INSTITUTO POLITÉCNICO NACIONAL

CENTRO DE BIOTECNOLOGÍA GENÓMICA

“MOLECULAR MONITORING AND ASSESSMENT OF ONCHOCERCIASIS CONTROL IN MEXICO WITH THE APPLICATION OF CURRENT AND NOVEL DIAGNOSTIC APPROACHES”

TO OBTAIN THE DEGREE OF DOCTOR OF PHILOSHOPY IN BIOTECHNOLOGY

BY

HEMAVATHI GOPAL

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SECRETARÍA DE INVESTIGACIÓN Y POSGRADO

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"Molecular monitoring and assesment of onchocerciasis control in México with the application of current and novel diagnostic approaches"

Presentada por el alumno:

Gopal	---------	Hemavathi
Apellido paterno	Apellido materno	Nombre(s)

Con registro: B 0 9 1 3 0 5

aspirante de:

Doctorado en Ciencias en Biotecnología

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Dedicated to my beloved parents.....

CERTIFICATE

I hereby certify that the PhD thesis entitled "**Molecular monitoring and assessment of onchocerciasis control in Mexico with the application of current and novel diagnostic approaches**" submitted by **Ms. Hemavathi G**., for the degree of **DOCTOR OF PHILOSOPHY** in **BIOTECHNOLOGY** to University of IPN, Mexico is the result of the research work carried out by her in the laboratory of Biomedicine, CBG-IPN, Mexico under my guidance and supervision during the period of August 2009-June 2012.

I further declare that the results of this work have not been previously submitted either partially or fully for any other degree or fellowship.

Dr. Mario A Rodríguez-Pérez

Guide and Supervisor
Department of Biomedicine,
CBG-IPN, Mexico

Date:

Place: Mexico

DECLARATION

I hereby declare that the thesis entitled "**Molecular monitoring and assessment of onchocerciasis control in Mexico with the application of current and novel diagnostic approaches**" which is submitted herewith for the degree of **DOCTOR OF PHILOSOPHY** in **BIOTECHNOLOGY** to university of IPN, Mexico is the result of the research work done by me in the laboratory of Biomedicine, CBG-IPN, Mexico under the guidance of **Dr. Mario A. Rodríguez-Pérez** during the period of August 2009-June 2012.

I further declare that the results of this work have not been previously submitted either partially or fully for any other degree or fellowship.

Date:

Place: Mexico **Hemavathi G.**

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Date:

Place: Mexico ***Hemavathi G.***

ABBREVATIONS

AIDS	acquired immunodeficiency syndrome
APOD	acute papular onchodermatitis
APOC	African Programme Control Of Onchocerciasis
ATP	annual transmission potential
BSA	bovine serum album
bp	base pairs
cm	centimeter
CPOD	chronic papular onchodermatitis
Co	company
$CHCl_3$	chloroform
˚C	degree Celsius
CA	California
DEC	diethycarbamazine
DTT	d-thiothreitol
DPM	onchocercal depigmentation
DNA	deoxyribo nucleic acid
dNTP	deoxynucleotide triose phosphate
EIA	enzyme immuno assay
ELISA	enzyme linked immunosorbent assay
EDTA	ethylene di amine tetra acetic acid
Fig	figure
GABA	gamma-amino butyric acid
g	gram
H_2SO_4	sulphuric acid
HCl	hydrochloric acid

h	hour
Kg	kilogram
Km	kilometer
LOD	lichenified onchodermatitis
L3	third stage larvae
Log	Logarithm
mM	mili molar
$MgCl_2$	magnesium chloride
mg	milligram
µg	microgram
ml	milliliter
MDP	Mectizan Donation Program
mf	microfilariae
µm	micrometer
mm	millimeter
NaCl	sodium chloride
$(NH_4)_2SO_4$	ammonium sulphate
$NaHCO_3$	sodium bicarbonate
$NaCO_3$	sodium carbonate
NaOH	sodium hydroxide
Na_2SO_3	sodium sulphate
NaI	sodium iodide
NaH_2PO_4	di sodium phosphate
nm	nanometer
ng	nanogram
#	number
OD	optical density

OEPA	Onchocerciasis Elimination Program for Americas
OCP	onchocerciasis control program
PCR	polymerase chain reaction
P-C	phenol chloroform
PMPs	paramagnetic particles
pH	negative logarithm of hydrogen ion
%	percentage
RT	room temperature
RIA	radio immuno assay
rpm	revolution per minute
SDS	sodium dodecyl sulphate
SD	standard deviation
TDR	tropical disease research
Taq	thermus aquaticus DNA polymerase
USA	United States of America
UV	ultraviolet
v/v	volume over volume
WHO	world health organization

RESUMEN

El objetivo del presente estudio fue evaluar la transmisión de *O. volvulus* en los focos endémicos del Sur y Norte de Chiapas en México.

En el foco Norte de Chiapas el tratamiento fue suspendido en 2008 basado en datos entomológicos del año 2006 donde se muestran transmisiónes bajas o nulas de *O. volvulus*. La vigilancia epidemiológica pos-tratamiento fue realizada en el año 2010 en cuatro comunidades; Nueva Esperanza, Altagracia, Chimix y El Ámbar. Las moscas fueron colectadas de febrero a mayo de las 10 a las 5. Un total de 207 lotes de cuerpos que contenía 50 cuerpos cada uno fueron evaluados por PCR. La tasa de infección por 2000 moscas varió de 0 (UCI 95 % 0.5-12.2). El potencial anual de transmisión varió de 0 (UCI 95% 5.4- 8.6). Estos resultados demuestran que no hubo contacto vector parásito y la transmisión fue 0. Por lo tanto OEPA declaró la eliminación de la Oncocercosis en el foco Norte de Chiapas en el año 2011.

En el Foco sur de Chiapas se utilizo la PCR para monitorear la transmisión de *O.volvluls* analizando lotes de cabezas y cuerpos de S.ochraceum. Los insectos fueron colectados en las siguientes comunidades: Amplación Malvinas, Estrella Roja, Nueva Costa Rica, Morelos, Brasil, Mexiquito, Santa Rita, Loma Bonita, Montowa, La Granja, La Soledad, Finca Victoria y Finca Santa Malia. Un total de 3,185 lotes de cabezas y cuerpos fueron evaluados. En general para el foco Sur de Chiapas la tasa de infectividad por 2,000 moscas fue 0 (UCI 95% 0.3-4.1). El potencial estacional de transmisión fue de 0 (2.3-12.8). Estos resultados demuestran que en el año 2011 la transmisión ha sido

interrumpida. Basados en estos resultados el programa de control basado en la distribución masiva de ivermectina ha sido suspendido en el año 2012 y se inició con la vigilancia post tratamiento.

Las pruebas entomológicas son esenciales para determinar si la transmisión de *O.volvulus* ha sido interrumpida o eliminada. En la actualidad la detección por PCR se ve limitada por el tamaño del lote y por el número de moscas procesadas. Los métodos de purificación de ADN basados en la unión de oligonucleótidos con pruebas de ADN fue capaz de detectar una mosca infectada en lotes de 100 a 200 moscas. Lo anterior indica una mejora de 2 a 4 veces con respecto al método tradicional. Este método en el futuro puede ser utilizado por los programas de vigilancia epidemiológica para un gran número de moscas en un menor tiempo.

Palabras Claves: Oncocercosis, Transmisión, Chiapas, México, Vigilancia entomológica, PCR, cuentas magnéticas, interrupción, eliminación.

ABSTRACT

The main objective of the present study was to evaluate the transmission of *O. volvulus* in the endemic communities of Southern Chiapas and Northern Chiapas in Mexico. In N. Chiapas, the ivermectin mass treatment had been halted in 2008 based on the entomological data collected from this focus in 2006 showing low or no evidence for the ongoing transmission of *O. volvulus*. The post-treatment entomological surveillance was carried out in 2010 in four communities namely Nueva Esperanza, Alta Gracia, Chimix and El Ambar. The flies were collected from these regions for a period of four months from February to May from morning 10:00 AM to 5:00 PM. A total of 207 body pools containing 50 bodies each were subjected to pool screen PCR assay. The prevalence of infective rate per 2000 flies being 0 with 95% UCI (0.5-12.2). The seasonal transmission potential was found to be 0 and 95% UCI ranged from 5.4-8.6. The above results indicated that there was no-ongoing parasite-vector contact and zero transmission was observed. Hence, OEPA declared the elimination of onchocerciasis from N. Chiapas focus of Mexico in 2011. The PCR monitoring of onchocerciasis transmission was estimated in the pools of heads and bodies of the black flies (*Simulium ochraceum*) collected in the communities of Amplacio Malvinas, Estrella Roja, Nueva Costa Rica, Morelos, Brasil, Mexiquito, Santa Rita, Loma Bonita, Montowa, La Granja, La Soledad, Finca Victoria and Finca Santa Malia of S. Chiapas focus. A total of 3,185 pools (sampling time was 7.00AM-5.00PM) were tested from the above communities and grouped into head and body pools. The prevalence of infective rate per 2000 flies was found to be zero with a 95% UCI ranging from 0.3-4.1, followed by seasonal transmission potential of zero with 95% UCI from 2.3-12.8 for the communities of S.

Chiapas. The results obtained from PCR monitoring of the *O. volvulus* in S. Chiapas focus during 2011 showed that the transmission has been interrupted. Based on the above results the control program would halt the ivermectin mass distribution from 2012 and initiate post-treatment surveillance in S. Chiapas. Entomological survey of black flies is crucial to estimate whether onchocerciasis transmission has been interrupted or eliminated. At present PCR pool screening is used, which is limited by the pool size and number of flies processed. Oligonucleotide based DNA purification methods based on oligonucleotide binding of *O. volvulus* DNA tested was able to detect one infected fly/larvae in pools of 100-200 flies. Thus, indicating a 2-4 fold improvement over the traditional method. This method can be in future used by the control programs for the entomological surveillance where large number of flies can be tested in short time.

Key words: Onchocerciasis, Transmission, Chiapas, Mexico, entomological surveillance, Pool screen PCR, magnetic beads, interrupted, elimination.

MOLECULAR MONITORING AND ASSESSMENT OF ONCHOCERCIASIS CONTROL IN MEXICO WITH THE APPLICATION OF CURRENT AND NOVEL DIAGNOSTIC APPROACHES

1: INTRODUCTION

Onchocerciasis or river blindness is a considerable public health problem affecting millions of people worldwide mainly in Africa, South America and Central America (Rodríguez-Pérez et al., 2004). The disease is caused by a filarial nematode known as *Onchocerca volvulus* which is transmitted by black flies belonging to the Genus *Simuliid* (Little et al., 2004). Despite the colossal resources committed by the international communities to its control, the disease still remains a major public health problem as one of the major causes of blindness in the affected areas. Onchocerciasis eradication has been the main goal of the three international disease control programs namely, the Onchocerciasis Control Program (OCP) in West Africa, which was later, replaced by the African Program for Onchocerciasis Control (APOC) and the Onchocerciasis Elimination Program for the Americas (OEPA). Each program has its own set of strategies and approaches that differ from each other in order to achieve their goal of eliminating onchocerciasis as a major public health problem. The overriding goal of these programs was to suppress the transmission to the extent that the *Simulium* vectors will not be able to acquire the parasite from humans thus abolishing the disease (WHO, 2001).

According to a recent estimation, about 37-40 million people are infected worldwide of which approximately 779,000 had been blinded by onchocerciasis (Basáñez et al., 2007). In Mexico, onchocerciasis is distributed in three foci, Southern Chiapas, Northern Chiapas and Oaxaca. It was originally estimated that approximately 630,000 people were at risk, with 286,000 inhabitants residing in the endemic areas of which 25, 645 individuals were infected (Martin Tellaeche et al., 1998; WHO 1991, 1995). However, after the initial report of 112 cases of blindness as a result of infection with *O.volvulus* in 1989, no new cases of blindness has been recorded in Mexico (Martin Tellaeche et al., 1998; Rodríguez-Pérez et al., 2008b).

Presently, the control of the disease relies on chemotherapeutic treatment of the affected communities through annual or biannual distribution of microfilaricidal drug. Ivermectin does not have potency over adult worms and its distribution should be for about 15 years, the average life span of adult worms of *O. volvulus.* Ivermectin is given orally (150 mg/kg) (Bassissi et al., 2004). The evidence abounds that the prevalence of the infection has been significantly reduced after the introduction of Ivermectin for the treatment of onchocerciasis in Mexico. Hence, for the detection of the remaining transmission level, a very large population of flies has to be processed to determine the current status of the disease. The traditional method apart from being tedious and cumbersome, it makes impractical to examine large number of flies to be examined (Yameogo et al., 1999; Adeleke et al., 2010). Therefore, reliance on such method will be a clog towards speeding results for monitoring transmission and evaluating success of

control programs. Moreover, it will require many years to satisfy the required number of flies needed to be examined. However, the use of Polymerase Chain Reaction method has been optimized in the field and validated that it produces similar results to classic dissection, with the advantages that it is less cumbersome and permits large number of flies to be screened (Yameogo et al., 1999).

The general objective of this study is to determine the current level of transmission of *O.volvulus* as a post-treatment surveillance by using PCR-ELISA in two endemic foci in Mexico. The main purpose of the present study was to examine the transmission levels of the onchocerciasis in various parts of Mexico, where the control programmes have been successful due to mass treatment with ivermectin. The structure of the thesis contains a total of four objectives. The first objective describes the surveillance of onchocerciasis transmission in Northern Chiapas after successful control of transmission due to ivermectin treatment. This chapter comprises of post ivermectin treatment surveillance in order to provide evidence on whether there is or no possibility of parasite re-establishment in the area.

The second objective was to compare the transmission levels in two coffee fincas of Southern Chiapas after 11 years of ivermectin treatment. This chapter describes the prevalence of *O. volvulus* transmission in the local vector populations. The findings are

also discussed based on epidemiologic data of the number of infected resident population as well as the itinerant populations (migrants).

Third objective was to monitor for *O. volvulus* transmission in the communities of Southern Chiapas focus for onchocerciasis using PCR-ELISA methods and to evaluate the ongoing ivermectin treatment in Southern Chiapas. Southern Chiapas was considered as one of the major communities endemic for onchocerciasis. In the State of Chiapas, the affected localities belong to 23 municipalities, in an area of 12,640 Km^2 with over 18,000 registered cases of onchocerciasis.

Fourth objective was to evaluate an improved DNA purification protocols based on magnetic bead particles for the detection of parasite of *O.volvulus* DNA in pools of black flies and its validation in endemic communities. The experimental strategy for the novel protocol was to increase the pool size of flies tested by O-150 PCR-ELISA using the genomic DNA purified using the silica coated paramagnetic, general magnetic and oligonucleotide based Dynal magnetic bead capture method, thus decreasing the labour involved during the isolation and purification of DNA and increases the number of flies per pool compared with the gold standard Phenol Chloroform method. The different beads used in paramagnetic DNA preparations in this study are silica coated and shows that the method is not a significant departure from the standard PC method which also

utilizes the conventional Whatman plate containing the glass filters as a silica matrix. In the other two methods for DNA preparation, the beads used are general magnetic beads using Nucleo mag Tissue DNA kit and oligonucleotide based Dynal magnetic method M-280 beads, these beads are very specific and coated with streptavidin that captures parasite DNA. The principle involved in the oligonucleotide based Dynal magnetic bead capture method is based on reversible adsorption of nucleic acids to paramagnetic beads under appropriate conditions. Field study has been carried out in one of the community in Las Golondrinas located in Southern Chiapas using automated silica coated magnetic bead method.

2: CHAPTER I: LITERATURE REVIEW

2.1. Historical background of Onchocerciasis

Onchocerciasis is an infection caused by filarial parasite known as *O. volvulus*. In humans, this infection is caused by the bites of *Simulium* blackflies. One of the major symptoms of this disease is blindness. It is endemic to Africa, Yemen, Central and South America (WHO, 2004). Onchocerciasis is found in Africa, some parts of South America and one country (Yemen) of the Arabian Peninsula. Onchocerciasis was likely introduced from Africa to Americas during slave trade (Gustavasen et al., 2011). John O'Neill, a scientist in the Gold Coast studying a filarial parasitic infection known as "craw-craw" or dermatitis, first observed the microfilaria of *O. volvulus* in 1875 (John O'Neill, 1875). Nearly twenty years later in 1893, the adult worms and their morphology were discovered and identified by Patrick Manson, a Scottish medical scientist and a pioneer of Tropical Medicine. The German Zoologist, Rudolf Leuckart, using samples collected by missionaries from Ghana described the morphology of the adult worms.

The major symptoms of the disease are blindness, scaly itchy, nodular skin, which is known as Kru kru or Craw Craw in West Aftrica. The discovery of experimental observations are listed in Table 1. A Guatemalan physician, Rodolfo Robes using case studies of coffee plantation workers of Guatemala in 1915 discovered and identified the life cycle and transmission pattern of the parasite (Clark, 1947). Later in 1926, the Scottish parasitologist Blacklock working in Africa collected *S. damnosum* flies and

infected them by placing close to infected individuals. He then traced the development of the parasite in the gut, thorax, head and proboscis of the flies thus providing the final piece of the information on the life cycle of the disease (Cox, 2002).

All the above findings laid the foundation for later discoveries. In 1946, a scientist Jean Hissette working in Congo linked the blindness in onchocerciasis to microfilariae even though Ghanaians living along the Red Volta River had long ago associated the biting flies with skin lesions and blindness. The discoveries made in after years led to deep investigations into the distribution of vector species as well as the transmission patterns and possible characteristic symptoms of various strains of the disease. At present, many studies have suggested that there are at least two strains of *O. volvulus;* a savanna strain and a forest strain. The savanna strain is more common, found in the woodlands and savannas of West Africa. The most serious cases of ocular pathology and blindness are associated with savanna strain.

In the West African rain forest the latter or endemic forest strain are found, moreover scientists have noted that endemic areas within these regions have more cases of hyper pigmentation and other skin diseases related to onchocerciasis. In the recent years these observations have been confirmed with the advent of new technological systems that have allowed scientists to utilize DNA probes to prove the existence of two pathogenic strains of the disease (Toè et al., 1994).

Table 1. History of the discovery of *Onchocerca volvulus*

Year	Scientist	Discovery
1904	Emile Brumpt	Infection occurs along riverbanks and skin microfilaria in the skin come from deeper cutaneous nodules, where adult filariae reside
1915	Rodolfo Robles	Sheds light on the life cycle and transmission of the parasite
1917	Rodolfo Robles	New disease like subcutaneous nodules, anterior ocular lesions, dermatitis and microfilariae
1920	Jean Montpellier & A.Lacroix	Role of microfilariae in causing skin lesions
1923	Breadalbane Blacklock	Role of blackflies in the transmission of onchocerciasis
1932	Jean Hissette	Role of microfilariae in blindness
1972	Neafie	Description of adult male and female worms

2.2. Distribution of onchocerciasis

Onchocerciasis is one of the major infectious blindness in the world. This disease is predominant in the areas with population living near flowing rivers/ turbulent waters where black fly vectors can breed. Studies in this regard have shown a correlation between the proximity of villages to the water and prevalance of onchocerciasis. At present, onchocerciasis has affected mainly the African continent, with smaller fractions distributed in Central and South American continents. The world wide distribution of onhcocreciasis is shown in the Fig. 1. The World Health Organization has put the present estimation of onhocerciasis infected people at 18 million of which 500,000 are with visual impairments and 270,000 are blind (WHO, 1995; Winnen et al., 2002).

Twenty two thousand villages in Africa have been newly surveyed identifying many new foci where onchocerciasis was prevailing in 2005. Currently, it has been estimated that 37 million people are infected with *O.volvulus* with 90 million people at risk in Africa. Onchocerciasis is the second most preventable disease leading to blindness in Sub-saharan Africa. The prevalence and clinical manifestation of onhcocerciasis is marked by geographical variations that have direct influence of estimates of the disease burden arround the world (Kale, 1998).

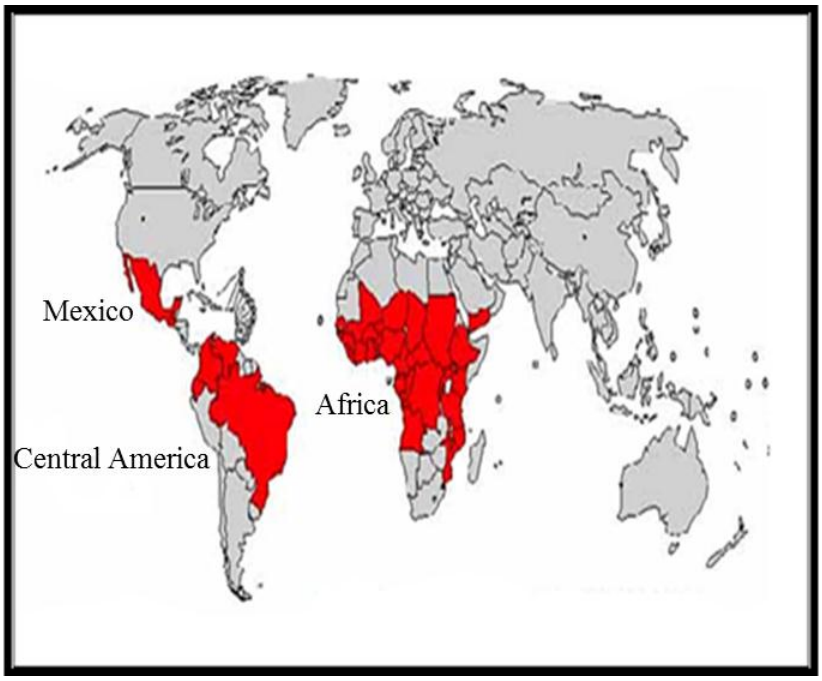


Fig. 1: Distribution of onchocerciasis in the world

In Central and South America, onchocerciasis is distributed in six foci namely Mexico, Guatemala, Ecuador, Colombia, Venezuela and Brazil. The schematic distribution of onhcocerciasis in Latin America is shown in Fig. 2. Guatemala, Mexico and Venezuela all together account for 93% of the Latin American cases of onchocerciasis. In Americas, the most affected country is Guatemala, where the active foci are located on the Western slopes of mountain ranges and Northwestern border to Mexico.

In Mexico, onchocerciasis is distributed over the states of Southern Chiapas, Nothern Chiapas and Oaxaca (Fig. 3). These areas are endemic to onchocerciasis. In Southern Chiapas, 17 muncipalities are affected with an area of 12,640 Km^2 and population of 109,617 at risk. This contained a total of 559 communities of which 39 localities were hyperendemic, 209 were mesoendemic and 311 were hypoendemic. In northern Chiapas focus of onchocerciasis was discovered after other two foci in Mexico were identified in 1952. In 1989, there were 72 endemic communities with 351 clinical cases of onchocerciasis, whereas in 1993, there were 42 endmeic communities with 180 clinical cases identified by local programs. In 2000, this was reduced to 13 endemic communities with 83 clinical cases. During 2001-2005, low annual incidence of exposure to onchocerciasis was observed (Rodríguez-Pérez et al., 2006). It has been shown that the Oaxaca focus had 30 affected muncipalities with a risk population of 44,919 in 98 communities of which 11 are mesoendemic and 87 are hypoendemic.

Fig. 2: Distribution of onchocerciasis in Latin America

Fig. 3: Distribution of onchocerciasis in Mexico

2.3. General biology of the parasite

O. volvulus is a filarial parasitic worm whose name is derived from the Greek word, onchos *means* hook, barb; kerkos *means* tail and *volvo, volutus (Latin) means* turn, roll (involve, revolve, evolve). The common names for onchocerciasis are river blindness, White water disease, Blinding filarial disease, Sowda (Arabia), Galfilarienne and craw craw (Africa) (Clark, 1947).

2.3.1. Taxonomy

Phylum: Aschelminthes (roundworms)
Class: Tissue-dwelling nematode, Secernentea
Subclass: Spriruria
Order: Sprirurida
Superfamily: Filarioidea
Family: Onchocercidae
Genus: *Onchocerca*
Species: *O. volvulus*

2.3.2. Morphology

The worm looks like unsheathed eggs, with sharply pointed, curved tails and measures about 150-350 µm long and 5-9 µm in diameter. The lifespan of the microfilaria is about 6-30 months. Microfilariae are released from the nodules, which are created around the adult worms and migrate through the dermis and connective tissue, and around the vicinity of the nodules. The morphology of the adult worm is shown in

Fig. 4. They are found in the fluid within the nodules and dermal skin layer. Sometime, they are also found in urine, blood sputum and the eye, when infection is heavy. The adult microfilaria is white colored and thread like in appearance. They are typically coiled together in pairs in the subcutaneous tissue, are characteristically slender and blunt at both ends. Female adult worms may be as long as 50 cm in length and as thick as 0.5 mm in diameter. The male adult worms are shorter and measure less than 5cm in length and 2.2mm in diameter. These worms lack lips and buccal capsules but display two circles of four papillae around the mouth and the esophagus lacks conspicuous divisions. In females, the vulva lies behind the posterior end of the esophagus, and in males the tail is a curled ventrad that lacks alae (Markell et al., 1999).

2.3.3. Life cycle of *Onchocerca volvulus*

The life cycle of *O.volvulus* includes two stages: one in humans and the other in blackflies. The schematic representation of the life cycle of *Onchocerca volvulus* is shown in Fig. 5. The parasite is transmitted through the bites of female blackflies or Buffalo gnats of the genus *Simulium* in humans that requires blood for oviposition. The blackfly serves as intermediate host required for the larval development and humans serving as a definitive host where the adult stage of the parasite occurs. The two major stages of the parasite namely the adult and offspring are manifested in skin and muscle tissue of infected man. Nodules are formed by the aggregation of tissues in which female and male worm live together. Female worms are viviparous producing offspring continuously known as microfilaria (mf). Clinical manifestations of the disease develop after latent period of one to two years from exposure (Vachon, 1993).

Fig. 4: Morphology of adult worms

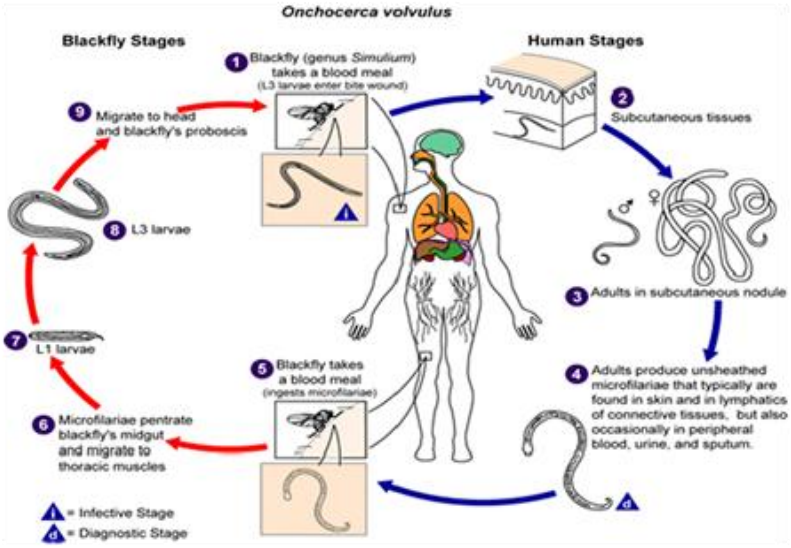


Fig.5: Life cycle of Onchocerca volvulus

1 During a blood meal, an infected blackfly (genus *Simulium*) introduces third-stage filarial larvae onto the skin of the human host, where they penetrate into the bite wound.

2 In subcutaneous tissues the larvae develop into adult filariae, which commonly reside in nodules in subcutaneous connective tissues.

3 Adults can live in the nodules for approximately 15 years. In the subcutaneous nodules, the female worms are capable of producing microfilariae for approximately 9 years.

4 In the subcutaneous nodules, the female worms are capable of producing microfilariae for approximately 9 years. The microfilariae, measuring 220 to 360 µm by 5 to 9 µm and unsheathed, have a life span that may reach 2 years. They are occasionally found in peripheral blood, urine, and sputum but are typically found in the skin and in the lymphatic of connective tissues.

5 A blackfly ingests the microfilariae during a blood meal.

6 After ingestion, the microfilariae migrate from the blackfly's midgut through the hemocoel to the thoracic muscles.

7 The microfilariae develop into first-stage larvae.

8 Finally into third-stage infective larvae.

9 The third-stage infective larvae migrate to the blackfly's proboscis and can infect another human

1

Further development of the microfilaria occurs only when ingested by the blackfly. The female blackfly ingests microfilaria from the blood meal when it inserts the proboscis into infected human skin tissue for feeding. On entering the insect stomach, the microfilaria need to migrate quickly into the thoracic muscles to avoid being encapsulated the peri-trophic membrane. This change in the environment from human skin to insect stomach initiates the further development of microfilaria into the larval stages, which takes about ten days for the microfilaria to become infective larvae. In due course, the insect feeds on humans for blood meal and lay eggs. Once the larvae enter the humans, they mature into adult worms thus completing their life cycle (Vachon, 1993).

Microfilariae induce the clinical symptoms of the disease. The larval nematodes then spread all over the body releasing *Wolbachia* symbionts on their death triggering the immune response leading to itching, loss of elasticity, sagging and destruction of the nearby skin tissues, inflammation and scarring of the corneal and optic nerves of the eyes gradually leading to blindness. It may take ten or more years for the significant damages to occur to the eyes before leading to blindness. Filarial products were taught to be the major stimulus for the inflammatory reactions in the body. Recent research has indicated that the endotoxin like molecules from *Wolbachia* play an important role in pathogenesis of the disease and adverse reactions during the treatment. It has been found that the *Wolbachia* is essential for nematode fertility and hence have been identified as a potential target for chemotherapy (Hoerauf et al., 2003).

Adult worms of *O.volvulus* live approximately for eight to nine years, but it can stay up to 15 years (Roberts et al., 1967). Fertilized female worms can produce around ten million microfilariae during their lifetime, but of these heavily infected ones may harbor 50-200 million microfilariae. Later, microfilariae will develop by migrating into the host skin, where they can be ingested by the competent insect vector (*Simulium*). Approximately in human host the microfilariae can stay for one to two years (Eberthard, 1986).

2.3.4. Transmission of Onchocerciasis by *Simulium* vectors

The *Simulium* vectors that transmit onchocerciasis vary significantly depending on the region and ecosystem. The important vectors from different regions are *S. sirbanum, S. damnosum* and *S. neavei* in African savanna region, *S. yahense* and *S. squamosum* and S. *soubrense* in rainforest region of Africa, *S. leonense* in lowlands of Sierra Leone, *S. sanctipauli* in large coastal rivers of West Africa. In Latin America, *S. ochraceum* are found in densely forested areas while *S. metallicum* and *S. exiguum* are common in hilly, sparsely wooded areas. Among these species, *S.ochraceum* s.I is one of the primary vectors in the American region, as it responsible for over 50% of all cases of onchocerciasis reported in the region (WHO, 1991). The different and important vectors in Mexico are shown in Fig. 6. However, *S. exiguum*, and *S. guianense* (vectors in South-America) are as competent vectors as *S. damnosum* s.l. in Africa (Gustavsen et al., 2011). Around fast- flowing streams and rivers, *Simulium* flies breed, as they prefer highly oxygenated areas. The reason is the larvae have an obligatory aquatic stage,

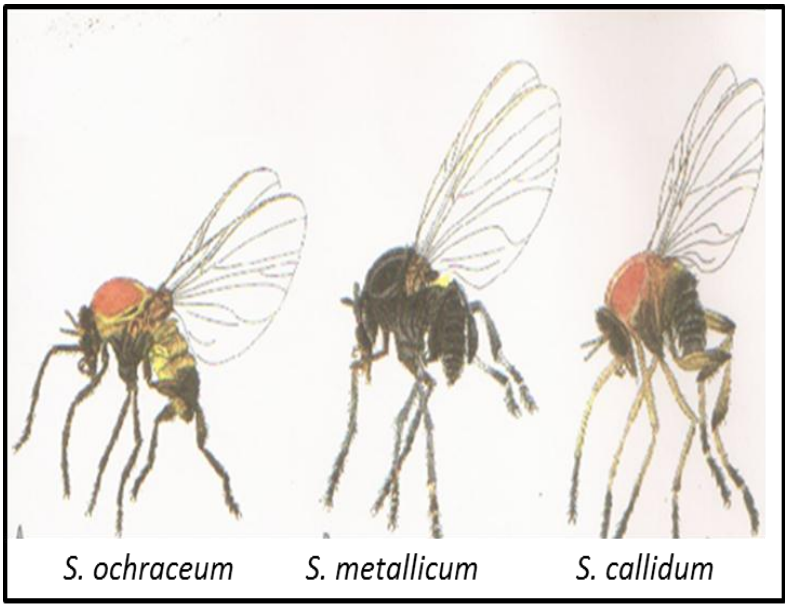


Fig. 6: Three important *Simulium* species in Mexico

which requires high oxygen tension. The larvae and pupae attach to submerged rocks or vegetation, in the aquatic stage and certain African species attach themselves to fresh-water crabs. Adult flies will emerge after 8-12 days. The *Simulium* flies survive for 4 weeks and they travel for several kilometers for the suitable areas for mating and reproduction. Once blackflies find their breeding site, they tend to stay within 2 km of the site. In order to complete their life cycle, most female *Simuliids* require warm blood meal from the warm-blooded vertebrates. In such cases, it has a good opportunity to spread skin or blood dwelling vertebrate pathogens. For each ovarian cycle the process is repeated.

2.3.5. Influence of migrants on the transmission of onchocerciasis

Onchocerciasis came to America with the slaves around the 16th century. These slaves were mainly from Africa who harbored *O. volvulus* parasite in their body. It has been assumed that the infected slaves serving as reservoirs of infection migrated within the Americas and between coffee plantations thus spreading the onchocerciasis wherever they migrated. The disease spread with the help of a suitable *Simulium* species in this region, which was different from that of the African origin thus making onchocerciasis spread more effectively into the local population (Gustavsen et al., 2011). Migration of people from the contiguous border country of Guatemala has also been reported to exhibit enhanced spread this disease further into various parts of Mexico.

Migration of labours across Mexico-Guatemala border spread the disease within the endemic foci (WHO, 1995). Onchocerciasis in this region is mainly associated with coffee plantation and the transmission peaks with the harvesting season (Brandling-Bennett et al., 1981; Vásquez Castellanos, 1991). The origin of the onchocerciasis in Southern Chiapas was due to the migration of laborer's working at the coffee plantations from Guatemala, which shares its borders with Southern Chiapas. The disease spread to Northern Chiapas during the annual visits of the residents from Northern Chiapas focus for the coffee harvest to Southern Chiapas where the transmission levels were high. Whereas, the infection with the filarial parasite in Oaxaca might be due to movement of people from Oaxaca to other endemic regions of Chiapas and Guatemala performing religious pilgrimages (Vásquez Castellanos, 1991).

With the initiation of the ivermectin treatment, it was possible to control the disease but to curtail it has been a very tedious task. It is necessary to prevent the transmission of infection from vectors to humans and coffee laborers serving as transmission reservoirs increase the intensity of disease recurrence in the areas under regular ivermectin treatment. For this reason, the biannual treatment with ivermectin was modified to four times a year in order to achieve higher coverage rates and to include all the temporary coffee workers for treatment. This would surely accomplish the goal of interrupting the transmission.

2.4. Clinical manifestations

Onchocerciasis is major disease that triggers different host immune responses to the micro filarial stage of *O.volvulus* (Alonso et al., 2009). The most important clinical manifestations of the disease are dermal (hyper-pigmentation), intense itching, lymphadenitis that leads to hanging groins and elephantiasis of the genitals and severe visual impairment. There are other symptoms of pathogenesis like low body weight, general debility and diffuse musco-skeletal pain (WHO, 1987). The first and most common clinical symptom of the disease is prurutis and it may occur on its own or with association of onchocercal skin disease (Alonso et al., 2009). There are other clinical symptoms occurring in certain endemic areas in Africa that includes epilepsy, growth arrest, general malaise and debilitation. The symptoms may visualize between one to three years after the initial infection with *O.volvulus*. The most typical and important disease of *O.volvulus* includes:

2.4.1. Dermatological lesion (dermal pathology)

The skin infection of the onchocerciasis is highly variable. The important and principal site of the infection is the skin. When body responds to *O.volvulus* antigens, the irritation and itching of the skin occurs. Thickening and cracking of the skin automatically occurs due to this inflammation. Due to thickening of the skin there is loss of elasticity, rise in atrophy and wrinkling of the skin. The presence of microfilariae in the skin gradually destroys the elastic tissue over a period of time (years), by causing the

formation of redundant folds in the host. Skin lesions are the most pervasive of onchocerciasis. In African country, the survey was conducted in seven endemic areas, where 40-50% of all adults had reported severe itching. The skin diseases are classified in to seven (Fig. 7) main categories based on cutaneous changes associated with the type of onchocerciasis that developed (Enk, 2006).

i) Acute papular onchodermatitis (APOD): the disease is severe in condition, wide spread eczematous rash with multiple tiny pruritic papules growing to vesicles and pustles (Enk, 2006). It is a type of solid speckled papular rash with the possibilities of vesicles. APOD often affects the face, trunk and the extremities.

ii) Chronic papular onchodermatitis (CPOD): it is more severe than APOD in condition. This disease is concerned with severe itching, maculo-papular rash that contains spreader flat-topped papules and hyper-pigmented rashes with larger papules.

iii) Lichenified onchodermatitis (LOD): this disease is hyper-keratotic in condition with increased hyper-pigmentations of the confluent plaques often affecting the lower extremities and related with lymphadenopathy.

iv) Onchocercal depigmentation (DPM): this disease is also known as “leopard skin”, where the loss of pigment mainly around the hair follicle and found on the shins. It leads to vitiligo-like lesions with hypo-pigmented patches, which contains perifollicular spots.

v) Lymphadenitis: lymph nodes are inflamed resulting in swelling. In some cases of African onchocerciasis the femoral and inguinal nodes (groin area) gets infected and in Americas the lymph nodes of the head and neck are affected.

vi) Sowda: here the skin gets dark, thick, itchy and covered with scaly papules, one of the severe cutaneous diseases. This disease was originally seen in Yemen but now it is been found in Central America, West Africa and Sudan.

vii) Erisipela de la costa: is one of the macular rashes with edema of the face, towards one side of the face. These lesions are found in Latin America.

Onchodermatitis is endemic in population and is most common symptoms. There are different clinical patterns that are not mutually exclusive because two or more patterns may represent simultaneously and one pattern may evolve into another pattern (Enk, 2006).

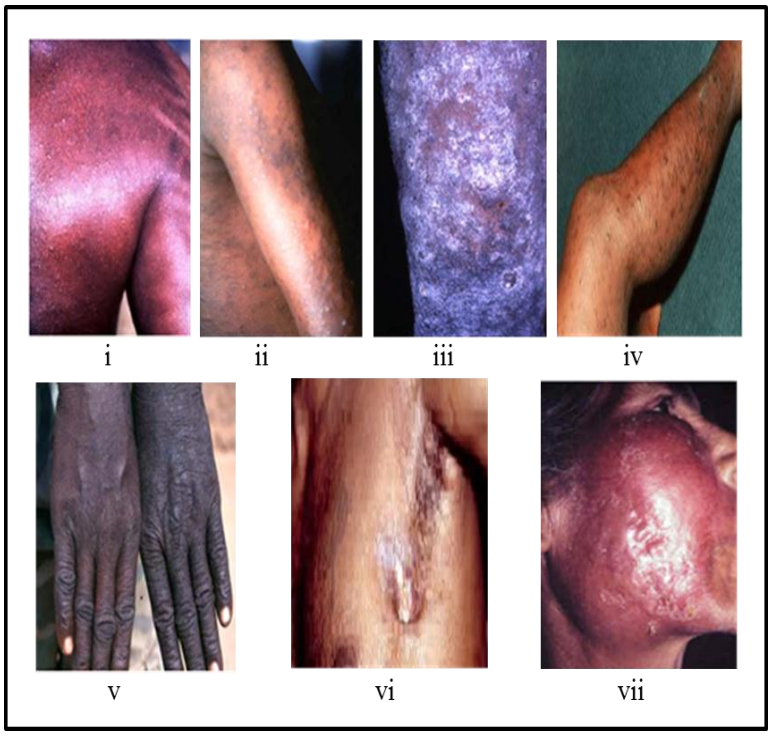


Fig. 7: Seven different types of skin disease

2.4.2. Ocular lesions (ocular pathology)

Wide spectrum of symptoms of onchocerciasis that covers ocular lesions, ranging from simple mild symptoms like itching, redness, pain, photophobia, diffuse keratitis and blurring of vision to more severe symptoms of corneal scarring, night blindness, intraocular inflammation, glaucoma, visual field loss and eventually blindness (Enk, 2006). It takes several years to develop the lesions of the eye and occurs mostly in individuals over the age 40 in the eye. The various structures of the anterior and posterior segments of the eye can be affected, since the ocular lesions are bilateral. The living or dead microfilaria can be present in the anterior segment of the eye. Microfilaria can be observed with a slit lamp if it is present in the anterior chamber of the eye. Severe anterior uveitis with formation of synaechiae, cataract and glaucoma can be caused by dead microfilaria. In such cases it may affect major portions of the cornea that lead to sclerosingleratitis with fibro vascular and marked reduction of the visual functions (Enk, 2006).

The main cause of the blindness in the case of onchocerciasis is sclerosingleratitis. Along the sheaths of the ciliary vessels and nerves from under the bulbar conjunctive, microfilaria will pass directly into the cornea of the eye through nutrient vessels present in the optic nerves. Sclera or white of the eye gets inflamed with the invasion of the microfilariae in the cornea, which is followed, by invasion of fibrous tissue. This leads to high vascularization of the cornea and critically impairs the vision of the host. The immune responses and subsequent invasions completely lead to blindness.

Almost 500,000 people are blind or having impaired vision and 270,000 people suffering from irreversible blindness by onchocerciasis. Sclerosing keratitis, iridocyclitis, choroid-retinitis, choroid-retinal atrophy, optic neuritis and optic atrophy are the most potent and severe blinding ocular lesions of onchocerciasis (Etyá ale, 2002).

2.4.3. Onchoceromata (nodules)

Almost 30% of the cases in onchocerciasis show symptoms of onchocercomas. The development of the nodule takes over 18-months' time and varies in its size and numbers. Depending on the bite of the black fly the nodules are located. Mostly the nodules are found on the lower trunk and pelvic region in Africa, and in Americas in the head and neck. In the nodules the adult worms are encapsulated and found in the regions like bony prominences on head, scapular girdle, pelvic girdle, knees, ribs and ankles (Duke, 1990). Studies have shown that few female worms will be hidden in deep-lying tissues and are not visible (Schulz-key, 1990). These worms are enough to maintain large number of microfilaria in the skin and other parts of the body like eyes, which could be responsible for the visual impairment (Duke, 1990).

2.5. Control of Onchocerciasis

Onchocerciasis was first identified in Mexico in the year 1923 with the positive diagnosis of a child of a German finca owner, who lived in Chiapas before going back to Germany (Ruiz Reyes, 1979). The Mexican Institute of Hygiene issued a bulletin in 1925 alerting the public about the probable existence of onchocerciasis in Southern Mexico. Efforts to reduce black fly population were started in 1932 using larvicides. Fairchild and

Barreda carried out the first successful control of the vector of onchocerciasis in 1945 in Guatemala using DDT. However, no widespread attempts were made in Mexico for eradication of onchocerciasis and therefore only limited success was achieved. The limitation for the success of the control was largely due to inaccessibility of breeding site, less expensive preventive measures taken like denodulation.

There are various control programs to monitor onchocerciasis of which the onchocerciasis control programme in West Africa (OCP) is one of the longest and largest running programs in Africa, maintaining active control operations from 1975 through 2002. The control strategy of the OCP was based on weekly aerial larviciding of all breeding sites in the rivers in the program area (Ba et al., 1987, Hogard et al., 2001). The program was started with seven member countries in West Africa (Niger, Burkin faso, Ghana, eastern Mali, Ivory Coast and Togo) and was extended in 1986 to include Guinea, Guinea Bissau, Western Mali, Senegal and Sierra Leone (Liese et al., 1991). In late 1980's OCP program was based on vector control strategy, later in 1987 the decision was made to make ivermectin available for free of cost from the Mectizan Donation Programme (MDP), making additional change in the strategy of the OCP control (Thylefors, 2004). The OCP virtually eliminated transmission throughout 90% of its original area (Ba et al., 1987; Hougard et al., 2001).

The microfilaricidal drug ivermectin used as a tool to eliminate and control onchocerciasis morbidity has resulted in the development of two new programs namely the OEPA (Onchocerciasis Elimination Programme for Americas) (Sauerbrey, 2008) and APOC (African Programme Control of Onchocerciasis) (WHO, 1995; Boatin, 2008). APOC was followed in the countries of Sub-Saharan Africa. The main objective of the APOC programme was to distribute ivermectin in the 19 countries, outside the OCP, where it is an important public health problem, and suffering from onchocerciasis (Angola, Burundi, Cameroon, Chad, the Central African Republic, the Congo, the Democratic Republic of the Congo, Ethiopia, Equatorial-Guinea, Gabon, Kenya, Liberia, Malawi, Mozambique, Nigeria, Rwanda, Uganda, Sudan and Tanzania). It was found that in these countries around 6.4 million people who are heavily infected live in areas where blindness is caused by parasite strains and around 6 million people of heavily infected live where strain produce severe itching and skin disease (Remme, 2004).

The OEPA program was launched in 1992, with its Headquarters in Guatemala, to act as a supporting and technical body of multinational, multi-agency coalition that includes the endemic countries, the Bill and Melinda Gates Foundation, Merck & Co and with other partners (Sauerbrey, 2008). The OEPA programme was conducted to eliminate onchocerciasis in the 13 foci in the 6 endemic countries and to interrupt the transmission of onchocerciasis in these countries by eliminating the reservoir of infection from all the endemic countries of Latin America (OEPA, 2008). The 3 main objectives of the OEPA were (i) the coverage of the ivermectin treatment to at least 85% of those

eligible to receive drug in endemic communities, (ii) controlling new ocular morbidity by the infection caused by *Onchocerca volvulus,* iii) complete elimination of the transmission of parasite wherever feasible (Sauerbrey, 2008). The six countries that were included in OEPA'S programme were: Brazil, Ecuador, Guatemala, Columbia, Mexico and Venezuela (Shibuya et al, 2000).

OEPA's plans were aimed at controlling the morbidity and gradually interrupting transmission by ivermectin treatment twice or quarterly per year in Mexico and Guatemala (Burnham and Mebrahtu, 2004). In the communities like Brazil, Ecuador and Venezuela the aim was to control morbidity, where treatment was being given once or twice a year (WHO, 1995). At present, it is twice a year and there are plans to implement the four times a year scheme. It was known that distribution of ivermectin even eliminated vector flies in the Western hemisphere (Alonso et al., 2009). OEPA has made substantial progress towards the goal of eventual elimination. Transmission has been interrupted in 8 out of 13 foci in Latin America to date, including Lopez de Micay in Columbia, Esmeraldas in Ecuador, Escuintla-Guatemala, Santa Rosa and Huehuetenango foci in Guatemala, North-central in Venezuela, Northern Chiapas and Oaxaca in Mexico (Rodríguez-Pérez et al., 2010a, b; WER 2011; Sauerbrey, 2008; Lindblade, 2007). There is on-going ivermectin distribution in five endemic countries of Latin America.

2.5.1. Control through nodulectomy

Removal of the adult worms from the nodules is one of the important control measures for onchocerciasis. Nodulectomy, a therapeutic measure varies in Latin America, as a function of onchocerciasis prevalence (Guderian et al., 1997). Intensity of the infection can be reduced in the hypo-endemic areas by nodulectomy treatment, although new nodules can be observed in hyper-endemic areas (Guderian, 1988). The microfilarial load and skin pathology can be reduced by nodulectomy. During chronic infection, the deep nodules can occur and however, palpable nodules do not indicate majority of the active parasite. By control through nodulectomy, the prevention of blindness is less, but it may reduce the number of microfilariae entering into the eye (Aoki et al., 1983).

2.5.2. Control through Vector

One of the oldest control program OCP, started in 1974 aimed at vector control by aerial spraying organophosphate Temephos (Abate) over the breeding sites of savannah *S.damnosums* species. When it became resistant, Cholrophoxin was used, and when this became ineffective was replaced by biocide *Bacillus thruingiensis* serotype H-14. Few cases were reverting to susceptibility of the fly larval population of the organophosphates. Permethrin and carbosulfan was used instead of phosphates. Finally, ivermectin was supplemented with these actions; over 20 million people were protected from onchocerciasis.

2.5.3. Control through chemotherapy

A series of new drugs were being developed during World War II. US servicemen suffering from tropical parasites in pacific regions of the war were treated with Diethycarbamazine (DEC). After 1949, it was used in Mexico on patients following surgical removal of the adult worms every four months. It was also used in small doses to elicit the immune response in people carrying the microfilaria. From 1981 to 1990, more than 3.2 million doses of DEC were used to diagnose the disease (Programa de Control de la Onchocerciasis, 1990). A major effort to fight onchocerciasis in Chiapas was initiated by 1962. The state had then seventeen brigades, each was provided with a nurse, technician and support personnel to carry out visits to communities in the endemic areas every alternate month. From 1981-1989 more than 60,000 worms were surgically removed and DEC administered, thus reducing the frequency of blindness (Programa de Control de la Onchocerciasis, 1990).

2.5.4. Control through Ivermectin

Ivermectin has a potent, rapid action against skin microfilariae. It is the anthelmintic drug for the treatment of onchocerciasis (Basáñez et al., 2008). Ivermectin is a mixture of avermectins B1a and B1b. It is a broad-spectrum antiparasitic agent. It acts on gamma-aminobutyric acid (GABA) receptor complex, which is an inhibitory neurotransmitter (Goa et al., 1991). The avermectin mixtures (B1a and B1b), are macrocyclic lactones synthesized by the actinomycete *Streptomyces avermectilis* (later renamed *S. avermectinius*). It is formulated as six-mg tablets and given orally. The

recommended dose is 150-µg/kg weight taken annually or biannually. Children under five years of age or under 15 kg body weight, pregnant women, breast-feeding mothers within one week of delivery and individuals with neurological disorders or severe intercurrent disease are excluded from treatment with ivermectin. It has been shown to activate glutamate-gate ion channels resulting in hyper polarization of the membranes of neurons causing paralysis in *C. elegans* and *H. contortus* (Ardelli et al., 2009). In nematodes, ivermectin competitively inhibits specific 3H-GABA sites (Ros-Moreno et al., 1999) and thus paralyses *O. volvulus* mf that leads to suppression in reproduction.

Ivermectin has been known to be less permeable through the blood-brain barrier and does not interfere with the GABA production in the central nervous system (Taylor & Greene, 1989). Ivermectin exhibits potent microfilaricidal activity against many major filarial parasites in humans, including *Wuchereria bancrofti*, *Brugia malayi*, *Loa loa* and *Mansonella ozzardi*, but not against *M. perstans.* It is also active against *Ascaris lumbricoides*, *Trichuris trichiura*, *Enterobius* spp., and *Strongyloides stercoralis*. It has potent activity against cutaneous larva migrans, for which treatment is not available and has the potential to become the drug of choice for ectoparasitic infestations (mites and lice) in humans (Õmura, 2008). The repeated doses of ivermectin on ocular microfiliarial loads indicated a reduction by about 70% (Rodríguez-Pérez et al., 1995), which may result in beneficial effects like regression of early lesions in the anterior segment of the eye, onchocercal optic nerve disease and visual field loss. Table 2 shows various methods used in controlling the onchocerciasis in Mexico.

Table 2: Methods of controlling onchocerciasis in Mexico

Control parameters	Mode of action
Prevention of human-vector contact	Use of insecticides, sleeping nets, protective cloths
Containing the vector	Application of larvicides at the breeding sites of Simulium flies
Vaccination	Not at present
Nodulectomy	Central America and Africa (children)
Available drugs	
Ivermectin	Microfilaricides
Amocarzine	Macrofilaricides

Although, ivermectin is known to be a better microfilaricidal drug than DEC, is inactive against adult worms. Histological studies of onchocercal nodules from Mexico, Guatemala, and Ecuador from individuals with recurrent ivermectin treatments have shown dramatic effects on adult female viability and fertility and marked reductions in the frequencies of male worms (Cupp et al., 2004), leading to the conclusion that long term ivermectin treatment has a profound effect on survival and reproduction of this species (Cupp and Cupp, 2005). Unfortunately, the adult worms, which have an average estimated lifespan of 10 years (Bradley et al., 2005), are not killed by the treatment. This together with the fact that infections are multiple and cumulative, and in some individuals asymptomatic, underlines the problems involved in elimination and control of the parasite. This has been observed in Africa mainly, but not in Latin America. Macrofilaricidal effect can be observed when ivermectin given four time every year for consecutive years (Duke, 2005).

2.5.5. Development of new drugs

The studies over two decades on ivermectin treatment have revealed that ivermectin was very effective in suppressing major symptoms of the disease onchocerciasis from the public domain. But still, the elimination of onchocerciasis completely has been very difficult to achieve (Borsboom et al., 2003) in Africa where only one annual dose of ivermectin was given. Ivermectin alone may not be good enough to completely eliminate onchocerciasis as it does not kill the adult worms. Therefore

there is an urgent need for the development of newer drugs and methodologies to monitor and control the transmission. Suramin, a true macrofilaricidal drug that was good, but it is too toxic for the use and the drug is occasionally fatal on its treatment. Some of the studies in Latin America and Africa (Awadzi et al., 1997) shown that Amocarzine, a piperazinyl derivative of monoscanate, have good macro and microfilaricidal activity (Nutman et al., 1996; Poltera et al., 1991). This drug is not well established to assess the adulticidal efficacy, more clinical trials are necessary to confirm. Later it was planned to combine the amocarzine with ivermectin in order to introduce longer intervals for distribution in mass chemotherapy (Poltera, 1998).

A number of chemicals like pyrimidinylguanidines, imidazolinylhydrazones and thiadiazole were evaluated for treating *O.volvulus* using in vitro culture systems, but very few showed activity on adult worms. However, new drugs that would effectively control the adult worms by killing or sterilizing would be ideal for eradication of the disease. Recent studies have indicated that the endo-symbiont bacterium *Wolbachia* might play a role in the ocular and skin disease (Taylor, 2003). Post- treatment reactions might also be related to *Wolbachia* products that mediate inflammatory responses (Keiser et al., 2002). Some of the studies have shown that the *Wolbachia* is necessary for the survival of adult worms, which got killed when these symbionts were killed using antibiotics like tetracycline and doxycyclines. Thus, *Wolbachia* has been looked for as a potential target for the treatment of *O.volvulus* infected individuals (Hoerauf, 2008; Hoerauf et al., 2008a, b). Also moxydectin has been shown to affect the embryogenesis and has been

identified as potential drug (Molyneux, 2009). Still the pathogenesis of onchocerciasis is poorly understood and a present ivermectin is the only safe drug for the treatment of onchocerciasis. By use of the ivermectin drug, around 90% of the ocular microfilarial loads were reduced within 2-4 years, 50-30% reduction in sclerosing keratitis and early iridocyclitis (WHO, 1995).

Closantel is an anthelmintic drug and has been shown to be a potent inhibitor of chitinase OvCHT1 of *O. volvulus* and also inhibits the molting of the infective L3 stage larvae of *O. volvulus* which happens to be essential for the parasite (Gloeckner et al., 2010). *Wolbachia* bacteria are endosymbionts for the filarial parasite and have been shown to be important for the survival of *O. volvulus.* Treatment with deoxytetracycline has shown that the killing of *Wolbachia* bacteria ultimately lead to the death of *O. ochengi* in infected cattle's (Langworthy et al., 2000). Hence, *Wolbachia* has been identified as a potential target for the development of new drugs that are more potent. However, the exact role of *Wolbachia* in the life of parasite is not clear but it has been assumed to be important in embryogenesis (Hoerauf et al., 2008a, b).

2.6. Diagnosis of *Onchocerca volvulus*

Onchocerca volvulus, a parasitic nematode infects 18 million people in Africa and Latin America. Many attempts to control this disease pointed on controlling *Simulium* fly vectors and ivermectin treatment (Boatin et al., 1998). The control programmes requires

accurate and specific diagnostic methods for finding endemic areas and to detect residual or new infections (Sharong et al., 2000). The diagnosis can be carried out as mentioned below.

2.6.1. Parasitological diagnosis for *Onchocerca volvulus*

Traditionally, the main diagnosis of onchocerciasis was based on identification of microfilariae (mf) in skin snips by microscopy (Maizels et al., 1990). As already mentioned, mf may be seen in the cornea and anterior chamber of the eye and sometimes they are found in urine and blood in heavy infections (WHO, 1987). In brief, the skin snips is removed from inflamed area iliac crests from African patients and in shoulders from American patients using scalpel blade. Later, skin biopsy is weighed and incubated overnight in 0.1 ml saline buffer in microtitre plate. After 30 minutes of incubation around 60% of microfilariae will emerge and later to 75% after 24 h. Using microscope the mf are counted by staining with Giemsa stain the species can be easily distinguished. However, for this type of test, at least two skin snips is necessary to determine the infection intensities. This method is highly sensitive and specific for the communities with high infection and prevalence (Taylor et al., 1987). In the communities where disease is endemic skin snips are becoming unpopular (Boatin et al., 1998) and skin-snip microscopy has lower sensitivity than newer biochemical methods including skin-snip PCR, ELISA, EIA and antigen surveys (Boatin et al., 2002). Detecting *O.volvulus* based on DNA methods may be useful specifically for diagnosis of onchocerciasis in low prevalence or treated human population (Harnett, 2002). Different diagnosis tests are shown in Fig. 8.

2.6.2. Detection of adult worms

Adult worms can be examined or detected by excising the palpable nodules under local anesthesia. Ultrasonographic techniques can also be used to distinguish an onchocercal nodule from lymph nodes, lipomas, fibromas and foreign body granulomas. It detects impalpable nodules and number of nodules in conglomerate mass by counting the number of worm thus helping in determining the worm burden accurately or precisely.

2.6.3. Mazzotti Test

Mazzotti test can use only when onchocerciasis is suspected with no detection of the parasite in the skin or eye. In this test diethylcarbamazin (DEC) is used. The patient is administered 50 mg of DEC orally and observed for the effects of microfilarial death for the symptoms such as itching, rashes that could occur within 24hrs. This test is rarely used in recent times because it can cause severe allergic reactions, sometimes leading to death and hence is replaced by DEC patch test.

2.6.4. DEC patch test

This is an alternative to Mazzotti test, which was associated with serious side effects due to large doses of DEC. In patch test a gauze pad is soaked in 20% DEC

solution and placed on the hip, followed by examination for skin inflammation due to DEC induced microfilariae death. It is the best method for testing re-emergence of onchocerciasis infection and is less invasive than nodulectomy.

2.6.5. Immunological diagnosis

Although these methods are used widely for diagnosis their sensitivity and acceptability for control of this disease in huge populations necessitates newer approaches that would help control programme mapping, monitoring assessment and surveillance. Several rapid and sensitive techniques have been developing for these reasons. Antigen detection is the preferred method for diagnosis as it detects latent infections using immunological tests (Taylor et al., 2010; Rodríguez-Pérez et al., 1999).

For the detection of parasite specific antibodies in the blood sample or in the serum radio immuno assay (RIA) and enzyme linked immuno sorbent assay (ELISA) are commonly used. These are sensitive, simple and indirect assays for detecting the antibody bound to the parasite lysate or extracts. These methods utilize two antibodies namely the primary antibody that binds to the specific antigen found in the parasite and secondary antibody that is conjugated to an enzyme or radioisotope.

Fig. 8: Different type of parasitological diagnosis tests for Onchocerciasis

ELISA tests for the presence of antigens to *O. volvulus* parasite requires a small blood sample. Although this method could diagnose *O. volvulus* infection its accuracy was hampered due to its extensive cross reactivity among filarial antigens and its inability to distinguish between past and current infections (Zimmerman et al., 1993). Due to the lack of specificity and sensitivity of these sero-diagnostic techniques attempts were made to identify specific antigens from the parasites. Many recombinant polypeptides from *O. volvulus* antigens have provided the required specificity for definitive diagnosis. These antibody-based assays could not distinguish past and present infection nor could assess the efficacy of chemotherapy.

Identification and characterization of the *Onchocerca* specific non coding 150 base pair tendemely repeated DNA sequence (O-150) allowed the identification of specific primers for PCR amplification and development of species and strain specific DNA probes (Zimmerman et al., 1993). The overall schematic representation of the various steps involved in the diagnosis and treatment of onchocerciasis is shown in the Fig. 9.

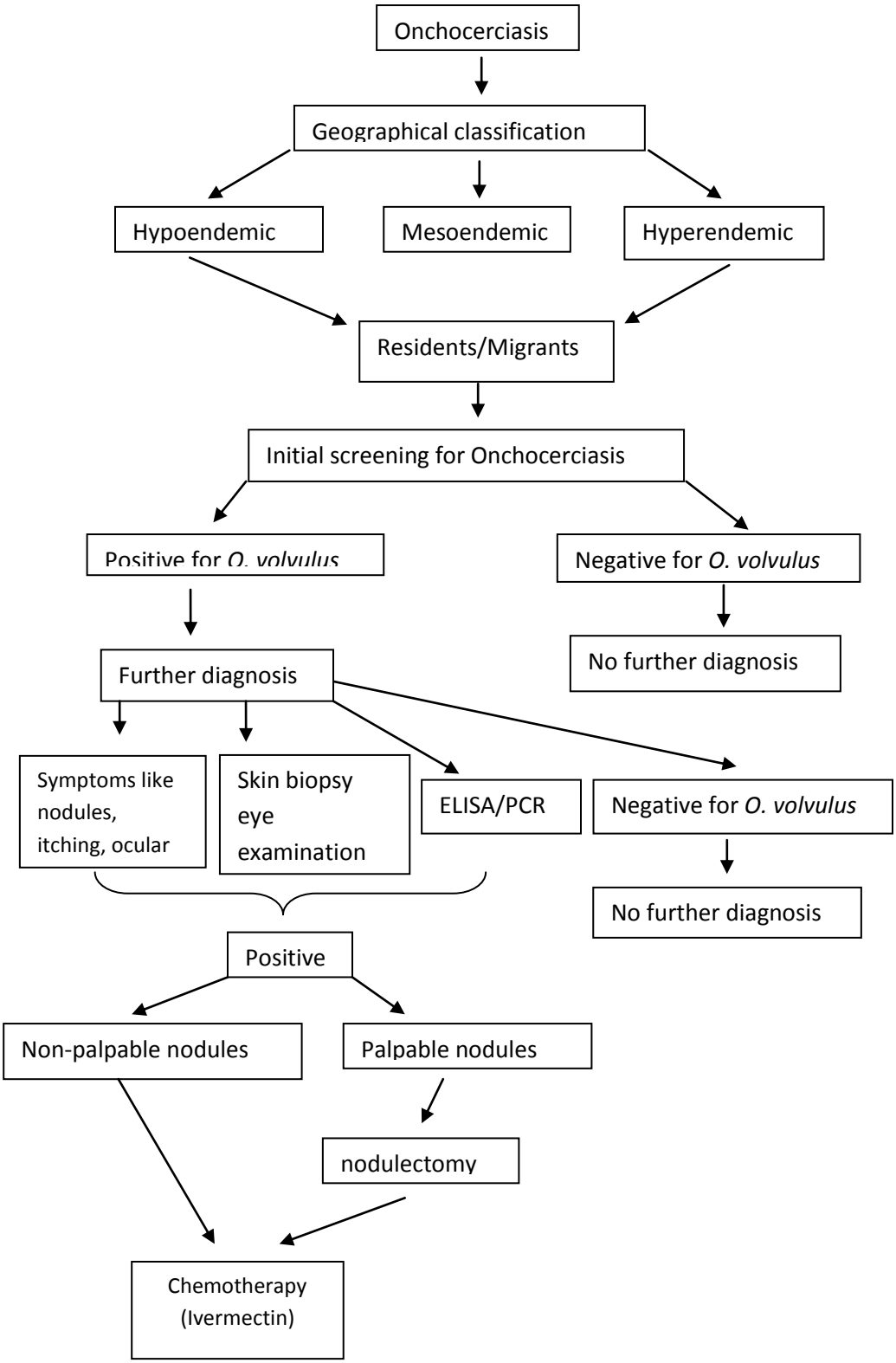


Fig. 9: Schematic representation of overall diagnosis and treatment of onchocerciasis

2.7. Treatments for onchocerciasis

Drugs that are used to treat and control filariasis include diethycarbamazine, suramin, ivermectin and albendazole. Ivermectin is the only drug for onchocerciasis that is used by the control programmers and administered annually or bi-annually, or quarterly through community directed treatment. Ivermectin is an anthelmintic drug for the treatment of onchocerciasis. The standard dose for onchocerciasis is 150 microgram/kg for adults and children, administered once every year in Africa. Ivermectin causes hyper-polarization of glutamate sensitive channels and immobilization of microfilariae (Wolstenholme and Rogers, 2005).

2.7.1. Diethylcarbamazine (DEC)

If used for treating onchocerciasis ivermectin should be administered first and DEC given only after clearing microfilariae from skin and eyes. This drug is no longer used in treating onchocerciasis due to its toxicity and side effects.

2.7.2. Doxycycline

O volvulus serves as a host to *Wolbachia* endosymbiont that is essential for its growth, development and survival. In the onchocerciasis control programme 100mg/day doxycyclines administration for 6 week resulted in long-term sterilization of female worms and reduction of skin microfilariae. Doxycycline is a microfilarialcidal used in treating onchocerciasis that doesn't have any fatal adverse effects that were normally observed with suramin therapy.

2.7.3. Suramin

Suramin is the only microfilaricidal drug currently in use for onchocerciasis. The recommended dosage of suramin is 4g per adult weighing 60kg or more. Suramin is administered by intravenous injection, as it is not absorbed from gastrointestinal tract and causes irritation when administered intramuscularly. Suramin mainly acts by inhibiting many metabolic processes but mainly acts by interfering with binding of general growth and angiogenic factors to their cellular receptors. This is no longer used now.

2.7.4. Amocarzine

Amocarzine is a piperazinyl derivative of amoscanate; it has a good microfilaricidal activity and has been developed to the stage of phase II and III clinical trials mainly carried out in Ecuador and Guatemala. It's mainly administered orally with a dosage of 3mg/kg body weight twice for three days. It mainly acts on mitochondria by inhibiting the electron transport chain.

2.8. CUREENT TREND OF ONCHOCERCIASIS IN MEXICO

The endemic regions in Mexico are located in Southern states of Oaxaca and Chiapas. Southern Chiapas is the most endemic foci as this region shares its border with Guatemala. In Oaxaca, there are 29 municipalities covering an area of 4,200 square km of which 25 are hyperendemic, 34 are meso-endemic and 77 are hypo-endemic (WHO

weekly epidemiological record, 2009). An estimated 44,919 individuals are at risk in Oaxaca focus. In the Chiapas state there are 23 municipalities that are affected by onchocerciasis covering an area of 12,640 square km. It is estimated that 109,279 individuals are at risk in Southern Chiapas and 7,125 individuals in Northern Chiapas region.

The onchocerciasis control program in Mexico was started in year 1989 treating the symptomatic individuals at the beginning. Later on the strategy was modified to provide the treatment bi-annually to all the eligible residents of the at risk communities in 1995 and in 2003 to provide quarterly treatments in the hyperendemic communities. The studies have shown that the transmission of *O. volvulus* has been suppressed after the elimination of microfiladermia in S. Chiapas (Rodríguez-Pérez et al., 2008a). In N. Chiapas no microfilariae have been found in the eye or skin snips in 2006 from the two sentinel communities examined suggesting that there is no transmission of *O. volvulus* going on, hence ivermectin treatments were halted in 2008 and post-treatment surveillance phase has been started. In Oaxaca, the studies conducted in 2007-2008 revealed absence of parasite DNA in *Simulium* samples indicating the absence of morbidity and transmission of the parasite. Thus, ivermectin treatment was halted in 2009 and post-surveillance phase was started (Rodríguez-Pérez et al., 2010a, b). The current status of onchocerciasis in Mexico is shown in Fig. 10.

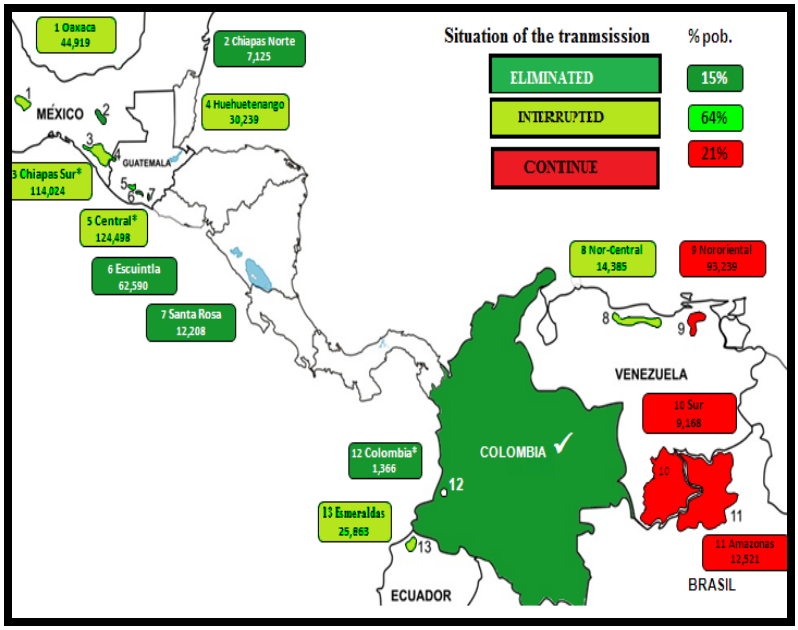


Fig. 10: Current situation of onchocerciasis in Latin America

3: JUSTIFICATION

The entomological monitoring and surveillance of onchocerciasis from S. Chiapas and N. Chiapas will enable the control programmes to evaluate the situation in these foci of Mexico. This study will provide the control programmes the evidence of the effectiveness of ivermectin treatment, based on the data collected, the Health authorities would decide whether to continue or halt the on-going ivermectin treatment. Also in foci where the ivermectin treatment has been halted already, this would provide sufficient data to declare the elimination of onchocerciasis and certify it onchocerciasis free.

The high-throughput automated oligonucleotide based Dynal and silica coated magnetic bead method with 2-4 folds more efficient than the standard PC method for genomic DNA purification was validated. This will lead to a faster PCR pool screen assay and large number of flies can be processed during entomological surveillance.

4: OBJECTIVES

4.1. GENERAL OBJECTIVE

To detect the transmission of *O.volvulus* in *S.ochraceum* population in endemic communities in Southern Chiapas focus, and post treatment surveillance in Northern Chiapas, which could provide the valuable data for making the strategy to eliminate the Onchocerciasis from Mexico.

4.2. SPECIFIC OBJECTIVES

1. To do the entomological surveillance of post-treatment of transmission in the Northern Chiapas focus for Onchocerciasis.
2. To compare the levels of transmission in two coffee fincas of the Southern Chiapas focus after 11 years of treatment with ivermectin.
3. To monitor by PCR for *O. volvulus* transmission in black flies in the Southern Chiapas focus.
4. To evaluate and validate an improved DNA purification protocols based on magnetic bead particles for *O. volvulus* host DNA in pools of black flies.

5: HYPOTHESIS

The transmission of onchocerciasis in the endemic communities could still exist in Chiapas foci.

An efficient and improved DNA purification method would speed up the process of PCR pool screen assay used for screening the vector populations for the presence of *O. volvulus*.

6: CHAPTER II: MATERIALS AND METHODS

In this chapter, the experimental procedures used throughout the studies of the present thesis were explained. The immunological and molecular techniques to the diagnosis of *Onchocerca volvulus* were described in detail.

6.1. Materials

Sodium Chloride (NaCl), Sodium bicarbonate ($NaHCO_3$), Sodium Carbonate ($NaCO_3$), Sodium Iodide (NaI), Sodium Hydroxide (NaOH), Ficoll 400, Sodium Sulphite (Na_2SO_3), Agarose, Di Sodium Phosphate (Na_2HPO_4), Sodium dodecyl Sulphate (SDS), Isopropanol, Ethanol, Tris-HCl, Ethylenediaminetetraacetic acid (EDTA), Salmon sperm DNA, Proteinase K, Dithiothreitol (DTT), Phenol, Chloroform ($CHCl_3$), Isoamyl alcohol, Ammonium Sulphate [$(NH_4)_2SO_4$] Magnesium Chloride ($MgCl_2$), dNTP'S (Adenine, Guanine, Thymine and Cytosine), Streptavidin, Tris base, Tween20, Taq polymerase, N-lauryl sacrosine, Polyvinylpyrrolidone, Hydrochloric acid (HCl), Sulphuric acid(H_2SO_4), Disodium EDTA, and streptavidin paramagnetic particles (M-280).

All the chemicals used were of analytical grade. Some chemicals were purchased from Sigma Aldrich Company, St. Louis, MO, USA and few from Research Organics and Promega Co Madison, USA. Sterilized Mili-Q and double distilled water was used for all the experiments.

6.2. Methods

6.2.1. Study Area

In Mexico, onchocerciasis has been distributed in Southern Chiapas, Northern Chiapas and Oaxaca. The flies were collected from different communities from the above three foci. There are 559 total communities in Southern Chiapas, of which 39 communities were indicated as hyper-endemic. In this study, 11 hyper-endemic communities from southern Chiapas were selected for the entomological evaluation carried out during 2009-2011. The communities selected were Costa Rica, Amplacio Malvinas, Barrio Brasil, Barrio Mexiquito, Santa Rita, Montowa, Estrella Roja, La Soledad, La Granja, Loma Bonita, Finca Victoria, Finca Santa Malia and Las Golondrinas.

From Northern Chiapas, Alta Gracia, El Ambar, Nueva Esperanza, and Chimix were included for the entomological surveillance post-treatment in the year 2010. Alta Gracia and El Ambar have historically shown clinical cases of onchocerciasis. During 1995, the prevalence of infection was shown to be 0.8 % with 146 clinical cases, mostly imported, from 18,891 total populations among 42 communities.

6.2.2. Entomological studies

Using the procedure below the black flies were collected from November 2009 to April 2010 and November 2010 to April 2011, coinciding with the peak *O.volvulus*

transmission season (Rodríguez-Pérez et al., 1994) and from two different foci (Southern Chiapas and Northern Chiapas), where onchocerciasis is endemic.

6.2.3. Collection of flies

Flies were collected by the volunteers of communities and collectors of the national Mexican program. Flies collectors received Mectizan (ivermectin) one week before the beginning of collection process. Flies were collected from a nearby coffee plantation and within the center of the community. The collection of flies was carried out during the first 50 minutes of each hour starting at 7.00 AM and ending at 5.00 PM. The flies were collected before they began feeding. The landing rate measured from the collection was taken as an estimate of the biting rate. The collected flies were preserved in absolute isopropanol and transferred to laboratory for further processing. The flies were preserved at 4 ºC until processed.

6.2.4. Migrant studies

Onchocerciasis came to Americas with the slave trade mainly from Africa and migrated to various places like coffee plantations spreading the infections. This was further enhanced by the migration of indigenous people from border countries of Guatemala, Columbia and Venezuela. Southern Chiapas was more endemic than other places and three villages namely Las Golondrinas, Rosario Zacatonal, and Nueva America were selected for the since they are the most endemic communities. These

communities received a large influx of migrants from neighboring states to work in coffee plantations. Las Golondrinas and Rosario Zacatonal were close to each other separated by a distance of 7 km and were 2.5 and 3.0 km away from the coffee finca (Palestina), respectively. Coffee finca La Victoria, La Fortuna and Santa Fe surrounds the village Nueva America with a distance between them being 2 and 5 km. The nearest breeding sites of the Simuliids were located at approximately 0.5 to 6 km from these villages and directly came under the flight range of black flies (Dalmat, 1955; Collins et al., 1992).

The temporary laborers migrate to these villages biannually who could be eligible to receive ivermectin treatment by the control program that would have already received ivermectin treatment at home or could have missed it as they were away when treatments were given. Thus, missing treatments in both places of infected coffee workers would serve as possible reservoirs for the simuliid. Each year coffee cultivation occurs in two seasons namely planting-clearing and harvesting carried out from May-July and November-February, respectively. The harvesting season coincides with the onchocerciasis transmission season in Guatemala which is at its highest. Migrant laborers working in these coffee fincas were mainly targeted for the ivermectin treatment since 2003. The parasitological examinations were carried out on both the residents and the migrants before they started their work from May and November.

6.2.5. Processing of black flies

Separation of head and bodies

The preserved flies were placed in liquid nitrogen and subjected to vigorous agitation to separate the heads and bodies. The tubes containing flies were tapped hard to separate heads from bodies. The heads were separated from the bodies by sieving through a 25-mesh sized sieve and each fraction was thereby processed separately as described by Yameogo et al (1999). The other method used for the separation of flies was based on automated tissue lyser. The tubes containing flies were placed on the metal plates and inserted into the tissue lyser and allowed to shake at 4000 rpm for 4 minutes. The heads were separated from the bodies by sieving through 25-mesh sieve filter and each of the fractions (head and bodies), collected separately, and stored in alcohol. The isolation of DNA from black flies has been carried out using different methods like Standard phenol-chloroform method, Silica coated paramagnetic method, nucleomag DNA kit and Dynal beads method. These methods have been assessed, compared, optimized and quantified in order to optimize the pool size.

(i) Isolation of DNA from pools of heads and bodies using Phenol-Chloroform (PC) method

The method based on P-C for parasite DNA extraction in pools of blackflies connected to a PCR-ELISA is the standard procedure extensively used for routine monitoring and surveillance post-treatment of transmission in Latin America. The

maximum pool size of 50 flies in one pool was standard for using phenol-Chloroform procedure. Stored flies were placed in 1.5 ml screw-cap micro centrifuge tubes and rinsed three times with 95% ethanol. As much as possible ethanol was removed and the remainder was evaporated for about 10 min at room temperature (RT). Flies were then homogenized in 300 µl of lysis buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.1% SDS) along with 2 µl of carrier Salmon sperm DNA and the whole mixture was pulverized thoroughly. The homogenate was incubated at 55 °C for 1 h with 100 µg/mL of proteinase K solution. To disrupt the parasite cuticle, the whole mixture was boiled for 30 min in the presence of 10 mM dithiothreitol (DTT) and followed by a series of freeze-thaw steps to release the DNA from the parasites. The DNA was then purified from the individual preparations by two cycles of extraction with 1:1 (v/v) phenol-chloroform, followed by one extraction with chloroform.

The aqueous layer formed in the chloroform solution was extracted and then transferred into a micro centrifuge tube and the volume was measured. Three volumes of NaI solution was added and incubated at 4°C for 15 min. The solutions were transferred into deep well micro-titer plate and washed with 500 µl of the ethanol wash solution (200mM Tris-HCl, pH 7.5, 2mM NaCl, 20mM EDTA, 50% [v/v] ethanol). The washing step was repeated three times and centrifuged at 13,000 rpm for 1 min. The genomic DNA was re-suspended in 50 µl of sterile water and stored at -20 °C (Rodríguez–Pérez et al, 1999).

(ii) Isolation of genomic DNA using automated silica coated paramagnetic bead based method

The automated method provides automated nucleic acid purification for multiple analyses. The primary principle of this method is to use sample lysis and binding to paramagnetic particles. Flies were placed in 1.5 ml screw-cap micro centrifuge tubes and rinsed three times with 95% ethanol. As much as possible ethanol was removed and the remainder was evaporated for about 10 min at room temperature (RT). This protocol provides automated nucleic acid purification for multiple analyses. The primary principle of this method is based on sample lysis followed by DNA binding to silica coated paramagnetic beads. The samples were inserted into the cartridge supplied with the Maxwell® 16 Tissue DNA Purification kit (Cat. No. AS1030, Promega). The kit contained buffers for lysis, washing and elution, along with elution tubes, silica-coated MagneSil® Paramagnetic Beads (PMPs) and a purification plunger. The genomic DNA of samples was isolated without any cross contamination within 45 min according to the procedure supplied by the manufacturer. It is completely automated for the purification process.

(iii) Isolation of genomic DNA using general magnetic bead (nucleomag tissue DNA kit)

The basic principle of this method is based on reversible adsorption of nucleic acids to paramagnetic beads under appropriate buffer conditions. The samples are lysed

with T1 lysis buffer which contain SDS. For the adjustment of the binding conditions under which nucleic acids bind to the paramagnetic beads buffer MB2 and the nucleomag-B-beads binding are added to the lysate. After magnetic separation, the paramagnetic beads are washed twice to remove contaminants and salts using wash buffers MB3 and MB4. There is no need for drying step as ethanol from previous wash steps is removed by a final incubation of the beads in buffer MB5. Finally, highly purified DNA is eluted with low salt elution buffer (MB6) and can directly be used for downstream applications. The nucleomag 96 tissue kit can be used either manually or automated on standard liquid instruments or automated magnetic separators.

The flies were homogenized in 200µl of T1 buffer, 25 µl of proteinase K provided in the kit (nucleomag tissue DNA kit cat #1102/001, Machery- Nagel GmbH & Co, Bethlehem, PA, USA). The homogenates were incubated at 56^0C for 30 minutes and centrifuged for 5 minutes to clear the lysate. 225µl of the supernatant was taken and added to 24µl B-beads and 360µl MB2 buffer; shake for 5 min at RT. The magnetic beads were separated against the side of the wells by placing the separation tube on the magnetic separator. We waited for 2 min until all the beads has been attracted to the magnet. The supernatants were removed and discarded and 600µl of wash buffer MB3 was added to each sample and the bead/DNA complex washed by shaking for 5 min at RT. The washing was followed by MB4 and MB5 wash buffer. During MB5 wash buffer the samples were incubated for 60s to remove the traces of ethanol eliminating the drying

step. Finally 100µl of elution buffer was added to each sample, shake for 5 min at RT and separated by magnet plate and DNA was collected into a new eppendorf tube.

(iv) Isolation of genomic DNA using Oligonucleotide based Dynal magnetic beads M-280

Dynabeads® are super paramagnetic, monosized polymer beads. Each bead has an even dispersion of super magnetic material ($Fe_2O_3$and Fe_3O_4) coated with a thin polymer shell to encase the magnetic material. This provides a specific and defined surface for the adsorption and coupling of bioreactive molecules (ligands). The physical characteristics of M280 Dynal beads used for the experiments were 2.5 µm in diameter with 4-8 m2/g surface and 1.4 g/cm3 density.

Genomic DNA was isolated from pools of flies using a protocol for Dynabeads M-280 streptavidin® (Dynal, AS Oslo, Norway). In brief, pools of black flies were homogenized in 100µl of TE buffer, 20 µl of 10mg/ml of proteinase K. Homogenates were incubated at 56^0C for 2 h, and 10 µl of 1M dithiothreitol (DTT) was added and adjusted the volume to 500µl with TE. The samples were incubated at 95^0C for 30 min and subjected to three cycles of freezing and thawing. The solution was then centrifuged and supernatant was collected. Further, supernatant was mixed with 1M Tris-HCl (pH 7.5), 4M NaCl and 0.5 µM OVS-2-biotin 5´-B-GCNRTRTAAATNTGNAAATTC- 3 followed by heating at 95^0C for 3 min and cooled to < 35^0C. During this period, streptavidin magnetic beads were prepared and beads were washed with binding buffer:

100mM Tris-HCl (pH 7.5) 100mM NaCl using 200µl per wash and placed on the magnet and allow the beads to settle for 2 minutes between the each wash. Finally, the beads were suspended in the original volume of binding buffer. 10µl of bead solution was added to each sample and followed by overnight incubation on roller/shaker at room temperature. The beads were allowed to settle for 2 min and continued by washing for 6 cycles with 150µl of binding buffer per wash. After the final wash as much as solution was removed and the beads were suspended with 20µl of PCR water and followed by incubation at 80^0C for 2 min and immediately chilled on ice. Finally, the solution was placed on magnet and beads were allowed to settle for 2 min, collected the DNA and discarded the beads. The collected sample DNA was stored at -20^0C.

6.2.6. PCR amplification for O-150 repeated sequence

The purified genomic DNA was used to amplify by PCR. All PCR reactions were carried out in a PCR micro titer plate. 3µl of the purified genomic DNA was used as a template for the PCR amplifications and the total volume PCR reaction mixture was 50µL.

(i) Design of Primer

The sequence of the two primers are 1632: (5´-GATTYTTCCGRCGAANARCGC-3´) and the second primer 1632: (5´-B-GCNRTRTAAATNTGNAAATTC- 3´), where B = biotin; N =A, G, C, or T; Y = C or T; and R = A or G). Reaction mixtures contained 60 mM Tris-HCl, pH 9.0, 15 mM

$(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP and dTTP, and 2.5 units of *Taq* polymerase (Roche Diagnostics, IN, USA).

(ii) **PCR Cycling**

PCR conditions consisted of five cycles of one minute each at 94°C, 2 min at 37°C, and 30 sec at 72°C, followed by 35 cycles of 30 sec each at 94°C, 37°C, and 72°C, respectively. The reaction was completed by incubating at 72°C for 6 min. Row A was reserved for 10 negative controls and two positive controls. One positive control contained the minimal amount of positive control DNA found to be consistently detected by the PCR amplification conditions, as determined by an initial titration study. This control was carried out to ensure that all the reaction sets were operating at peak efficiency. The second positive control contained the same minimal amount of positive control DNA mixed with 1µl of positive control and 2µl of old sample containing positive DNA preparation from a pool that tested negative in a prior set of reactions. This control ensured that no inhibitors were present in the DNA preparations (Rodríguez–Pérez et al., 2004).

6.2.7. Analysis of PCR products by agarose gel electrophoresis (AGE)

Agarose gel electrophoresis is used to separate DNA fragments by their sizes and visualize them. Using 1.5% agarose gel diluted with TE buffer that contain 0.1 µg/ml ethidium bromide, the amplified products were separated. The products were

electrophoresed at 90 volts for approximately 45 min in an electrophoresis unit and the gel was visualized under UV light and picture was taken for further interpretation.

6.2.8. Enzyme linked immunosorbent assay (ELISA)

The PCR amplification products were detected by PCR enzyme-linked immunosorbent assay (ELISA). Briefly, the microtitre ELISA plate was bound to a streptavidin (1 µg/mL with 1X coating buffer- 50mM $NaHCO_3$, 2mM $NaCO_3$) coated ELISA plate, incubated at 4^0C overnight. The ELISA plate was washed 6 times with TBS/Tween 20, followed by emptying plate on to a paper towel between each wash. Firstly, 20 µl of 50 ng/ml hybridization buffer [20X SSPE, 10X Denhardt's, 1% (W/V) N-lauryl sacrosine, 20 % SDS] with 10% of each PCR amplification reaction (5 µl) was incubated at RT (room temperature) for 30 min. Later, the DNA strands were denatured by treatment with alkali (NaOH) and incubated for 1 min. The bound PCR fragments were then hybridized to a fluorescein-labeled *O. volvulus*-specific oligonucleotide probe (OVS2: 5´-AATCTCAAAAAACGGGTACATA-FL-3´). The bound probe was detected with an alkaline phosphatase (Fig.11) labeled anti-fluorescein antibody (fragment FA; Roche Diagnostics). Bound antibody was detected using the ELISA amplification reagent kit (Invitrogen/BRL, Carlsbad, CA, USA) following the manufacturer's instructions. Colour development was stopped by the addition of 25µl of 0.3 M sulphuric acid and the plates were read using ELISA plate reader set at 450 nm. The cut-off for classifying a sample as putatively positive was set at the mean of the 10 internal negative controls plus three standard deviations, or 0.1, whichever was greater. A second independent PCR and

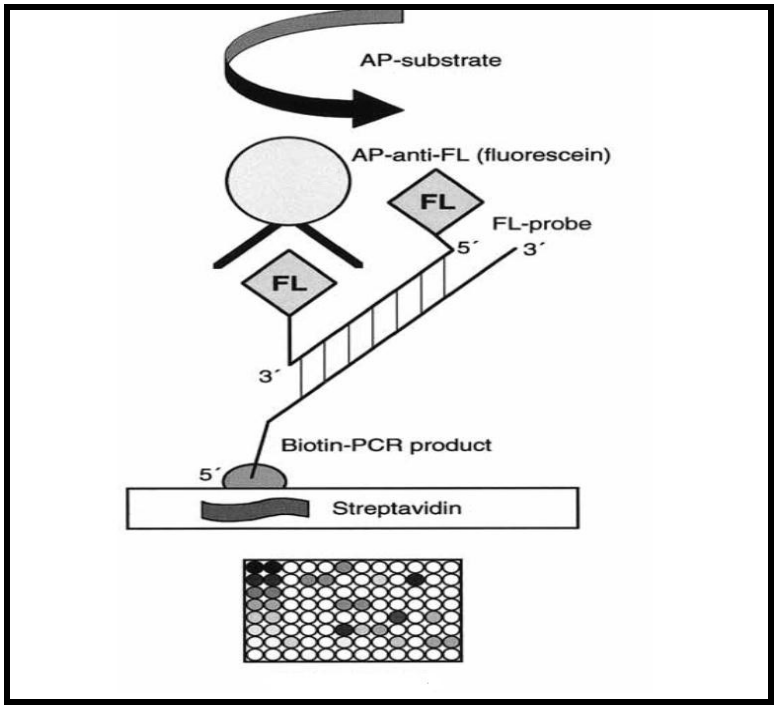


Fig.11: Schematic representation of ELISA

ELISA was then carried out on the putatively positive samples. Only putative samples classified as positive in both independent reactions were scored as confirmed positive (Rodríguez–Pérez et al., 2004).

6.2.9. Data analysis

In case of onchocerciasis, the collection of *S. ochraceum* s.I. females was not carried out throughout the year due to random biting pattern. Hence, it would be difficult to calculate precisely the annual transmission potential (ATP). In Mexico, the transmission levels during the peak transmission period were very low due to effect of 12 years of ivermectin treatment. From this perspective, the transmission potential value outside the peak transmission period was assumed to be zero or near to zero. The seasonal transmission potential during the peak transmission period of January through May is considered to be fairly accurate for the determination of the ATP. Due to multiple rounds of ivermectin treatment it can be assumed that the number of infective larvae in each infective black fly would be near to 1 (Rodríguez-Pérez et al., 2006).

Depending on the study area and the specific combination of *Onchocerca* and *Simulium* that is prevailing in that area, the prevalence of infection is determined in the thoraces of the vector which usually contains the non-infective L1 and L2 stages which is higher than that found in the heads which contains only the infectious L3 larvae. This fact determines the parasite-vector contact and the prevalence of infection in the bodies of

flies would be a very good indicator for monitoring the presence of mf in untreated coffee fincas and other villages. For the control programs, it has been indicated that the assessment of the transmission levels can be estimated from the total number of *O. volvulus* larvae in the bodies and detection of L3 larvae in the heads of the flies (Basáñez et al., 1998). The prevalence of infection can be calculated as number of flies positive for any of the *O. volvulus* larval stage divided by the total number of parous flies examined.

The values for the prevalence of infection in the bodies and heads pools and their associated 95% CIs were determined using the computer program Pool Screen version 2.0. (Katholi et al., 1995). The prevalence of infection in the head pools were used for the calculation of seasonal transmission potential (STP) as a product of the seasonal biting rate, the number of flies carrying L3 larvae in the study season and the average number of L3 larvae in each infective fly. The seasonal biting rate was determined as the product of the geometric mean (Williams, 1937), the number of flies collected per person per day and the total number of days in the months of January through May.

The new pool screen version 2.01 is used to obtain the number of vectors collected in the field, number of pools gathered, number of pool tested, seasonal transmission potential, prevalence of infected flies and seasonal transmission potential and their associated 95% CIs. In order to obtain the above parameters, the data has to be formatted according to the instructions obtained from the manufacturer and fed to this

software. In the first step, excel sheet is created with number of flies collected in the field, the results obtained by PCR and the file is saved in CVS /doc format only as the program does not accept any other format. In the next step, atp hour (multiple hour calculator) is calculated depending on the latitude and longitude of the community area, date of flies collection from starting till the end in order to obtain the total number of day light hours. All the above parameters can be obtained using pool screen version 2.01 application tool upon providing the calculated day light hour and loading the excel data.

7: CHAPTER III: ENTOMOLOGICAL POST-TREATMENT SURVILLANCE OF TRANSMISSION IN THE NORTHERN CHIAPAS FOCUS FOR ONCHOCERCIASIS

Human onchocerciasis is caused by the infection with filarial parasite *O.volvulus*. It is one of the main causes of infectious blindness worldwide and is an important public health problem. In Mexico, there are three endemic foci namely Oaxaca, Northern Chiapas and Southern Chiapas affected by onchocerciasis. The onchocerciasis control program in Mexico was started in 1989 by treatment with ivermectin. At the beginning, only symptomatic individuals from hyper endemic communities were treated. Later on annual treatments were undertaken from 1991-1997 to treat all symptomatic residents of meso-endemic and hypo-endemic communities. From the year 1997 onwards this treatment strategy was modified to provide bi-annual mass treatment to all those eligible residents of all the communities at risk, regardless of endemicity.

Northern Chiapas is one of the thirteen foci of onchocerciasis in Latin America. There were 72 endemic communities with 351 clinical cases of onchocerciasis in 1989, mostly imported cases. Onchocerciasis in Northern Chiapas was discovered after the identification of two foci in Mexico, mostly in 1951 when health personnel started giving systematic health care in this area. Hence, there is no exact information on when onchocerciasis was discovered in Northern Chiapas (Fernández de Castro, 1967; 1979).

The two sentinel communities in northern Chiapas showed the highest prevalence of onchocerciasis in the region that were identified by health authorities in 1995. These sentinel communities included Altagracia (17°01’51’N, 92° 46’02’ W; elevation =1,300m above sea level) and El Ambar (17°01’29’’N, 17°01’29’’ W; elevation = 1,610 meters), and were epidemiologically classified as hypo endemic for onchocerciasis. A total of 146 clinical cases of onchocerciasis, mostly imported cases, in a total population of 18, 891 individuals from 42 communities were identified. The local programmes identified 180 clinical cases of onchocerciasis in 1993 from 42 endemic communities with. Further, this dropped to 13 endemic communities with 83 clinical cases in 2000. There has been a gradual decrease in the number of endemic communities in Northern Chiapas over a period of time due to various control programmes that were undertaken in this region. In 2007, only 13 endemic communities with 77 clinical cases of onchocerciasis were detected

Over the last two decades there have been tremendous changes in the social and economic status of the indigenous population of Northern Chiapas focus due to the abandoning of the endemic regions and migration to industrialized cities in Mexico and United States. As a result, the seasonal migration of workers from Northern Chiapas to coffee plantations in Southern Chiapas has declined. This gradual decline in the seasonal migration to the endemic area might have had an effect on the transmission of onchocerciasis with no individual being detected to harbor nodules in Northern Chiapas focus from 1996. It has been suggested that the onchocerciasis infection in Northern

Chiapas was mainly due to seasonal migration of people and no independent transmission of parasite was observed in this focus. This hypothesis was found to be true and was supported with the fact that the Northern Chiapas focus is free of infection if indigenous populations do not travel outside the endemic area (Fernández De Castro, 1967; Davis, 1968; Vásquez Castellanos, 1991). The entomological and serological studies conducted in 2001 and 2005 have shown that autochthonous transmission of *O.volvulus* could be occurring in Northern Chiapas focus, but at a very low rate (Rodríguez-Pérez et al., 2004, 2006).

Onchocerciasis elimination program for the Americas (OEPA) started to eliminate onchocerciasis from the America. In this regard to reach its aim OEPA has set certain goals to eliminate new cases of onchocerciasis induced ocular morbidity due to infection with *O.volvulus* and also to interrupt the transmission of the parasite by 2012. To achieve this objective, WHO and OEPA have chartered a series of epidemiologic and entomologic criteria to declare the elimination of onchocerciasis. The important criteria set by WHO/OEPA are (i) to eliminate new ocular morbidity, which is defined as prevalence of less than 1% of *O.volvulus* microfilariae in the anterior chamber of the eye or cornea, (ii) to reduce new infections of occurrence rate to less than one new case per 1000 individuals i.e., lack of specific antibodies to *O.volvulus* in children below the age of 10. To calculate one sided 95% confidence intervals (CI) for the point prevalence that excludes 0.1 % requires a sample size of 3000 children (Lindblade et al., 2007; WHO, 2001). This means that all the children of that age residing in the focus will be tested in

which over than 3000 children of below the age of 10 reside in order to check that there is no evidence of parasite exposure and (iii) absence and near absence of the infective stage larvae of *O.volvulus* in the vector population i.e., prevalence of infective flies of less than 1/2000.

In 2005, follow up studies were conducted which indicated that none of the flies bodies collected had the parasite DNA indicating the absence of vector-parasite contact (Rodríguez-Pérez et al., 2006). After 11 years of ivermectin mass treatment in Northern Chiapas focus there was no evidence of microfilariae found in the anterior chamber of the eye or cornea and in skin snips in the residents examined in 2006 in the two sentinel communities. None of the children below the age of 10 were found to harbor the antibodies anti *O.volvulus* thus, suggesting that there is no ongoing transmission of *O.volvulus* in Northern Chiapas focus. As a result, these treatments with ivermectin were halted in 2008 and the post treatment surveillance phase was initiated. In this chapter the data obtained from the epidemiological follow up study of onchocerciasis in Northern Chiapas conducted throughout the year 2010 is presented.

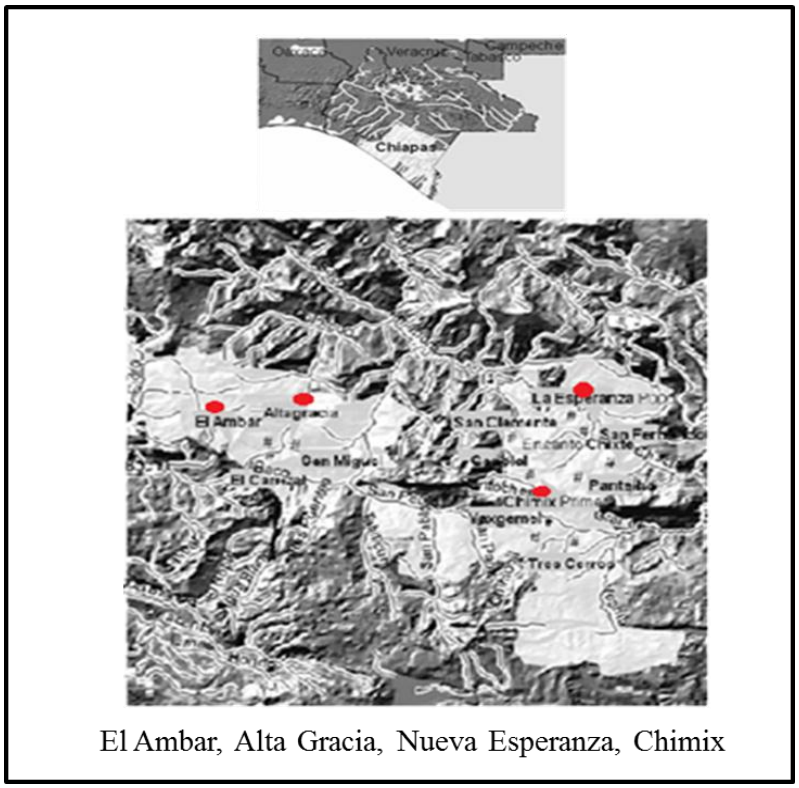
Study area

Two sentinel (Altagracia and El Ambar) communities from the Northern Chiapas were selected by local health authorities in 1995. The selected communities exhibited highest prevalence of clinical onchocerciasis cases historically in these regions.

Altagracia and El Ambar were epidemiologically identified as hypo-endemic for onchocerciasis, corresponding to other communities in these foci. In 2006, the total population in these two communities Altagracia (578) and El Ambar (813) were figured as 1,391 individuals. In the present, epidemiological study was conducted in 2010 in four sentinel communities from the Northern Chiapas focus; Nueva Esperanza, El Ambar, Altagracia, and Chimix (Fig. 12) where the post ivermectin treatment surveillance was carried out from 2008.

RESULTS

The *Simulium ochraceum s.I* flies were collected during the peak transmission period February through May 2010 from the four sentinel communities in the Northern Chiapas focus endemic for *O.volvulus*. A total of 207 bodies were pooled as each containing 50 bodies (Nueva Esperanza: N=135; Altagracia: N=39; El Ambar: N= 27; Chimix: N=6) were tested by PCR. The results of this analysis were shown in Table 3. All the body pools tested were negative for *O.volvulus* DNA. Thus, head pools were not tested further for these communities.



El Ambar, Alta Gracia, Nueva Esperanza, Chimix

Fig.12: Geographical distribution of study area in Northern Chiapas, Mexico

Table 3: Number of *Simulium ochraceum* flies collected from sentinel communities in northern Chiapas during 2010 in Mexico

Communities/year	Number of pools	Number of positive head pool
Nueva Esperanza	135	0
Altagracia	39	0
El Ambar	27	0
Chimix	6	0
Total	207	0

The parasite DNA detected in pools of bodies and the parasite DNA detected in pools of heads were evaluated by using PCR assays as explained in materials and methods. In Nueva Esperanza, the estimated point prevalence of infective flies was zero (0.5; 95%-UCI) followed by seasonal transmission potential being 0 (5.4), in Altagracia the prevalence of infective flies was 0 (1.9; 95% CI) followed by STP 0 (8.6; 95% CI), in El Ambar the prevalence of infective flies was 0 (2.8; 95%CI), followed by STP 0 (6.5 95% CI), in Chimix the point prevalence of infective flies was 0 (12.2; 95% CI) followed by STP was 0 (6.7 95% CI) (Table 4) The samples collected were less, since density of the flies in this focus is very low.

DISCUSSION

The entomological data shown above will be very useful for onchocerciasis control programs, which aims to control and eliminate the transmission of the onchocerciasis in endemic areas where ivermectin treatment is in progress. The elimination is based on the hypothesis that ivermectin can reduce the microfilariae in skin and to lessen the presence of microfilariae in the corneal chamber of eye that results in the reduction of transmission rate to being below the significant level for the parasite population to maintain itself (Collins et al., 1995).

Table 4: Transmission intensity of *O.volvulus* in four sentinel communities of northern Chiapas, Mexico

Community	Seasonal biting rate	Infected rate	Infective rate* IC(1/2000)	Seasonal transmission potential
Nueva Esperanza	20,971	**0**	**0**	**0**
	(23,038-19,076)	(0.5)	(0.5)	(5.4)
Altagracia	10,225	**0**	**0**	**0**
	(11,751-8,866)	(1.9)	(1.9)	(8.6)
El Ambar	5,580	**0**	**0**	**0**
	(6,614-4,654)	(2.8)	(2.8)	(6.5)
Chimix	1,338	**0**	**0**	**0**
	(1,599-1,104)	(12.2)	(12.2)	(6.7)
Total	8,732	**0**	**0**	**0**
	(8,064-9,446)	(0.3)	(0.3)	(1.3)

* Expressed as rate per 2000 flies examined.
*Bold numbers represent point estimated and value in parentheses represents 95% upper limit confidence interval surrounding point estimate

The distribution of mass ivermectin treatment usually affects the microfilarial load in the skin of human host and therefore parasite uptake by the simuliid vector (Cupp et al., 1989; Basánez et al., 1994). The entomological indicators recommend that the impact of ivermectin on transmission is not based upon the prevalence of all forms of the parasite found in vector, but only the infective larvae (Basáñez et al., 1998).

During the last decades there was no evidence of new clinically defined cases of onchocerciasis in Northern Chiapas focus. These evaluations indicate that the onchocerciasis is no longer a serious health problem to the endemic communities in Northern Chiapas. However, due to the migration from different regions of Mexico where onchocerciasis is still continuing such as Southern Chiapas, it may be still possible that onchocerciasis infected migrants could still introduce new cases. As in the earlier studies, onchocerciasis in Northern Chiapas was hypothesized due to annual seasonal migration of coffee workers from Southern Chiapas (Davies, 1968; Vásquez Castellanos, 1991).

The entomological criteria for the absence or near absence of infective stage larvae of *O. volvulus* in the vector population is defined as < 1 infective fly per 2,000 flies tested (WHO, 2001). For the year 2001, the entomological data in Altagracia, after 4 years of mass bi-annual treatment with ivermectin showed that out of 125 pools tested one positive head pool was identified. The prevalence of infective flies was 0.32/2,000

(0.008-1.65/2,000 95%CI) following the seasonal transmission potential 1.5 L3 (0.04-7.7 95 %CI) /person/season (Rodriguez-Perez et al., 2006). In the year 2005, the prevalence of infective flies was 0/2,000, following STP 0 (0.9/2,000 95% CI), below the threshold of 1/2,000. In the present study, the prevalence of infective flies was 0 (0.3; 95% UCI) followed by STP being 0 (1.3; 95% UCI).

Meanwhile, the serological studies carried out in 2001, among 922 individuals aged 16 were tested for IgG4 antibodies to Ov16 using prototype Immuno chromatographic card test (Weil et al., 2000) from 10 endemic and non-endemic communities, none of the samples were positive leading to a post-exposure incidence of 0% (95% UL = 0.3%). The serum samples from the children of 10 years above and younger (1,241 individuals) were examined in the sentinel communities (Northern Chiapas), none of the serum samples were positive leading to 95 % CI of a maximum prevalence of 0.2%, clearly indicating that there is no parasite contact among children (Rodríguez-Pérez et al., 2010b). The present evidence of zero infectivity of the fly confirmed the earlier information that there is cessation of onchocerciasis transmission in Northern Chiapas.

According to the data published by Rodríguez-Pérez et al., (2006) in 2001, a total of 197 including both head and body pools from Altagracia and El Ambar were screened. None of the head or body pools were positive to PCR assays. Continuing with

entomological studies, in 2005, a total of 71 body pools from these communities were examined. According to the OEPA program acceptance of the procedure for PCR screening of vector pools, the data obtained in 2005 was the first evidence of fly-parasite contact, as prevalence of the infection in bodies is constantly greater than the prevalence of parasite in head pools (Rodríguez-Pérez et al., 2006). Since none of the body pools tested was PCR-positive, it was declared that absence of parasite vector contact existed, hence none of the head pools were tested further.

In El Ambar, the total of 101 pools in 2001 and 17 pools in 2005 were examined. In 2001, the calculated prevalence of infective flies was 1.5 per 10,000 (95% CI; 0.01-3.9 per 10,000) following the seasonal transmission potential 1.4 L3/person/year. Furthermore, there was no evidence of infection in vector population in this community in 2001 or 2005, following the seasonal transmission potential zero (Rodríguez-Pérez et al., 2006).

The entomological data represented in the year 2005, showed that there is no more evidence of ongoing transmission of *O.volvulus* in Northern Chiapas focus. As a result, ivermectin distribution was halted in the year 2008 and post treatment surveillance were initiated in this region (Lindblade et al., 2007; Rodríguez-Pérez et al., 2010b; WHO, 2009).

The serological data presented by Rodríguez-Pérez et al., (2010b) suggests that the estimated incidence of *O. volvulus* exposure in sentinel communities (Altagracia and El Ambar) was 0%. Among 682 residents tested of 1,391 individuals in these sentinel communities for ophthalmogic studies also indicates the absence of microfilariae in corneal chamber. From 986 residents the skin biopsy were collected and examined, none of the skin biopsy was positive (Rodríguez-Pérez et al., 2010b).

In order to certify the elimination of onchocerciasis, it has to pass through four phases (WHO, 2001). In phase, I until transmission is suppressed, ivermectin treatment is included. In phase II, suppression is maintained through treatment if the mean reproductive lifespan of the adult female. In phase III the adult parasite population should have died by senescence, and removal of ivermectin treatment will not result in resumption of the transmission. Phase IV contains the studies on parasite transmission in the post-treatment era, where treatment has been ceased. From 2008, Northern Chiapas focus has entered the Phase IV, where post treatment surveillance is being carried out (Fig.13).

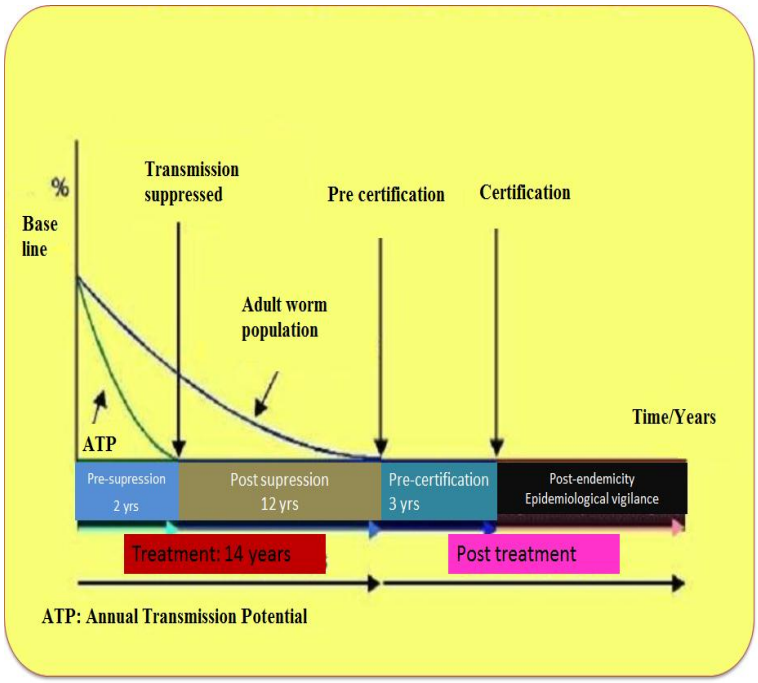


Fig.13: Certification process for elimination of onchocerciasis

CONCLUSION

The earlier entomological, serological and parasitological data presented in this study showed that there is no ongoing transmission of *O.volvulus* in this focus, therefore from 2008 ivermectin treatment was suspended and intensive surveillance program was initiated. Currently, the result of entomological surveillance data represents no evidence of ongoing transmission in Northern Chiapas. Thus, the local, federal Mexican health authorities along with OEPA should certify that the human onchocerciasis is successfully eliminated from Northern Chiapas.

8: CHAPTER IV: COMPARING THE LEVELS OF TRANSMISSION IN TWO COFFEE FINCAS OF THE SOUTHERN CHIAPAS FOCUS AFTER 11 YEARS TRETAMENT WITH IVERMECTIN

Onchocerciasis is also known as river blindness, is one of the world's major parasitic diseases affecting eyes often leading to blindness when it reaches to advance stage in humans. Onchocerciasis also causes extensive skin disfiguration leading to extensive itching and rashes. This is further characterized by dermatitis, atrophy of skin, pigmentation etc (WHO, 1988; Liese et al., 1991). Onchocerciasis apart from its devastating effect on the human body it also causes eradication of the whole communities where it is endemic.

The recent estimates indicates that at least 37 million people are infected; mostly in Africa (Basánez et al., 2006). A number of control programmes aimed at controlling the river blindness have been undertaken with local governments and united nation agencies like WHO, APOC, OEPA and OCP. The three main onchocerciasis endemic foci in Mexico are Southern Chiapas, which is contiguous with Guatemalan endemic foci, Northern Chiapas and Oaxaca. In these foci the *O.volvulus* is mainly transmitted by *Simulium ochraceum* senso lato (Ortega et al., 1992).

The pathological and entomological studies on onchocerciasis have revealed that the ecological and behavioral factors and people's intervention play important role in control of the disease. A number of attempts have been made to draw relationship between onchocerciasis and migration of people. Migration in simple terms takes place where certain conditions in the place of origin are inferior to that of the destination place, which has a greater influence on people moving away from their homes. The rural areas are backward underdeveloped and lacks basic facilities thereby not attracting economic investment thus affecting the overall development. Therefore, people migrate to other cities and towns in anticipation of better opportunities that offer better social and economic status. The rural to urban migration often caused by economic considerations and rural deprivation have been playing important role inducing migration from the endemic communities. In the America the infected migrants who do not receive the ongoing ivermectin treatment may serves as an infection reservoir playing a significant role in the transmission of the disease and eventually affecting parasite elimination from the region (Maia-Herzog et al., 1999). However the actual impact of such temporary migration of population has seldom been ascertained but there were, fortunately, few interesting studies.

Migration and infectious disease have always been interacting with each other over centuries, long before researchers had the ability to understand the causes of disease and disease agents that caused migration of people to safer location. In spite of several attempts being made to control onchocerciasis by various control programmes which

were able to eliminate severe pathological manifestations and reduce its morbidity through mass distribution of ivermectin in certain areas but transmission still continue in few areas (Meridith and Dull, 1998; Cupp et al., 1992). Transmission have been declared eliminated in the Northern Chiapas focus in 2007, but interruption, and suppression of transmission in Oaxaca, and the Southern Chiapas focus was not documented until 2008 and 2010, respectively (Rodríguez-Pérez et al., 2008a, 2010a, b).

In Mexico, Southern Chiapas or Soconusco is the main onchocerciasis affected focus. It shares its borders with the Northwest Guatemalan focus and hence is included in a single endemic region (WHO, 1991). The *O. volvulus* is mainly transmitted by *S. ochraceum sensu* lato. The endemic area in the Soconusco (Chiapas) is in the Sierra Madre de Chiapas, a mountain range that runs parallel to the Pacific coast. The fly vectors are not able to survive in the atmosphere of the coastal plains making these areas free of the disease. The relevant environments for the vector *S. ochraceum* are the zone above the sea level with 500-1500 meters on the Western slopes of the mountains and the coffee producing zone. In 1846, the coffee production began in the Sierra de Chiapas, followed by development of large fincas, or coffee plantations, with annual yield of more than 216 million kilograms of coffee beans (Secretaria de agricultura, 1990).

Migration of laborers across Mexico-Guatemala border could spread the disease within the endemic foci (WHO, 1995). It has been shown that onchocerciasis is

associated with coffee plantation in these areas and transmission peaks with the harvesting season (Brandling-Bennett et al., 1981; Vásquez Castellanos, 1991). The origin of the onchocerciasis in Southern Chiapas was due to the migration of laborer's working at the coffee plantations from Guatemala, which shares its borders with Southern Chiapas. The disease spread to Northern Chiapas is possibly due to the annual visits of the residents from Northern Chiapas focus for the coffee harvest to Southern Chiapas where the transmission level was high, whereas the infection with the filarial parasite in Oaxaca might be due to the movement of people from Oaxaca to other endemic regions of Chiapas and Guatemala performing religious pilgrimages (Vásquez Castellanos, 1991). The migration of laborers from endemic and non-endemic areas along with people who do not receive treatment under the mass treatment programme contribute in transmission of the disease serving as parasite reservoirs (Rodríguez-Pérez et al., 1995).

The main occupation of the residents in Las Golondrinas, Nueva America and Rosario Zacatonal is coffee plantation. These villages receive temporary laborers bi-annually or quarterly who could be eligible for mass treatment with ivermectin but at the time of treatment, some temporary migrants would not be present or receive treatment and or not in the records of the onchocerciasis elimination programmes. These migrants might have been treated with ivermectin while at home or it is also possible and most likely that these people could have missed ivermectin treatments at home as they were away when treatment were given. Hence temporary coffee workers could have missed treatments at both home and away (Guatemala and Mexico). The coffee plantation and

cultivation includes two seasons each year namely, planting-clearing and harvesting. Normally planting-clearing is carried out from May to July, whereas harvesting is done from November to February. The harvesting season coincides with onchocerciasis transmission found to be high in Guatemala (Rodríguez-Pérez et al., 1999).

Most of these laborers arrive in groups mainly from Guatemala before the start of each coffee season and return after completion of their farming season. Coffee workers were investigated in order to access the vector population and effect of human migration in transmission of onchocerciasis from 1997-1999. All examined migrant laborers who were positive were offered ivermectin treatment. The migrant workers who stopped receiving treatment were immediately reported to Mexican Onchocerciasis Elimination Programme. This programme continues the bi-annual treatment of residents in villages and in 2003 onwards-migrant laborers working at the coffee plantations were mainly targeted for treatment in order to control the transmission of the disease.

The onchocerciasis control programmes in Mexico begin in 1989 with the treatment with ivermectin (Mectizan). In the beginning, treatment was given only to patients who should positive for nodulectomy or Mazzoti reaction and those who were resident of hyper endemic villages. Bi-annual treatment with ivermectin from 1991-1994 was extended to all eligible residents of meso and hypo endemic villages. The coverage of ivermectin treatment in hypo-endemic villages was increased in order to reach all

eligible residents of the village from 1995-1997. From 1997 onwards the strategy was modified to provide bi-annual mass treatment of ivermectin to all eligible residents from all the at risk villages that is from hypo to hyper endemic villages and more emphasis was given to eliminate onchocerciasis form these regions.

This chapter describes the results of the study conducted to evaluate the prevalence of intensity of the micro filarial infection in the residents and migrant population in two coffee fincas of Southern Chiapas focus in order to ascertain the factors affecting the transmission of the disease from humans to vectors of migrants (untreated workers). This study also includes the entomological survey that was carried out during the planting-clearing and harvesting season of 2009-2010 from May through July and November through February, respectively.

Study area

In this chapter the two coffee fincas included for the entomological studies were Finca Victoria and Finca Santa Amalia. The Geographical distribution is shown in the Fig. 14.

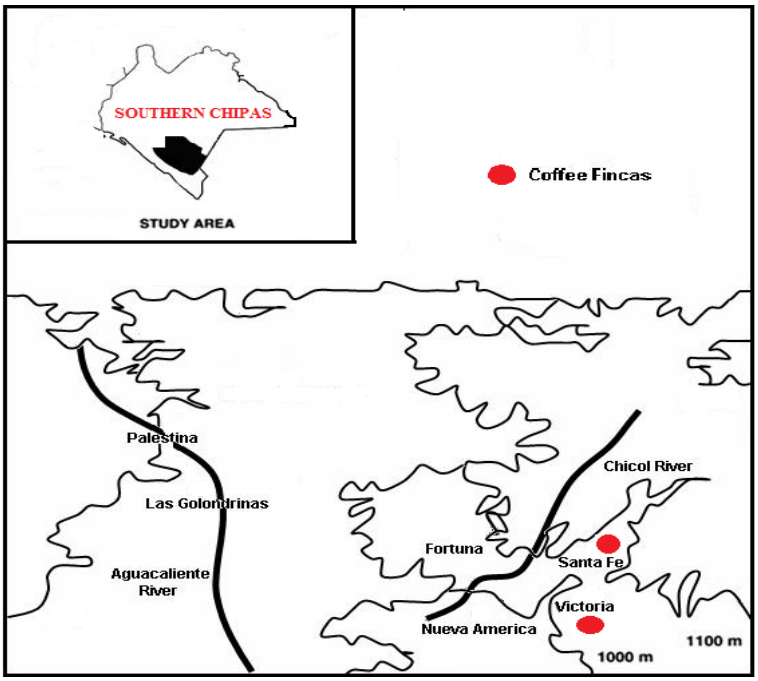


Fig. 14: Distribution of study areas in S. Chiapas (Courtesy: Rodríguez-Pérez et al., 2007)

RESULTS

In this study, a total of 22,561 host seeking *S.ochraceum* flies were collected during the period of November through April (2009-2010) respectively from Finca Victoria and Finca Santa Amalia endemic communities for *O.volvulus* in Southern Chiapas (Table 5). The results obtained by PCR screening were used to calculate a prevalence of infected and infective flies in the vector population along with 95% CIs. To calculate the seasonal transmission potential, the prevalence of infective flies was combined with biting rate. The results are summarized in Table 6. In the community, Finca Victoria a total of 233 pools (n=233 pools, 11,540 flies) each pool containing 50 heads or bodies were examined by PCR. None of the body/head pools were positive for *O. volvulus* DNA, which represent a low or non-existence rate of parasite vector contact and assimilating lack of transmission. Thus, the prevalence of infective flies was 0 with (95% upper limit (UL) is 0.3/2,000 flies). Correspondingly, the seasonal transmission potential was 0 with upper bound of the 95% CI (7.5) L3 per person. In Finca Santa Malia (n=223 pools, 11,021 flies), all body pools tested were negative for *O.volvulus*. However, the prevalence of infective flies was 0 (95% upper limit (UL) is 0.4/2,000 flies).

From each community, more than 10,000 flies were collected which comply with the WHO guideline and also the samples were enough to exclude 1/2000 in the UL of the 95% CI. The point prevalence of infective flies and seasonal transmission potential was

0, the situation met OEPA criteria for “absence or near absence” of transmission. The two coffee fincas included in this study receive a peripatetic population of migrant workers twice a year. In 2007, Rodríguez-Pérez et al., (2007) published the infection in migrants, resident populations and entomological studies and infection in the vectors, in which flies collected during 1997-1998. As described, a total number of 35-850 individual migrants in the coffee fincas of each season in 1997-1998 and 1998-1999 varied widely. During each coffee season, new people are hired for working in coffee field.

Table 5: Total number of *Simulium ochraceum s.I* flies examined from two communities in Southern Chiapas, Mexico

Communities/year	Number of pools	Number of Flies	Number of positive heads
Finca Victoria	233	11,540	-
Finca Santa Amelia	223	11,021	-
Total	456	22,561	**-**

Table 6: The prevalence of infective flies, seasonal biting rate and seasonal transmission potential estimated in coffee fincas, Southern Chiapas, Mexico, evaluation 2009- 2011

Focus/Year 2009-2010	Number of positive heads	Seasonal biting rate	Prevalence of Infective rate *IC(1/2000)	Seasonal transmission potential
Finca Victoria	0	50,282 (43,079-58,642)	**0** (0.3)	**0** (7.5)
Finca Santa Amalia	0	36,250 (32,588- 40,303)	**0** (0.4)	**0** (7.3)

* Expressed as rate per 2000 flies examined.
* Bold numbers represent point estimated and value in parentheses represents 95% upper limit confidence interval surrounding point estimate

In all the coffee fincas, the infected migrants, and the microfilariae prevalence was ranging from 14% (10-20%) in 1997 and 7% (2-16%) in 1998 (Finca Victoria, Finca Santa Amalia and Nueva America). The prevalence of skin microfiladermia was found to be 2.8% and 0.3% for the years 2004 and 2006 respectively after 13 and 15 years of ivermectin treatments for the formerly hyperendemic communities Las Golondrinas and Las Nubes.

In the coffee fincas the proportion of parous flies was significantly higher. The percentage of the parous flies in fincas (Finca Victoria, Finca Santa Amalia and Nueva America) was 68.2% (95 CI = 67.5-68.8%). Despite, the number of flies harboring *Onchocerca volvulus* larvae of any stage per 1,000 parous flies was low 2.3 (1.5-3.2) in these localities. Subsequently, the number of infected *Simulium* was higher during the former compare to the latter season of the coffee season.

DISCUSSION

From the data of the present studies, the transmission of *O.volvulus* has been dramatically reduced in the Southern Chiapas focus in Mexico, where no evidence of current infection or recent transmission found in these two fincas in Southern Chiapas focus. However, in these studies, multiple (annual, bi-annual & quarterly) treatments with ivermectin have been shown to reduce transmission of *O. volvulus* from 92% to 95%. The ivermectin treatment in Chiapas has reached at least 85% of the eligible population at

risk since 2004; (Yamagata et al., 1986). The required rounds of the ivermectin treatment were completed before conducting evaluations during 2009-2010.

The entomological data is very useful to the Mexican National Onchocerciasis Control Programme, which aims to eliminate onchocerciasis transmission with quarterly and bi-annual treatments with ivermectin. This achievement is based on the attribution that ivermectin can reduce the skin microfilariae load level that results in a reduction of transmission to a rate that is below what is necessary for the parasite population to maintain itself (Collins et al., 1995). The most important fact that measurement of transmission levels is one of the most direct methods of assessing a programme progress toward the major key goal of preventing transmission (Yameogo et al., 1999).

The endemic area for Onchocerciasis in the Soconusco (Chiapas) is generally in the Sierra Madre de Chiapas, a mountain range that runs parallel to the Pacific coast. The disease is free in the coastal plain, where fly vectors cannot survive in these atmosphere, since hydrology does not support their reproduction. The relevant environments for the vector *S. ochraceum* are the zone above the sea level with 500-1500 meters on the Western slopes of the mountains and the coffee producing zone with annual yield of more than 216 million kilograms of coffee beans (Secretaria de agricultura, 1990). In 1846, the coffee production began in the Sierra de Chiapas, consequently development of large

fincas, or coffee plantations. Thus, the origin of the disease in the Sierra de Chiapas was associated with migratory patterns determined by economic factors.

The proportion of the infected flies was found to be higher during the planting period from May through July than during the harvesting period. An earlier study on the parous flies during 1990-1991 has shown that the parous flies were found in higher proportions in the coffee finca areas of the villages than in the village. This was carried out before the introduction of ivermectin mass treatment (Basáñez et al., 1998). In 2003 the biannual ivermectin treatment strategy was modified in many of the hyperendemic foci to four times per year to attain a higher coverage and interrupt the ongoing transmission. A new strategy was adopted to include and treat all temporary coffee workers (Rodríguez-Pérez et al., 2007).

By the migration of the infected flies and infected people from endemic to non - endemic areas, there is a chance of spreading disease. The migrant population working in the fincas varies during each coffee season. Migration can result in new foci of the disease if sufficiently large reservoirs of parasites accumulate and adequate vectors are present. In Guatemala, already it has been reported that the migration of infected persons, travelling from non-endemic areas to north make annual visit to the Yepocapa focus usually become infected with onchocerciasis during coffee harvesting season, when the biting rate of *S.ochraceum* is at its peak (Dalmat , 1955). Studies on migrants carried out

during two coffee harvest seasons showed a prevalence of 0.6% (95% CI= 0-3.2%). Thus, indicating that migrants are unlikely to play a major role in transmission of onchocerciasis and that transmission in the endemic area of Guatemala is decreasing rapidly (Lindblade et al., 2009)

The prevalence of infective rate determined in the present is 0 with 95 % CI 7.3/2,000 flies), and in FSA 0 (95%CI= 7.5/2,000 flies). According to the results obtained during this evaluation, the infection has decreased compare to the earlier entomologic data. The prevalence of infective flies estimated in 2004 was found to be 0.31 and 0.8 for Las Golondrinas and Las Nubes with a seasonal transmission potential of 14.5 and 10.6 L3s per person per year respectively. In 2006 it was found to be 0 and the estimated STP was also 0 L3s per year per person for the two regions. The pre-treatment prevalence of infection was 78% (95% CI=72.9-82.6%) in Las Golondrinas, which was reduced to 0.3% with effective ivermectin treatment for 15 years.

In Ecuador because of the migration of infected populations from the Santiago focus, the focus has been infected with onchocerciasis (Shelley and Arzube, 1985). One has to consider that migration is a serious problem for the disease control despite multiple treatments with ivermectin. The transmission persists in the Rio Cayavas of Ecuador, and more or less it is partly due to significant migration of potentially infected individuals

from hyperendemic communities along the Rio Cayapas to Santiago and vice versa (Vieira et al., 2007).

In Southern Chiapas focus, after seven years of bi-annual treatment of ivermectin, the prevalence of transmission was still not reduced, hence the bi-annual treatment was modified to quarterly treatment in the year 2004 for the suppression of the infection. The data obtained from entomological studies conducted during 2001 in Southern Chiapas demonstrated that, many villages had occurrence of infected and infective S.ochraceum in the host seeking vector population showing that transmission has not been interrupted or suppressed (Rodríguez-Pérez et al., 2004 ; Rodríguez-Pérez et al., 2006).

All the accumulated data suggest that the ivermectin distribution program has been a big success in suppressing the overall transmission levels of onchocerciasis and significantly reduced the skin microfilariae, corneal morbidity and the presence of microfilariae in the anterior lobe of the eye. The absence of new clinical cases of onchocerciasis in Southern Chiapas focus over the last decade indicates that there is no parasite transmission occurring in these foci and also that it's no longer a serious threat to the endemic communities. But there is still a possibility of new cases being identified in this region due to migration of people from other areas and neighboring states, however, with successful suppression of the onchocerciasis transmission from Northern Chiapas and Oaxaca foci making these chances very superficial.

CONCLUSION

The occurrence of transient infected labors in and out the Southern Chiapas, appearing failure of people to receive regular treatment retains the transmission of the parasite from humans to the plenteous local vector populations. Another reason could be that due to the reduction in coffee price in the worldwide, movement of coffee laborers has extremely decreased in Southern Chiapas. Since the infection rate and transmission is zero in these two coffee fincas, the program may halt ivermectin treatment during 2012. In these communities in Southern Chiapas OEPA program has declared that the transmission has been successfully suppressed. Further surveillance should be carried out in order to identify the transmission rate and to declare interruption of transmission of onchocerciasis in these communities and surrounding coffee fincas.

9: CHAPTER V: PCR MONITORING OF *ONCHOCERCA VOLVULUS* TRANSMISSION IN THE SOUTHERN CHIAPAS ONCHOCERCIASIS FOCUS

Onchocerciasis or river blindness is a chronic and slow progressive disease. This infection has been one of the major causes of blindness in the world (Hotez et al., 2008). Despite being partially controlled by international prevention programs, it still remains the major health hazard and is endemic in Africa, Arabia and the Americas. Elimination of onchocerciasis in Latin America is targeted by OEPA through mass distribution of ivermectin. It has been assumed that this drug will be able to reduce the microfilarial load to a level that is below the level that is required to maintain the transmission, thus leading to elimination of the infection. To achieve this goal, WHO has put forward a set of criteria for certifying an area free of onchocerciasis (WHO, 2001). At present two different measures of transmission suppression have been suggested by WHO.

The suppression of infectivity is defined as 99% reduction based on the pre-treatment data available for some areas with the pre-treatment rates. For areas where pre-treatment data is not available, suppression of transmission is defined as absence or near absence of L3 larvae in the vector population and absence of infection in the humans i.e., infectivity level not more than one in 10,000 flies carrying the infective larvae.

Southern Chiapas (Soconusco) is considered as one of the major endemic foci of onchocerciasis in Mexico. Southern Chiapas is contiguous with the Huehuetenango focus

in Guatemala forming the endemic region. The onchocerciasis elimination program for the Americas (OEPA) has been successful in eliminating the severe pathological manifestations and also in reducing the morbidity through mass ivermectin distribution, the only drug of choice that kills microfilariae (mf) (WHO, 2006). The main goal of OEPA is to eliminate the disease and reduce the ocular morbidity through mass distribution of ivermectin drug (Cupp et al., 2004).

In order to use chemotherapy as a mode of suppressing the transmission of onchocerciasis and to eliminate onchocerciasis it is important to monitor the levels of transmission. Estimation of the transmission levels of *O. volvulus* is also important for evaluating the overall effectiveness of the National Onchocerciasis Control Programmes. Manual dissection of the flies was used as standard traditional method for the detection of larvae to estimate the intensity of transmission of *Simulium* vector (Walsh et al., 1978). This method holds good for measuring the transmission levels in the areas where infections rates are higher i.e., high prevalence of infection in the vector population and in areas that are not under the influence of control programs. Moreover, with the success of the control programs in reducing the infection rates, the prevalence of infection in the vector population has also reduced. Therefore, the traditional method for detecting the infection rates is not efficient and is relatively labor intensive making it impractical for the routine surveillance of the vector.

A new method based on the Polymerase chain reaction (PCR) amplification of the *Onchocerca* specific repeated DNA sequence (O-150) was developed as an alternative

for detecting the *O. volvulus* and to differentiate between the blinding and the non-blinding strains of the parasite (Meredith et al., 1991; Zimmermann et al., 1992). In this method, the PCR amplification of the *Onchocerca* repeated sequence (O-150) is done followed by hybridization with *O. volvulus* species-specific DNA probe (OVS2) and its detection by ELISA (Nutman et al., 1994; Toè et al., 1994). The O-150 PCR can detect a single *O. volvulus* infected fly in the pools containing about 100 non-infected flies with a sensitivity and specificity more than 90% (Katholi et al., 1995; WHO, 1995; Merriweather & Unnasch, 1996). It is possible to calculate the prevalence of infection in the black flies based on the pool size and the percentage of negative pools found using the mathematical model (Katholi et al., 1995). A method for separating the heads and bodies of the black flies in bulk has been developed (Yameogo et al., 1999). Thus, allowing one to estimate the prevalence of L3 or vertebrate infectious stage parasite in the heads and developmental stage L1 and L2 larvae in the bodies. The field studies carried out in Africa and Mexico using O-150 PCR assay method for estimating the prevalence of infected and infective flies in the vector populations have been in accordance with the traditional dissection methods that were routinely used (Rodríguez-Pérez et al., 1999). Thus, allowing the use of O-150 PCR for monitoring the transmission in the areas with low levels of infection in the vector populations due to success of the control programs would be beneficial.

In Mexico, the onchocerciasis control program started treatment-using ivermectin in 1989. In the beginning only the individuals showing the symptoms were treated in the hyper-endemic communities. The biannual treatment with ivermectin was then extended

from 1991-1995 to cover all the symptomatic individuals and residents of the meso-endemic and hypo-endemic communities. In 1995, mass treatment with ivermectin was made available to all eligible residents of all the communities that were at risk regardless of the endemicity. The data collected in 2001 suggested that the onchocerciasis transmission was still continuing in this focus. Therefore, this strategy was further modified in 2003 to cover most of the formerly hyperendemic communities of Southern Chiapas focus to provide the mass treatment with ivermectin four-times per year for 50 communities. The bi-annual treatment was continued in meso-endemic and hypo-endemic communities of this focus (Rodríguez-Pérez et al., 2004, 2006).

Onchocerciasis has been mainly associated with the coffee plantations in the Mexico-Guatemala region and the peak transmission of L3 larvae occurs in late dry season in association with the presence of old multiparous *Simulium ochraceum* sensu lato female flies (Brandling-Bennett et al., 1981; Rodríguez-Pérez and Reyes-Villanueva, 1994; Vásquez Castellanos et al., 1991). The origin of onchocerciasis in Southern Chiapas has been attributed to the migration of coffee plantation workers from Guatemala (Davies 1968; Vásquez Castellanos et al., 1991). With the use of microfilarial drug ivermectin the severe clinical symptoms of the disease have been eliminated. The adult worms are not affected but on repeated exposure to ivermectin, fertility and survival of adult worms is affected (Cupp et al., 2004).

The entomological and parasitological data collected in 2001 suggested that the transmission was still going on in Southern Chiapas focus. In spite of achieving

consistent reduction in the transmission rates, new cases have been appearing in these communities and mainly amongst the children less than 5 years of age. During the past four year (2003-2006) the ivermectin program has achieved higher rates of coverage in the endemic communities of S. Chiapas. The ivermectin coverage has been above 85% for each year from 2003-2006 with an average coverage being 90.1%. The recent data suggest that the transmission has been suppressed in this focus (Rodríguez-Pérez et al., 2008a).

Study area

The flies were collected from different communities from the Southern Chiapas foci (Fig. 15). In this study four sentinel communities; Amplacio Malvinas (92°28´24 ″W, 15°20´36 ″N, 1000 masl), Estrella Roja (92°28´48 ″W, 15°16´11″N, 660 masl), Jose Maria Morelos (92°27´35 ″W, 15°13´48 ″N, 1400 masl), Nueva Costa Rica (92°48´46 ″W, 15°28´01 ″N, 600 masl), and seven extra-sentinel communities; Brasil (92°28´48″ W, 15°13´48″ N, 1056 masl), Mexiquito (92°27´35 ″W, 15°15´0″N, 1143 masl), Coronado Santa Rita (92°28´19″W, 15°13´04″N, 1375masl), Loma Bonita (92°26´29″W, 15°35´22″N, 1405 masl), Montowa (92°28´04 ″W, 15°35´22″N, 1034 masl) , La Granja (92°28´50″W, 15°14´41″N, 920 masl) and La Soledad (92°27´42 ″W, 15°13´33″N, 1075 masl) of Southern Chiapas have been included. Ivermectin mass treatment was provided to all the eligible residents of all the communities in Southern Chiapas focus. From 1994-2010, biannual ivermectin treatment has been provided i.e., 22 rounds of treatment given over the last 13 years with coverage being greater than 85% every year from 2001 to 2008.

Black flies were collected between January and May, 2009 to 2011 coinciding with the known peak of in the area in accordance with the standard procedures (Rodríguez-Pérez et al., 1995, 2004; Walsh et al., 1978). The flies were collected from two different sites for each community simultaneously, one from the nearby coffee plantation and the other from within the community. *S. ochraceum* s.l. was collected by teams of volunteers using landing-bait method. Collectors received ivermectin treatment one week before the starting the collection process. Black flies were collected before they start feeding. An estimate of the biting rate was determined by measuring the landing rates during the collection of flies.

Black flies were divided into pools with 50 individual flies per each pool followed by separation of heads and bodies as described previously (Rodríguez-Pérez et al., 2004). The heads and bodies that were separated as mentioned in the section of materials and methods. The proportion of infection in the vector population was determined from the proportion of the body pools that were positive in the PCR assays and was further expressed as the number of positive flies per 2000 flies tested. If none of the body pools were found to be positive then it was assumed that there is no detectable parasite-vector contact and the associated prevalence of infectious flies would be zero. The results obtained from PCR were used to calculate the prevalence of infective flies in the vector population along with 95% CI's. The prevalence of infective flies was then combined with estimates of the biting rate to calculate an estimated seasonal transmission potential (STP).

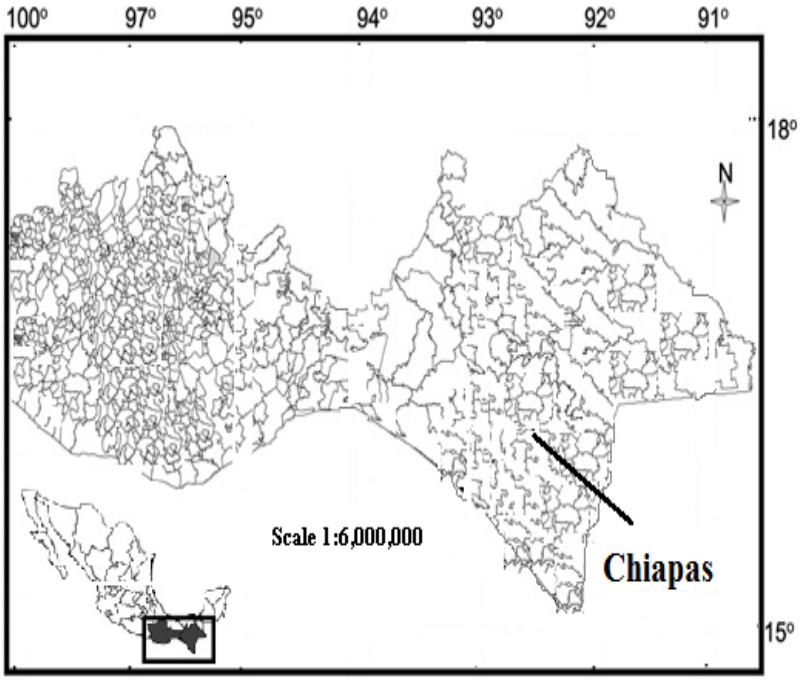


Fig.15. Geographical representation of Chiapas in Mexico

Results

In the present study, a total of 42,931 flies were subjected to analysis by PCR in 922 pools (Amplacio Malvinas N=207; Estrella Roja N=223; Jose Ma. Morelos =242; Nueva Costa Rica N=250) in the sentinel communities in the year 2009-2010 (Table 7). In the communities of Estrella Roja, Nueva Costa Rica and Morelos the calculated prevalence of infective rate was 0 (0.4; 95% -UCI) while the seasonal transmission potential being 0 (5.4, 7.8 and 18.4 95% UCI). In the community Amplacio Malvinas the calculated prevalence of infected rate was 0 (0.5; 95%-UCI) while the seasonal transmission potential 0 (3.7 95% UCI). The results were shown in the Table 8.

In extra-sentinel communities during 2009-2010 flies were collected from different communities (Table 9) and examined by PCR in 893 pools (Brasil N=239; Mexiquito N=223; Santa Rita N=205; Loma Bonita N=57; L Granja N=19 and La Soledad N=66). One head pool in Brasil and two head pools in Mexiquito were found to be positive to PCR-ELISA, all the body pools tested for rest of the communities were found negative. In the negative communities, the overall prevalence of infective flies of 95%-UCI ranged from 0.3- 4.1 (Table 10). Upper confidence interval limits of the STPs ranged from 5.7 to 12.6 L3 per person per season. In a few of the tested communities the flies collected were not sufficient to comply with the criteria of WHO/OEPA which demands at least 10,000 flies. The prevalence of 95%-UCI of infective flies in Brasil was 0.3 (0-0.7 95% UCI) and estimated STP was 7.5 (0-21.1 95% UCI) per person per season. In the community Mexiquito, the calculated prevalence of infective flies was 0.4

Table 7: *Simulium ochraceum* s.I. collected and number of flies examined from four sentinel communities in Mexico, 2009-2010

Communities	No. of pools tested	No. of Flies examined	No. of positive head pools
Amplacio Malvinas	207	8,517	-
Estrella Roja	223	9,435	-
N.Costa Rica	250	12,069	-
Morelos	242	12.910	-

Table 8: Prevalence of infected, infective flies and seasonal transmission potential estimated in sentinel communities, Southern Chiapas, Mexico, evaluation 2009- 2010

Communities	Seasonal biting rate	Infective rate (1/2000)	Seasonal transmission potential
A. Malvinas	14,111	**0** (0.5)	**0** (3.7)
Estrella Roja	14,628	**0** (0.4)	**0** (2.9)
N. Costa Rica	38,753	**0** (0.4)	**0** (7.8)
Morelos	102,020	**0** (0.4)	**0** (18.4)

*Bold numbers represent point estimated and value in parentheses represents 95% upper limit confidence interval surrounding point estimate

Table 9: *Simulium ochraceum* s.I. collected and number of flies examined from seven extra- sentinel communities in Mexico, 2009-2010

Communities	No. of pools tested	No. of positive head pools
Brasil	239	1
Mexiquito	223	2
Santa Rita	205	-
Loma Bonita	84	-
Montowa	57	-
Granja	19	-
Soledad	66	-

Table 10: Prevalence of infected, infective flies and seasonal transmission potential estimated in extra-sentinel communities, Southern Chiapas, Mexico, evaluation 2009-2010

Communities	Seasonal biting rate	Infective rate (1/2000)*	Seasonal transmission potential
Brasil	60,183	**0.3** (0-0.7)	**7.5** (0-21.1)
Mexiquito	83,038	**0.4** (0.2-1.0)	**16.6** (8.3-42.3)
Santa Rita	51,720	**0** (0.4)	**0** (10.3)
Loma Bonita	14,278	**0** (0.9)	**0** (6.4)
Montowa	9,390	**0** (1.3)	**0** (6.1)
Granja	3,142	**0** (4.1)	**0** (6.4)
Soledad	10,311	**0** (1.1)	**0** (5.7)

*Bold numbers represent point estimated and value in parentheses represents 95% upper limit confidence interval surrounding point estimate

(0.2-1.0 95%-UCI) followed by estimated STP 16.6 (8.3-42.3 95 % UCI) L3 per person per season.

Further evaluation was carried out during 2010-2011 a total of 45,363 flies were collected from different communities and tested by PCR in 914 Pools (Brasil N=252; Mexiquito N=265; Morelos N=275; and Estrella Roja N=122) in extra-sentinel communities. All the body pools tested in extra-sentinel communities were found to be negative (Table 11) for the *O. volvulus* DNA and these results indicate the absence of parasite vector contact hence, none of the head pools were tested further. The overall prevalence of infective flies of 95%-UCI ranged from 0.3- 0.6 (Table 12). Upper confidence interval limits of the STPs ranged from 2.3 to 12.8 L3 per person per season. The total number flies collected from each community were increased from 11,021 to 13,174 flies. The number of vectors collected complied with the WHO guidelines of having more than 10,000 flies tested from each community. In all the cases, the samples collected were sufficient to exclude 1/2000 in the UL of the 95%.

Table 11: *Simulium ochraceum* s.I. collected and number of flies examined from seven extra- sentinel communities in Mexico, 2010-2011

Communities	No. of pools tested	No. of Flies examined	No. of positive head pools
Brasil	252	12,491	-
Mexiquito	265	13,174	-
Morelos	275	13,692	-
Estrella Roja	122	6,006	-

Table 12: Prevalence of infected, infective flies and seasonal transmission potential estimated in extra-sentinel communities, Southern Chiapas, Mexico, evaluation 2010-2011

Communities	Seasonal biting rate	Infective rate (1/2000)	Seasonal transmission potential
Brasil	58,732	**0** (0.3)	**0** (8.8)
Mexiquito	81,338	**0** (0.3)	**0** (12.2)
Morelos	85,657	**0** (0.3)	**0** (12.8)
Estrella Roja	7,530	**0** (0.6)	**0** (2.3)

*Bold numbers represent point estimated and value in parentheses represents 95% upper limit confidence interval surrounding point estimate

The new pool screen version 2.01 developed recently by Katholi and Unnasch was used to analyze the entomological monitoring data of 2010 from S. Chiapas (Table 13) as an example in order to evaluate its application. This version is a more realistic model that can be used to calculate the annual biting rates based upon actual collection data from Ecuador and Mexico. The old pool screen version was a very simplistic method for calculating seasonal biting rate, which was calculated from the geometric means of the counts per day and multiplied by the number of days in the season. Whereas, the new pool screen version 2.01 can calculate the Seasonal transmission potential, infective rates, prevalence of infection, seasonal biting rates with 95% UCI at one go. The new version can be used to process large number of samples in more efficient way.

Discussion

The epidemiological parameters for onchocerciasis transmission and its morbidity obtained recently strongly suggest that the transmission of *O. volvulus* is reaching a stage of permanent interruption in Southern Chiapas focus of Mexico. The continuous use of semi-annual treatment with high coverage rates of ivermectin have been very important in order to meet the criteria of OEPA's goal of declaring an area being interrupted of transmission and achieve the elimination of onchocerciasis. The control programs have been successful in interrupting the transmission of onchocerciasis in the nearby foci with progress being made for its elimination from these areas. This area includes the foci of Northern Chiapas and Oaxaca in Mexico, Santa Rosa, Huehuetenango and Escuintla-Guatemala in Guatemala, Lopez de Micay in Columbia.

Table 13: Prevalence of infected, infective flies and seasonal transmission potential estimated in few communities of Southern Chiapas, Mexico, evaluation 2009- 2010 using new pool screen version 2.01

Communities	Seasonal biting rate	Infective rate (1/2000)	Seasonal transmission potential
Brasil	148,350	**0.16** (0-0.5)	**16.7**(0-51.6)
Mexiquito	200,490	**0.36** (0.2-1.0)	**45.1** (0-97.9)
Santa Rita	130,251	**0** (0.5)	**0** (31.6)
Loma Bonita	117,395	**0** (1.4)	**0** (4.8)
Montawa	80,028	**0** (2.0)	**0** (4.7)
La Soledad	98,030	**0** (1.8)	**0** (5.0)

*Bold numbers represent point estimated and value in parentheses represents 95% upper limit confidence interval surrounding point estimate

The entomological and epidemiological evaluation of the effectiveness of ivermectin treatment of *O. volvulus* transmission in the endemic foci of onchocerciasis in Southern Chiapas in Mexico is presented in this chapter. All the accumulated data suggest that the ivermectin distribution program has been a big success in suppressing the overall transmission levels of onchocerciasis and significantly reduced the skin microfilariae, corneal morbidity and the presence of microfilariae in the anterior lobe of the eye. The absence of new clinical cases of onchocerciasis in Southern Chiapas focus over the last decade indicates that there is no parasite transmission occurring in these foci and also that it's no longer a serious threat to the endemic communities. But still there is a possibility of new cases being identified in this region due to migration of people from other areas and neighboring states, however, with successful suppression of the onchocerciasis transmission from Northern Chiapas and Oaxaca foci making these chances very superficial.

The Mexican National Onchocerciasis Control Program aims to eliminate the onchocerciasis transmission by using bi-annual ivermectin treatments. This is based on the hypothesis that ivermectin can decrease the skin mf to a level that can reduce the transmission to a rate that is less than that required for the parasite to maintain itself. The suppression effect of ivermectin has been found to be more effective in case of *S. ochraceum* s. I. is the vector as in Mexico as this vector needs to feed on people having high microfiladermia in order to form enough infective stage larvae (Collins et al., 1995).

The infection and transmission was not distributed evenly due to differences in the parous infection rate (body pools) and infective rates (head pools) from different points where flies were collected from the same state. In Morelos and Amplacio Malvinas of Southern Chiapas, the infective rate in the parous flies was found to be 5.9 and 22.8, respectively per 10,000 parous flies. The overall vector infection and infective rates were higher for Southern Chiapas than for other two foci. The higher rates may be due to the migrant population. The migrant population working in the coffee plantations was not included for the ivermectin treatment and this represents about 40% of the total Southern Chiapas population during the peak transmission period. Thus, migrants form a significant parasite reservoir that has to be included in the ivermectin treatment program (WHO, 1995; Boatin et al., 1997).

In the tested sentinel and extra-sentinel communities of Southern Chiapas, skin biopsy, and parasitological and ophthalmologic evaluation data are available and all were found to be negative for *O. volvulus*. The entomological studies conducted in 2009-2010 year suggested that infection rates were significantly less and the criteria set by OEPA to regard an area free of infection were met for all the above-mentioned areas in foci of S. Chiapas. Therefore OEPA has decided to continue the administration of ivermectin in these areas for another year and then stop the treatment with ivermectin. Thus, later on the post-treatment surveillance phase would be initiated in order to monitor the reoccurrence of the disease.

CONCLUSION

After coverage with Mectizan/ivermectin treatment conducted from 1995 to 2011 and according to the various indicators of morbidity and entomological studies conducted during 2009-2010 in Chiapas, it can be declared that the transmission has been suppressed. Further evaluations conducted from 2010-2011, coverage of quarterly ivermectin/Mectizan treatment in this focus and applying the criteria for certification of the elimination established by WHO, in southern Chiapas, it can be concluded that the transmission of onchocerciasis has been interrupted. The ivermectin may be halted during 2012 and further post treatment surveillance should be carried out in this focus for testing the disease re-occurrence.

10: CHAPTER VI: TO EVALUATE DNA PURIFICATION PROTOCOLS BASED ON SILICA AND OLIGO NUCLEOTIDE MAGNETIC BEAD METHOD FOR PARASITE *O.VOLVULUS* DNA IN POOLS OF BLACK FLIES

The major challenge ahead of the Onchocerciasis Elimination Program for the Americas is to be able to assist the participating countries to detect the residual activities when this program approaches winds up activities by the end 2012. To monitor the epidemiological surveillance and to access the success of the control program, an effective tools or methods for detecting the recrudescence of *O. volvulus* infection is needed. For this, the highly needed of these methods of surveillance of *O. volvulus* is to detect new infections in the endemic populations. Hence, several new methods are being tested and quantified in order to replace the age-old skin-snip method that is becoming less acceptable and be a subject of ethical criticism because of the fear of HIV/AIDS and other blood borne-diseases (Bradley and Unnasch, 1996). Surveillance activities to detect the new infections are useful to detect *O. volvulus* infective larvae in the vector population because the presence of *O. volvulus* infective larvae is the direct indicator of the transmission of infection. Early detection of the transmission is very important to rapidly identify the recrudescence of infection as development of infection in humans takes 18 months to 2 years (WHO, 1987).

O. volvulus transmission has been traditionally determined by dissection of wild caught vector black flies (Walsh et al., 1978; Walsh, 1983). This method can efficiently detect the transmission in areas where onchocerciasis is hyper-endemic or meso-endemic

with prevalence of infection in the vector population is very high (Cheke et al., 1992). However, with the success of control programs the prevalence of infection in the vector populations has drastically reduced. Hence, the classical dissection method to estimate the prevalence of infection is not efficient in areas where the infection rates are less.

Few years ago, a method based on PCR amplification of an *Onchocerca* DNA sequence (O-150) was developed to identify *O. volvulus*. It has been extensively used to detect and identify the specific repeated sequence of *O. volvulus* (Katholi et al., 1995; Merriweather and Unnasch, 1996). In a population of vectors with a low prevalence of infection, as it occurs currently in areas with a successful elimination programmes based on mass ivermectin treatment, large vector samples have to be processed in order to obtain an accurate estimate of the infection and infectivity. The development of O-150 PCR-ELISA assay and the Katholi´s algorithm (Katholi et al., 1995) have become the gold standard in determining the point of prevalence of infected and infective flies together with the associated confidence intervals in the black fly vector populations in Africa and Latin America (Rodríguez-Pérez et al., 2010a; b; Viera et al., 2007; Gonzalez et al., 2009). However, it has to be investigated to find out what is the maximum value for the upper bound of the 95% confidence interval for transmission rates in areas where transmission is no longer detectable.

The genomic parasite DNA from black flies pools were usually isolated and purified by the standard method. This method is time consuming and required skilled technicians to carry out the work. Currently, there are several novel automated

approaches developed for DNA isolation. Compared to the standard method, the automated methods are faster, time saving and less tedious. This would allow testing of significantly large number of black flies for detecting the parasite DNA in as many communities as possible per endemic area during the post-treatment entomological surveillance (ES). This study optimizes the isolation of parasite DNA in pools of black flies and further detection of the O-150 gene by PCR connected to an ELISA by using silica coated paramagnetic (Promega/Uniparts Model # MX3031, Cat. # AS1200**)**, general bead (nucleomag tissue DNA kit cat #1102/001, Machery- Nagel GmbH & Co, Bethlehem, PA, USA) and oligo nucleotide based Dynal M-280 streptavidin® coated (Dynal, AS Oslo, Norway) magnetic bead methods.

One of the automated silica coated bead method was validated under a natural transmission setting, using *S. ochraceum* s.l. flies collected in an endemic Mexican community for onchocerciasis when parasite transmission was ongoing in the year of 1994. The advantage of the automated method for parasite DNA isolation from the black flies for PCR pool screen could be used in entomological monitoring and surveillance thus saving lot of time and more over large number of flies can be processed in short period. Thus this will speed up the monitoring process.

Our goal was to identify and develop a high-throughput automated method for detecting the *O. volvulus* DNA in pools of black flies that would allow us to process and test a large number of flies. Such a novel method has to be rapid, reproducible and amenable for screening large samples of black flies. Automated method would equip

onchocerciasis programmes with a powerful tool to estimate the parasite transmission in black flies during routine monitoring and surveillance of onchocerciasis in endemic areas. Here, the effectiveness of automated method with silica coated, general and oligo nucleotide based Dynal magnetic beads was carried out and one of the method was compared with the standard phenol-chloroform method and was validated using *S. ochraceum* s.l. flies collected from a Mexican endemic village for onchocerciasis which had ongoing transmission of *O. volvulus* in 1994.

Study area and experimental infection of flies

Presumed uninfected *S. ochraceum* s.l. flies were collected from endemic villages, Nueva Reforma (15° 33′ 05″ N; 92° 45′ 04″ W; 500 m above sea level masl), Las Golondrinas (15° 26′ 06″ N; 92° 39′17″ W; 920 m above sea level masl) and Morelos (15°13′48″ N, 92°27′35″ W; 1,400 m above sea level), located in the Southern Chiapas focus in the year 1994 at early morning sessions from 07:00 h to 10:00 h when the rate of nulliparous female flies was high and the risk of filarial infection was minimal (Rodríguez-Pérez et al., 1995, 2006, 2008a; Rodríguez-Pérez and Reyes-Villanueva, 1994). Collectors received Mectizan (ivermectin) one week before beginning the collection process. Presumed infected flies were obtained by directly feeding the flies on an infected consenting adult male resident of the community. Flies were allowed to feed on areas of the torso below the shoulder of each volunteer for about 3 min to satiation. The collections of flies were carried out according to the ethical and biosafety protocols

followed by the committees of the National Institute for Public Health of the Mexican Health Ministry, Cuernavaca, Morelos, Mexico (Rodríguez-Pérez et al., 1995, 2010a).

Flies were collected in 75 x 12mm polypropylene tube with snap-on caps, maintained according the procedure given by Figueroa, et al., (1977) and incubated for 9 days. The flies were preserved in absolute isopropanol at 4 ºC until processed for PCR which was done in 2010. Three years since the ivermectin program was started in Mexico in 1989 (Rodríguez-Pérez et al., 2007), most communities had yet on-going transmission, thus *S. ochraceum* s.l. were collected during routine vector monitoring in the endemic village Las Golondrinas located in the Southern Chiapas focus at the peak *O.volvulus* transmission season lasting from February to April, 1994.

Optimization of pool size and genomic DNA using different magnetic bead methods

There is a possibility that substances from the cuticle content of the fly may inhibit the PCR reaction. Thus, the genomic parasite DNA isolated from pools of black flies is needed to optimize the detection methods. The combinations of presumed uninfected flies were assayed as 50, 100, 150 and 200 spiked with one infected fly in each pool using silica coated paramagnetic and standard PC method. Similar combinations of presumed uninfected flies were assayed as 50,100,150 and 200 spiked with one infected larvae in each pool using general magnetic bead method. We tested the sensitivity of the O-150 PCR/ELISA to detect a single *O. volvulus* infected

S.ochraceum s.l fly and larvae from a set of different pools of flies, using different magnetic bead method. To determine the optimal pool size without affecting the sensitivity of the assay, these methods have been assessed and compared in order to optimize the pool size. Besides, according to the section 6.2.5 in materials and methods, the black flies were processed for genomic DNA and further tested by PCR-ELISA.

Results

It is important to have a method to distinguish between the infectious and infective flies in the pool screen assay method, which is based on the separation of heads from the thoraces and bodies of *S. ochraceum* s. I. To estimate the efficiency of different methods, the heads and bodies collected from collection sites are separated .The separated heads and bodies were used in the pool screen PCR assay method to quantify the performance of the different parasite DNA isolation methods using combinations of infected and non-infected black fly and larvae samples were determined. There were no problems in isolating parasite DNA from pools of black flies that had been preserved in absolute isopropanol for over 10 years. Pool sizes ranging from 50 to 200 flies were assayed for all the methods employed in this study.

The mixing experiment results using the silica coated paramagnetic method showed positive results only in the body and head pools of 50 and 100 flies, respectively. As each pool contained only one infected fly to be detected the sensitivity of detection

method to detect the genomic parasite DNA in different pools will be analysed. The results obtained with the general bead method were similar to that estimated using silica coated paramagnetic bead method. While using oligonucleotide based magnetic method all the body and head pools ranging from 50 to 200 flies were shown to be positive for PCR-ELISA, indicating the sensitivity of detection (Table 14).

Silica coated paramagnetic method with pool size of 100 flies was chosen and validated in the field contrasting the results obtained with the standard PC method using a pool size of 50 flies. A pool size of 100 flies for the silica coated paramagnetic beads method was chosen because in our mixing experiments, we obtained good results of amplified PCR products using pool of flies less than 150. The number of flies processed, and the number of positive heads and bodies of flies collected from Las Golondrinas during 1994 is summarized in Table 15.

Table 14: Optimization of pool size for isolation of *O. volvulus* genomic DNA from black flies using different magnetic bead methods

Pool Size	Phenol chloroform method		Silica coated Paramagnetic beads		Nucleo-magnetic beads		Oligonucleotide magnetic beads	
	Heads	Bodies	Heads	Bodies	Heads	Bodies	Heads	Bodies
50	+	+	+	+	+	+	+	+
100	-	-	+	+	+	+	+	+
150	-	-	-	-	-	-	+	+
200	-	-	-	-	-	-	+	+

Table 15: The number of bodies and heads *S.ochraceum* s.l. pools examined and the number of positives for *O. volvulus* DNA when using two methods of DNA isolation

	Phenol chloroform	**Silica coated paramagnetic beads**
Number of flies per pool	50	100
Total number of flies examined	6,700	6,700
Number of bodies and heads pools examined	134	67
Number of positive body pools	12	7
Number of positive head pools	3	5

A total of 13,400 flies were collected and divided into two equal number of flies for isolation of DNA by the two methods and further tested by O-150 PCR-ELISA. At the community level, three head pools were found positive (*ps* = 50; *p* = 1.0; 95% confidence intervals CI = 0.14-2.16) by the standard PC method, while five head pools were found positive (*ps* = 100; *p* = 1.69; 95% CI = 0.45-3.13) by the automated method. Twelve body pools were found positive (*ps* = 50; *p* = 3.78; 95% CI = 1.89-6.07) using the standard PC method and seven body pools were positive (*ps* = 100; *p* = 2.35; 95% CI = 0.83-4.06) in the automated method (Table 16). In both methods, the rates of infected flies are similar at $\alpha = 0.05$ because the point estimator using the automated method (= 2.30) lies between 1.89 and 6.07, the CIs estimated for the body of flies assayed by the standard PC method (Korin, 1977). There was no significant differences ($P > 0.05$; Z = -0.57) between the two methods according to a wise-pair Mann-Whitney type non-parametric test. The same procedure also applies for the infective rate in which both methods produced no statistical variation. The seasonal transmission potential (STP) when using the standard PC method was 5.5 L3s per person per season which is similar to the STP of 8.8 L3s per person per season obtained using the automated method (Table 16).

In order to improve the pool size and DNA isolation in the laboratory, the new isolation method based on oligo nucleotide magnetic beads coated with streptavidin and OVS2-B (biotin) probe (specific for *Onchocerca volvulus*) was used for the isolation of DNA. The magnetic beads used in this method are oligonucleotide based and more specific. For the optimization of the pool size the black flies were divided into pools of 50, 100, 150 and 200 each of heads and bodies.

Table 16: The infective and infected rates and the seasonal transmission potentials estimated in Las Golondrinas, Mexico when using two methods of parasite DNA isolation

Year of peak transmission season and the estimated biting rate	DNA extraction method	Prevalence of infected rate*	Prevalence of infective rate#	Seasonal transmission potential
1994 10,522 bites per person per season	Phenol chloroform	**3.78** (1.89 - 6.07)	**1.05** (0.14 - 2.16)	**5.5** (0.73 - 11.36)
	Silica coated paramagnetic beads	**2.30** (0.83 - 4.06)	**1.69** (0.45 - 3.13)	**8.8** (2.3 - 16.46)

* Expressed as rate per 2,000 flies examined.
# The bold numbers represents the point estimate for the value in question and the numbers surrounding the point estimate represent the 95%-confidence intervals,

Each pool was spiked with single/two infected L3 larvae and DNA extraction was carried out as mentioned in the materials and methods section. The extracted DNA was assessed for its suitability to the PCR assay. The use of DNA purified from oligonucleotide based magnetic bead purification method gave very good PCR amplification of *S. ochraceum* DNA that was more consistent. The positive signals were obtained in all pools containing up to 200 heads or bodies in the PCR-ELISA assays (Fig. 16). The initial experiments suggested that the oligonucleotide based method was capable of detecting one L3 in pools up to 200 heads or bodies. To further explore the sensitivity of the assay, the experiment was repeated using 10 separate pools containing 200 heads or bodies spiked with single L3. All the pools were found to be positive suggesting that the oligonucleotide based magnetic method was capable of consistently detecting a single L3 in pools of up to 200 heads or bodies of S.ochraceum s.I. (Fig. 16). None of the negative pooled samples gave a positive reaction indicating the high specificity for the assay and that the pool size of 200 flies is suitable for the PCR assay.

To further analyze, the PCR amplified products were electrophoresed on a 1.5% agarose gel with 100 base pair (bp) marker DNA, stained with ethidium bromide and observed for band specific for *O.volvulus* for each pools screened at 150bp and thus all pools indicating positive amplification of the *O. volvulus* specific DNA. This method has been optimized in laboratory with Latin American black flies; further this method will be very useful for control programmes for entomological surveillance.

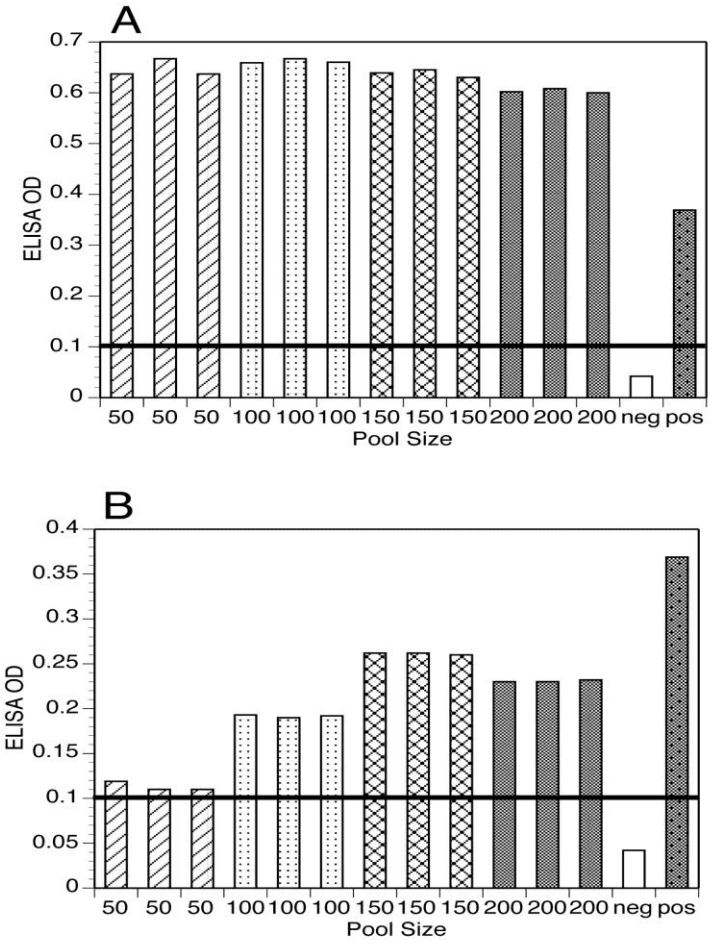


Fig. 16: Performance of oligonucleotide based purified DNA on different size pools of *S. ochraceum* heads and bodies spiked with one *O. volvulus* L3: Pools of containing different numbers of *S. ochracuum* heads (Panel A) and bodies (Panel B) were spiked with one *O. volvulus* L3 and DNA prepared from the spiked pools by oligonucleotide capture. Purified DNAs were used as templates in O-150 ELISA PCR assays as described in Materials and Methods. Neg = sham extraction prepared in parallel with the pools. pos = PCR positive control.

Discussion

The selection of a DNA isolation and purification method depends on the objectives and strategies of a detection system. None of the DNA isolation protocol meets all these strategies of the detection system. The factors like performance of the kit, time and maximum number of samples assayed are necessary to be considered. Many protocols have been used and recommended for DNA isolation and purification from plants, insects, and animals. These DNA isolation protocols are complicated and time consuming, utilizing high amount of toxic materials like phenol, and with complex procedures like absorptive column and sometimes detergents like acetyl trimethyl ammonium bromide (Reineke et al., 1998).

Fly´s heads and bodies contain a number of complex biological compounds which cause problems when PCR is used as diagnostic methods due to presence of numerous polysaccharides and lipids that cannot be removed completely during the classical extraction protocols. Any remaining contaminants in the final DNA preparation will hamper the DNA purity and in turn act as strong PCR inhibitor. Samples containing EDTA can chelate divalent cation like Mg that is essential for PCR. The presence of RNase or DNase can also affect PCRs by degrading nucleic acid targets, primers. Direct inhibition of the DNA polymerase is the best-known reasons of PCR inhibition. Several recent articles have shown that heme, heparin, phenol, polyamines polysaccharides, urine, and calcium alginate inhibit PCRs in this manner (Rådström et al., 2004). Thus, several methods have been developed to avoid DNA contamination by diluting the

sample extraction using chloroform and using thermostable DNA polymerase such as Tfl or Tth DNA polymerase instead of Taq DNA polymerase (Weidbrauk et al.. 1995; Bextine et al., 2004). For *O. volvulus* parasite DNA, the standard PC method has been modified to include two successive DNA purifications so that most *S. ochraceum* s.l. inhibitors are eliminated to improve the sensitivity of the PCR-ELISA for the detection of a single positive fly when screening areas with extremely low transmission levels (Rodríguez-Pérez et al., 1999, 2004, 2006, 2010a, b).

Another method relies on dilution of the genomic DNA to remove contaminants that inhibit the PCR reaction, but it is very difficult to estimate the effects as such dilution may have on the detection of a single infected fly, and cannot be used for pool sizes greater than about 20 flies (Davies et al., 1998). This method differs from the assays used in the case of *S. damnosum* s.l., where a single round of purification was sufficient for the standard PC method (Katholi et al., 1995). In preparing extracts of *S. ochraceum* s.l., the amount of pigments obtained was greater than that extracted from an equivalent number of *S. damnosum* s.l., making it necessary to carry out an additional round of purification in the PC method. This prolonged the time needed to process each sample. In contrast to the above conditions, the present work has shown the silica coated paramagnetic and general beads method to be efficient for *S. ochraceum* s.l. at a pool size of 100 flies and Dynal beads method to be more efficient for *S.ochraceum* s.I. at a pool size of 200 flies.

The beads used in one of the paramagnetic DNA preparations are silica coated so this method is not a significant departure from the standard PC method, which also uses the conventional Whatman plate containing the glass filters as a silica matrix. This means that the method reported here from Promega is similar to the standard method and is automated and much faster than using standard PC method. Thus, paramagnetic approach rapidly purified DNA from black fly heads or bodies without losing sensitivity for the detection of *O.volvulus*.

The automated method increased the pool size of detection, decreased the labour and reduced the time involved in the whole process. The standard PC method takes about 2 days for processing 20 pools of 50 flies each using considerable amounts of phenol and chloroform which are toxic chemicals while the silica coated paramagnetic bead method takes just 45 min to process about 16 pools of 100 flies each operating automatically without any toxic chemicals. The oligo nucleotide based Dynal magnetic bead method takes 1hr to process 50 pools of 200 flies. Thus, the latter method speed-up the processing time, particularly under the circumstance of large amounts of flies necessary to be examined. The silica coated paramagnetic and oligonucleotide magnetic beads method could be used as an effective and reproducible alternative to other isolation/purification DNA protocols (animal tissue and plant cells), with the additional advantage of avoiding considerable DNA losses and use of extra volume of toxic materials and time for pool size of 100 and 200 flies. It has fewer steps and manipulations, thus reduces the risk of contamination with foreign DNA with high

reliability. A commercially available kit designed to chemically lyse the flies for PCR purposes yielded only small amount of DNA of high quality suitable for PCR.

Each of these methods has its own merits and pitfalls. Standard PC isolation method was inexpensive with readily available reagents but is laborious (Chan et al., 2001). The above mentioned methods do not require the use toxic phenol/chloroform chemicals for isolation, and there is no risk of cross contamination. These methods are simplest but are quite expensive. Overall results showed that all these methods have performed well for the detection of a single infected fly and infected larvae. Although some combinations of presumed infected and uninfected flies resulted negative to the PCR, probably due to the fact that only 80% - 90% of flies obtained microfilariae in the blood meal when fed on the participating volunteers (Basáñez et al., 1994), the PCR/ELISA method could detect PCR products of one infected *S. ochraceum* in a pool size of 100 when using the silica coated paramagnetic beads, general bead and 200 pool size of Dynal bead methods.

However, the level of detection was similar to that (1 infected *S. damnosum* s.l. in a pool of 100 uninfected flies) of radioactive DNA probes (Meredith et al., 1991, Katholi et al.,1995). Thus, the standard PC method can be used for pool sizes of 50 to ensure that the method would detect any infected fly when screening the wild-caught population and the silica coated paramagnetic, general beads method has advantages on the ease of performance and less time for processing effectively with the pool size of 100. The PCR-

ELISA protocol was able to detect *O. volvulus* DNA in samples of wild-caught *S. ochraceum* s.l. that had been preserved in absolute alcohol for over 15 years, indicating no degradation of DNA, as demonstrated elsewhere (Davies et al., 1998).

In the field/validation study, the prevalence of infection in flies detected by PCR using the DNA isolated by both methods was statistically similar. In the standard PC method, 12 body pools (3 head pools) out of 134 assayed (6,700 flies) were positive (= 3.78; 95%-CI = 1.89-6.07). In the silica coated paramagnetic beads method, 7 body pools (5 head pools = 2.30; 95%-CI = 0.83-4.06) were positive in an equivalent number of flies. A variation of parity rates between populations could explain the apparent differences in the number of infected flies between the two methods (Basáñez et al., 1998); however, as both populations were collected at similar time and collection site, the apparent differences could be as a result of other factors not yet investigated.

In addition, the infected rates estimated by PCR-ELISA in this study were also statistically similar to the infected rate of 2.40 (95%-CI = 1.55-3.66) estimated by dissection in a study performed in 1993-1994 in Las Golondrinas (Basáñez et al., 1998). Using both methods of DNA isolation and detection by PCR-ELISA, we also re-evaluated the STP in Las Golondrinas that had occurred in 1994, after 3 years of the commencement of the mass ivermectin distribution programme. In a previous evaluation conducted in Las Golondrinas in the dry season of 1993, using the dissection method, the STP was of 14 L3s per person (Rodríguez-Pérez et al., 1995). In the present study, a STP

of 5.5 (8.8 for the silica coated paramagnetic beads method) L3s per person was estimated. The ongoing transmission in Las Golondrinas in 1994, despite of 3 years under compliance to ivermectin treatment could have occurred because the high human biting rate of the local vector species. However, after 12 years (i.e. in 2006) under high levels of ivermectin coverage in Las Golondrinas, the STP was zero (95%-UCI = 9.8) L3s per person per season, indicating that the transmission had been suppressed (Rodríguez-Pérez et al., 2008a, b).

Although, these magnetic bead methods are quite more expensive than the standard P-C method, the latter employs two working days for testing a similar number of samples. The weekly salary of a well-trained technician could be of around USD $240 if the standard method is performed. But the local laboratory can provide faster results to the onchocerciasis programmes. However, it could serve as a reference for molecular diagnosis of other parasites and pathogens if an automated method is established. Hence, the specialized equipment for automated DNA isolation can be used for the purpose of research and diagnosis of many other infections in the local laboratory making the initial expenditure worth-while. The local laboratory could also process entomological materials from other endemic countries and may serve as a reference laboratory.

When the manual isolation DNA method of phenol chloroform (PC) was applied for amplification of DNA from insects, it was found to be inefficient due to the presence of PCR inhibitors in insect tissues. Because of this, two rounds of DNA purification have

been incorporated to a standard PC method in order to overcome the inhibition for extracting parasite DNA in pools of *S. ochraceum* s.l. flies (Rodríguez-Pérez et al., 1999, 2004, 2006). More efficient DNA isolation for high-throughput methods such as those based on silica coated paramagnetic, general and oligonucleotide magnetic beads specific for *O. volvulus* would enhance the performance of the molecular detection techniques such as PCR and also get rid of the shortcomings of the standard method.

Conclusion

In conclusion, the use of the new high-throughput automated methods based on silica coated paramagnetic , general and oligonucleotide based Dynal magnetic beads allows the PCR pool screen assay to reflect the level of transmission in a given area, which is less time-consuming but more expensive than standard PC method. These methods based on magnetic beads can be used for high number of flies per pool. The automated approach could be very useful in areas where the level of transmission is extremely low as a result of successful control interventions, or in areas where the interventions have been suspended and a large number of flies from many communities are necessary to be assayed during the intensive entomological surveillance.

11: GENERAL CONCLUSION

The present work describes the monitoring and entomological surveillance data on the transmission of onchocerciasis in two endemic foci, Northern Chiapas and Southern Chiapas of Mexico. Northern Chiapas is the second smallest of all the foci in Latin America for onchocerciasis. The entomological data conducted in 2006 indicated that there was no ongoing transmission of *O.volvulus* in this focus and therefore the mass distribution of ivermectin treatment was suspended in 2008 and intensive surveillance program was initiated to ensure that the disease does not re-appear in this focus. The present study reports the post-treatment surveillance data conducted in 2010 in N. Chiapas focus of Mexico. The results showed that there was no evidence of transmission occurring in this focus. Thus, based on the surveillance results the local and federal Mexican health authorities along with OEPA have certified that the onchocerciasis has been successfully eliminated from Northern Chiapas focus of Mexico.

In the Mexico-Guatemala region, onchocerciasis was mainly associated with coffee plantations and the seasonal transmission occurs during the dry season often associated with the presence of multiparous *S. ochraceum* s.l females. Onchocerciasis in Southern Chiapas has been commonly associated with the migration of coffee laborers from Guatemala. In this thesis the results of the entomological studies carried out from 2009-2010 indicate that the prevalence of infection and seasonal transmission potential was very low in coffee fincas, sentinel and extra-sentinel communities of S. Chiapas. Based on these data, OEPA program was able to declare that the transmission in S.

Chiapas focus has been successfully suppressed. Further, the results of PCR monitoring carried out in 2011 were negative for all the communities indicating that the transmission has been successfully interrupted in S. Chiapas. The entomological data would encourage the OEPA/WHO to halt ivermectin treatment during 2012 and post treatment surveillance will be initiated to identify the transmission rate and to certify the elimination of onchocerciasis in Southern Chiapas for testing the disease re-occurrence.

For post-treatment entomological surveillance to be effectively performed, large numbers of black flies have to be processed for each community to certify the elimination. The infection level in simulid population has been classically detected by the dissection of the wild caught flies. Although this technique was useful in estimating the parity rates and the transmission levels, it was ineffective for estimating infection rates in the vector population in the endemic areas where the infection has been drastically reduced due to successful ivermectin mass treatment. The PCR assay for detecting the larvae was develop based on the amplification of onchocerca repeated sequence (O-150) followed by hybridization of the amplified products with O.volvulus species specific DNA probes. For the successful utilization of this method highly pure vector DNA is required. Various DNA purification methods have been evaluated to extract high quality *O. volvulus* DNA from the pools of black flies from Mexico for using in the pool screen PCR assay.

Phenol chloroform method is normally used by the control programs for purification of DNA from the vector population and has certain limitations. Therefore, new DNA isolation methods have been tested and validated so that one of these methods could be in-future used for entomological surveillance in Mexico as a replacement for more tedious and time consuming P-C method. Here, high-throughput automated methods for *O. volvulus* DNA isolation from black fly pool screen for PCR assay have been tested and validated. DNA isolation methods based on magnetic beads namely silica coated, general and oligonucleotide magnetic beads showed that it can detect one infected fly in a pool of 100-200 flies as compared with PC which does effectively in a pool of 50 flies. This new, improved automated method is more sensitive and has loads of advantages over PC method.

The new automated methods can be implemented in the control programs to assess the level of transmission in a given area using PCR pool screen assay in less time. Although this method is more expensive than standard PC method, it can process large numbers of flies per pool in a relatively short time which is indeed the need of the hour. The automated approach could be very useful in areas where the level of transmission is extremely low as a result of successful control interventions or in areas where the interventions have been suspended and a large number of flies from each community are required to be tested during the intensive entomological surveillance.

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13: ANNEXURE

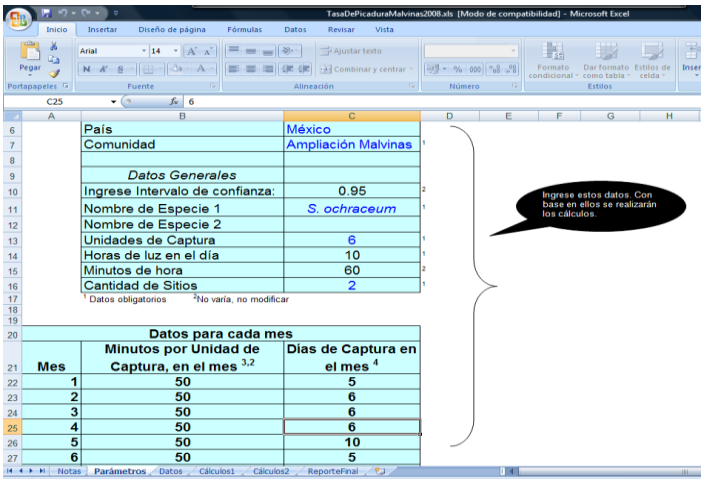
13.1. Registration format to capture Simulids

LOCALITY: _____ DATE: _____HABITAT: _____

CAPTURE PERSON: _____BAIT: _____

TIME	*S.ochraceum*	*S.metallicum*	*S.callidum*	TOTAL/HOURS	OBSERVATIONS
7:00-7:50					
8:00-8:50					
9:00-9:50					
10:00-10:50					
11:00-11:50					
12:00-12:50					
13:00-13:50					
14:00-14:50					
15:00-15:50					
16:00-16:50					
TOTAL FLIES/DAY					

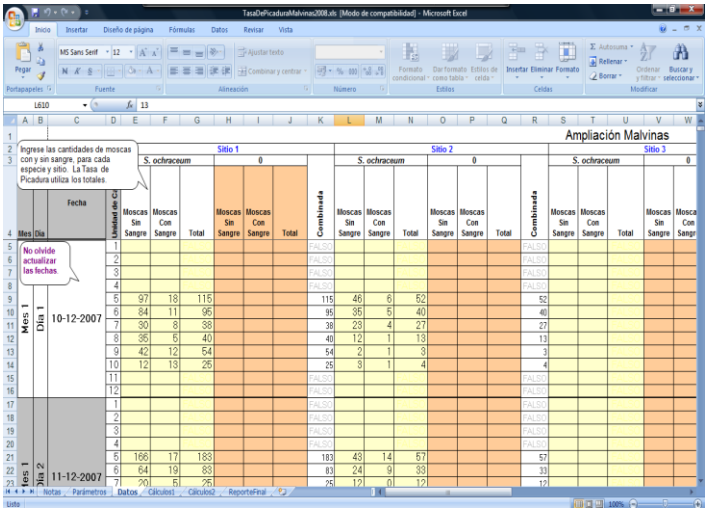
13.2. Data analysis format of seasonal transmission potential by using computer programmes

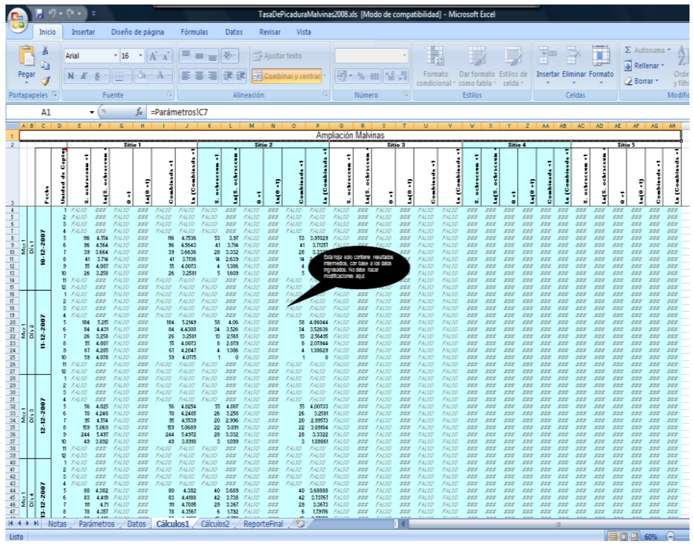


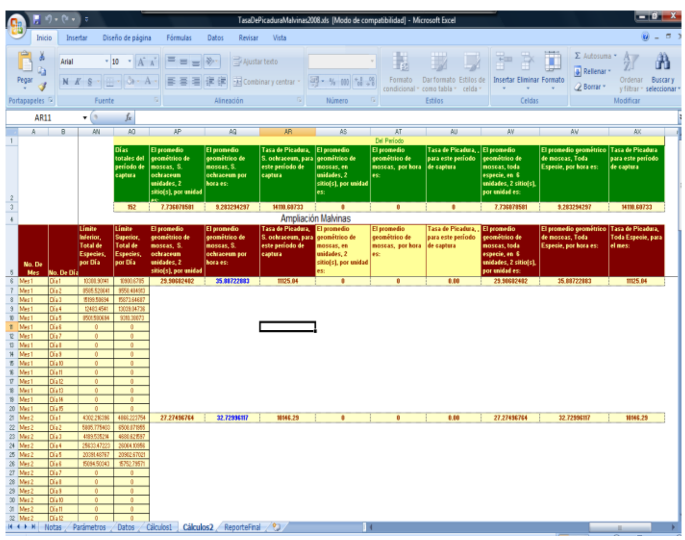
País	México
Comunidad	Ampliación Malvinas
Datos Generales	
Ingrese Intervalo de confianza:	0.95
Nombre de Especie 1	*S. ochraceum*
Nombre de Especie 2	
Unidades de Captura	6
Horas de luz en el día	10
Minutos de hora	60
Cantidad de Sitios	2

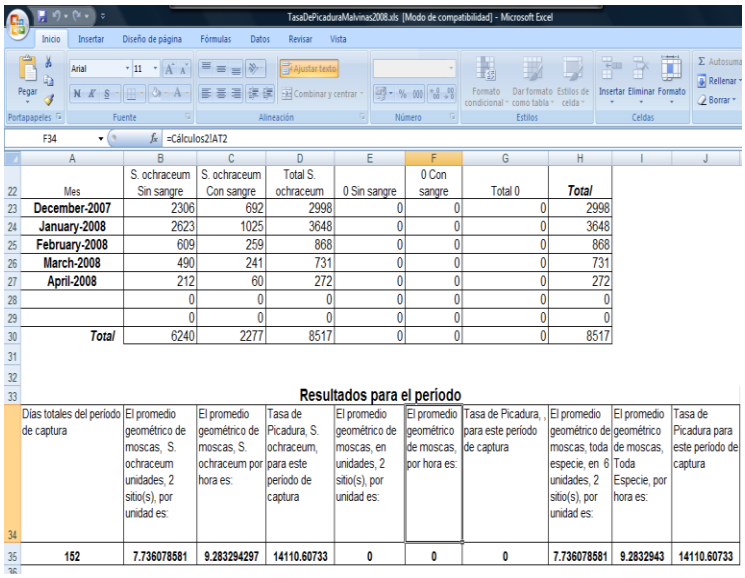
[1] Datos obligatorios [2]No varía, no modificar

Datos para cada mes		
Mes	Minutos por Unidad de Captura, en el mes [3,2]	Dias de Captura en el mes [4]
1	50	5
2	50	6
3	50	6
4	50	6
5	50	10
6	50	5




TasaDePicaduraMalvinas2008.xls [Modo de compatibilidad] - Microsoft Excel
Ampliación Malvinas

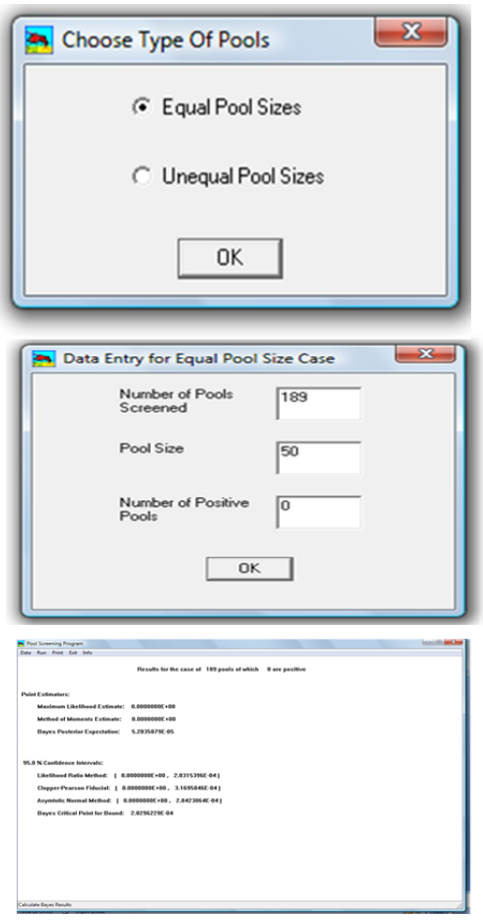

TasaDePicaduraMalvinas2008.xls [Modo de compatibilidad] - Microsoft Excel
Ampliación Malvinas



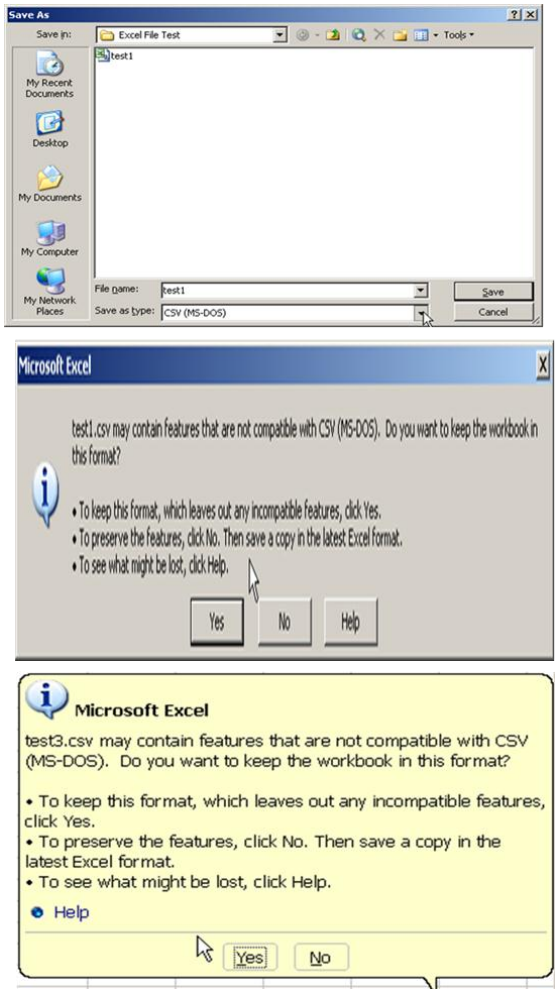
	A	B	C	D	E	F	G	H	I	J
22	Mes	S. ochraceum Sin sangre	S. ochraceum Con sangre	Total S. ochraceum	0 Sin sangre	0 Con sangre	Total 0	*Total*		
23	**December-2007**	2306	692	2998	0	0	0	2998		
24	**January-2008**	2623	1025	3648	0	0	0	3648		
25	**February-2008**	609	259	868	0	0	0	868		
26	**March-2008**	490	241	731	0	0	0	731		
27	**April-2008**	212	60	272	0	0	0	272		
28		0	0	0	0	0	0	0		
29		0	0	0	0	0	0	0		
30	***Total***	6240	2277	8517	0	0	0	8517		
31										
32										
33	**Resultados para el período**									

34	Días totales del período de captura	El promedio geométrico de moscas, S. ochraceum unidades, 2 sitio(s), por unidad es:	El promedio geométrico de moscas, S. ochraceum por hora es:	Tasa de Picadura, S. ochraceum, para este período de captura	El promedio geométrico de moscas, en unidades, 2 sitio(s), por unidad es:	El promedio geométrico de moscas, por hora es:	Tasa de Picadura, para este período de captura	El promedio geométrico de moscas, toda especie, en 6 unidades, 2 sitio(s), por unidad es:	El promedio geométrico de moscas, Toda Especie, por hora es:	Tasa de Picadura para este período de captura
35	**152**	**7.736078581**	**9.283294297**	**14110.60733**	**0**	**0**	**0**	**7.736078581**	**9.2832943**	**14110.60733**

13.3. Schematic view for the calculation of infected and infective rate using pool screen version 2.0



13.4. Schematic representation for the calculation of and infected and infective rate using new pool screen version 2.01



Data Summary

Number of Pools Gathered = 360
Total Number of Vector Insects Collected = 10921
Number of Positive Pools Observed = 0

Maximum Likelihood Parameter Estimates

Parameter	Estimate	Standard Error
Proportion of Vectors Infected	0.0000000E+00	Not Estimable
Expected Biting Rate (h)	3.6403348E+01	1.6592756E+00
Seasonal Transmission Potential	0.0000000E+00	Not Estimable

95.0 Confidence Intervals for the Parameters

Parameter	Lower	Upper
Proportion of Vectors Infected	0.0000000E+00	2.7427172E-04
Expected Biting Rate (h)	3.3151227E+01	3.9655468E+01
Seasonal Transmission Potential	0.0000000E+00	1.0116369E+01

13.5. Preparation of Buffer and Reagents

1. Homogenization buffer

NaCl	100mM
Tris-HCL	10mM
EDTA	1mM
SDS	0.1%

2. Coating buffer

$NaHCO_3$	50mM
$Na_2\ CO_3$	2mM

3. Hybridization buffer

SSPE	20x
Denhardt's	10X
N-lauryl sacrosine	1%
SDS	20%
Water	500ml

4. Antibody dilution buffer

NaCl	0.4M
Tris-HCL	0.1M
BSA	0.5%

5. SSPE/SDS buffer

SSPE	1X
SDS	0.1%

6. Binding Buffer

Tris HCl	100mM
NaCl	100mM

7. TE Buffer

Tris HCl	1M
EDTA	0.5M

8. NaI stock

NaI	90.3g
$Na_2 SO_3$	1.5g

9. Ethanol Wash (20X stock)

NaCl	2mM
Tris-HCL	200mM
EDTA	20mM

20X stock	50ml
Absolute alcohol	500ml
Distilled water	450ml

10. TBS 10X

Tris base	30.2g
NaCl	43.8g

Dissolve in 350ml distilled water. Adjust pH to 7.5 with HCl and makeup the volume to 500ml.

11. SSPE 20X

NaCl	174g
NaH_2PO_4	27.6g
Disodium EDTA	7.4g

Dissolve in 700ml distilled water. Adjust pH to 7.4 with NaOH. Bring volume to 1 liter.

12. Denhardt's 10X

BSA	0.2%
Polyvinylpyrrolidone	0.2%
Ficoll 400	0.2%

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