# Detection of *Onchocerca volvulus* infection in *Simulium ochraceum sensu lato*: comparison of a PCR assay and fly dissection in a Mexican hypoendemic community

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SUMMARY

Detection of *Onchocerca volvulus* larvae in vector populations is of prime importance in the assessment of the effectiveness of onchocerciasis control programmes. Traditionally, detection of larvae is attained by the dissection of flies, but this time-consuming method cannot easily discriminate between species of *Onchocerca*. The genome of all *Onchocerca* species has a unique 150 bp repeat, which can be amplified by PCR, and *O. volvulus*-specific DNA probes can detect these products by Southern blot (SB). This study optimizes a PCR/SB assay, and compares it with fly dissection to estimate the prevalence ($p$) and intensity of infection ($m$) in the local vector population of a Mexican community that has become hypoendemic as a result of 7 years of treatment with ivermectin and nodulectomy. The PCR detected 1 infected fly in a pool of 99 uninfected flies, but the optimal pool size was 50 flies. At the community level, 1 out of 10550 flies was positive ($p = 0{\cdot}0095\,\%$, 95 % confidence intervals CI = 0·00024–0·05280 %; $m = 0{\cdot}00027$ larvae/parous fly, CI = −0·00026–0·00081) by PCR, and 4 out of 10772 flies ($p = 0{\cdot}0371\,\%$, CI = 0·01012–0.09505 %; $m = 0{\cdot}00107$ larvae/parous fly, 95 % CI = 0·00002–0·00212) by dissection (observed $m = 0{\cdot}0005$). Both methods produce statistically similar estimates of the prevalence and intensity, indicating that pool screening is a viable alternative for entomological surveillance in areas where the intensity of transmission is becoming extremely low as a result of control interventions.

Key words: onchocerciasis, entomological surveillance, ivermectin, PCR, *Simulium ochraceum s.l.*, Mexico.

## INTRODUCTION

Monitoring *Onchocerca volvulus* transmission levels is of paramount importance for evaluating the effectiveness of National Onchocerciasis Control Programmes. Traditionally, estimations of the transmission intensity by *Simulium* vector populations have relied on larval detection by manual dissection of flies. Because this method is extremely labour intensive, it is not practical for routine vector surveillance. Polymerase chain reaction (PCR)-based assays for *O. volvulus* present an alternative method for detecting larvae in flies (Merriweather & Unnasch, 1996; Unnasch & Meredith, 1996). The method is based on PRC amplification of an *Onchocerca* repeated sequence gene family (O–150), followed by hybridization of the amplified products with an *O. volvulus* species-specific DNA probe: OVS2 (Zimmerman *et al*. 1992; Zimmerman, Toè & Unnasch, 1993), and detection by either an ELISA test or Southern blot: SB (Nutman *et al*. 1994; Toè, Merriweather & Unnasch, 1994). This approach has produced an assay with a specificity and sensitivity exceeding 90 % (WHO, 1995; Merriweather & Unnasch, 1996). The method allows the O–150 assay to be used to detect a single *O. volvulus* infected black-fly (Diptera: Simuliidae) with at least 1 parasite larva in pools containing up to 100 uninfected flies (Katholi *et al*. 1995).

A PCR/ELISA-based assay has been successfully used for the detection of *O. volvulus* in pools of *S. exiguum s.l.*, which were collected before and after a 6-year control programme using ivermectin plus nodulectomy to treat an endemic community in Ecuador (Guderian *et al*. 1997). A different approach, involving PCR and detection of amplified products on an agarose gel, has been recently developed for the identification of *O. volvulus* (Oskam *et al*. 1996), and the method used to evaluate the impact of intervention with ivermectin on infection rates of *S. ochraceum s.l.* populations (Davies *et al*. 1998). However, this assay relies on dilution of fly genomic DNA to remove contaminants that inhibit the PCR reaction and de-

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tection of PCR products on the gel, making it difficult to estimate the effects such dilution may have on the detection of a single infected fly, and it cannot be used for pool sizes greater than about 20 flies (Davies *et al*. 1998).

The present study reports the application of a PCR/SB-based assay to investigate *O. volvulus* infection rates in pools of *S. ochraceum s.l.* flies collected from a Mexican endemic community that has been treated with ivermectin plus nodulectomy for a period of 7 years. Results obtained using the PCR/SB assay were similar to those obtained from individual fly dissection, indicating that the assay may represent an accurate and efficient method to determine prevalence of *O. volvulus* infection in samples of *S. ochraceum s.l.* populations in Central America.

## MATERIALS AND METHODS

### *Study site and insect collection*

Entomological surveys were conducted between April 1997 and February 1998 (Table 1), in a formerly hyperendemic community (Las Golondrinas: 92° 39′ 06″ W, 15° 25′ 59″ N; altitude of 890 masl) in the southern onchocerciasis endemic focus of Chiapas, Mexico. The skin infection prevalence in this community in 1991 (before intervention with ivermectin) was 78 % (community microfilarial load of 12 mf/skin snip), but it dropped to 13 % (community microfilarial load of 0·04 mf/ss) in 1997 after 11 rounds of mass ivermectin treatment plus nodulectomy (Rodríguez-Pérez *et al*. 1999). Insect collections were made as previously described (Rodríguez-Pérez *et al*. 1995). Biting flies were sampled simultaneously at 2 collection sites, one in a nearby coffee plantation and the other within the community, and the detection of infected *S. ochraceum s.l.*, routinely performed *in situ* by dissection and microscopical examination of individual flies once their parous status had been ascertained. Two extra collection sites were simultaneously surveyed; one was located within the community at 250 m from the other community site, but at a similar distance from the border of the village. The other sampling site was located a 250 m from the coffee plantation site, within the same field. Insects from these collections were preserved in absolute isopropanol at 4 °C until processed for DNA recovery. All sampling sites were approximately 1500 m away from *S. ochraceum s.l.* breeding sites. The collections of insects in the 4 catching sites were performed simultaneously starting at 07.00 and ending at 17.50 h. Collections consisted of 20-min sampling units followed by a 10-min break. The host-seeking *S. ochraceum s.l.* females were captured upon alighting and before beginning to feed on the collectors. To ensure that none of the captured flies was able to ingest microfilariae from the collector, any flies showing evidence of a recent blood meal were discarded (Davies *et al*. 1998).

### *Optimization of the fly pool size*

In order to determine the optimal fly pool size in our PCR/SB tests, the presence of *O. volvulus* DNA was investigated in pools combining different proportions of infected and uninfected *S. ochraceum s.l.* A collection of uninfected flies was carried out in the study locality in the early morning, when the rate of nulliparous females is high and the risk of filarial infection minimal (Rodríguez-Pérez & Reyes-Villanueva, 1994). Infected flies were obtained by directly feeding these flies on an informed consenting adult male resident of the community (27·19 mf/mg, as determined by 6 skin biopsies; Rodríguez-Pérez *et al*. 1995, 1999). An mf skin load between 20 and 30 mf/mg was expected to produce a percentage of infection in flies between 80 and 90 % (Basáñez *et al*. 1994). Blood-engorged flies were placed in absolute isopropanol. Five combinations of presumed infected : uninfected flies were assayed as follows: 0 : 50, 1 : 59, 1 : 79, 1 : 89, 1 : 99.

### *Preparation of genomic DNA* (Katholi *et al*. 1995)

Flies were placed in 1·5 ml snap-cap microcentrifuge tubes, and rinsed 3 times in 95 % ethanol. As much ethanol as possible was removed, and the remainder allowed to evaporate for about 10 min at room temperature (RT). Flies were homogenized in 300 $\mu$l of lysis buffer (100 mM NaCl, 10 mM Tris–HCl, pH 8·0, 1 mM ethylenediamine tetra-acetic acid EDTA; Integra Biosciences, UK, 0·1 % sodium dodecyl sulphate (SDS); Sigma) along with 2 $\mu$l carrier human placental DNA; Sigma (500 ng/ml) in the mixture. The homogenate was incubated at 55 °C for 1 h in 100 $\mu$l of proteinase K solution; Sigma (400 $\mu$g/ml in fresh lysis buffer), and boiled for 30 min in the presence of 4 $\mu$l of 1 M dithiothreitol DTT; Sigma. The homogenate was subjected to 2 cycles of freezing in liquid nitrogen and thawing at RT, 1 $\mu$l of carrier DNA (500 ng/ml) was added, and the lysate extracted twice with a 1/1 mix of phenol and chloroform. Following a final extraction with chloroform, the volume of the aqueous layer was measured, and after adding 3 volumes of NaI solution (Bio 101, Inc. 1001-401) and 5 $\mu$l of glass slurry (Sephaglass; Pharmacia Biotech 27-9285-01), the extract was placed at 4 °C for 15 min. To purify DNA, the glass slurry was pelleted out and washed in 500 $\mu$l of ethanol wash solution (10 mM Tris–HCl, pH 7·5, 100 mM NaCl, 1 mM EDTA, 50 % (v/v) ethanol). The washing step was repeated 3 times, and the pellet dried at RT for 5 min. The pellet was

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Table 1. The total number of *Simulium ochraceum s.l.* examined females and those parous females infected with *Onchocerca volvulus* collected during 74 days (collections between 07.00 and 17.50 h) within the village and coffee plantations of Las Golondrinas (Chiapas, Mexico) after 7 years of intervention with ivermectin and nodulectomy

| | No. of female *S. ochraceum* examined | No. of (percentage) parous in village | No. of (percentage) parous in coffee plantation | No. of infected parous females collected at* | |
|---|---|---|---|---|---|
| | | | | 07.00–09.30 h | 10.00–14.30 h |
| Dissection method | 10772 | 4215 (0·83) | 4669 (0·82) | 1 V–1 C | 1 V–1 C |
| PCR method | 10550 | N.D. | N.D. | 0 | 1 V |

* V, village; C, coffee plantation.
N.D., No data.

Table 2. The total number and percentage of parous flies infected with *Onchocerca volvulus*, and the estimated mean (95 % confidence intervals) of larvae per parous fly in biting *Simulium ochraceum s.l.* collected in areas of Las Golondrinas (Chiapas, Mexico) after 7 years of intervention with ivermectin and nodulectomy

| | No. of parous females | Percentage infected parous females | Estimated mean no. of larvae per parous fly | 95 % confidence intervals of estimated mean no. of larvae per parous fly | |
|---|---|---|---|---|---|
| Dissection method | 8884 | 0·045 | 0·00107 | 0·00002 | 0·00212 |
| PCR method | 8701* | 0·011* | 0·00027 | −0·00026 | 0·00081 |
| Both methods | 17584* | 0·028* | 0·00068 | 0·00008 | 0·00127 |

* Assuming a parity rate of 82.47 % from Table 1.

suspended in 100 $\mu$l of 10 mM Tris(hydroxymethyl) aminomethane-1 mM EDTA, pH 8·0, TE; Sigma at 55 °C for 5 min. The glass slurry was pelleted out from this solution by centrifugation for 2 min, and the supernatant placed in a fresh tube. The glass purification step was then repeated on the supernatant, and the DNA (genomic DNA) re-suspended in 50 $\mu$l of TE and stored at −20 °C.

### *PCR amplification of the 150-repeat family present in the genome of* Onchocerca *parasites, and separation by agarose gel electrophoresis* (Zimmerman *et al.* 1992, 1993)

Five $\mu$l of DNA prepared from pools of flies were used as template DNA in each PCR reaction including: 5 $\mu$l 10× $NH_4$ PCR buffer without $MgCl_2$, 6 $\mu$l of 50 mM $MgCl_2$ (final concentration: 6 mM), 0·8 $\mu$l of 12·5 mM dNTP mix (Bioline, UK) (final concentration: 0·2 mM each of 4 nucleotides), 1·25 $\mu$l of 20 $\mu$M primer 1: 5′-GATTYTTCCGRC-GAAXARCGC-3′, and primer 2: 5′-GCXRTRT-AAATXTGXAAATTC-3′ (Unnasch & Meredith, 1996; Nutman *et al.* 1994) (final concentration of primers: 0·5 $\mu$M), 0·25 $\mu$l of 500 U (5 U/$\mu$l) *Taq* DNA polymerase (Bioline UK), 1·25 $\mu$l of 100 % formamide (final concentration 2·5 %; Sarkar, Kapelner & Sommer, 1990), and 29·2 $\mu$l of filter distilled water. DNA extracts were substituted in negative control reactions by 5 $\mu$l of distilled water and in positive controls by 1 ng of the plasmid pOVS134 (final concentration: 20 pg/$\mu$l) which contains an insert consisting of 12 tandem copies of the O–150 repeat family (Zimmerman *et al.* 1992, 1993). Each mixture was overlaid with 75 $\mu$l of light mineral oil. PCR conditions were 25 cycles of amplification, each of 1 min at 94 °C, 2 min 37 °C, and 30 s at 72 °C. An initial and final step of 95 °C for 5 min and 72 °C for 5 min were performed to ensure complete denaturation and elongation of the template DNA, respectively (Pharmacia Biotech, PCR beads instructions p. 16). The PCR products were stored at −20 °C until electrophoresed on 2 % agarose gels containing 0·1 $\mu$g/ml ethidium bromide (Unnasch & Meredith, 1996).

### *Southern blot for detection of PCR products* (Southern, 1975; DIG-Genius system protocol from Boehringer–Mannheim)

After electrophoresis, the DNA in the gel was denatured using 0·5 M NaOH, 1·5 M NaCl for 30 min at RT, followed by neutralization with 0·5 M Tris–HCl, 1·5 M NaCl, pH 6·8. Denatured DNA was

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blotted on a nylon transfer membrane NM (Hybond-$N^+$ of Amersham, UK) (Southern, 1975). The NM was rinsed with 2× saline sodium citrate SSC, pH 7·0, for 5 min followed by baking at 80 °C for 2 h. The NM was pre-hybridized with a solution of 6× SSC, 5× Denhardt's solution, 20 mM $NaH_2PO_4$, and 500 μg/ml salmon sperm DNA at 42 °C for 5 h. The NM was treated overnight at 42 °C with 25 ng/ml digoxigenin-labelled *O. volvulus* specific oligonuleotide probe (OVS2-DIG: 5′-CCCTAAT-CTCAAAAAACGGG-3′). The NM was washed twice with 2× SSC, 0·1% SDS at RT for 30 min, followed by 0·1× SSC, 0·1% SDS at 52 °C for 45 min. The next steps were carried out at RT. The NM was equilibrated with Tris-buffered saline, pH 7·5–0·3% Tween 20 TBSTT, for 1 min. After blocking with 5% non-fat dried milk in TBSTT for 60 min, the NM was treated for 30 min with an anti-digoxigenin AP-conjugated antibody (anti-DIG, final concentration 150 mU/ml; Boehringer–Mannheim BM-84338428) and washed with TBSTT for 30 min. The NM was equilibrated in detection buffer (100 mM Tris–HCl, pH 9·5, 100 mM NaCl) for 2 min, and developed in darkness in colorimetric buffer (10 ml detection buffer containing 15 μl of 4-nitroblue tetrazolium chloride NBT from BM in 70% dimethyl sulphoxide ($Me_2SO$) (100 mg/ml), and 35 μl of x-phosphate/5-bromo-4-chloro-3-indolyl-phosphate BCIP from BM in 100% $ME_2SO$ (50 mg/ml)). The reaction was stopped with TE.

### *Statistics*

A Mantel–Haenszel test was used to determine association between parity rate and sampling site (Basáñez *et al*. 1998). The software program (Poolscreen™) of Katholi *et al*. (1995) was used to determine the overall frequency of infected flies and its 95% confidence interval (CI) from both pool screening and individually dissected flies. Statistical comparison of proportions infected between pooled and dissected flies was made using the $2 \times 2 \chi^2$. Fisher's exact test using the Epi-Info statistical package (Centres for Disease Control, Atlanta, GA). An indirect estimate of mean number of larvae per parous fly using either dissection or PCR was derived from the observed proportion of positive parous flies according to Basáñez *et al*. (1998). Because sampling was conducted simultaneously in similar sites, fly populations examined by the 2 methods were considered the same and parity data obtained from the dissected fly population was directly extrapolated to that processed by PCR. A simple comparison was performed between the indirect estimate of mean larval intensity analysed by PCR and the observed estimate from dissected flies. The latter was also compared with the 95% CI obtained for the former (Korin, 1977).

## RESULTS

The total number of flies collected in 74 days of entomological surveys was 21322 (Table 1). Of these, 10772 flies were individually dissected: 5091 and 5681 flies trapped within the community and the coffee plantation, respectively. The estimated parity rate at the 2 sites was 0·83 and 0·82, respectively (overall parity rate of 0·825; summary overall value of odds ratios = 0·96; Mantel–Haenszel value of 0·68, with 7 D.F., $P = 0{\cdot}40$) (Tables 1 and 2). Four infected females (0·037% and 0·045% in the total biting and parous population, respectively) were collected, 2 of them within the community. One fly harboured 1 L1 and the second a single L2. The other 2 infected females were collected in the coffee plantation. One harboured 1 L1 and the other 1 L2.

In the preliminary mixing experiments, the PCR/SB method was able to detect the pool containing 1 infected female in 99 uninfected females. However, the bands from PCR products were more conspicuous on the nylon membrane for those pools with fewer flies, but the 1/89 experimental pool was negative. No PCR products were detected in the pool of 50 uninfected flies. Based on these results, a pool size of 50 was selected to ensure that our method would detect any infected fly when screening the wild-caught population.

The 10550 flies preserved in isopropanol (4650 and 5900 collected in the community and coffee plantation sites, respectively) were separated in pools of 50 each. A total of 211 pool samples were processed. From these, only 1 positive sample (50 flies collected within the community) showed specific hybridization to the OVS2-DIG probe (Table 1).

The prevalence of infection and CI from pool screening ($n = 211$ pools of 50 flies each with 210 negative pools) estimated by the Katholi method was of 0·0095% (95% CI = 0·00024–0·05292%). The Katholi method was also used to estimate the prevalence of infection and CI of data obtained by the dissection method. In this case, it was assumed that each fly dissected represented 1 pool. Thus, the number of pools examined was 10772, and the number of negative pools = 10768, resulting in an estimated prevalence of 0·03713% (95% CI = 0·01012–0·09505%). It is accepted that both rates of infection are similar at $\alpha = 0{\cdot}05$ because that of dissected flies (0·037%) lies between 0·0002% and 0.05%, the confidence interval estimated for the flies analysed by PCR (Korin, 1977). There were also no significant differences ($P = 0{\cdot}194$; one-tailed probability) between the number of infected flies by PCR (1/10550) and the number of infected flies by dissection (4/10772) estimated by the $2 \times 2 \chi^2$ Fisher's exact test.

The mean intensity of larval infection in the dissection method was 0·00045 (estimated of 0·00107)

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larvae per parous fly. The direct estimate, 0·00045, is contained within the confidence interval for the indirect estimate: 0·00002–0·00212 (i.e. between 0·04 and 4·7 larvae per infected fly, with a precision of 50 % in the estimation of the mean). In reality, there was only 1 larva per infected fly, but the estimate takes into account a given degree of larval aggregation, with $k = 0{\cdot}273$ assumed to be a known parameter for *S. ochraceum s.l.* (Basáñez *et al*. 1998). The estimate of larval intensity equal to 0·00027 larvae per parous fly, obtained from the proportion of positive parous flies analysed by PCR, compares well with the direct estimate from dissected flies and is also contained within the 95 % CI of the indirect estimate (this gives approximately 2 larvae per positive fly). When both methods are combined, the proportion of parous flies infected is 0·028 %; this gives an estimate of 0·00068 larvae/parous fly (95 % CI = 0·00008–0·00127; i.e. between 0·32 and 4·5 larvae per infected fly and a precision of 44 % in the estimation of the mean) (see Table 2).

## DISCUSSION

DNA detection methods based on PCR assays and algorithms to estimate prevalence of infection have been developed for *O. volvulus* in single and pooled infected blackflies (Meredith *et al*. 1991; Katholi *et al*. 1995; Merriweather & Unnasch, 1996; Oskam *et al*. 1996). These approaches have been used to estimate the prevalence of infection of *O. volvulus* in wild-caught *Simulium* spp. under field conditions (Toè *et al*. 1994; Guderian *et al*. 1997; Davies *et al*. 1998). Although the use of PCR in fly pool screening is not new, the present report is the first to compare the prevalence and larval intensity estimated by PCR pool screening to that obtained from dissections of *S. ochraceum s.l.* populations. The results indicate that, as in a recent study carried out on *Simulium damnosum s.l.* in West Africa (Yamèogo *et al*. 1999), the prevalence and larval intensity estimates obtained by both methods are statistically similar. These findings, taken together, validate this molecular test as a tool to estimate the prevalence and larval intensity of *O. volvulus* infection in the different vector populations.

Although, the 1/89 experimental pool was negative, probably due to the fact that only between 80 and 90 % of flies obtained microfilariae in the bloodmeal when fed on the participating volunteer (Basáñez *et al*. 1994), the PCR/SB method, using a digoxigenin-labelled DNA probe, could detect PCR products of 1 infected *S. ochraceum* in a pool of 100 uninfected flies. This level of detection was similar to that (1 infected *S. damnosum* in a pool of 100 uninfected flies) of radioactive DNA probes (Meredith *et al*. 1991; Katholi *et al*. 1995). However, it was necessary to carry out 2 successive DNA purifications using glass beads to improve the sensitivity of the digoxigenin assay to detect 1 positive fly. This contrasts with assays using *S. damnosum s.l.*, where a single round of glass purification was required (Katholi *et al*. 1995). In preparing extracts from *S. ochraceum*, the amount of pigment extracted from the flies was greater than that extracted from an equivalent number of *S. damnosum s.l.* (T. R. Unnasch, unpublished observation), making it necessary to carry out an extra round of purification. Although the additional step does not add appreciably to the overall material costs of the assay, it does prolong the time needed to process each sample. It is possible that other methods of DNA purification (e.g. Chelex binding; Bilton & Jaaarola, 1996) may prove to be more efficient for *S. ochraceum s.l.*

Several methods of detection of DNA from PCR products have been applied which differ in their sensitivity. Guderian *et al*. (1997) used the same amplification method as the one reported here, with the exception that they used an ELISA test to detect the amplified product (Nutman *et al*. 1994). Another method (Davies *et al*. 1998) relies on the dilution of samples to remove contaminants that inhibit both the PCR and DNA detection on agarose gel. Although this method does not require detection with DNA probes, it is difficult to estimate the effects such dilution would have on the detection of a single infected fly. This will be particularly important in areas where infection levels are low (Basáñez *et al*. 1998), and it is likely to be an unreliable method at low density infections or with pool sizes greater than 20 flies (Davies *et al*. 1998).

Using a PCR/SB method we evaluated the effect of 7 years of intervention with ivermectin on *O. volvulus* infection rates of *S. ochraceum* in Las Golondrinas. In a previous evaluation conducted after 4 years of intervention, the infection rate in biting parous *S. ochraceum s.l.* populations was 0·19 % (0·12 % in the whole population) (Basáñez *et al*. 1998) and we expected a further reduction of this value as a result of the cumulative effect of successive treatments. A pool size of 50 flies was chosen to be optimal for 2 reasons: in our preliminary mixing experiments we obtained a very good resolution of amplified PCR products using any pool of flies less than 100, and because simulations with the pool screen programme suggested that 50 was a good pool size to accurately measure prevalence down to about 0·1 % (T. R. Unnasch, unpublished observation). The estimated infection rate in the total parous *S. ochraceum* populations sampled (dissection and DNA detection) was of 0·028 % (0·023 % in the whole biting population). Taking into account a pre-treatment natural infection rate of 0·70 % in the total biting population (1·68 % in parous flies) (Rodríguez-Pérez *et al*. 1995) this represents a reduction of approximately 97 % (98 % in the parous population). This infection rate is lower than that of 0·08 %, estimated from biting *S. exiguum s.l.* populations in

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an endemic focus from Ecuador after 6 years under high levels of compliance to ivermectin treatment (Guderian *et al*. 1997). Assuming a parity rate of 82 % and a prevalence of infection of 0·028 % in our study area, the estimated mean intensity of infection would be of 0·00068 larvae per parous fly (approximately 12 larvae in 5 flies makes about 2 larvae per positive fly) (Basáñez *et al*. 1998). Despite low prevalence and intensity of infection levels, significant transmission still occurs, given the high human biting rate of the local vector species (Rodríguez-Pérez *et al*. 1999).

Although the prevalence detected by PCR screening and fly dissection was statistically similar, the PCR assay detected only 1 positive pool out of 211 assayed (10550 flies), while dissection detected 4 infected females in 10772 flies examined. A variation of parity rates between populations could explain this observation (Basáñez *et al*. 1998), but similar timing and collection site of both populations make this possibility unlikely. On the other hand, some of the larvae observed by dissection could be other *Onchocerca* species as they are difficult to distinguish morphologically (Toè *et al*. 1994). To the best of our knowledge, no other *Onchocerca* spp. infecting animals have been described in the region and *S. ochraceum s.l.* is a highly anthropophilic species (Dalmat, 1955; Rodríguez-Pérez & Rivas-Alcalá, 1991). However, a human blood-index varying between 0·75 and 0·99 has been used in mathematical models of transmission by *S. ochraceum s.l.* (Basáñez, 1996), and the possibility of infections with *Onchocerca* spp. of animal origin cannot be completely ruled out. Finally, different infection rates may result from aggregation in fly infection (Basáñez *et al*. 1998); *a posteriori*, our sample size allows a precision of only 0·45 in the estimation of the mean infection intensity and the apparent differences in infection intensities could result from the reduced sample size used in this study.

In contrast to dissection, the PCR/SB method using fly pools, permits the processing of larger numbers of flies and it is highly specific for *O. volvulus* (Merriweather & Unnasch, 1996), eliminating false positives originating from the presence of other *Onchocerca* species, and making it a viable surveillance alternative in areas where the prevalence and intensity of infection in the vector population are extremely low. Other advantages of the PCR method for routine entomological surveillance are in terms of costs and time involved. Although an initial investment of about US$ 45000 is needed in specialized equipment by the reference laboratory, only US$ 3·00 per 100 black-flies assayed is expended. This is in favour of the cost needed for the dissection method of US$ 15·00 per 100 black-flies assayed (Yamèogo *et al*. 1999). The PCR equipment can be used for the purposes of research and diagnosis of many other infections in the reference laboratory making the initial expenditure worthwhile. The reference laboratory could also process entomological material from regional or national onchocerciasis control programmes.

As the presence of *O. volvulus* infective larvae is a direct indication of transmission, the pool screening technique can be restricted to the analysis of the fly head capsules, although thoraces and abdomens may also be examined in order to detect other parasitic larval stages (Basáñez *et al*. 1998). By restricting the analysis to only heads, it is possible to detect only infective flies, as only the infective stage (the L3) is found in the head capsule. A method for the mass isolation of heads from the African vector *S. damnosum s.l.* has recently been reported, which should be applicable to *S. ochraceum s.l.* as well (Yamèogo *et al*. 1999). Applying this method to the pool screen assay will restrict the analysis to the detection of infective flies.

As only parous flies will be infected with *O. volvulus*, it is also important to obtain accurate estimates of the infection and/or infectivity rate in the parous fly population, as opposed to the fly population as a whole. This is because the parous rate, and therefore the proportion of the vector population potentially exposed to the parasite, may vary greatly from season to season. As there is no indirect method that permits determination of parous rates on pools of flies, it will be necessary to dissect a subsample of any collection to assess parity. However, this may be done on a relatively small number of flies in the field. Once this is done, it is possible to correct for parous rate in the pool screen PCR assay, by adjusting the pool size in the Katholi algorithm to reflect the proportion of parous flies in the population (e.g. for a parous rate of 80 %, and a pool size of 50 flies, it is possible to adjust for parous rate by entering a pool size of 40 in the Katholi algorithm, reflecting the average number of parous flies in each pool) (Yamèogo *et al*. 1999). Use of isolated heads and correction of the pool size for parous rates will allow the pool screen assay to reflect accurately the level of transmission in a given area, with a method which is less time-consuming and more economical than dissection in areas where the level of transmission is extremely low as a result of control interventions.

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