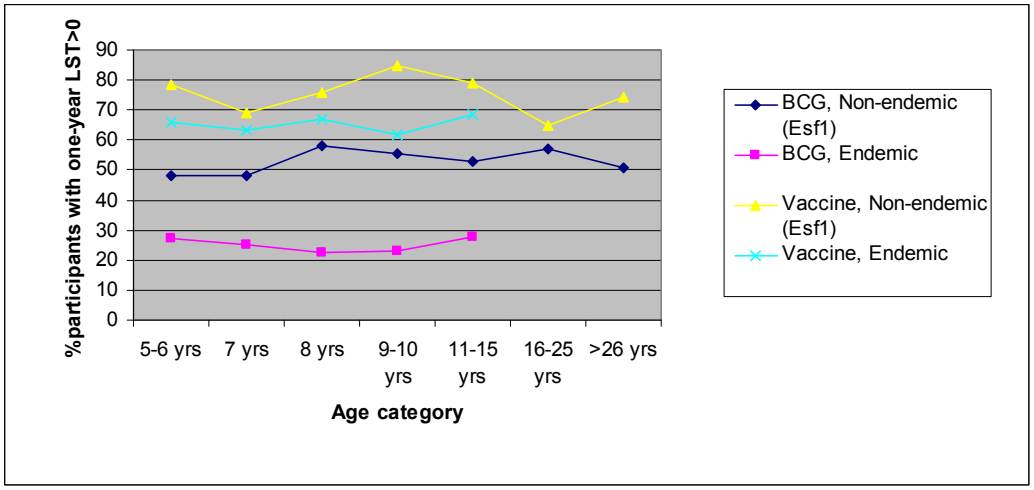
LST		Female	Male
Day 80	N	217	182
	% with LST= 0	62.7	50.5
	% with LST> 0	37.3	49.5
One-year	N	191	161
	% with LST= 0	72.3	53.4
	% with LST> 0	27.7	46.6

Fisher's exact: day 80 p=0.019, one-year p<0.0005

5.2.3 One year LST

Graph 5.11 below, depicts changes in LST from day 80 to the one-year evaluation. Overall, the change does not seem to be age-dependent, regardless of the endemic origin of participants, or study treatment. For all age groups combined, the frequency of LST response in the BCG arm of Esf1 (non-endemic participants) increased significantly from day 80 (as discussed earlier, tables 5.2 and 5.3). In Bor3 (endemic participants) other than a slight drop in day 80 LST measurements, no sizeable change was observed in LST from day 80. This observation points at a state of equilibrium with respect to LST response in endemic participants. This state of equilibrium is missing in non-endemic participants who respond massively to natural exposure.

Figure 5.11 - Age-based one-year LST>0 in participants of endemic and non-endemic origin in vaccine and control arms (Endemic participants were limited to school age children-- all pre-vac LST=0)



Trend test for age: all p> 0.3

5.3 Summary

Immune response and LST development over time as a result of vaccination and natural exposure was assessed overall and in demographic groups. As expected, ALM+BCG vaccine is a significantly greater immune response stimulator than BCG alone, as demonstrated by day 80 and then one-year LST evaluation (which do not differ significantly in endemic participants, but an increase from day 80 to one-year is observed in non-endemic participants due to natural exposure).

Overall, the hypothesis of no difference in immune response being associated with demographic characteristics can be rejected.

Naturally developed LST (pre-vaccination LST) was not an exclusion criterion in Bor3 and Zav3; therefore, participants with screening LST>0 were included in these trials. In comparison to Bor3 LST is less prevalent but has larger average induration among the non-endemic participants in Zav3. By contrast, in Bor3 naturally induced LST is observed more frequently but with moderate induration.

LST due to natural exposure is, as expected, age dependent, both in its prevalence and induration. In Zav3, where participants were as old as 59 years, the prevalence of pre-vaccination LST>0 peaks at the 11-15 age group and drops thereafter. This phenomenon may be confirmation that the site in Zav3 is a new endemic area and the population do not demonstrate the same age/exposure characteristic as in an endemic focus (where LST prevalence is expected to increase with age). In Bor3 the more or less linear age/LST correlation is expected since the trial site had been an endemic focus for many years. However, this could only be demonstrated within the trial age range (6-13 years). Therefore, it can not be compared with Zav3 for ages above 13 years.

Pre-vaccination LST is more frequent in females in Bor3; however, induration is significantly larger in male participants in both trials. Additionally, male participants in the control arm tend to maintain their LST reactivity more than females.

An interesting observation with respect to pre-vaccination LST in Borkhar was that some of the participants with pre-vaccination LST>0 had LST=0 readings on day 80. This suggests that LST has a volatile nature and not always a reliable indicator of exposure.

Post-vaccination LST was measured in most trials in 80 days and then one year after vaccination. As mentioned, stronger LST response was observed in the vaccine arms of trials. In general, the one-year LST results diminished slightly but did not change dramatically from day 80 except that the average induration in vaccine and BCG arms converged somewhat. In trials in *L. major* foci, in Esf1 with non-endemic participants average day 80 LST results were similar to Bor3 with endemic participants; one-year LST results however, were much stronger in Esf1, presumably due to *leishmania*-naive participants' response to the natural exposure during the year. The fact that LST>0 proportion among endemic participants does not continue growing during the follow up (despite continuous exposure) may indicate a point of equilibrium (or maximum) for LST conversion in a population.

A common pattern of LST response could not be identified for trials with multiple doses of the vaccine/BCG.

CHAPTER 6

LST RESPONSE AS A CORRELATE OF IMMUNITY

Immunization with leishmanial antigen, BCG injection and natural exposure could, to varying degrees, stimulate cellular response to *Leishmania* and increase LST (Modabber and Reed, 2004). Exposure to the parasite in populations of endemic foci is generally life-long and repeated. In a newly endemic area (or during an epidemic in a non-endemic area) exposure is not repeated over a long period of time. The dogma maintains that significant immune system stimulation (DTH) due to natural exposure (and the ensuing positive LST response) is linked to protection.

Repeated exposure (i.e., asymptomatic, subclinical infection) and previous clinical infection are common in leishmaniasis endemic foci and are believed to be detectable via LST≥5 (Jumaian et al., 1998;Weigle et al., 1993), which in turn is believed to be associated with immunity (endemic immunity) (Ben Salah et al., 2005;Davies et al., 1995;Guirges, 1971). In other words, LST≥5 (generally accepted as "positive"), if not indicative of a developing current infection, would be associated with protection.

Bor3 and Zav3 provide pre-vaccination LST measurements and the opportunity to test the dogma; i.e., protection associated with pre-vaccination LST>0 or LST>5 in endemic and non-endemic populations (after boosting with BCG alone or ALM+BCG vaccine). The opportunity is also provided to assess LST as a reliable/lasting indicator of pre-vaccination exposure by assessing the change in LST measurement during the follow-up.

It has been observed in some of the trials (Antunes et al., 1986;Khalil et al., 2000a;Momeni et al., 1999) that LST≥ 5mm measured 40-80 days after vaccination is associated with a reduced incidence of disease. However, this has not been suggested by all vaccine trials -- either because this effect was not present or because different trials were not equally able to indicate it, owing to their differences in incidence rate, levels of endemic immunity, etc. It is of interest to further test whether disease incidence is reduced in LST responsive participants using the large combined sample size of the present study and to observe whether it holds in various subgroups.

In this chapter incidence and vaccine efficacy will be studied in connection with the DTH response as measured by LST at different points in time, before and after vaccination, in the vaccine or the BCG arm.

The overall objective is to evaluate the association between LST response and immunity (due to intervention or natural exposure) and examine LST's merits as a correlate of immunity.

6.1 Naturally developed (pre-vaccination) LST and protection

To test that LST≥5 due to natural exposure is associated with immunity, the incidence in participants with pre-vaccination LST≥5 or LST<5 in the Bor3 and Zav3 trials were compared. The important caveat is that all participants received either BCG or the

vaccine, ALM+BCG. Therefore, the comparison of the clinical outcome between LST<5 and LST≥5 groups does not show the pure effect of naturally acquired LST≥5 but rather its joint effect with BCG (which could augment the effect of previously activated DTH).

The hypothesis to be tested should take BCG into account, as follows:

H0: % cases among those with pre-vaccination LST≥5+BCG equals % cases among those with pre-vaccination LST<5+BCG
H1: % cases among those with pre-vaccination LST≥5+BCG is less than % cases among those with pre-vaccination LST<5+BCG

The following table displays the % cases in the two studies associated with pre-vaccination data:

Table 6.1 - Incidence in pre-vaccination LST groups (BCG and vaccine arms) in Zav3 and Bor3, combined

Pre-vaccination LST		**Bor3+ Zav3**
LST < 5		12.9%
	N	3595
LST ≥5		4.4%
	N	210
TOTAL		12.5%
	N	3805
Fisher ex. p		< 0.001

This analysis leads to the rejection of the hypothesis as formulated; i.e., it leads to the conclusion that LST≥5 due to natural exposure is in fact associated with significant protection, when considering the data from both trials combined (keeping in mind that all participants had received BCG either with or without ALM antigen and these results could partially reflect its effect)

6.1.1 Further observations in relation to naturally developed LST

Further examination of the number of cases at LST<5 and LST≥5, reveals that in Bor3, LST≥5 was associated with significant protection, regardless of vaccination status; in Zav3, LST≥5 is associated with borderline significant protection. This is depicted in the table 6.2 below. This difference is linked to the smaller prevalence of LST≥5 in Zav3 (55 compared to 148) in spite of the higher annual incidence rates in Zav3 (figure 4.8). Although the difference in % cases among LST+ participants between the two trials is not statistically significant, the difference in the % of LST≥5 between Bor3 and Zav3 is quite significant ($p < 0.001$). This difference probably reflects the difference in endemicity between the two trial sites: Zavareh, a new endemic area, has smaller overall prevalence of exposure. The are further differences between Bor3 and Zav3 in their patterns of exposure and incidence. These differences will be discussed in the next section.

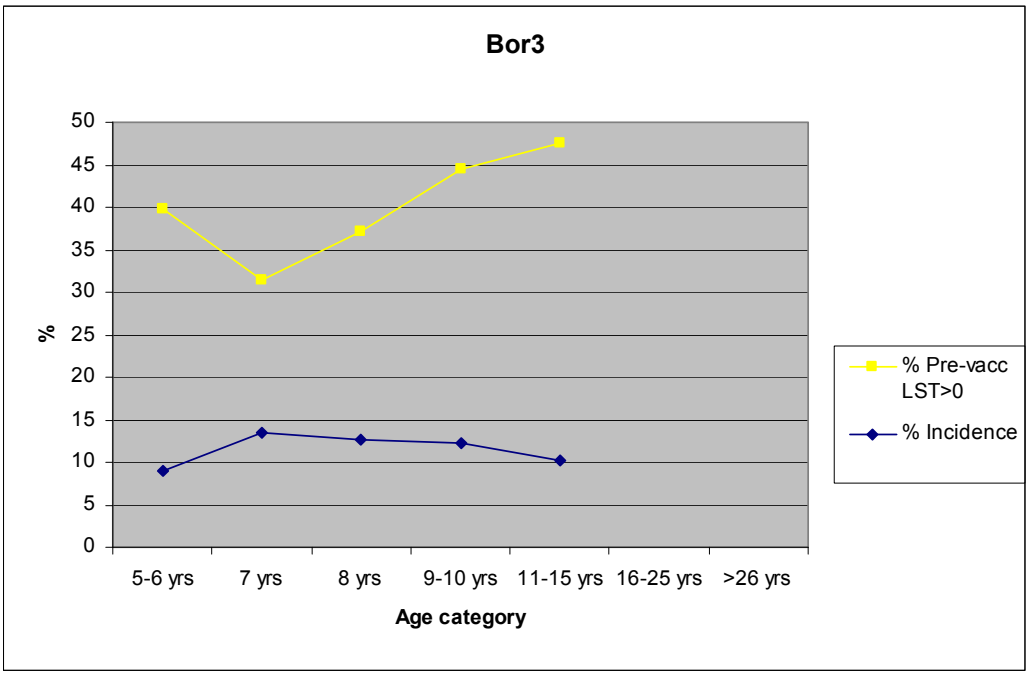
Table 6.2 - Cases in pre-vac LST groups in Zav3 and Bor3

Pre-vaccination LST		Zav3	Bor3
LST<5		13.2%	12.6%
	N	1620	1975
LST≥5		4.8%	4.1%
	N	62	148
TOTAL		12.9%	12.0%
		1682	2123
Fisher's exact p		0.053	0.001

6.1.1.1 Differences in endemic exposure and immunity between Bor3 and Zav3

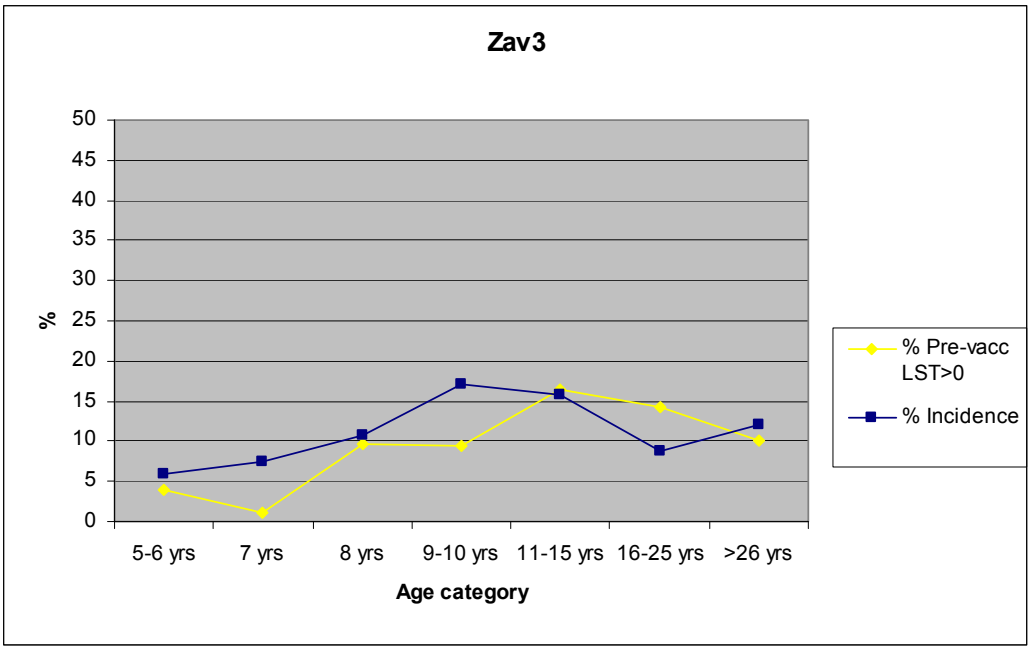
As discussed in Chapter 5, pre-vaccination exposure (detectable by LST) is age related in both Bor3 and Zav3 (up to age 13 - see figure 5.7). In Bor3, as suggested by figure 6.1, the protective effect of endemic exposure (detectable by pre-vaccination LST) appears age-based, as would be expected of an endemic focus. However, this is not the case in Zav3, the endemic focus with non-endemic population (figure 6.2).

Figure 6.1 - Pre-vaccination LST and incidence by age in Bor3



Trend test significant for LST ($p<0.0005$) but not for incidence

Figure 6.2 - Pre-vaccination LST and incidence by age in Zav3



Trend test significant for LST (p=0.020) but not incidence

This observation should be considered in conjunction with another finding discussed in Chapter 5 in relation to the induration and prevalence of pre-vaccination LST. As discussed in Chapter 5, compared to Zav3, pre-vaccination in Bor3 is more prevalent but with more moderate induration, on average (see table 5.1). Together these findings suggest distinct profiles associated with naturally developed, protective DTH and LST results in endemic and non-endemic populations under high incidence conditions.

6.2 Post vaccination LST and protection

6.2.1 Day 80 LST and protection

In some of the previous vaccine trials, LST conversion to ≥5 in 40-80 days after vaccination has been linked to lower incidence in the vaccine arm. In these trials, lower incidence was observed in the subset of participants whose immune system was stimulated by the vaccine (evident by the LST≥5). To test whether the current data support the notion that vaccine induced immune system stimulation could lead to protection, the following hypothesis is formulated:

H0: % case in participants with day 80 LST conversion to ≥5 in the vaccine arm equals % case in participants in the BCG arm or those with day 80 LST<5 in the vaccine arm

H1: % case in participants with day 80 LST conversion to ≥5 in the vaccine arm is less than % case in participants in the BCG arm or those with day 80 LST<5 in the vaccine arm

The hypothesis formulated above is in line with and addresses the same question as in the study by Antunes and Mayrink (Antunes et al., 1986). Khalil (Khalil et al., 2000a) and Momeni (Momeni et al., 1999) tested somewhat different hypotheses. In Esf1, Momeni et al limited their analysis to the first year follow up results. Furthermore, both Momeni and Khalil included with the LST≥5 group not only participants from the vaccine arm but also the LST converted participants from the control arm. Effectively, they tested the efficacy of LST conversion rather than the vaccine. The hypothesis formulated above tests the reduction in incidence associated with the ALM+BCG vaccine in LST converted vaccine arm participants against all others. The group of interest is not defined by LST conversion but LST conversion due to the vaccine.

Examining incidence in individual trials, after excluding those with pre-vaccination LST>0, does not provide statistically significant evidence that vaccine was efficacious in conjunction with day 80 LST≥5. These results are presented below.

Bam1: Reduced incidences observed in the vaccine arm (in all LST categories and overall) are not statistically significant, as demonstrated in the following table.

Table 6.3 - Incidence in day 80 LST groups in vaccine and BCG arms in Bam1

Bam1		LST= 0	0< LST< 5	LST≥5	Total
BCG		3.0%	4.5%	5.2%	3.4%
	N	1282	421	58	1761
Vaccine		2.8%	2.9%	2.7%	2.8%
	N	355	1154	298	1807
Total		2.9%	3.3%	3.1%	3.1%
	N	1637	1575	356	3568
Fisher ex. p		0.526	0.074	0.260	0.212

		Vaccine arm		
		LST≥5	All others	Fisher Ex.
	%	2.7%	3.1%	p=0.411
	N	298	3270	

Bam3: Similar to Bam1, differences are not significant, as depicted in the table 6.4. The only difference between the design of Bam1 and Bam2 trials was the number of study injections which may be the reason for higher incidence in LST>5 in the vaccine arm (possibly due to stronger stimulation of cases in incubation by the vaccine compared to BCG alone -- this will be discussed later).

Table 6.4 - Incidence in day 80 LST groups in vaccine and BCG arms in Bam3

Bam3		LST= 0	0< LST< 5	LST≥5	Total
BCG		4.9%	4.6%	2.6%	4.8%
	N	1459	434	39	1932
Vaccine		3.6%	3.5%	5.8%	3.9%
	N	469	1150	361	1980
Total		4.6%	3.8%	5.5%	4.3%
	N	1928	1584	400	3912
Fisher ex. p		0.160	0.182	0.346	0.134

		Vaccine arm LST≥5	All others	Fisher Ex.
	%	5.8%	4.2%	p=0.099
	N	361	3551	

Bor3: In Bor3, similar to Bam3, results are not significant (table below). However, when comparing LST positives and LST negatives, significant protection is associated with the former, regardless of vaccine arm.

Table 6.5 - Incidence in day 80 LST groups in vaccine and BCG arms in Bor3

Bor3		LST= 0	0< LST< 5	LST≥5	Total
BCG		12.3%	11.3%	3.0%	11.5%
	N	381	124	33	538
Vaccine		9.2%	13.4%	8.2%	11.0%
	N	119	307	182	608
Total		11.6%	12.8%	7.4%	11.3%
	N	500	431	215	1146
Fisher ex. p		0.228	0.342	0.260	0.390

		Vaccine arm LST≥5	All others	Fisher Ex.
	%	8.2%	11.8%	p=0.098
	N	182	964	

Esf1: Similar to Bor3, results are not statistically significant, as displayed in the following table.

Table 6.6 - Incidence in day 80 LST groups in vaccine and BCG arms in Esf1

Esf1		LST= 0	0< LST< 5	LST≥5	Total
BCG		19.2%	18.5%	14.9%	18.7%
	N	741	276	87	1104
Vaccine		16.7%	20.1%	17.5%	18.2%
	N	318	427	423	1168
Total		18.4%	19.5%	17.1%	18.4%
	N	1059	703	510	2272
Fisher ex. p		0.191	0.329	0.344	0.393

		Vaccine arm LST≥5	All others	Fisher Ex.
	%	17.5%	18.7%	p=0.315
	N	423	1849	

Overall, no significant reduction in incidence can be observed in individual studies among the immunologically responsive (LST≥5) vaccine arm participants compared to others or between vaccine and BCG arms within the same LST group.

The next step in this analysis is to estimate via meta-analysis the reduction in incidence in all Iran trials associated with both ALM+BCG vaccine and subsequent day 80 LST≥5. As stated earlier, this analysis is directed at testing reduction in incidence associated with the vaccine. Therefore, it focuses on the effect on incidence of LST response associated with ALM+BCG vaccine but not with BCG alone. Including day 80 LST conversion in the BCG arm would test not LST conversion due to the vaccine but rather LST conversion, regardless of the stimulus. Incidence rates and risk ratios in different trials are presented in table 6.7 and 6.8. Figure 6.5 displays the forest plot for this analysis.

Table 6.7 - Incidence in the treatment/LST groups

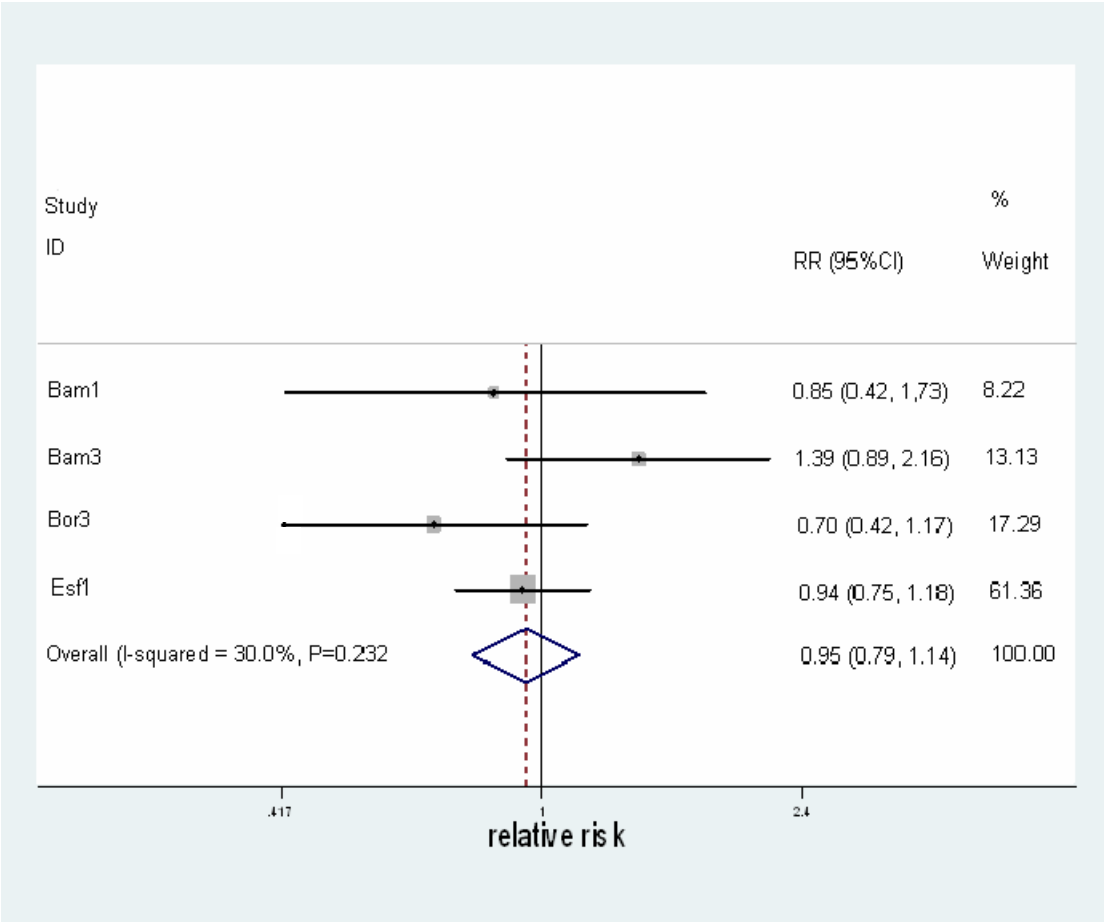
Study	BCG or LST< 5 Total N	BCG or LST< 5 Cases	Vaccine & LST≥5 Total N	Vaccine Cases	% Case (BCG or LST< 5)	% case (Vaccine & LST≥5)
Bam1	3270	103	298	8	3.15	2.68
Bam3	3551	149	361	21	4.20	5.82
Bor3	964	114	182	15	11.83	8.24
Esf1	1849	345	423	74	18.66	17.49

Table 6.8 - Meta-analysis of RR results between (LST≥5 in vaccine) and all others

Study	RR	95% Conf. Interval		% Weight
Bam1	0.852	0.419	1.733	8.22
Bam3	1.386	0.889	2.161	13.13
Bor3	0.697	0.417	1.166	17.29
Esf1	0.938	0.747	1.177	61.36
M-H pooled RR	0.948	0.79	1.137	100

Heterogeneity chi-squared = 4.29 (d.f. = 3) p = 0.232
I-squared (variation in RR attributable to heterogeneity) = 30.0%
Test of RR=1 : z= 0.58 p = 0.565

Figure 6.3 - Forest plot of effect in LST responsive vs non-responsive subsets in Iran clinical trials



Meta-analysis results (overall RR=0.948, p=0.565) do not lead to the rejection of the null hypothesis; i.e., they do not support the notion that significant protection is associated with vaccination even when LST converts on day 80. It should be emphasized, however, that a reformulated hypothesis (for example first year follow up results only, etc) could potentially lead to different results.

6.2.1.1 Day 80 LST response and protection

Increased LST after vaccination (80 days) could be due to different reasons. Just as a raised LST could show immune system stimulation by vaccine or BCG, it could also show a developing infection destined to become a case. As a result, compared to those with LST<5, the pool of LST≥5 participants would include a larger percentage of those who would later be identified as clinical cases. Due to the stronger stimulation by the ALM+BCG compared to BCG alone, this imbalance would be more pronounced in the vaccine arm, leading to the observation that LST stimulation by the vaccine that would lead to LST<5 could lead to protection. In fact, such an imbalance should not be viewed as indicative of protection by the study treatment but rather the result of the LST≥5 classification criterion.

Accordingly, caution should be exercised in analysing the association between day 80 LST of 0<LST<5 and protection when combining the data from all clinical trials. This is indicated in the following table. As mentioned previously (tables 6.3 to 6.6), comparison of the two arms within the same LST group in each trial separately does not lead to significant findings. However, when combining the data from different trials, vaccination appears to be linked with a significant reduction in cases in the LST response group of 0<LST<5, as indicated in the table below.

Table 6..9 - Incidence in day 80 LST groups in vaccine and BCG arms in all trials combined (pre-vaccination LST=0)

All trials	LST= 0	0< LST< 5	LST≥5	Total
BCG	7.7%	8.3%	8.3%	7.9%
N	3863	1255	217	5335
Vaccine	7.2%	6.6%	9.3%	7.4%
N	1261	3038	1264	5563
Total	7.6%	7.1%	9.2%	7.6%
N	5124	4293	1481	10898
Fisher ex.	0.304	0.029	0.366	0.306

In the above table, incidence in the BCG arm is the same in both 0<LST<5 and LST≥5 groups while in the vaccine arm, it is significantly lower in the medium LST group compared to:

- Vaccine arm LST≥5 group (p=0.002)
- BCG arm LST<5 (0.047)
- Entire BCG arm combined (p=0.030)
- All other cells in the table (BCG or vaccine), combined (0.012)

The difference is not significant between the 0<LST<5 and LST=0 groups in the vaccine arm.

Initially, this observation may appear to indicate protection among those whose LST on day 80 was minimally stimulated by the vaccine. However, this finding may be at least partially explainable by the LST-based grouping of study subjects: cases who received the vaccine have higher LST than healthy individuals who received the vaccine. Therefore, in the vaccine arm, more cases end up in the high LST group and fewer in the medium LST group. This is not because more individuals have been protected but because they have been grouped into the high LST category.

This can be seen by observing the distribution of cases in the LST groups in the BCG and vaccine arms (% LST group within case/healthy participants), indicated below:

Table 6.10 - Day 80 LST distribution among healthy and infected in four trials (throughout the follow-up duration)

	BCG			Vaccine		
Day 80 LST	Healthy	Case	Total	Healthy	Case	Total
N	4,915	420	5,335	5,154	409	5,563
0	72.5%	71.0%	72.4%	22.7%	22.3%	22.7%
0< and < 5	23.4%	24.8%	23.5%	55.1%	48.9%	54.6%
≥5	4.1%	4.3%	4.1%	22.2%	28.9%	22.7%
Fisher's Exact p:			0.755			0.008

As can be seen in the above table, an imbalance of about 6-8% of cases is observed between the medium and the high LST groups only in the vaccine arm and not in the BCG arm. This imbalance leads to a lower proportion of cases in the medium LST group in the vaccine arm that is statistically significant and could be interpreted as protection.

6.2.1.2 Lower first year incidence in day 80 LST positive participants in L. major foci

In Esf1, the *L. major* foci with non-endemic participants, stimulation by day 80 of the immune system (LST≥5) is associated with significantly reduced number of cases compared to those not stimulated (in the first year of follow-up only). This effect is observed in both study arms (table 6.11).

Table 6.11 - Incidence (first year of follow-up) in Esf1 (L. major focus with non-endemic participants)

Day 80 LST		BCG	Vaccine	Total
< 5		10.03%	11.28%	10.56%
	N	1017	745	1762
≥5		3.45%	7.33%	6.67%
	N	87	423	510
Total		9.51%	9.85%	9.68%
	N	1104	1168	2272
P (incidence diff)		0.0446	0.0296	0.0089

Bor3, conducted in endemic participants in another *L. major* focus, shows identical trends but with much smaller incidence in all cells of the table (possibly due to left over endemic immunity, even after excluding all pre-vaccination LST>0). As a result, Bor3 results are not significant. However, combining first year follow up results in Bor3 and Esf1 leads to highly significant results consistent with Esf1, as displayed in tables 6.12 and 6.13.

Table 6.12 - Incidence (first year of follow-up) in Bor3 (pre-vaccination LST=0)

Day 80 LST		BCG	Vaccine	Total
< 5		1.98%	1.41%	1.72%
	N	505	426	931
≥5		0.00%	0.55%	0.47%
	N	33	182	215
Total		1.86%	1.15%	1.48%
	N	538	608	1146
P (incidence diff)		0.4145	0.3632	0.1706

Combined Bor3 and Esf1 results are presented in table 6.13.

Table 6.13 - Combined incidence (first year of follow-up) in Bor3 and Esf1

Day 80 LST		BCG	Vaccine	Total
< 5		7.36%	7.69%	7.50%
	N	1522	1171	2693
≥5		2.50%	5.29%	4.83%
	N	120	605	725
Total		7.00%	6.87%	6.93%
	N	1642	1776	3418
P (incidence diff)		0.0447	0.0584	0.0119

Overall, these observations share an important aspect: reduced incidence is not observed only in the vaccine arm, but also in the BCG arm. This points at the possibility that BCG could be an important protective component of the vaccine.

It is noteworthy that the two *L. major* foci show similar results of reduced first year incidence not seen in Bam trials. This is consistent with the fact that Bam is a focus of *L. tropica* which a typically longer period of latency and disease duration. Therefore, first year incidence could not be expected to show the effect of BCG, as most cases could not be identified until the second year of follow-up.

Further examination of incidence over the entire follow up period in Bor3 reveals that after removing those with pre-vaccination LST>0, the number of cases is significantly smaller among those with day 80 LST≥5, when combining the two study arms (difference between BCG and vaccine not significant), as presented in table 6.14. This finding was not observed in any other trial and may in fact show the effect of sensitization due to natural exposure or vaccination in the first year + subsequent booster effect by BCG or vaccine at the end of the first year.

Table 6.14 - Incidence associated with day 80 LST (in pre-vaccination LST=0)

Bor3 Day 80 LST		BCG	Vaccine	Total
< 5		12.1%	12.2%	12.1%
	N	505	426	931
≥5		3.0%	8.2%	7.4%
	N	33	182	215
Total		11.5%	11.0%	11.3%
	N	538	608	1146

P (comparison of LST groups in the total column) = 0.0496

In summary, evidence of protection associated with LST≥5 is observed in the two trials in *L. major* foci in both study arms (ALM+BCG or BCG alone).

6.2.2 One year LST and protection

The likelihood of the LST reactivity one year after vaccination correlates with
1) vaccination status (longer lasting LST reaction in the vaccine arm due to ALM)
2) chance of sub-clinical infection
3) chance of clinical infection.

There is a higher chance of infection among LST reactive participants because infection leads to LST response. It is therefore not surprising that in both study arms, incidence is higher in higher LST groups (see the table below). In this context, a reactive one-year LST works as a partial retrospective proxy for infection over the first year of follow-up period.

At the same time, given equal chances of natural exposure in the BCG and vaccine arms during the first year of follow-up, the percent of LST reactivity should be greater in the vaccine arm. This has been pointed out previously (chapter 5) and can be verified in the following table.

Table 6.15 - First year incidence in one year LST groups in Bam1, Bam3, Bor3 and Esf1 (in pre-vaccination LST=0)

		LST= 0	0< LST< 5	LST≥5	Total
BCG		0.9%	5.6%	28.7%	3.8%
	N	3472	1124	317	4913
Vaccine		1.2%	2.1%	14.3%	3.4%
	N	1662	2717	665	5044
Total		1.0%	3.1%	18.9%	3.6%
	N	5134	3841	982	9957
P (BCG vs Vacc):		0.346	0.000	0.000	0.314

The important observation in the above table is that within LST reactive groups (0<LST<5 and LST≥5) the incidence among vaccine participants is significantly lower than that among BCG participants (this observation was verified in *L. major* and *L. tropica* trials separately but is not indicated here). This could be interpreted as the ability of the vaccine to confer protection among those with high LST reaction. However, the overall incidence in the two study arms, regardless of LST, are not significantly different. This observation raises the following question: given the random assignment to the study arms and the expectation for roughly equal incidence, does the vaccine really confer protection if it cannot reduce the overall incidence in the vaccine arm?

To answer this question one should consider the LST-based classification rule in table 6.16. The reduced incidence in the vaccinated LST reactive groups may reflect the mixing of participants with similar LST measurement without regard for the reason for their LST reactivity: vaccine or exposure. In calculating incidence within each LST group, those not infected but with the raised LST (due to natural exposure, vaccine or BCG) all contribute to the denominator but only those infected due to natural exposure contribute to the numerator. Given equal exposure in both arms, the number of such participants is far greater in the vaccine arm, since the vaccine has a greater ability than BCG to raise LST response for one year. As a result, for roughly equal number of cases (which would be expected if the vaccine was not effective), a larger denominator in the vaccine arm leads to lower calculated incidence in LST reactive groups, while the overall incidence in the two arms are the same.

6.2.2.1 One-year LST and protection in the second year of follow up

When considering second year incidence in different one-year LST groups, a lower relative reduction in incidence among those with 0<LST<5 is observed (table 6.17). This is a similar situation to that explained earlier for the day 80 LST; i.e., classifying

based on LST result would place infected cases out of the 0<LST<5 group and in the LST≥5 group. LST in this case is a prospective marker.

Table 6.16 - Second year incidence in one year LST groups (in pre-vaccination LST=0)

		LST= 0	0< LST< 5	LST≥5	Total
BCG		3.7%	5.5%	4.1%	4.1%
	N	3472	1124	317	4913
Vaccine		3.4%	3.8%	4.8%	3.8%
	N	1662	2717	665	5044
Total		3.6%	4.3%	4.6%	4.0%
	N	5134	3841	982	9957
P (BCG vs Vacc):		0.644	0.019	0.618	

6.2.3 Incidence associated with LST reactivity both, 80 days and one year post vaccination

Continuous stimulation of the immune system has been stated as a requirement for protection (Breton et al., 2005;Scott, 2005;Selvapandiyan et al., 2006;Zinkernagel et al., 1996). In mice, it is clear that persistent parasite stimulation (with live parasite) maintains the effector CD4+ T cell pool which in turn mediates DTH and contributes to protection (Scott, 2005). It could, therefore, be hypothesized that for protection during the first year of follow-up, neither the LST response after 80 days nor that after one year would be individually sufficient. Rather, a continuous immune stimulation over the follow-up period would be necessary. For protection, therefore, LST>0 both on day 80 and one year post vaccination would be a minimum requirement.

Table 6.17 indicates incidence (entire follow-up) in various day 80 and one year LST groups. Restricting to first year cases does not significantly change these findings:

Table 6.17 - Incidence in day 80 and one year LST groups (pre-vaccination LST=0)

		One year LST< 5		One year LST≥5		Total	
		d 80 LST= 0	d 80 LST> 0	d 80 LST= 0	d 80 LST> 0	d 80 LST= 0	d 80 LST> 0
BCG		6.0%	6.8%	40.4%	23.8%	7.7%	8.7%
	N	3275	1168	171	143	3446	1311
Vaccine		5.0%	5.6%	28.7%	18.1%	7.0%	7.4%
	N	1010	3232	94	552	1104	3784
Total		5.8%	5.9%	36.2%	19.3%	7.5%	7.8%
	N	4285	4400	265	695	4550	5095

Within treatment pairs of difference between day 80 LST groups in the one year LST≥5 are significant as follows: BCG: p=0.0018, vaccine: p=0.0168, overall: p=0.0000. Pairs of difference between BCG vs Vacc are not significant.

The table above indicates lower incidence in the subset of participants with one year LST≥5 whose day 80 LST>0 (either by BCG or vaccine). An argument, similar to the one presented above, suggesting that the abundance of healthy LST-positive individuals (due to vaccine or BCG) augmented the denominator in the positive LST groups, thereby lowering their calculated incidence, would not hold in this case since the reduced incidence is observed in both the vaccine and the BCG participants. Unlike the vaccine, BCG tends not to produce a long lasting immune stimulation.

Therefore, many of the one-year LST positive individuals in the BCG arm (and a similar number of their vaccine counterparts) must have received additional stimulation through natural exposure; i.e., a natural boost for the priming with BCG or vaccine. Such a prime-boost approach could be a practical method of immunization in endemic foci.

The above table also provides support for the notion that a continuously stimulated immune system (evidenced by both day 80 and one year LST) is associated with lower incidence. At the same time, it is important to note that there is no significant difference between vaccine and BCG. Therefore, the lower incidence which could be indicative of protection, could be attributed to the BCG in both study treatments. Although, as pointed out before, the ALM in the vaccine leads to significantly higher LST response, this form of stimulation does not bring about a significantly larger protection in comparison to the BCG. It was also observed that some cases in the vaccine arm had values of LST in excess of 5 mm or 10 mm or higher.

The idea that ALM+BCG and BCG alone both resulted in protection in participants is also suggested by the following table. The two treatments were for the most part indistinguishable with respect to their protectiveness: they appear to have both protected study participants against their immediate risk of infection. The natural development of the disease is that in *L. major* infection clinical manifestation usually occurs in less than one year after exposure while in *L. tropica* infection clinical manifestation takes over one year (figure 6.6). As table 6.19 indicates, in Iran clinical trials in *L. major* foci (except in Esf1), in the first year (year of vaccination) incidence was significantly lower than the second year. By contrast, in *L. tropica* foci, the second year incidence was lower (table 6.19). , it appears that immunization by BCG or ALM+BCG may have been protecting against the immediate risk of infection (first year in *L. major* and second year in *L. tropica*) by a overall factor of roughly RR= 0.55, or efficacy of about 45%. This effect may have been strengthened by multiple injections of BCG (alone or in vaccine) as suggested by the significantly lower first year incidence in Bor3 and Zav3 (3 injections) but not in Esf1 (1 injection).

Figure 6.4 - Expected exposure/infection during follow up in ZCL and ACL trials

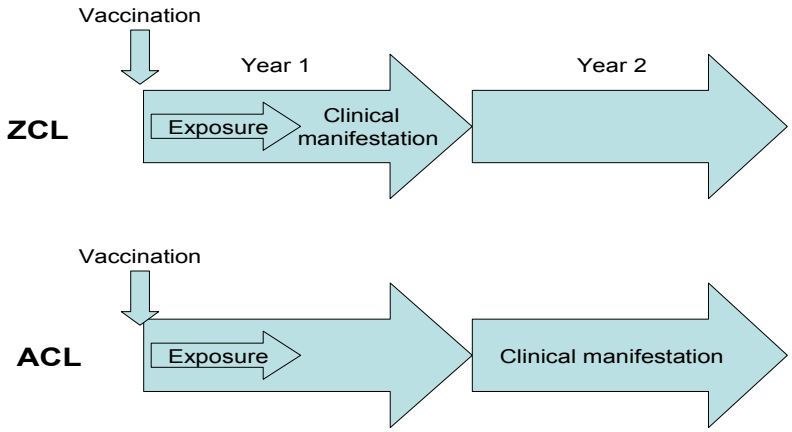


Table 6.18 - Protection by BCG and vaccine against the immediate risk of infection in pre-vaccination LST=0

Incidence in:	Bam1	Bam3	Bor3	Esf1	Zav3	Overall
First year	1.90%	2.54%	1.33%	9.59%	1.23%	3.36%
Second year	1.18%	1.71%	9.80%	8.64%	11.69%	4.76%
N	3633	4087	1275	2314	1463	12772
p (1st yr-2nd yr)	0.013	0.009	0.000	0.261	0.000	0.000

Note: second year incidence in Bor3 represents second and third year combined

6.2.4 LST as a marker of immunity

LST provides an accessible and simple diagnostic tool and is widely used for identifying exposure to leishmanial exposure. Nevertheless, LST is not an exact measure of immune response and can not be considered a highly sensitive or specific tool. The problems with LST include:

- LST could change dramatically over multiple measurements in a period of a few weeks and thus could be a source of misclassification. This was pointed out earlier in the discussion of pre-vaccination LST>0 in Bor3 which changed to zero by day 80 in about one third of those with pre-vaccination LST>0. Therefore, at any point in time, LST measurement is subject to a certain level of unexplainable variability and unreliable sensitivity and specificity. In fact, it is not known how many endemically exposed participants were missed in the screening evaluation in Bor3 or in other trials where screening criteria called for LST=0.
- Additionally, LST could rise in response to different stimuli: inactivated leishmanial antigen, immune stimulation by the adjuvant (in this case BCG), natural exposure without subsequent disease, or clinical infection. Therefore, it seems impossible, without other forms of testing or more detailed information about the circumstances, to judge based on LST results alone whether a person is exposed, infected, vaccinated or immune -- i.e., LST is a marker of different things.
- The only instance when LST provided a fairly reliable marker of immunity was in the case of pre-vaccination LST in endemic participants in Bor3. significant protection was observed among these endemically exposed participants (who also received BCG). However, even in this instance the subsequent LST measurement in some of these participants equalled zero; unexpected in exposed individuals). Furthermore, LST is not able to identify all cases of endemic immunity.
- LST induration is not directly correlated with immunity. In Bor3, for example, pre-vaccination LST protection was associated with more prevalent but on average smaller-sized LST reactions compared to Zav3 where less prevalent but larger LST responses were not associated with significant protection.

Whether LST can be considered a marker of immunity depends on the type of immunogenic stimulation and the immune response behind it. LST measurement without the knowledge of what gave rise to it does not necessarily correlate with protection since it could just as easily correlate with unfolding CL. As indicated earlier, compared to BCG alone, the ALM+BCG vaccine leads to much more frequent

and persistent DTH response, as measured by LST. Ultimately, however, as far as these trials suggest, the superior immunogenicity by the vaccine and the subsequent rise in LST do not directly translate into better protection compared to BCG. The protective outcomes of immunization with BCG or ALM+BCG are indistinguishable despite the higher LST associated with the latter.

6.3 Summary: LST, as a measurement of DTH, is believed to have the potential for assessing the strength of immune response and immunity in exposed and vaccinated individuals and be used as a marker of exposure and immunity.

Individuals living in endemic areas are continuously exposed to the antigen. This leads to a form of immunity detectable by LST. Generally, it is believed that endemic exposure leads to immunity in a certain percentage of residents. The analysis of two trials Bor3 and Zav3 confirms this notion and sheds more light on the LST and endemic immunity:

- There are differences in the LST manifestation of immune response and its associated immunity between inhabitants of a long-term endemic focus and residents of a new endemic area. LST in the former is more prevalent but also more moderate in its average induration.
- Observations in Bor3 suggest that natural endemic exposure (LST≥5) in a long-term endemic focus leads to significant immunity once boosted by BCG.
- In a new endemic area, where residents have experienced exposure for only a few years immunity associated with LST≥5 due to natural exposure is not significant but is close. This is probably because too few individuals are exposed with LST≥5. Therefore, LST positivity alone (regardless of how and where it was developed) can not be considered a sufficient correlate of immunity.
- Endemic immunity in a long-term endemic focus is clearly age related. This relationship is dictated by the accumulated risk of exposure in older individuals. This age relationship is seen even in children under 14.

Several trials have reported protection in the immunologically responsive (LST>5) subset of participants; Brazil studies indicated such protection only in the vaccine arm. Meta-analysis of all trials comparing the incidence in immunologically responsive subset against all other participants did not lead to the same conclusion.

The role of LST was assessed as a tool for evaluating vaccine effectiveness in various immunologically responsive groups. The important finding was that since LST could indicate immune response not only to vaccination but also to developing infection (at least in CL), it can not be freely used to classify trial participants for efficacy assessment in various LST groups. Classifying based on inappropriate LST measurement could lead to spurious conclusions of efficacy. For example, LST measurement one year after vaccination should not be used to evaluate vaccine efficacy in LST+ vs LST- groups, since the denominator in the LST+ group is inflated by all those with vaccine induced LST+ but the numerator consists only of LST+ due to exposure, leading to a smaller calculated incidence in the LST+ group. A different but related situation exists for classifying based on day 80 LST. By definition, the high LST group is where most infected individuals are placed. As a result, the moderate LST response would have relatively few infected cases, not because of

vaccine efficacy, but because of the LST classification rule. It should be pointed out that the LST-based incidence comparison in the way that has been done by Antunes, Mayrink, Momeni and Khalil (Antunes et al., 1986;Khalil et al., 2000a;Momeni et al., 1999), is valid.

Continuous exposure has been stated as a requirement for developing immunity. This has been presented as an argument in favour of live vaccines. In the current study, participants with day 80 LST response of 0<LST<5 mm to vaccine or BCG who also had a positive LST response to either treatment one year after vaccination were found to have half the risk of those who were LST+ one year after injection but had not responded on day 80 LST evaluation. They were more likely than those whose one-year LST was negative; but this is to be expected since the one year LST directly correlates with the disease incidence during the first year of follow up. In addition, in Bor3 and Esf1, trials in L. major foci, protection was observed in first year incidence independently of treatment (BCG or vaccine) in those whose day 80 LST had converted to LST>5. The important implications of this analysis are:

- BCG and vaccine are indistinguishable in the protection they may afford. It could be concluded that in fact most of the protection from the vaccine is due to the BCG adjuvant.
- LST is significantly more strongly stimulated by the ALM in the vaccine compared to BCG alone. However, this increased LST is irrelevant to protection.
- BCG or vaccine injection (if followed by LST>0 response) provides the priming that when coupled with natural exposure in endemic foci, could reduce the risk of disease by about 50%. This could be viewed as a practical approach in many endemic foci.

Additional support for the role of BCG in protection comes from yet another observation: protection against the most immediate infection, regardless of the LST value or vaccination status. Bam1 and Bam2 trials were conducted in ACL foci with significantly longer course of infection development compared to ZCL. Bor3, Esf1 and Zav3 were conducted in ZCL foci where the infection is manifested and healed faster. In both Bam trials, particularly Bam3 (with 3 study injections) second year incidence was significantly lower than the first year. In ZCL trials (with the exception of Esf1 where only one study injection was administered) incidence in the first year was lower than the second year. These observations are consistent with the natural development of the local disease. Furthermore, these findings suggest possibly better protection associated with multiple injections.

Overall, the protective effect of BCG in clinical trials has not been the target of analysis. Furthermore, as stated by other investigators (Modabber and Reed, 2004) both the vaccine and BCG stimulate DTH and it would be difficult to measure the effect of one in presence of the other as the two treatments in the same trial.

Another general conclusion is that since LST correlates not only with protection but with disease, BCG or antigen exposure, etc, it should be viewed as a marker of protection only under circumstances when the nature of the immune stimulus is well defined. As an example, increased LST in response to ALM+BCG does not correlate with increased protection. However, LST>0 on day 80 continued over the course of

the follow up does correlates with protection. Similarly, LST≥5 due to endemic exposure boosted by BCG leads to protection.

Additionally, as observed in these trials, LST measurement is subject to substantial variation over time due to immunological reasons or measurement error and should be used with this variability in mind. Although variation due to measurement error is immunologically meaningless, it nonetheless could cause significant variation with consequences in the interpretation of the results and their immunological implication.

CHAPTER 7

DISCUSSION

Leishmaniasis constitutes a major public health problem in many countries in the world and is among the most neglected diseases, mainly afflicting the poor. Diversity of causative parasite species, epidemiology, immunology and clinical manifestations make leishmaniasis a serious public health challenge in endemic countries. Urbanization, deforestation and other changes to the environment, ecological changes due to global warming, displacement of human populations as in mass migrations due to war and famine could all give rise to leishmaniasis epidemics and the expansion of endemic foci. Large scale control measures are difficult to plan, implement and sustain and case identification and therapy are difficult and costly. First line therapy in almost all endemic countries consists of antimonials; relatively old, toxic compounds that are now facing resistance by the parasite in many areas. Prevention by an efficacious vaccine would be a desirable control method. Randomized, controlled clinical trials in search of an efficacious prophylactic leishmaniasis vaccine have been conducted since the 1970's.

The results of these studies vary from trial to trial, owing to the design and conduct of individual trials, causative parasite species, local disease epidemiology and immunology, the vaccine candidate, etc. Problems such as lower-than-expected incidence, misclassification of exposed/protected trial volunteers as unexposed and occasional problems with the conduct of a trial could all have a negative impact on a given trial's statistical power and the ability to detect the difference between trial arms. Other problems such as the length of the required follow-up period could also affect the final sample size and power. Combining and comparing the results of various clinical trials and simultaneously analysing them could help bypass problems with individual trials.

The present study used meta-analysis and other techniques to test the data from 9 efficacy clinical trials of first generation leishmaniasis candidate vaccines to re-assess the vaccine role in protection, immune response (measured by LST) to the vaccine and to natural exposure, and the merits of LST as a marker of protection in various demographic groups.

The data used in this study represent trials that are different with respect to:

1. Parasite species used in the vaccine (*L. amazonensis* in South American vaccines, *L. major* in Iran and Sudan trials),
2. Parasite geographical origin; e.g., local parasites used in Equador vaccine
3. Antigen preparation (merthiolate vs autoclaved),
4. Same antigen but different vaccine dose; e.g., single, double or triple injection of ALM+BCG in various trials,
5. Disease causing parasite and its epidemiology; e.g., *L. tropica* in Bam, *L. donovani* in Sudan, etc.
6. Study population; e.g., age and gender make up of the sample, endemic/non-endemic origin of participants.

Vaccine efficacy

With the exception of the trial of a locally produced tri-valent vaccine in Ecuador (Armijos et al., 1998), leishmaniasis vaccine trials have demonstrated no prophylactic efficacy when comparing the vaccine and the control arms as originally randomized. However, significantly lower infection rates in the immunologically responsive (LST conversion 40-80 days post vaccination) subset of the randomized sample has been demonstrated in trials by Mayrink, Momeni and Khalil. Additionally, Sharifi, et al showed a significant difference in protection between vaccine and BCG in boys, compared to girls in a trial using a single vaccine dose but not in a trial using triple doses of the vaccine. On the therapeutic side, several trials have demonstrated the efficacy of killed parasite vaccines. Based on these observations, it was hypothesized that combining the data from various trials could better assess the role of vaccination with first generation vaccines on protection due to vaccination.

At the first step, meta-analysis was used to examine the efficacy all trials simultaneously, without explicitly considering their differences and heterogeneities. Results suggested no overall efficacy. Meta-analysis was subsequently used to also examine the effect of vaccination in more homogeneous subsets of trials. Grouping trials by factors such as the causative parasite (e.g., *L. tropica* or *L. major*) or use of BCG as adjuvant did not lead to significant efficacy results. Similarly, combining trials with the same antigenic content (*L. major* or *L. amazonensis*) did not suggest significant protection for either vaccine.

The exception was in the analysis of *L. amazonensis* trials including trials done in Brazil, Colombia and Ecuador. In this analysis, exclusion of the Colombia study (the largest in South America) led to significant efficacy for the Brazil and Ecuador results combined. This approach can be disputed since it could be viewed as an arbitrary exclusion of a trial. Additionally, excluding the Colombia trial would ignore the information from the largest trial in South America. On the other hand, it could be argued that the Colombia trial has an overly dominant effect when combined with other, much smaller *L. amazonensis* vaccine trials in South America, imposing its possible trial-specific conditions/limitations on the overall results. One such trial-specific condition in Colombia was the possibility of ineffectiveness *L. amazonensis* in creating cross immunity with *L. panamensis*, a dominant causative species in Colombia. There is some evidence epidemiologically that those infected with *L. amazonensis* are still susceptible to infection by *L. panamensis* (R. Killick-Kendric, personal communication). Another trial specific factor in Colombia could be the inclusion of a number of participants with pre-vaccination LST between 0 and 3. If any protection could arise as a result of the pre-vaccination LST>0, then this would have had an undetermined adverse effect on the study power and efficacy assessment. The efficacious results of Brazil+Ecuador-Colombia suggests a potential prophylactic advantage for the *L. amazonensis* vaccines and the merits of further investigation of vaccines based on this parasite in South America.

Vaccine efficacy was further studied in demographic groups identified by age, gender and the origin of trial participants (i.e., originally from endemic or non-endemic areas). Isolated indications of efficacy in some demographic groups (such as vaccine efficacy in boys in Bam1 or in older than 9 year olds in Bam3) could not be verified in other

trials. Furthermore, meta-analysis of homogeneous demographic groups did not reveal significant and consistent efficacy in any of the groups.

Despite the observation in some of the individual trials (Brazil, Iran and Sudan) that protection associated with vaccination could be observed in the immunologically responsive participants (LST>5 in 40-80 days after vaccination), no such protection was observed in the current study (based on the meta-analysis of 4 trials in Iran: Bam1, Bam2, Bor3, Esf1). Comparisons were made between the LST≥5 group in the vaccine arm and all others combined.

These results are consistent with the fact that the candidate vaccines in these clinical trials have been relatively weak immune stimulants. Moreover, they did not provide on-going stimulation, which is believed to be necessary for long term protection. It should be noted that when continued LST reactivity was observed (in individuals whose LST was reactive both 80 days after vaccination (LST>0) and one year after vaccination (LST≥5)), significant protection was observed. This protection was however not different between the vaccine and the BCG arms. This strongly points to the fact that BCG was not a true placebo or a negative comparator for all the trials using BCG as a control, since it seems to induce a cross reactive immune response.

Role of demographic characteristics in exposure and incidence

- Background:

It is generally believed that in the context of leishmanial infection, immunity is correlated with previous/repeated exposure to the parasite and the resulting clinical or sub-clinical infection; a usual event in endemic foci. This is also seen in other diseases such as malaria; where in endemic areas, a form of acquired immunity develops with continuous/repeated exposure. Endemic immunity is a consequence of exposure and is promoted by behaviour and characteristics that increase exposure risk. Demographic characteristics, primarily age, gender and residence in endemic foci are correlated with the chance of exposure and its consequences of infection and/or immunity in the following ways:

- In an endemic focus, age is directly correlated with the length of time of exposure. Over time, inhabitants of endemic foci have a continuous and cumulative chance of exposure, infection and the resulting immunity. Therefore, it is expected that immunity be higher and incidence lower in older individuals in endemic foci.
- By contrast, non-endemic, *Leishmania*-naive populations moving into an endemic focus (or if a previously non-endemic area changes its characteristics), would be exposed to the parasite for the first time with no age-related background immunity. In this case, age affects incidence only to the extent that certain age groups engage in risk behaviour more than others; as an example, 8-10 year old children (boys more than girls in some communities) may generally play outdoors and be exposed more frequently than adolescents. This exposure is not due to age (=length of exposure time from birth) but rather due to the risk behaviour associated with the particular age bracket. Therefore, an age-related continuous trend in incidence and immunity is not

necessarily expected (although after a number of years of exposure to endemic conditions, age-related immunity may settle in).
- Endemic and non-endemic populations may be genetically different in their response to the antigen.
- Gender, similarly to age, if associated with behaviour that would augment the risk of exposure and/or infection, could affect immunity and incidence. Playing and working outdoors, sleeping habits, coverage of the body and so on, are all age/gender related behaviour and can affect risk in an endemic area.
- Genetic differences between males and females may potentially influence their response to the leishmanial antigen.

Consequently, it is important to investigate exposure, incidence and the effect of intervention (vaccine or BCG) in relation to demographics. Clinical trials in Iran (Bam1, Bam3, Bor3, Esf1, Zav3) provide participant level data and a good mix of age, gender and endemic origin for the demographic analysis of efficacy and incidence. Additionally, they all evaluated the same vaccine (ALM+BCG) compared to BCG as the control treatment. Moreover, two trials (Bor3 and Zav3) included participants with LST>0 at screening, providing the opportunity to investigate the effect of previous natural exposure on incidence. Despite consistencies, Iran trials are different with respect to the following important factors. These differences were addressed and accounted for in the analysis and helped identify the role of some of these factors on infection and immunity:

- Causative parasite and its related epidemiology/pathology/immunology (*L. tropica* in Bam trials vs *L. major* in others).
- Participants age limitation (6-13 or 15 in Bam1, Bam3 and Bor3, 5-59 or 72 in Esf1 and Zav3).
- Endemic origin of participants (Endemic in Bam1, Bam3 and Bor3, non-endemic in Esf1 and Zav3).
- Additionally, unspecified/unmeasured variation in incidence in trials in the same region with similar disease epidemiology or in different years of follow-up in the same trial could potentially confound the effect of study treatment (effect of multiple injection vs single injection in different trials, for example), endemic origin, etc.

- Incidence and demographics in Iran trials:

Despite isolated findings in some of the trials (such as age related incidence in Esf1), separate and grouped analyses (meta-analysis) of Iran trials did not reveal consistent and significant differences in incidence (combined or separately in control and vaccine arms) in different age or gender groups. However, they did indicate substantial differences in incidence (regardless of study treatment) between trials in endemic vs non-endemic populations who had moved to an endemic focus, or residents in new epidemics.

A positive, consistent and statistically significant age-based trend in incidence was not observed in all trials. This is contrary to the general belief that age affects immunity and, therefore, incidence. However, this absence should not be interpreted as no

relationship between incidence and age. Two enrolment criteria in these trials worked against detecting the effect of age on incidence:

- Exclusion of LST>0 at screening: previous exposure and resulting endemic immunity is correlated with demographics (age and gender) and is manifested by LST>0 at screening. Eliminating LST>0 volunteers would eliminate many instances of endemic immunity and would minimize/remove from the trial sample the effect of age and gender on exposure, endemic immunity and consequently incidence (in participants of endemic background). By design, LST>0 volunteers were not enrolled in Bam1, Bam3 and Esf1 but they were enrolled in Bor3 and Zav3, once confidence was gained of the safety of vaccine in LST+ individuals.
- Exclusion of age>15 at screening: In parallel to the LST>0 exclusion criterion discussed above, age limitation of 13 or 15 (Bam1, Bam3, Bor3) could take away from the effect of age on incidence since older individuals (excluded at screening) would be the ones with longer exposure and higher chance of endemic immunity. Although infection and immunity in endemic foci starts early in life, it is likely that the prevalence of immunity (and paucity of infection) increases with age.

In trials with participants from endemic origin (Bam1, Bam3, Bor3), volunteers were recruited according to one or both of the above screening criteria which could minimize or remove the effect of age from the data. Consequently, the absence of a strong age/incidence relationship in these data does not rule out the existence of such a relationship.

Similarly, gender was not observed to be correlated with incidence.

In contrast to age and gender, participants' endemic origin is a significant determinant of observed incidence in each trial. The average annual incidence during study follow-up was 1.55%, 2.15% and 4.05% in Bam1, Bam3 and Bor3, trials with endemic participants, while it was 9.1% and 6.4% in Esf1 and Zav3, the trials consisting mainly of *Leishmania*-naive participants of non-endemic background. The lower rate in Bam trials was partially explained by the epidemiology of the local ACL (annual incidence of about 2%). However Bor3, Esf1 and Zav3 trials were conducted in ZCL foci in the Esfahan province. The significantly lower incidence in Bor3 compared to Esf1 and Zav3 is attributable to endemic exposure/immunity in the former. This is discussed later in more detail. Although a random surge in incidence during the follow-up in the Esf1 and Zav3 trial sites could not be ruled out as a cause of the higher incidence in these trials, it seems to be unlikely in both trial sites. Another important distinction between endemic and non-endemic populations when LST=0 is selected, is the selection of genetically "non-responsive" individuals in the endemic population who may have been exposed but did not respond to LST at screening or later.

Effect of natural exposure and vaccine on DTH (measured by LST)

A reactive leishmanin skin test (LST>0) indicates cellular response due to exposure to the leishmanial antigen naturally or via immunization. It could also indicate exposure to other immune stimulators with cross reactive antigens, such as BCG.

1) LST due to natural exposure:

Naturally developed immune response (pre-vaccination LST>0) was measured only in Bor3 and Zav3 and was found to be less prevalent but with larger average induration in the latter. This may be linked to the non-endemic origin of the participants in Zav3. By contrast, in Bor3 naturally induced LST was observed more frequently and with more moderate induration which may demonstrate the pattern of LST response under endemic circumstances.

LST due to natural exposure was found to be, as expected, age dependent, both in its prevalence and size of induration. This relationship was clearly observed in Bor3 despite participants' age limitation of 13 years. In Zav3, where the non-endemic participants as old as 59 years were enrolled, the prevalence and size of pre-vaccination LST>0 showed age dependence but not to the same degree as in Bor3. These findings indicate a possible correlation between age, LST pattern and endemic immunity in Bor3.

Pre-vaccination LST is more frequent with more moderate induration in females in Bor3; a pattern that also differentiates Bor3 from Zav3. In contrast to above, however, there is no difference in protection associated with pre-vaccination LST between males and females.

2) LST due to vaccination:

Post-vaccination LST was measured in most trials in 80 days and then one year after vaccination. As expected, ALM+BCG vaccine is a significantly stronger immune response stimulator than BCG alone, as demonstrated by day 80 and then one-year LST evaluation. Day 80 and one-year LST results do not differ significantly in endemic participants, while an increase from day 80 to one-year LST, possibly induced by natural exposure, is observed in non-endemic, *leishmania*-naive participants.

The fact that the proportion of LST>0 among endemic participants stayed relatively constant from day 80 to one year after vaccination may indicate a point of equilibrium (or maximum) for LST conversion in an endemic focus that is reached after vaccination and does not reach 100% even after further natural exposure during the follow up. On average, the maximum prevalence of LST>0 observed as a result of vaccination+natural exposure is between 70% to 80%. The difference between this value and 100% is primarily explained by those who are non-responsive to leishmanial antigen.

LST and Protection

1) Pre-vaccination LST and endemic immunity:

It is generally believed that previous clinical or sub-clinical infection, common in endemic foci, if associated with positive LST (LST≥5) could lead to some immunity (endemic immunity). In other words, LST≥5, when not indicative of a developing infection, could be associated with protection. Exclusion of LST reactive volunteers

from leishmaniasis vaccine clinical trials has the desirable consequence, among others, of allowing the assessment of the vaccine effect in *Leishmania*-naive individuals without confounding by the potentially protective effect of previous exposure. However, this limitation disallows estimating the vaccine effectiveness in those with previous exposure (who constitute a large part of the populations in endemic areas).

Bor3 and Zav3 trials allowed enrolment of volunteers regardless of their LST values; thus allowing the sample to include a more representative cross section of residents with respect to exposure. This enabled assessing disease prevalence associated with different values of screening LST.

In Bor3, as expected, significant protection was seen among those with LST≥5 in both study arms; i.e., ALM+BCG and BCG alone. BCG, the agent common to both study arms, was possibly also a factor in this protection. The conclusion from this observation is that naturally acquired LST≥5 (boosted by BCG) could produce sizeable protection. This finding may have practical ramifications for immunization in endemic foci where LST>0 is prevalent and BCG vaccination against TB a common practice.

In Zav3, the reduction in incidence in pre-vaccination LST≥5 participants was not statistically significant (borderline) possibly because the prevalence of LST≥5 was low. The low prevalence of LST≥5 and the average size of pre-vaccination LST in Zav3 was different from Bor3 probably because Zav3 is a newly endemic area. Nevertheless, lack of significant protection in LST positive individuals in Zav3 is contrary to the dogma that maintains LST≥5 is associated with protection. Comparing these results suggests that natural exposure and subsequent LST reactivity could lead to protection after boosting with BCG with somewhat more likelihood if it occurs under endemic conditions, over a long time and probably after repeated exposure.

In addition to the differences in the prevalence of immunity due to natural exposure, there are differences in the LST response between individuals in a long-term endemic focus and residents of a new endemic area, as discussed earlier. LST in the former is more prevalent but less severe with more moderate induration. The reverse is true of new endemic area residents. Contrary to expectation and the dogma, it is the more moderate LST reactivity in endemic participants which is associated with significant immunity once boosted by BCG.

Endemic immunity in a long-term endemic focus is age related. This relationship is dictated by the accumulated risk of exposure in older individuals. This age relationship is seen in Bor3 where LST positivity and incidence seem to correlate negatively in all age groups.

In summary, protective exposure seems to be facilitated by endemic conditions and time and it is associated with a certain age and LST profile distinct from that seen in newly exposed non-endemic populations.

2) Post vaccination LST and protection:

LST measurement after vaccination has been used in vaccine clinical trials as an indicator of immunological response and potentially a correlate of immunity.

Several trials have reported protection in the immunologically responsive (i.e., LST converted) subset of participants in the vaccine arm. However, meta-analysis of all trials (restricted to LST=0 at screening) comparing the incidence in immunoligcally responsive subset against all other participants did not lead to the same conclusion.

In the present study, to better investigate the possible correlation between LST measurement and protection due to vaccination, the association of post-vaccination LST response with subsequent protection was assessed: for example comparing protection in vaccine and control arms in those with one-year LST≥5 or comparing incidence between LST+ and LST- in vaccine arm. Observations in the current study suggest that since LST could indicate immune response not only to vaccination but also to developing infection (at least in case of CL), one-year LST can not be freely used for this sort of comparison. This sort of classification and comparison could lead to spurious conclusion of efficacy. For example, comparing vaccine and control arms on protection observed in those with one year LST≥5, since in the vaccine arm the denominator in the LST≥5 group is inflated due to vaccination (but mainly due to vaccination and not necessarily due to exposure), leading to a smaller calculated incidence in the LST+ group. A different but related situation arises when classifying based on day 80 LST. The high LST group is where most infected participants are placed. As a result, the moderate LST response group would have relatively few infected cases, not because of vaccine efficacy, but because of the LST classification rule. It should be pointed out that the LST-based incidence comparison in the way that has been done by Antunes, Mayrink, Momeni and Khalil, is valid but could not be observed in the combined data of all 10 trials used in the analysis.

Continuous exposure has been stated as a requirement for developing immunity. This has been presented as an argument in favour of live vaccines. In the current study, participants with moderate day 80 LST response to vaccine or BCG who also had a positive LST measurement one year after vaccination were found to have half the risk of those who were LST+ one year after injection but had not responded on day 80 LST evaluation. They had a higher risk of infection compared to those whose one-year LST was negative; but this is to be expected since the one year LST directly correlates with the disease incidence during the first year of follow up.

The important implications of this analysis are:

- LST is significantly more strongly stimulated by the ALM in the vaccine compared to BCG alone. However, this increased LST is irrelevant to protection.
- BCG and vaccine are associated with similar reduction in infection prevalence in endemic participants who were naturally exposed prior to the study. In this case BCG seems to provide an effective booster after natural priming.
- On the other hand, injection of BCG or vaccine (if inducing LST>0 response) provides the priming that when coupled with natural exposure in endemic foci, could reduce the risk of disease by about 50%. This could be viewed as a practical approach in many endemic foci.

LST as a marker of immunity:

LST is an inexact indicator of immune response. Although it can provide information about immune stimulation, it has shortcomings as a reliable marker of immunity or exposure:

- LST magnitude is subject to great variation that is not always explainable. For that reason, it is generally accepted that LST is not an accurate quantitative test. For example, LST>0 mm in about 38% of exposed endemic individuals at trial screening in Bor3 was found to equal zero just a few weeks later (at day 80 measurement). Such a change from non-zero to zero is important since it points at a fundamental difference in immune response and could be a source of misclassification of exposed and unexposed individuals (for example allowing exposed, possibly immune individuals into trials with LST=0 screening criteria thus leading to bias in effect estimation). Whether this sort of change reflects measurement error or actual immunological variability may not be so important since the final result is similar: confusion about the immunological status of the person under evaluation.
- In addition, LST reflects immune system stimulation without providing any information about the stimulus. Any of a number of stimuli could cause LST reaction including injection with inactivated leishmanial antigen, Th1 immune response stimulants such as BCG and previous or current infection.
- As indicated earlier, compared to BCG alone, the ALM+BCG vaccine leads to much more frequent and persistent DTH response, as measured by LST. Ultimately, however, as far as the results of the current study suggest, the superior immunogenicity (LST response) of the vaccine does not translate into superior protection compared to BCG in the converted population. The protective outcomes of immunization with BCG or ALM+BCG are indistinguishable.

Although an inexact indicator, LST can still provide the basic information about immune system stimulation in most individuals. However, LST measurement without the knowledge of the stimuli and conditions that gave rise to it is insufficient information and can not be viewed as a reliable marker of immunity. For example, LST reading of individuals in an endemic focus who have received a dose of BCG may be considered a partial correlate of immunity. Conversely, LST reaction in *Leishmania*-naive individuals who received a dose of inactivated antigen would not be a correlate of immunity In summary, LST could be used as a marker of immunity if it measures the relevant immune response after appropriate stimulation. As such it is a better marker for inhabitants of endemic foci.

Protection due to BCG:

In the clinical trials used in this study, BCG was used as the control treatment to enable blinding and presumably also to provide an estimate of how much immunogenicity and protection in the ALM+BCG arm was attributable to the BCG adjuvant. However, as mentioned above, BCG and ALM+BCG were equally observed to be associated with significantly reduced incidence in conjunction with previous exposure (positive LST) in a manner similar to prime boost vaccination

(priming with natural exposure and boosting with BCG). This highlights the significant contribution of BCG (the agent common to both treatments), either alone or as adjuvant added to ALM, in conferring protection. This is despite the fact that BCG alone led to generally much more moderate LST reaction compared to ALM+BCG in all trials.

Protection was also observed in individuals with LST>0 on day 80 and LST≥5 one year after vaccination. This protection was observed equally in the BCG and the vaccine arm. This is another indication of the role of BCG in protection since BCG was the common agent in both study treatments.

Also in Esf1 and Bor3 trials, protection associated with positive day 80 LST was observed in both BCG and ALM+BCG arms.

Additional support for the significant role of BCG in protection comes from another observation: protection against the most immediate risk of infection (after study injection), in both study arms combined. Due to the clinical development of the disease, in both Bam trials conducted in a focus of *L. tropica*, first year exposure should predominantly lead to clinical manifestation in the second year. Contrary to this expectation, in these trials, particularly Bam3 (with 3 study injections) second year incidence was significantly lower than the first year. By contrast, in *L. major* trials, first year exposure should normally show clinical signs in the same year. Again, contrary to expectation, in *L. major* trials incidence in the first year was lower than the second year (with the exception of Esf1 where only one study injection was administered). These observations suggest that exposures in the same year as vaccination lead to fewer clinical infections compared to exposures in the year after or before vaccination. As stated, these results are the same for the vaccine and the BCG arms and, therefore, point at BCG as the possible explanation for incidence reduction.

CHAPTER 8

CONCLUSIONS AND RECOMMENDATION FOR FURTHER RESEARCH

8.1 Conclusions:

With respect to the stated objectives of this research the following conclusions can be made:

Objective 1. Efficacy of first generation vaccines and/or BCG:

- Clinical trials are subject to possible shortcomings and problems that could occasionally limit their ability to show effects that are in fact present. Analysing the available body of efficacy clinical trial data for all first generation leishmaniasis vaccines combined was hypothesized to bypass trial-specific limitations and bring about the advantages of a larger sample size in testing the efficacy of these vaccines as well as searching for clues to identify important factors in developing new vaccines. In the present study, the data from almost all leishmaniasis vaccine efficacy trials conducted in various countries were analysed via meta-analysis. Results did not support the hypothesis of efficacy when all vaccine trials were considered together without explicitly accounting for their differences (differences based on antigenic composition, use of adjuvant, causative parasite).
- Similarly, even after accounting for such differences by combining groups of more homogeneous vaccines together, efficacy could not be concluded. For example, combining the data from clinical trials of vaccines made with the same antigen (e.g., *L. major*), same adjuvant (i.e., BCG), or same causative parasite in the trial site did not suggest efficacy for any of these groups.
- The exception was in the analysis of *L. amazonensis* vaccines conducted in Brazil, Colombia and Ecuador. Although combining all these trials did not demonstrate efficacy, further analysis indicated a large heterogeneity between the trial conducted in Colombia and others. Excluding Colombia data from this group suggested overall efficacy for all other *L. amazonensis* vaccines combined. This could reflect the ineffectiveness of vaccination with *L. amazonensis* against *L. panamensis* infection dominant in Colombia. Since the Colombia trial had several times the sample size of any of the other trials, when combining all data this trial could have the effect of drawing all results in its own direction, not allowing the results of other trials to significantly contribute to the overall results. It may be warranted, therefore, to further investigate the effect of vaccines based on *L. amazonensis* against *L. braziliensis or L. amazonensis* but perhaps not *L. panamensis.*
- When considering the results of *L. major* vaccine (ALM+BCG) trials in different demographic groups formed based on age, gender and endemic origin of participants, again, no efficacy was observed for the vaccine.
- Furthermore, the observation in some clinical trials that the immunologically responsive subset of the vaccine arm participants (identified by post vaccination LST measurement ≥ 5) experience significantly reduced incidence compared to other trial participants could not be confirmed when combining results of Iran trials (for which individual level data were available).

- It was, however, observed that immunological reactivity (LST>0) 80 days after vaccination was associated with reduced incidence if LST measurement one year after study injection were LST≥5. This observation, was independent of the study treatment (i.e., no difference observed between the ALM+BCG and BCG alone arms).
- In addition, a level of protection associated with study participation (again, regardless of treatment) was observed in ALM+BCG trials in all participants of endemic origin with previous natural exposure (screening LST≥5).
- The two findings above indicate no difference between BCG and ALM+BCG in their association with reduced incidence. Since BCG was used in both trial arms, these findings may highlight the role of BCG, either alone or as adjuvant added to ALM, in conferring protection under endemic conditions.

Objective 2. Immunological response to natural exposure or immunization, measured by LST:

- By design, two of the trials used in this analysis enrolled volunteers with LST>0 at screening. Age dependence of exposure was seen in these trials although its significance was borderline. No significant gender dependence, however, was observed.
- Another characteristic affecting exposure is the endemicity of participants' geographical area of origin. Autochthonous inhabitants of an endemic focus were observed to have a different LST profile (more frequent with smaller induration) from residents of a newly endemic focus.
- Endemically exposed participants develop immunity against the disease following vaccination (either BCG alone or ALM-BCG), thus during the follow up, they develop clinical infection with lower frequency. As a result, studies done with participants from non-endemic origin should theoretically have higher overall incidence than those with participants from endemic area. This was verified in the present analysis. Comparison of infection rates in studies with endemic and non/endemic participants suggested that under endemic conditions, trials conducted with non-endemic participants show higher rates of infection than those with endemic participants.

Objective 3: LST as a marker of immunity

- LST has a number of shortcomings as a reliable marker of immunity including:
    - In general, LST readings are consistent with theoretical expectation. For example, compared to BCG recipients, vaccine recipients in all trials have significantly larger LST induration after vaccination; even after one year.
    - Ultimately, however, as far as the results of the current study suggest, this superior immunogenicity does not correlate with better protection than BCG alone
    - LST is subject to not only significant variability from measurement to measurement, but also deviation from theoretical expectation. For example, in Borkhar, 38% of participants with LST>0 at screening, had LST=0 eighty days after vaccination. This could constitute a source of misclassification of previous exposure.

- In addition, LST reflects immune system stimulation without providing any information about the stimulus (which could be inactivated leishmanial antigen, Th1 immune response stimulants such as BCG and previous or current infection).
- Compared to BCG alone, the ALM+BCG vaccine leads to significantly more frequent and persistent DTH response, as measured by LST. .

- Despite its variability, LST can still be considered a correlate of immunity if the stimuli and conditions that gave rise to it are known. For example, positive LST response (LST≥5) in a currently healthy individual from an endemic focus could indicate potential immunity, particularly if augmented with BCG injection. On the contrary, high LST readings due to vaccine are not strongly correlated with reduced infection rates. Therefore, if LST is supplemented with adequate information, it can be viewed as a correlate of immunity or infection.
- This study did not reveal a difference in the ability of LST as a marker of immunity for different age and gender groups.

8.2 Recommendations for further research:

Based on the conclusions of this study, further investigation in the following areas are recommended:

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1. In light of the significant protection observed after BCG or ALM+BCG? injection in LST positive individuals in Borkhar, Iran, long term effect of BCG vaccination of LST positives (LST≥5) vs unexposed individuals or those with LST<5 in endemic foci should be investigated further in a randomized controlled clinical trial setting. This information could help to reliably evaluate the effect of BCG in immunization in endemic foci.
2. Similarly, since a level of protection regardless of study treatment was observed among individuals with LST reactivity 80 days and one year after study injection, this phenomenon should be further investigated in clinical trial setting to better understand the effect of continued LST reactivity and the role of natural exposure and BCG injection as a booster or primer.
3. Further investigation is required for better understanding the reasons for the differences between endemic and non-endemic residents in their LST response patterns to natural exposure. These patterns should also be more closely investigated for better understanding of the immunological mechanisms that drive LST response and immunity.
4. Studying genetic factors in non-response to leishmanial antigens could shed further light on endemic immunity.
5. Evaluation of sensitivity and specificity of LST in detecting natural exposure and immunity in endemic areas should be further studied.
6. In light of the protection observed in some South American studies, further evaluation of *L. amazonensis* is recommended.

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ANNEX

- Articles submitted for publication
- Curriculum Vitae

FIRST GENERATION LEISHMANIASIS VACCINES: A REVIEW OF FIELD EFFICACY TRIALS

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Abstract

First generation candidate vaccines against leishmaniasis, prepared using inactivated whole parasites as their main ingredient, were considered as promising because of their relative ease of production and low cost. These vaccines have been the subject of many investigations over several decades and are the only leishmaniasis vaccine candidates which have undergone phase 3 clinical trial evaluation. Although the studies demonstrated the safety of the vaccines and several studies showed reasonable immunogenicity and some indication of protection, an efficacious prophylactic vaccine is yet to be identified. Despite this overall failure, these trials contributed significantly to increasing knowledge on human leishmaniasis immunology. To provide a collective view, this review discusses the methods and findings of field efficacy trials of first generation leishmaniasis vaccine clinical trials conducted in the Old and New Worlds.

Key words: Leishmaniasis, vaccine, clinical trial, review

Abbreviated title: Review of Leishmaniasis Vaccine Efficacy Trials

1 Introduction

Leishmaniasis is a vector-borne disease caused by several species of protozoan parasites of the genus *Leishmania*. Leishmanial infections have diverse clinical manifestations, including cutaneous (CL), mucocutaneous (MCL), diffuse cutaneous (DCL), visceral (VL or kala-azar), post kala-azar dermal leishmaniasis (PKDL) and recidivans (LR) [1]. Leishmaniasis is a public health problem in at least 88 countries, including some of the poorest in the world [2, 3]. The estimated global prevalence of all forms of the disease is 12 million, with 1.5 to 2 million added cases annually of CL (with average duration of few months to one year) and 500,000 of VL (with average duration of several months to more than one year) [3, 4, 4]. The geographical distributions of the diseases are concentrated geographically. 90% of visceral leishmaniasis cases occur in Bangladesh, Brazil, India, Nepal and Sudan; 90% of mucocutaneous leishmaniasis cases occurs in Bolivia, Brazil and Peru; and 90% of cutaneous leishmaniasis cases occur in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria [3].

Existing methods of vector or reservoir control cannot be used effectively by poor countries due to their high cost and problems with implementation and sustainability [5-7]. In addition, toxicity of pentavalent antimonials, the current treatments of choice in developing countries, and increasing parasite resistance [8-13] underline the need for an effective preventive vaccine [5, 14, 15] that not only would protect against leishmaniasis but could also interrupt the transmission cycle.

At the present time the only known and effective immunizing intervention for preventing cutaneous leishmaniasis (caused by *L. major*) in humans is Leishmanization (LZ). It is well known that the first natural infection with cutaneous leishmaniasis due to L .major is highly protective against subsequent infections. LZ is the practice of inducing the first infection by injecting live virulent parasites in an aesthetically acceptable site of the body in healthy individuals. It has been used in high incidence endemic foci to protect individuals against natural infection in an exposed body site (the face for example) and to establish infection at a scheduled time [16]. Leishmanization has been practiced in several countries, including Uzbekistan, the only country where the measure is in current use [17]. The first LZ campaign in Uzbekistan began in 1967 and was modelled after a similar campaign in Turkmenistan. The standard preparation, used in Uzbekistan for prophylaxis, consists of a mixture of live and killed promastigotes on the basis that such a mixture will be less virulent [6].

LZ was used in Iran (1980's) and Israel (1970's) as a prophylaxis against leishmaniasis, but is not currently practiced in either country [17, 18]. In Israel, the LZ program was discontinued because of loss of infectivity of the parasites used for LZ due to repeated sub-culturing, as well as immunosuppression that resulted in reduced responsiveness to diphtheria, pertussis and tetanus vaccine in children following LZ [17, 18]. In Iran, in the preliminary experiment with LZ, 80% protection was achieved [19, 20]. After this initial trial, nearly 2 million individuals, mainly army personnel, were leishmanized who were at risk of leishmaniasis after moving into endemic areas in the war zone as a result of the Iran-Iraq war. Despite its effectiveness, the LZ campaign in Iran was stopped in 1986 due to a small percentage of cases with complications such as protracted and non-healing lesions [20].

The refinement of methods of leishmanization using live, attenuated and/or drug-sensitive parasites (through culture, chemical, radiation or genetic manipulation) could lead to a significant improvement in LZ and enable its standardization, either as a vaccine, if sufficiently attenuated, or as a challenge system to evaluate other leishmaniasis vaccines in clinical trials. However, there may be concerns about the potential for attenuated parasites to revert to their original pathogenic state, and about the secondary transmission of parasites from leishmanized individuals, particularly in areas where the strains used for leishmanizing are not endemic [21]. Delivery of live *Leishmania* to the field in many endemic foci is a challenge, since *Leishmania* undergo metacyclogenic transformation in culture and must be conserved in a deep-frozen state.

First generation candidate vaccines against leishmaniasis consist of vaccines made with whole killed parasites. These vaccines are conceptually simple and could be relatively easy to produce in developing countries at low cost. This was one of the advantages that made them attractive candidates for vaccine development. However, the difficulty of standardization of vaccines derived from cultured parasites is a potential obstacle in the way of their registration. 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 Institute, Iran)[22, 23]. In addition, a trivalent preparation consisting of *L. brazilensis*, *L. guyanensis* and *L. amazonensis* antigens was evaluated in Ecuador [24]. 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 response.

We have reviewed data on all published Old World and New World field efficacy clinical trials of first generation leishmaniasis vaccines conducted between 1981 and 2007. In addition we have included data from three unpublished studies in Iran.

2 Efficacy trials of first generation vaccines

2.1 New World

First generation vaccines have been the subject of experiments in Latin America since the early part of the 20th century. The two main vaccines evaluated in the New World were the pentavalent preparation by Mayrink and colleagues in Brazil, known as Leishvacin®, and the simplified monovalent *L. amazonensis* vaccine; neither of which included adjuvant. Originally, merthiolate was used for parasite inactivation and preservation, but the autoclaved preparation of the same vaccine was shown to produce similar immunogenicity results [22, 25]. An autoclaved *L. mexicana* vaccine with BCG adjuvant was produced in Venezuela and used for immunotherapy of patients with cutaneous leishmaniasis. Prophylactic studies of this vaccine were inconclusive because of less-than-expected incidence of leishmaniasis in the trial areas [22]. Additionally, a trivalent preparation consisting of *L. brazilensis*, *L.*

guyanensis and *L. amazonensis* antigens was evaluated in Ecuador [24]. A brief description of these trials follows:

Brazil

Brazilian investigators conducted trials of different preparations of killed parasites as early as 1939 by Sales-Gomes followed by Pessoa and colleagues in 1940's evaluated 3 doses of a polyvalent vaccine of 18 strains of *Leishmania* in a trial involving 1127 healthy individuals (527 vaccine and 600 control) and observed 80% efficacy [26-29]..

These efforts were followed in the 1970's by Mayrink and colleagues who developed a pentavalent vaccine which, after two intramuscular injections (one week apart), was able to convert the leishmanin skin test (LST , also known as Montenegro skin test) results in 78.4% of vaccinated volunteers within three months, with no major side effects. Unfortunately, the efficacy of the vaccine could not be assessed in this trial since no cases occurred in the study area after vaccination, in either the vaccine or placebo group [26, 30, 31].

Suggestion of protection against leishmaniasis associated with vaccination was given by another trial of two injections of the same vaccine [32] involving 216 volunteers in the vaccine arm and 266 in the control arm. In the period two years after vaccination statistically significant differences in leishmaniasis incidence were observed between the vaccine and control arms (1.7% vs 8.9%, respectively, $P < 0.01$). However, the study was not a randomised double blind controlled trial, which makes the results difficult to interpret [26].

In 1981 and 1983 two randomized, double-blind, controlled trials of the pentavalent vaccine, using different doses, were conducted by Mayrink and colleagues in Brazilian army personnel. As in previous trials, two intramuscular injections, one week apart were administered [33]. In the 1981 trial, over 1300 volunteers were randomized to the vaccine and placebo arms. LST conversion among those vaccinated was 35%, considerably lower than in previous trials. The authors noted this might have been due to the immunosuppression effect of the routine army yellow fever vaccination which had been given about 60 days prior to the study injection. The trial results did not show a significant difference between the vaccine and the placebo arms. In the 1983 trial, over 1200 army recruits were randomly assigned to the vaccine and placebo arms. The LST conversion rate among those vaccinated was 68% (more than that in the1981 trial, possibly because the study started 120 days after routine yellow fever vaccination). Again, there was not a significant difference in the incidence of leishmaniasis among those in the two arms of the trial. Of interest in the 1981 trial was the finding that one group of vaccinees (who spent a longer time in the jungle and experienced greater incidence of the disease) whose LST converted after vaccination had a lower disease incidence compared to non-LST converted vaccinees or those who received placebo.

Despite promising results, due to the problems in its production and standardization, the 5-strain Leishvacin® presented difficulties in preparation and registration. Additionally, subsequent studies demonstrated the immunogenicity of a single-strain *L. amazonensis* vaccine. To address the development of a vaccine against CL, two Vaccine Advisory Groups, with the participation of representatives of national and

international organizations interested in developing an anti-leishmaniasis vaccine, were organized by World Health Organization (WHO)/ Special Programme for Research and Training in Tropical Diseases (TDR) in Washington DC, USA and Belo Horizonte, MG, Brazil in February and September 1991. The groups advised that studies with the vaccine should continue, using only one strain, the *L. amazonensis* (strain IFLA/BR/67/PH8). This strain was chosen because its antigens induced high stimulation indexes for lymphocytes from vaccinated volunteers, it was easier to grow in non-cellular media, it was internationally known and it was taxonomically well defined [26, 34].

It was later observed that 2 doses of *L. amazonensis* promastigotes killed by merthiolate and sonication (each dose containing 100mg of *Leishmania* protein vaccine plus 250mg of Corynebacterium parvum) have similar immunogenicity in mice as the 5-strain Leishvacin [34]. Immunogenicity of 2 and 3 doses of various concentrations of the monovalent vaccine in human volunteers was confirmed in a separate study [35].

Colombia

The immunogenicity and safety of three doses of the monovalent *L. amazonensis* vaccine was confirmed in Colombia in randomized, double-blind clinical trials. A phase 2 trial of vaccination in 296 army volunteers was conducted with intradermal vaccination and BCG as adjuvant and also intramuscularly without BCG. Three doses of vaccine with BCG were planned for each vaccinated participant but it was only administered twice due to BCG-induced lesions that were unacceptable to volunteers. Intramuscular administration proved to be safe and immunogenic and as a result a phase 3 trial was conducted with two injections of the vaccine without BCG [36]. The trial was conducted in army volunteers whose skin-test response to LST was <3 mm. Most infections in the trial area were due to *L. panamensis*. Participants in the vaccine arm (n = 1295) and the placebo arm (n = 1302) were followed up for 12 months. The vaccine was found to be safe and immunogenic but did not provide protection [37]. Participants in this study were not LST tested until the end of the trial and it is thus not possible to compare incidence rates in those who did or did not skin test convert to LST after vaccination.

Ecuador

The only leishmaniasis vaccine clinical trial in which efficacy was observed in the entire vaccinees' cohort (regardless of LST conversion after vaccination) was conducted in Ecuador [24]. In this study, safety, immunogenicity and efficacy of two intradermal doses of a locally prepared trivalent vaccine were assessed against two doses of BCG alone (n = 438 vaccine arm vs n = 406 controls). The vaccine consisted of *L. braziliensis*, *L. guyanensis* and *L. amazonensis* promastigotes originally collected from the lesions of patients living in the study area, mixed with BCG. After 12 months of follow up, the vaccine was shown to be safe, with 2.1% incidence of CL in the vaccine arm vs 7.6% in the control arm, corresponding to an efficacy 73%, [24].

The differentiating aspects of this trial from other leishmaniasis vaccine trials are 1) the origin of the vaccine being from locally obtained parasites, and 2) the young

average age of subjects, being around 5.5 years (5.4±3.9 vaccines and 5.7±3.9 controls). These aspects may partially explain the favourable results.

After the original 12 months of follow up, these subjects were followed up, in a separate study, for another 4 years. Results indicated that although the protective efficacy was still significant between the 13th and the 18th months, it was not so after the 19th month of follow-up [38]. The incidence in each 6-month period and the total incidence from the 19th to the 60th month were not significantly different between the vaccine and the control arm subjects who were followed-up.

However, the data in Armijos, et al, suggest that the loss of statistical significance in the difference between arms after the 19th month was not due to an increase in the number of cases in the vaccine arm, but rather a reduction in the number of new cases in the control group. In addition, the gradual reduction in the number of subjects followed up seems to be another factor contributing to differences that are not significant (number of subjects were calculated for each 6-month follow-up period based on incidence values reported in the article). It could, therefore, be argued that this study is inconclusive with respect to the erosion of the vaccine-derived protection after 18 months of vaccination (trend test of efficacy over the 5 years is not significant, P=0.406).

A problem with the first trial conducted by Armijos and colleagues was the unstandardised nature of the vaccine product tested. A subsequent trial compared two doses of vaccine (n = 750) against placebo (n = 756). The vaccine consisted of autoclaved *L. amazonensis* mixed with BCG. Although the vaccine was safe and immunogenic (significantly more LST conversion in the vaccine arm), the incidence of CL was similar in the two arms of the trial and the incidence of disease among vacinees who skin test converted was similar to that among those who did not convert [39]. The participants in this trial were older (average age 11± 10 years). The authors mention the possibility of the parasite killing method (heat), and the geographic origin of the parasite and the parasite species used in the vaccine as possible factors contributing to its ineffectiveness.

2.2 Old World

Following the cessation of the LZ program in Iran in the 1980's, the Iranian government created a national vaccine development program to develop a killed *Leishmania* vaccine at the Razi Vaccine and Serum Institute, Hessarak, Iran, using the same organism as used in the LZ program [17, 22]. Phase 1 and 2 studies of the safety and immunogenicity of different doses of inactivated *L. major* promastigotes with or without BCG as adjuvant were conducted in non-endemic areas in Iran. Initially full dose of BCG (as used in vaccination against tuberculosis) was used but subsequently it was reduced to 1/10 of the standard concentration. The results of these studies demonstrated that a low dose of BCG enhanced the immune response to the antigen [40, 41]. Vaccines produced by two methods of parasite inactivation (thimerosal treatment vs autoclaving) showed similar safety and immunogenicity results and because of its simplicity and lower cost, autoclaving was recommended as the preferred method of vaccine preparation for future trials [40]. The autoclaved *L. major* (ALM) preparation mixed with BCG adjuvant (10% the dose normally used in vaccination against TB) was used in several field trials in Iran and Sudan. The results

of some of these studies in the late 1990's (multiple injections in Bam, Iran, multiple injections in Zavareh, Iran and multiple injections in Borkhar, Iran) have not yet been published. This preparation was later replaced by a formulation involving precipitation of the ALM in aluminium hydroxide (alum), known as alum-ALM. Alum-ALM mixed with BCG showed significantly higher ability to convert the LST, but so far, Alum-ALM+BCG has only been studied in phase 1 and 2 (safety and immunogenicity) clinical trials and an LZ challenge study (see below).

Iran

In a randomized, double-blind, controlled field efficacy trial of ALM+BCG against zoonotic CL due to *L. major*, one injection of the vaccine (n = 1188) was compared with one injection of BCG alone (n =1122). Volunteers, whose age ranged from 5 to 72 years, were from healthy, LST-negative (LST=0), residents of an airbase in a part of Esfahan, Iran endemic for leishmaniasis. Participants had moved into the airbase from throughout Iran, including from areas not-endemic for leishmaniasis [42]. Post-vaccination LST was conducted on day 80 and after one year. The LST conversion rates following vaccination were higher compared to some other studies conducted in non-endemic or low endemic areas in Iran, but high rates were also observed in the control arm. The authors attributed this latter finding to natural exposure to leishmanial or cross-reacting antigens. Although after two years of follow-up there was no significant difference in the leishmaniasis incidence rates in the two arms, infection rates among LST converted individuals (both in the vaccine and the control arms) were lower than in those whose LST had not converted (7.3% vs 11.3 in the vaccine arm and 3.4% vs 10% in the control arm). This is consistent with findings in Brazil [33]. The difference is that in Brazil, the significant difference was between LST converted vaccinees and all others, while in Esfahan the difference was between participants whose LST converted and those who did not, regardless of BCG or vaccine treatment. This may reflect the fact that in Brazil the control treatment was not BCG. Another outcome of this study was the lower disease severity in vaccinated children less than 14 years old. A subsequent study of the immunogenicity data from this trial did not find a significant correlation between proliferation response to *Leishmania* antigens (IFN-γ production) and the magnitude of LST response [43]. The authors compared several different subgroups of the study but did not compare the vaccinated volunteers whose LST had converted with other groups. The authors raised the possibility that BCG may not be an ideal control since it could induce a similar immune response to that observed in those who received ALM+BCG.

Another randomized, double-blind, BCG controlled clinical trial in Bam, Kerman, assessed the efficacy of ALM+BCG in protecting against anthroponotic CL (ACL) caused by *L. tropica* in LST-negative (LST = 0) school children aged 5 to16 years [44]. One injection of the vaccine (n = 1839) was compared with one injection of BCG alone (n = 1798). Post vaccination LST was conducted on day 80 and again after one year. After two years of follow-up infection rates were similar in the vaccine and the control arms (2.8% and 3.3%, respectively). The LST conversion on day 80 after vaccination was significantly greater in the vaccine arm (16.5% vs 3.6%), but overall, less than that observed in Esfahan. The disease rates in the LST converted subgroups were not reported. Protection was observed in vaccinated boys, but not girls, though the difference in protection between the genders was not statistically significant. Also, it was noticed that through the 6th month of follow up

more cases were identified in the vaccine arm, while fewer were identified thereafter. This observation may point at the longer incubation time for the *L. tropica* infection.

Other studies were conducted in Iran with multiple injections of ALM+BCG. These studies were carried out in Bam, province of Kerman against anthroponotic lesihmaniasis caused by *L. tropica* and in Borkhar and Zavareh in the province of Esfahan against the zoonotic disease due to *L. major*. Results have not yet been published from these trials. Sharifi, et al (personal communication) conducted a study in LST = 0 school age children with 3 injections, one month apart, of ALM+BCG. BCG was used in the control arm. Two post vaccination LST measurements (day 80 and 1 year) were taken. Khamesipour et al (personal communication) conducted a three-dose trial in school age children in Borkhar, Esfahan province. The two main distinguishing aspects of this trial was 1) inclusion criteria allowed any initial LST values accepted into the trial and 2) although the first two injections of the vaccine were two month apart, the third injection was one year after the first. Several LST measurements were taken throughout the trial. Khamesipour et al (personal communication) also conducted a three-dose trial in Zavareh, in the province of Esfahan. Participants ages ranged from 5 to 59 years. In this study only the pre-vaccination LST was measured and, similar to the trial in Borkhar, participants with any value of LST were enrolled. Vaccine injections were one month apart. . In none of these trials was any evidence found of a protective effect of the vaccine against leishmaniasis.

Additionally, a clinical trial assessing the safety, immunogenicity and efficacy of alum-ALM (alum-precipitated ALM, an improved formulation of ALM) mixed with BCG followed by LZ challenge was conducted in a hyper-endemic area of Esfahan. This study (unpublished) did show the safety of alum-ALM+BCG but was inconclusive about the vaccine efficacy against challenge due to problems with the LZ reagent. A similar trial with an improved study design is currently underway in Iran. (Khamesipour, personal communication)

Sudan

The safety and immunogenicity of ALM+BCG was assessed in endemic and non-endemic areas of Sudan [45, 46]. Subsequently, a double-blind randomized field efficacy trial of two doses of ALM+BCG (n = 1155) vs BCG alone (n = 1151) was conducted in Sudan in an area endemic for VL due to *L. donovani* [47]. LST-negative (LST = 0) volunteers between the ages of 1 and 65 years were admitted to the study. Post vaccination LST conversion was significantly higher in the vaccine arm (30% vs 7% 42 days after vaccination). However, after two years of follow up, the rates of infection in the two study arms were not significantly different (11.5% in the vaccine arm and 12.3% in the control arm). Although the overall vaccine efficacy was not significant, individuals whose LST converted after vaccination (in each study arm separately or combined in both arms) had a significantly lower incidence of VL than non-responders (Vaccine arm: 8% vs 12.6%, P = 0.03; BCG arm: 3% vs 12.7%, P = 0.015; both arms combined: 7.2% vs 12.7%, P= 0.003). Reduced incidence in LST converted participants in Sudan is consistent with findings in Brazil and Esfahan studies, discussed earlier (with the difference that in Brazil the control treatment was not BCG and in Esfahan significantly reduced incidence was not observed separately in each study arms).

In addition to the studies of ALM+BCG, the safety and immunogenicity of different doses (10, 100, 200 and 400 μg of leishmanial protein) of alum-ALM+BCG was assessed in a small study in Khartoum [48]. LST conversion 42 days after vaccination was observed in all volunteers in the 10 μg, 100 μg and 400 μg doses but only in one of five volunteers in the 200 μg group. All doses were safe with minimal, local side effects [48]. Additionally, an extended phase 2 study [49] confirmed the safety and immunogenicty of alum-ALM+BCG compared to vaccine diluent as the control treatment in 544 leishmanin non-reactive children younger than 15 years old. Of interest is that the four cases of leishmaniasis observed in this study were all in the control arm. Alum-ALM+BCG was also evaluated in a hospital-based clinical trial for its immunotherapeutic effect in chronic PKDL patients in Sudan and demonstrated promising results [50].

Table 1 summarizes the background, trial design, vaccine profile and findings of prophylactic field efficacy trials of first generation leishmaniasis vaccine discussed above.

Table 1 - Leishmaniasis first generation vaccine efficacy trials

Author	**Sharifi**	**Momeni**	**Sharifi**	**Khamesi-pour**	**Khamesi-pour**	**Khalil**	**Antunes, Mayrink**	**Antunes, Mayrink**	**Velez**	**Armijos**	**Armijos**
Year Published	**1998**	**1998**	**N/P**	**N/P**	**N/P**	**2000**	**1986**	**1986**	**2005**	**1998**	**2004**
Study designation	Bam1	Esf1	Bam3	Bor3	Zav3	Sudan2	Brazil 1981-2	Brazil 1983-2	Colombia3	Ecuador2	N/A
Background											
Country, Area	Iran, Bam	Iran, Esfahan	Iran, Bam	Iran, Borkhar	Iran, Zavareh	Sudan, Gedaref	Brazil, Amazonas	Brazil, Amazonas	Colombia	Ecuador	Ecuador
Year(s) study conducted	1994-1997	1994-1997	1997-2000	1997-2000	1997-2000	1997-1999	1981	1983	2001-2003	1995-6?	2002?
Targetted parasite causing local disease	*L. tropica*	*L. major*	*L. tropica*	*L. major*	*L. major*	*L. donovani*			*L. pnamensis, L. braziliensis*	*L. panamensis, L. basiliensis, L.amazonensis*	*L. panamensis, L. basiliensis, L.amazonensis*
Expected annual incidence in controls	2%	5%	2%	6%	6% ?	9%	10%-25%	10%-25%	5%	7.5%	3%
Number of volunteers screened	12156	4712	6524	5869 [a]	2053	5093	?	?	3018	?	4164
Trial Design											
Number of volunteers accepted and randomized	3637	2453	4217	2191	2008	2306	1312	1274	2597 [b]	1042	1995
N in vaccine arm (original, received complete vaccination schedule ---if known)	1839	1256, 1118	2149, 2082	1107, 964	945	1155	667	658	1302, 1252	552, 438	1009, 750
N in control arm (original, final)	1798	1197, 1122	2068, 2008	1084, 956	1063	1151	645	616	1295, 1251	487, 406	986, 756
originally planned (nominal) power	80%	80%	90%	90%	90%	90%	90%	90%	80%	90%	90%
Hypothesized vaccine effectiveness (expected %reduction in annual incidence)	50%	50%	50%	50%	50%	50%	50%	50%	50%	50%	50%
LST requirement for inclusion (LST=0 mm, LST<5 mm , etc)	LST=0 mm	LST=0 mm	LST=0 mm	LST≥ 0 mm	LST≥ 0 mm	LST=0 mm	LST negative	LST negative	LST<3 mm	LST< 5 mm	LST< 5 mm
Length of time post vaccination for LST testing	80 days, 1 yr	80 days, 1 yr	80 days, 1 yr	80 days, 1yr, 1yr+80 days	Not done	42 days, 1 yr	40-45 days	40-45 days	Not done	1 month	2 months
Number of vaccine injections	1	1	3	3	3	2	2	2	3	2	2
Time between vaccine injections	single dose	single dose	30 days	75 days, 1 yr	30 dqys	28 days	7 days	7 days	20 days	30 days	56 days
Dose of *Leishmania* antigen injected (each injection)	1 mg ALM	1 mg ALM	1 mg ALM	1 mg ALM	1 mg ALM	1 mg ALM	240 ug Nitrogen	240 ug Nitrogen	11.11 mg protein/mL	72 mill promast.	240 ug Nitrogen
Injection method	ID (0.1 mL)	ID (0.1 mL)	ID (0.1 mL)	ID (0.1 mL)	ID (0.1 mL)	ID (0.1 mL)	IM (1 mL)	IM 1(mL)	IM (1 mL)	ID (0.1 mL)	ID (0.1 mL)
Control treatment	BCG	BCG	BCG	BCG	BCG	BCG	PBS + merthiolate	PBS+ mrthiolate	Saline (ph=7.4)	BCG	BCG
Duration of follow up (months)	12+12	12+12		36	24	24	12	12	12	12+48	12+14
Type of case detection during follow up (A=active, I=inactive)	A/I	A/I	A/I	A/I	A/I	A/I	A	A	A	A	A

[a] 2671 of 5869 were qualified for randomization but 492 participated in phase I/II trial.

[b] Recruitment was done in 3 groups: Sept-Nov/01 (989), Jan-March/)2 (1131), Jul-Aug/02 (477)

Table 1 (continued) - Leishmaniasis first generation vaccine efficacy trials

Author	**Sharifi**	**Momeni**	**Sharifi**	**Khamesi-pour**	**Khamesi-pour**	**Khalil**	**Antunes, Mayrink**	**Antunes, Mayrink**	**Velez**	**Armijos**	**Armijos**
Year Published	**1998**	**1998**	**N/P**	**N/P**	**N/P**	**2000**	**1986**	**1986**	**2005**	**1998**	**2004**
Product Profile											
Parasite species in vaccine	*L. major*	*L. major*	*L. major*	*L. major*	*L. major*	*L. major*	*L. guyanensis*	*L. guyanensis*	*L. amazonensis*	*L. braziliensis, L. guyanensis, L. amazonensis*	*L.amazonensis*
Parasite killing method	Heat	Heat	Heat	Heat	Heat	Heat	Merthiolate	Merthiolate	Merthiolate	Phenol	Heat
Antigen origin	Iran	Iran	Iran	Iran	Iran	Iran	Brazil	Brazil	Brazil	Ecuador	Brazil
Adjuvant	BCG	BCG	BCG	BCG	BCG	BCG	None	None	None	BCG	BCG
Vaccine Antigen manufacturer	Razi, Iran	Razi, Iran	Razi, Iran	Razi, Iran	Razi, Iran	Razi, Iran	Local	Local	Biobras	Local	Biobras
Adjuvant (BCG) manufacturer	Pasteur, Iran	Pasteur, Iran	Pasteur, Iran	Pasteur, Iran	Pasteur, Iran	Pasteur, Iran	N/A	N/A	N/A	Tokyo	
BCG concentration/injection	1/10 normal dose	1/10 normal dose	1/10 normal dose	1/10 normal dose	1/10 normal dose	1/10 normal dose	N/A	N/A	N/A	1/2 normal dose (500,000 organisms)	80000 bacilli
Leishmanin manufacturer	Pasteur, Iran	Pasteur, Iran	Pasteur, Iran	Pasteur, Iran	Pasteur, Iran	Pasteur, Iran	?	?	Colombian Nat'l Inst. of Health	Local	Biobras
Leishmanin composition	*L. major*	*L. major*	*L. major*	*L. major*	*L. major*	*L. major*	?	?	*L. amazonensis, L. panamensis*	same as vaccine	?
Findings											
Yr 1: Observed incidence in controls	2.17% [c]	N/A [d]	3.77%	1.75%	1.70%	N/A [f]	11.07% (gr 1), 1.40% (gr 2)	1.30%	6.80%	7.60%	0.13%
Yr 1: Observed incidence in vaccine arm	2.01% [c]	N/A [d]	3.16%	1.36%	1.40%	N/A [f]	8.70% (gr 1), 1.16% (gr 2)	0.61%	7.76%	2.10%	0.54%
Yr 1: Efficacy (= 1-(% case vaccine arm/%case control arm)	7.4%	N/A	16.2%	22.3%	17.6%	N/A	21.4% (gr 1), 17.1% (gr 2)	53.1%	-14.1%	72.4%	-315.4%
Yr 2: Observed incidence in controls	1.2% [c]	N/A [d]	1.76%	4.04% [e]	10.70%	N/A [f]	N/A	N/A	N/A	9.19% [g]	1.21%
Yr 2: Observed incidence in vaccine arm	0.83% [c]	N/A [d]	1.68%	4.67% [e]	11.40%	N/A [f]	N/A	N/A	N/A	4.41% [g]	1.22%
Yr 2: Efficacy	30.8%	N/A	4.5%	-15.6%	-6.5%		N/A	N/A	N/A	52.0%	-0.8%
Volunteers endemic origin	Low endemic	Mixed (army base)	Low endemic	Endemic	New endemic	different villages	Mixed (army concripts)	Mixed (army conscripts)	Mixed (army)	Endemic	Endemic
Age (range, mean)	6-15, 9.1	5-72, 18.2	6-12, 7.41	6-13, 8.45	5-59, 19.12	1-65, 6.9	18.6	18.6	>18, 19.8	5.6	10.7
Sex (% male)	50.60%	47.40%	50.80%	50.70%	46.80%	45.70%	100%	100%	100%	44.16%	44.90%
Protection observed in the overall sample	No	No	No	No	No	No	No	No	No	Yes	No
Protection in those with converted LST after vaccination	No	Yes	No	No	Not done	Yes	Yes	No	Not done	N/A	No
LST conversion rate (per protocol) 40-80 days post vaccination (vaccine arm, Controle arm)	16.5%, 3.2%	36.2%, 7.9%	18.2%, 2.0%	29.9%, 6.1% (Received 2 doses year 1)	Not done	30%, 7%	33%	70%	Not done	85.1%, 20.1%	74.4%, 14.7%

Footnotes for "Table 1 (continued)"

Bor3, Zav3, Bam3 findings are ITT. Other trials from published information.

[c] Cumulative 2-year incidence: Vacc=2.8%, BCG=3.3%, overall efficacy=15%

[d] Annual rate not reported. Cumulative 2-year incidence: vacc=18.0%, BCG=18.5%, overall efficacy=3%

[e] Third year incidence in Bor3: 6.64% (Control), 6.44% (Vaccine)

[f] Annual rate not reported. Cumulative 2-year incidence: vacc=11.5%, BCG=12.3%. Overall efficacy=6%

[g] Reported by Armijos 2003

3 Discussion

Overall, reproducible evidence of protective efficacy has not emerged from clinical trials of first generation leishmaniasis vaccines. In most of the randomized controlled trials reviewed (except Armijos 1998), no efficacy was demonstrated and this is consistent with the killed whole parasite preparations being inadequate to produce long lasting, relevant immune responses required for protection. In trials where post vaccination LST was measured, responses were notably larger in the vaccine group, but this evidence of immunogenicity induced by the vaccine was not carried over to a protective effect. This casts doubt on the merit of the vaccine induced LST response as a correlate of immunity. Nevertheless, conversion from negative LST reaction to LST>5 after vaccination has been observed to be associated with significantly lower infection incidence in Brazil, Iran and Sudan. In Brazil, where a true placebo (rather than BCG) was used in the control arm, significantly reduced incidence was observed only in the LST converted individuals in the vaccine arm (compared to all other participants in the vaccine or placebo arms). In Sudan (with BCG as control treatment)significantly reduced incidence was observed in the LST converted individuals in each study arm separately and also in the two arms combined, while in Iran, this was observed in both the vaccine and the BCG arms. This suggests that LST conversion may be associated with an immune response that can provide some protection. It certainly distinguishes a subpopulation of "responders" to leishmanial antigens or BCG after vaccination. On the other hand, there is a strong correlation between LST positivity and protection after recovery from the disease caused by several species [51] (although exceptions to this general statement exist); hence the immunological implication of the LST response depends on the factors and conditions that gave rise to it.

It is important to consider the possible role of BCG as not only was this used as an adjuvant but also served as the control vaccine in many of the trials. In the New World, the vaccines tested in Brazil and Colombia did not contain adjuvant. In Ecuador, Armijos used BCG (about 1/2 the dose normally used for vaccination against tuberculosis) in both his trials; with the locally produced trivalent vaccine and with the Brazilian Leishvacin. In immunotherapy, variable doses of BCG were used depending on the PPD results of each volunteer in Convit's initial protocol [52]. In the Old World different doses of BCG were tested in phase one trials [41] and subsequently 1/10 of the normal dose (used in vaccination against tuberculosis) was used in all leishmaniasis vaccine trials [40, 42, 44, 45, 47, 53].

An important aspect of all prophylactic clinical trials of killed parasites +BCG adjuvant was the use of BCG alone as the control, usually to preserve blinding in the trials, as BCG leaves a distinctive scar. Injection of BCG mixed with killed parasites significantly increases cell mediated immune responses to the vaccine measured by LST (but not interferon-γ production). Additionally, it has been observed that LST conversion due to vaccination is associated with reduced incidence of infection [33, 42, 47]. These observations point at the value of BCG as a vaccine adjuvant. However, in a clinical trial setting, the use of BCG in the control arm does not constitute a true placebo, since BCG induces LST conversion in some individuals in the control arm – albeit, to a lesser extent than in the experimental arm. Reduced incidence has been observed in participants whose LST converted post-vaccination, in both study arms (ALM+BCG or BCG alone)[42, 47]. This suggests a potential

association between BCG and reduced infection rates in the control arm. Therefore, on the one hand, BCG inclusion as an adjuvant increases the immunogenicity of the vaccine and its use is justified in the experimental arm. On the other hand, the use of BCG in the control arm can induce LST conversion and possibly modulates susceptibility to infection. Therefore, from the standpoint of assessing the efficacy of the vaccine, BCG may not be an appropriate candidate for use in the control arm of efficacy trials since it may detract from the ability of the trial to demonstrate the full effect of the vaccine [24, 53].

Surprisingly, in view of the prophylactic findings discussed above, therapeutic trials of first generation leishmaniasis vaccine have shown very encouraging results. From Convit's trials for treatment of patients in Venezuela and Machado Pinto's results in Brazil to Musa et.al's results on therapy of PKDL in Sudan, immunochemotherapy seems a promising mode of treatment [50, 54-56]. This justifies further investigation of first generation leishmaniasis vaccines for therapeutic purposes.

It should be mentioned that to date, only one second generation vaccine has been evaluated in phase 1-2 clinical trials [57]. Although previous trials with first generation vaccines did not result in identification of an efficacious vaccine, they did demonstrate the safety profile of these vaccine candidates. Moreover, these trials made a significant contribution to improving the overall quality of vaccine investigation in the endemic countries where they were conducted, training personnel and identifying particular issues related to vaccine development in general and vaccines against leishmaniasis in particular.

It is estimated that the total cost to conduct first generation vaccine trials through TDR over a period of 10 to 12 years in Iran, Sudan, Colombia and Ecuador (in addition to a follow up safety study of Myrink's vaccine in Brazil) was between 2 and 3 million dollars (Farrokh Modabber, personal communication). This also covered production of leishmanin for use outside of vaccine trials, training and some components of capacity building.

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EFFICACY OF KILLED WHOLE-PARASITE VACCINES IN THE PREVENTION OF LEISHMANIASIS - A META-ANALYSIS

Submitted for publication in the Vaccine

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Abstract

Despite decades of investigation in countries on three continents, an efficacious vaccine against *Leishmania* infections has not been developed. Although some indication of protection was observed in some of the controlled trials conducted with "first-generation" whole, inactivated *Leishmania* parasite vaccines, convincing evidence of protection was lacking. After reviewing all previously published or unpublished randomized, controlled field efficacy clinical trials of prophylactic candidate vaccines, a meta-analysis of qualified trials was conducted to evaluate whether there was some evidence of protection revealed by considering the results of all trials together. The findings indicate that the whole parasite vaccine candidates tested do not confer significant protection against human leishmaniasis.

Key words: Leishmaniasis vaccine, meta-analysis, clinical trial

Abbreviated title: Meta-analysis of efficacy trials of leishmaniasis vaccines

1 Introduction

Leishmaniasis is endemic in at least 88 countries, some of which are among the poorest in the world[1, 2]. The estimated global prevalence of all forms of the disease is 12 million, with 1.5 to 2 million cases of cutaneous leishmaniasis (CL) added annually (with duration of lesions typically from few months to a year) and 500,000 cases of visceral form of the disease (with duration of disease from several months to more than a year) [2, 3]. Current control measures, including environmental sanitation and drug treatment of cases, are expensive and cannot be sustained effectively by poor countries due to the problems of financing and implementation [4-6]. Moreover, toxicity associated with some of the most widely available drug treatments, including injections of pentavalent antimony compounds, and the resistance developed by the parasite [7-12], underline the need for development of effective methods of prevention, especially vaccines [4, 13, 14].

1.1 Historical perspective

To date, the only effective way of inducing immunity against leishmaniasis in humans is provided by leishmanization (LZ), the practice of injecting live virulent parasites in healthy individuals [15]. LZ has been practiced historically in high incidence endemic foci as a means of controlling the timing and site of the initial lesion, but it is no longer widely used because of rare complications and difficulties in standardization of the injected parasites [15-18].

During the first half of the 20th century researchers in Latin America investigated different antigens as potential vaccines [19, 20]. Beginning in the 1970s and 1980s, Mayrink and colleagues in Brazil and Convit and colleagues in Venezuela experimented with the use of whole, killed parasites, both for prophylaxis and therapy. Later studies were conducted with inactivated whole parasite vaccines in Ecuador (trivalent vaccine composed of three strains of locally obtained parasites), Colombia (Biobras single strain *L. amazonensis* vaccine), Iran and Sudan (autoclaved *L. major* with BCG included as an adjuvant: ALM+BCG) [21-28]. With the exception of the trial by Armijos in Ecuador in which a locally-prepared vaccine was used [21], none of the other trials demonstrated significant protection associated with vaccination [26].

Some investigators observed a lower incidence of leishmaniasis in the subset of those in the vaccinated group whose Leishmanin Skin Test (LST) had converted (from an induration of <5mm to >5 mm) after vaccination [24, 25, 29]. Also, researchers in Iran observed significant protection in school age boys but not in girls [27]. Evidence of potential clinical value of such vaccines for treatment, rather than the prevention, of disease was demonstrated in trials among leishmaniasis patients in the New World [30, 31].

We have re-examined the combined data from all except one published, and unpublished, randomized, controlled clinical trials (RCT) of prophylactic first generation leishmaniasis vaccine conducted to date to evaluate whether, overall, there is evidence of efficacy, or there is efficacy in some sub-groups of the trial populations.

2 Data selected for analysis

Definition of Studies:
All published and unpublished field efficacy trials of prophylactic candidate vaccines against leishmaniasis conducted to date were considered for inclusion. As a result, publication bias is not a concern in this meta-analysis.

Information sources:
Trial protocols and progress reports for studies in Iran, Sudan and Colombia were reviewed. For each clinical trial identified, the principal investigator was requested to provide the original trial database. Thus, individual-level data for trials conducted in Iran and Sudan as well as aggregated data for the trial in Colombia were obtained. These data were used to estimate or verify the effect statistics (relative risk) as well as age and gender composition in Iran studies. For other studies values reported in the published articles were used (see Included Studies, below).

Selection criteria:
1) Trial objective: efficacy of a first generation vaccine for prevention of leishmaniasis in healthy individuals in an endemic area.
2) Study design: randomized, double blind, controlled clinical trial designed to estimate vaccine efficacy.
3) Candidate vaccine: Killed, whole *Leishmania* promastigotes.
4) Normal field conditions during follow up: Sample size and power calculations are generally based on previously observed disease rates in the trial area. Unforeseen, major changes in environmental and climatic factors could lead to a significant change in disease incidence, affecting the study power and conclusions. Selected clinical trials were conducted under the usual field conditions that gave rise to the previously observed incidence rates.

Excluded Studies:
On the basis of the above criteria, one study [32] was excluded due to the unusual climatic changes that were attributed by authors to the El Nino phenomenon during the study follow up [32]. These changes led to significantly lower disease incidence rate than expected

Included Studies:
Data and reports from the randomized, blinded, controlled efficacy trials listed in table 1 were used. Trial details are presented in tables 1-3. Further details are available in Noazin et al., 2008[26].

Table 1 - Design characteristics of selected trials

	Study Label	Number of injections	Vaccine parasite	Inactivation method	Adjuvant	Control treatment	Causative parasite	Area, Country	Study population origin	Investigators, year published
A	Zav3	3	*L. major*	Autoclaved	BCG	BCG	*L. major*	Zavareh, Iran	Endemic	Khamesipour, et al., not published
B	Bor3	3	*L. major*	Autoclaved	BCG	BCG	*L. major*	Borkhar, Iran	Endemic	Khamesipour, et al., not published
C	Bam3	3	*L. major*	Autoclaved	BCG	BCG	*L. tropica*	Bam, Iran	Endemic	Sharifi et al., not published
D	Bam1	1	*L. major*	Autoclaved	BCG	BCG	*L. tropica*	Bam, Iran	Endemic	Sharifi et al., 1998
E	Esf1	1	*L. major*	Autoclaved	BCG	BCG	*L. major*	Esfahan, Iran	Endemic and non-endemic	Momeni et al., 1999
F	Sudan 2	2	*L. major*	Autoclaved	BCG	BCG	*L. donovani*	Gedarif, Sudan	Endemic	Khalil et al., 2000
G	Brazil 1981 and 1983	2	5-strain vaccine (species of brasiliensis and mexicana complexes, including *L. guyanensis and L. amazonensis*)	Merthiolate	None	Phosphate buffer + Merthiolate	*?*	Amazonas, Brazil - Exposure during military missions	Endemic and non-endemic- army recruits	Antunes et al., 1986
H	Colombia 3	3	L. amazonensis	Merthiolate	None	Saline	*L. Panamensis*	Colombia - Exposure during military missions	Endemic and non-endemic, army recruits	Velez et al., 2005
I	Ecuador2	2	3-strain vaccine (*L. guyanensis, L. brasiliensis, L. amazonensis*)	Merthiolate	BCG	BCG	*L. guyanensis, L. brasiliensis, L. amazonensis*	Rural rainforest, Ecuador	Endemic	Armijos et al., 1998

Individual level data were used for trials A-E, and published information was used for trials F-I (table 1).

Although most trials excluded individuals with LST>0mm, in the Borkhar (Bor3) and Zavareh (Zav3) trials (A and B in table 1) volunteers with any LST value at screening were enrolled. Since an LST>0 could indicate previous exposure to leishmaniasis and be associated with immunity, for these 2 trials we analysed data only on participants with a pre-vaccination LST of zero. This excluded 12% of trial participants in Zavareh and 40% in Borkhar.

The study conducted in Brazil in 1981 was conducted in two separate cohorts[29]. These cohorts were different in several respects, including the risk of disease, duration of exposure and previous vaccination history, and we have treated them as two separate studies (identified as Brazil 1981A and Brazil 1981B).

3 Statistical analysis

We used a meta-analysis approach based on relative risk (RR = incidence in the vaccine arm / incidence in the control arm) calculated for each study separately and then pooled across studies. Briefly, in calculating the pooled effect, an average of the trial-specific relative risks was calculated by weighting individual study effects according to their trial size (i.e., weighting by the relative quantity of information provided by the trial). These weights can be calculated using a variety of methods, including the inverse variance (I-V) and Mantel Haenszel (M-H) methods. If the relative risks in different studies are not widely different (i.e., studies are homogeneous), a fixed effect model would be appropriate. If the variation is more than would be expected by chance then a random effects model is more appropriate, for which a common method for calculation of the pooled effect is that of DerSimonian-Laird (D-L) [33]. To assess heterogeneity, we used a chi-square test of the Q statistic (Q = sum of squared deviations of weighted RR's from their overall mean); with degrees of freedom = k-1, where k is the number of studies. In addition, due to limitations of Q [34], I -squared was also used to assess heterogeneity. I-squared measures the percent of variation due to between-studies variability. A value of zero for I-squared indicates that all variability in relative risks is due to sampling error [34].

EpiInfo 2002, Stata 9 and MS Excel were used in the analyses. The "metan" program in Stata 9 was used to calculate relative risk (RR) estimates and corresponding 95% confidence intervals. The inverse variance (I-V) and the Mantel-Haenszel (M-H) methods were used separately to fit the fixed effect model and the DerSimonian-Laird (D+L) method to fit the random effect model.

4 Results

Age and gender breakdown of participants in vaccine trials included in this analysis are provided in table 2. Some trials were confined to children, whereas other included all ages and the trials among the military in Brazil and Colombia were confined to adult males. In two of the trials there was a significant excess of males in the vaccinated group and in two the vaccinees were significantly younger, on average.

Table 2 - Age and gender distribution of participants in trials selected for meta-analysis

Study	Bam1		Esf1		Bam3		Bor3		Zav3		Sudan2		Brazil1981 - 2		Brazil 1983 - 2		Colombia3		Ecuador2	
Trial arm	**V ***	**C ***	**V**	**C**	**V**	**C**	**V**	**C**	**V**	**C**	**V**	**C**	**V**	**C**	**V**	**C**	**V**	**C**	**V**	**C**
N	1838	1795	1190	1124	2149	2068	1107	1084	941	1055	1155	1151	667	644	658	616	1302	1295	438	406
Age (yrs)																				
Minimum	6	6	5	5	6	6	6	6	5	5	<3	<3								
Maximun	15	15	67	72	12	12	13	13	59	59										
Mean	9.1	9.1	18.0	18.8	7.4	7.4	8.2	8.7	19.0	19.2	6.5	7.2	18.6	18.6	18.6	18.6	19.8	19.8	5.4	5.7
P value (Kruskal-Wallis H)	0.822		0.118		0.132		0.000		0.666		0.010		--		--		--		0.271	
SEX																				
% Female	47.1	51.8	52.7	52.6	48.8	49.6	46.3	52.4	50.2	48.9	53.3	55.3	0.0	0.0	0.0	0.0	0.0	0.0	57.5	54.3
P value (Fisher Exact)	0.003		0.496		0.302		0.002		0.323		0.599		N/A		N/A		N/A		0.349	

* V=vaccine; C=Control

Participants included in the analysis of vaccine immunogenicity were restricted to those with negative pre-vaccination LST. LST measurements 42 to 80 days post-vaccination (depending on the trial) are displayed in Table 3. LST $\geqslant 5$ is generally accepted as an indication of a significant skin test response in volunteers after vaccination. In all trials where immunogenicity was assessed in both vaccinated and unvaccinated participants there was a significantly higher level of skin test conversion among vaccinated individuals. However, the level of skin test conversion varied substantially among trials, ranging from only 16% among those vaccinated in the Bam1 trial to 68% in the Brazil 1983-2 trial. In table 3 and thereafter, and in our analysis, we have treated the Brazil 1981-2 study as 2 distinct trials: Brazil 1981A-2 and Brazil 1981B-2. This approach was adopted due to the differences between the two cohorts of volunteers in this study in their duration and timing of exposure as well as the length of time after yellow fever vaccination that the vaccines were given (which could affect their immunological response) [29].

Table 3 - LST conversion 42-80 days post vaccination (among participants with LST=0 prior to vaccination).

Trial	Trial arm	N	% LST ≥5 mm
Bam1	V *	1807	16.5
	C *	1761	3.3
Esf1	V	1168	36.2
	C	1104	7.9
Bam3	V	1980	18.2
	C	1935	2
Bor3	V	608	29.9
	C	538	6.1
Zav3	V	772	--
	C	901	--
Sudan2	V	1919	30
	C	1005	7
Brazil1981A-2	V	311	33
	C	--	--
Brazil1981B-2	V	338	37
	C	--	--
Brazil 1983-2	V	611	68
	C	--	--
Colombia3	V	--	--
	C	--	--
Ecuador2	V	--	--
	C	--	--

* V = vaccine arm, C = control arm

-- = Not measured

The incidence rates of leishmaniasis in vaccine and control arms in the different trials are summarised in table 4 (participants in Zav3 and Bor3 with pre-vaccination LST≥0 mm were excluded from this analysis). Vaccine efficacy (VE) is calculated as (100*(1-RR)). The percent of the trial populations who developed disease varied from around 1% to 18%. In only one of the trials (Ecuador2) was the difference in incidences between the vaccinated and unvaccinated group statistically significant.

Table 4 - Incidence of leishmaniasis among vaccinated and unvaccinated individuals and estimated vaccine efficacy.

Study	Follow-up (months)	Vaccine Total N	Vaccine Cases	Control Total N	Control Cases	% Case (vaccine)	% Case (control)	Vaccine efficacy
Bam1	24	1838	52	1795	60	2.83	3.34	15%
Esf1	24	1190	214	1124	208	17.98	18.51	3%
Bam3	24	2082	81	2008	93	3.89	4.63	16%
Bor3	36*	604	64	561	63	10.60	11.23	6%
Zav3	24	742	102	868	109	13.75	12.56	-9%
Sudan2	24	1155	133	1151	141	11.52	12.25	6%
Brazil 1981A-2	12	322	28	289	32	8.70	11.07	21%
Brazil 1981B-2	12	345	4	356	5	1.16	1.40	17%
Brazil 1983-2	12	658	4	616	8	0.61	1.30	53%
Colombia3	12	1302	101	1295	88	7.76	6.80	-14%
Ecuador2	12	333	7	316	24	2.10	7.60	72%

* Although there were 3 years follow up in Bor3, only cases from years 2 and 3 are included in this analysis because vaccination was completed (3rd dose) at the end of year 1.

Table 5 shows the confidence intervals on the relative risk (RR) estimates from the trials and the relative weights derived for each study according to the method of pooling the results.

The weights assigned to the Ecuador trial vary substantially between the three estimation methods. This reflects the tendency of the fixed effect models (I-V and M-H) to give less weight to smaller trials.

Table 5 - Relative risks (incidence in vaccinated/incidence in unvaccinated), 95% confidence intervals and relative weights for each study according to the method used for meta-analysis.

					Weight (%)		
Study	N	RR	95% Conf. Int		I-V	M-H	D+L
Bam1	3633	0.846	0.587	1.22	6.32	7.26	8.33
Esf1	2314	0.972	0.818	1.155	28.37	25.59	19.66
Bam3	4090	0.84	0.628	1.124	9.94	11.32	11.41
Bor3	1165	0.944	0.679	1.31	7.84	7.81	9.72
Zav3	1610	1.095	0.851	1.408	13.34	12.02	13.66
Sudan2	2306	0.94	0.753	1.174	17.08	16.89	15.63
Brazil 1981A-2	611	0.785	0.485	1.271	3.64	4.03	5.39
Brazil 1981B-2	701	0.826	0.224	3.049	0.49	0.59	0.86
Brazil 1983-2	1274	0.468	0.142	1.547	0.59	0.99	1.02
Colombia3	2597	1.142	0.867	1.503	11.15	10.55	12.27
Ecuador2	649	0.277	0.121	0.633	1.23	2.95	2.05
					100	100	100

We sought evidence of heterogeneity in the results from the different trials. The heterogeneity statistics estimated by the three methods are very similar, as indicated in table 6 and in no case was there evidence of significant heterogeneity, providing

justification for using fixed effect models. A comparison of the RR's from the Old World and the New World trials indicated such heterogeneity as there is may be attributed to the latter group.

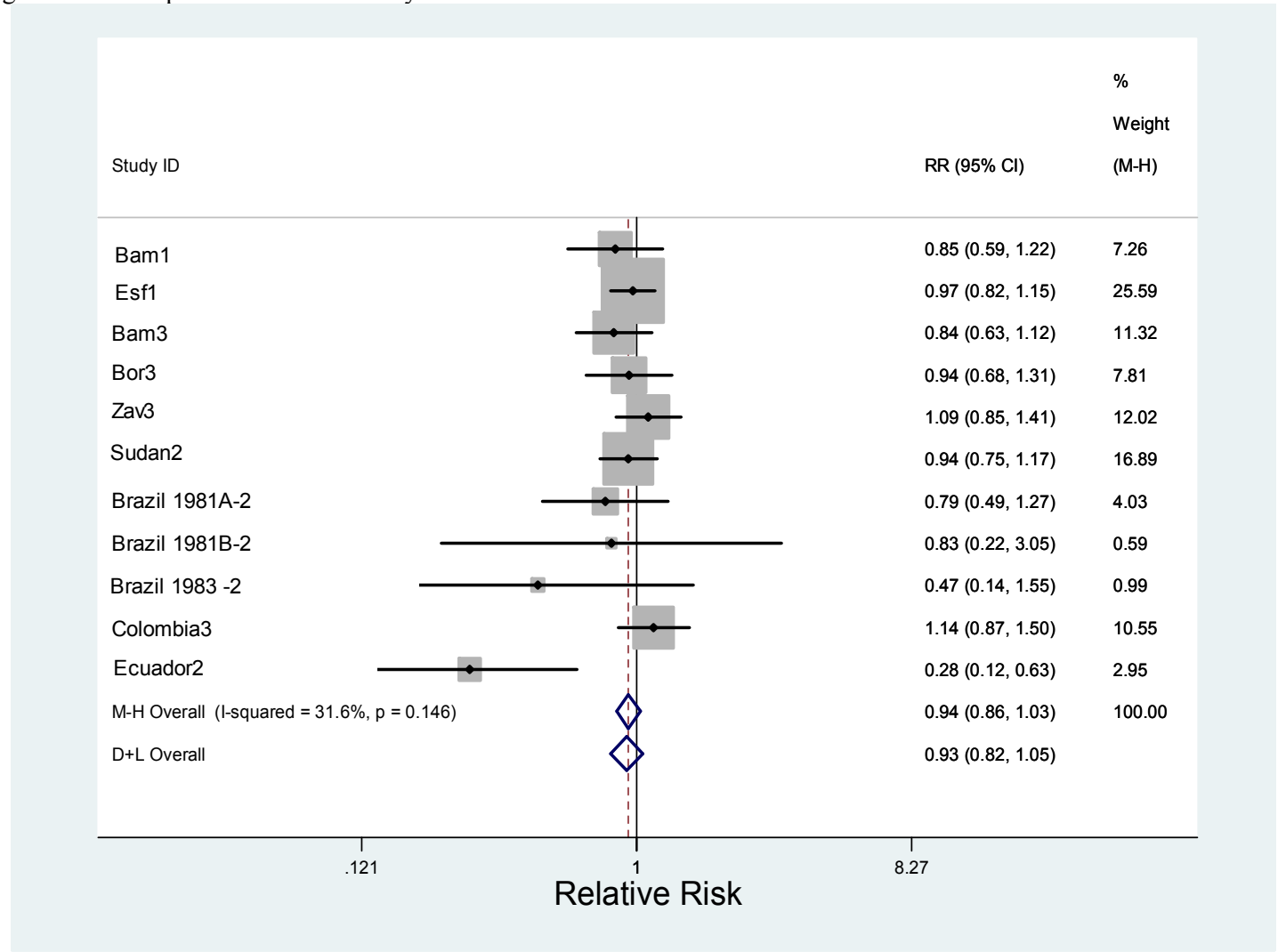
Pooled RR estimates and the 95% confidence interval (CI) estimated by the 3 methods (table 6) are very similar, regardless of the model used, providing little evidence to reject the hypothesis of no vaccine effect on leishmaniasis incidence.

Table 6 - Heterogeneity and effect statistics in the 3 methods of meta-analysis

	Heterogeneity statistics				Effect statistics			Test of RR=1	
Method	Chi square	d.f.	p	I-squared	Pooled RR	95% Conf. Int		z value	P
I-V	14.59	10	0.148	31.5%	0.947	0.864	1.038	1.16	0.246
M-H	14.62	10	0.146	31.6%	0.939	0.857	1.029	1.34	0.179
D+L	14.62	10	0.146	31.6%	0.928	0.821	1.049	1.2	0.231

Figure 1 shows the “forest plot” of the findings in the trials. The area of the gray square boxes represent the relative size of each trial with the centre dot and the line in the centre of each square representing the RR and its 95% CI. The overall RR is depicted by two blank diamond boxes, representing the M-H and the D+L estimates.

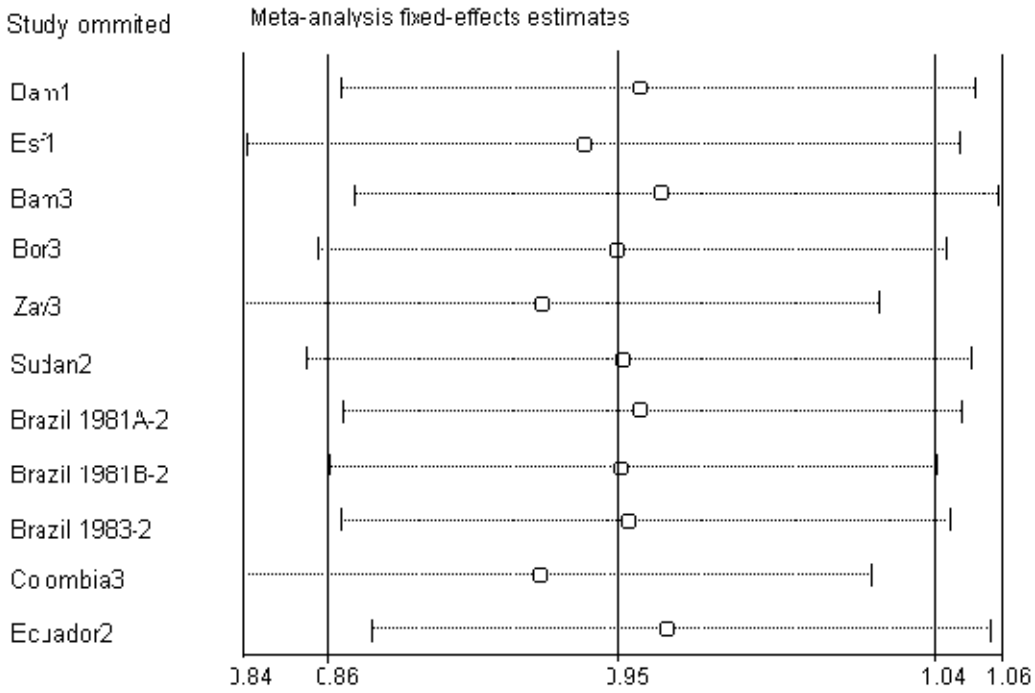
Figure 1 - Forest plot of vaccine efficacy measures in different leishmaniasis vaccine trials



While Old World trials are clustered around the vertical line of RR=1 (i.e., homogeneous but with minimal efficacy), the results from the trials conducted in Latin American tend to be scattered on the left of that line, suggesting more heterogeneity but also more efficacious results. The Ecuador trial, the only trial with significant results, is located in the far left of the forest plot. Despite their lower individual RR values, these trials have limited impact on the pooled RR due to their smaller sample sizes (and wide confidence intervals).

A graphical display of the influence of individual trials on the pooled RR is presented in Figure 2. This graph shows the values of the pooled RR, when studies are omitted one at a time. The reference line is the overall, pooled RR. Thus, the pooled RR is lower when Esf1, Zav3 or Colombia3 trials are omitted and the reverse is the case when any one of the Bam trials, Brazil 1981A-2 and the Ecuador2 are omitted

Figure 2 - Influence of individual trials on pooled RR The circles indicate the pooled relative risk estimate when each individual trial is omitted. 95% confidence intervals are also shown



5 Discussion

In some trials, vaccinated participants who skin test converted following vaccination were reported to have a lower incidence of leishmaniasis than other trial participants [24, 25, 29]. However, our meta-analysis clearly demonstrates the overall inability of first generation leishmaniasis vaccines evaluated to date in phase 3 clinical trials to protect vaccinated individuals against infection by the *Leishmania* parasite.

The apparent absence of efficacy of these vaccines may be due to a number of potential factors. First, the immune stimulation provided by a single dose, or even multiple doses, of inactivated parasite antigen, even when mixed with BCG as an adjuvant, may be inadequate. Secondly, BCG was used in the control arm in several of the studies and was also used as a vaccine adjuvant. BCG stimulates Th1 response and contributes to the immunogenicity of the vaccine [35]. However, when used as the clinical trial control vaccine (for blinding), and as a vaccine adjuvant, it induces Th1 response in both arms, thus making it potentially more difficult for any potential vaccine effect to be detected. To the extent that BCG alone might protect against leishmaniasis, the difference in incidence between the two study arms would be reduced and the statistical power of the study would be compromised. Thirdly, LST is an imprecise and highly variable indicator of previous exposure to leishmaniasis. This could lead to misclassification of some individuals with previous exposure and immunity as unexposed and allow their inclusion in both arms of the clinical trial. If such persons are still at some risk of leishmaniasis, but the vaccine confers no additional protection in such partially immune individuals, then the protective efficacy of the vaccine in "unexposed" individuals may be underestimated. Fourthly, it is possible that some genetically non-responsive volunteers in endemic areas would show no LST reaction while they could have been exposed and possibly immune. To the extent that this occurs, the resulting misclassification would contribute to a reduced difference between the two arms and contribute to misleading efficacy estimation.

Our use of the meta analytic approach is subject to the some limitations. The different vaccine candidates used in the trials were similar in their dependence on killed parasites, but the composition of the vaccines varied between trials, BCG was used as an adjuvant in some cases and the ecological setting of the different trials varied substantially. Thus it may be argued that combining the results from the trials should be done with great caution. We would not disagree with this view but have combined the findings to seek evidence that might encourage further work on first-generation vaccines. In this respect, our finding are depressing and suggest that other approaches to leishmaniasis vaccine development should be vigorously pursued.

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