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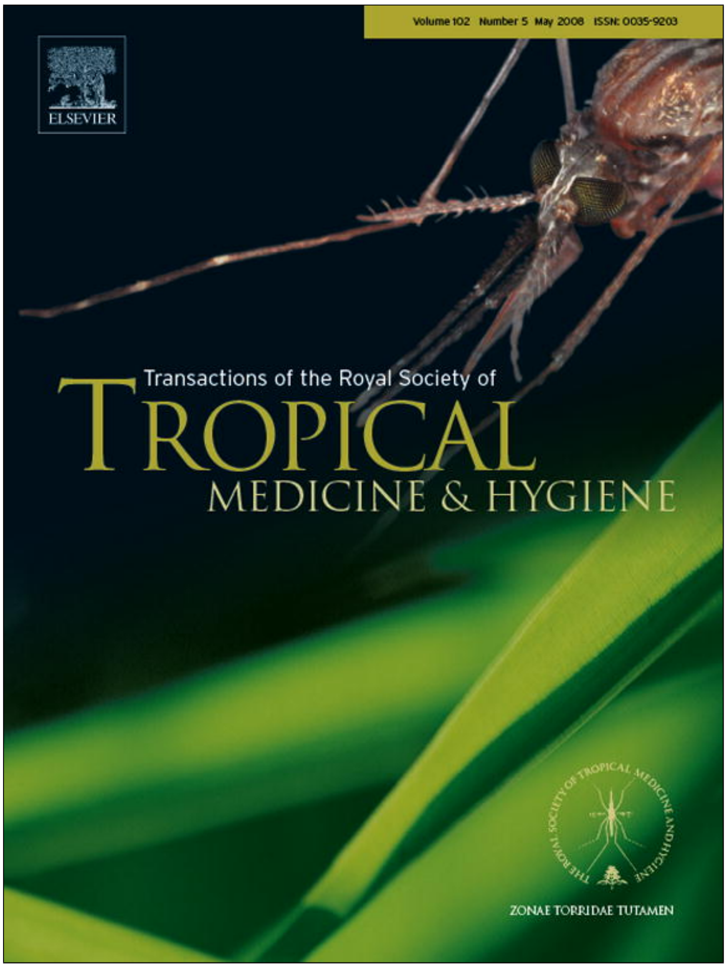
Volume 102 Number 5 May 2008 ISSN: 0035-9203
ELSEVIER
Transactions of the Royal Society of
TROPICAL
MEDICINE & HYGIENE
THE ROYAL SOCIETY OF TROPICAL MEDICINE AND HYGIENE
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Transactions of the Royal Society of Tropical Medicine and Hygiene (2008) **102**, 485—492

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Optimization of a semi-nested multiplex PCR to identify *Plasmodium* parasites in wild-caught *Anopheles* in Bolivia, and its application to field epidemiological studies

Frédéric Lardeux[a,b,*], **Rosenka Tejerina**[a,b], **Claudia Aliaga**[a,b], **Raul Ursic-Bedoya**[c], **Carl Lowenberger**[c], **Tamara Chavez**[b]

[a] *Caractérisation et Contrôle des Populations de Vecteurs, Institut de Recherche pour le Développement (IRD), CP 9214, La Paz, Bolivia*
[b] *Laboratorio de Entomología Medica, Instituto Nacional de Laboratorios de Salud (INLASA), Calle Rafael Zubieta No. 1889, Casilla M-10019, Miraflores, La Paz, Bolivia*
[c] *Department of Biological Sciences, Simon Fraser University, 8888 University Drive, Burnaby, BC V5A1S6, Canada*

Received 10 May 2007; received in revised form 7 February 2008; accepted 7 February 2008
Available online 20 March 2008

KEYWORDS
PCR;
Anopheles;
Plasmodium;
Prevalence;
Entomology;
Bolivia

Summary Without an adequate DNA extraction protocol, the identification of *Plasmodium* species in whole mosquitoes by PCR is difficult because of the presence of reaction inhibitors from the insects. In this study, eight DNA extraction protocols were tested, from which a chelex-based protocol was selected. Then a semi-nested multiplex PCR technique that detects and distinguishes among the four human *Plasmodium* species in single mosquitoes and in pools of up to 100 mosquitoes was optimized. The technique was used to detect *P. vivax* in wild-caught *Anopheles pseudopunctipennis* from a village in the Andean valleys of Bolivia in May 2003. The prevalence of infection was 0.9%. This is the first direct evidence of *P. vivax* transmission by this vector in this country. The extraction and PCR technique presented here can be useful to: (1) estimate *Plasmodium* prevalence in *Anopheles* populations in low prevalence areas where large numbers of individual mosquitoes would need to be processed to obtain a reliable estimate; (2) incriminate *Anopheles* species as malaria vectors; (3) identify all the circulating *Plasmodium* species in vectors from an area; (4) detect mixed infections in mosquitoes; and (5) detect mosquitoes with low-level parasite infections.

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[*] Corresponding author. Tel.: +591 2 278 28 80; fax: +591 2 278 29 44.
E-mail address: lardeux@ird.fr (F. Lardeux)

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doi:10.1016/j.trstmh.2008.02.006

1. Introduction

Since the work of MacDonald (1957), the understanding of malaria transmission dynamics by *Anopheles* species has relied on the estimation of various entomological and parasitological indices, of which the sporozoite rate (the proportion of infective mosquitoes, i.e. those with sporozoites in the salivary glands) is paramount. In general terms, a reliable estimate of the proportion of the infective mosquitoes in a vector population requires the processing of a relatively large number of insects, and the results depend on the sensitivity of the detection method employed.

Traditionally, the detection of malaria sporozoites in mosquito salivary glands occurs through the visualization of the parasites with a light microscope. This requires careful dissection and examination of individual mosquitoes. Along with the intensive labor required, another limitation of the method is that it cannot distinguish between the various species of *Plasmodium* and thus is of limited value in areas where more than one species is endemic; this is true of Bolivia, where *P. falciparum*, *P. vivax* and *P. malariae* are known to circulate sympatrically (Moscoso Carrasco, 1963). Some alternative methods to microscopy have been developed, which rely on specific monoclonal antibodies raised against circumsporozoite (CS) protein. Among these, the ELISA technique (Beier et al., 1988) is the most attractive alternative and has been used on various *Anopheles* species worldwide to identify *Plasmodium* species. Other methods based on nucleic acid hybridization of specific DNA probes have also been proposed (Baker et al., 1986, among others). However, all these techniques have their limitations in terms of sensitivity and/or feasibility in field conditions.

DNA amplification by PCR offers a practical and advantageous alternative to all previous techniques. It is a specific and sensitive method that can be carried out independently of time and place of capture of insects. PCR is routinely used to detect some pathogens in their vectors, such as dengue viruses (Sudiro et al., 1997), filarial nematodes (Nicolas et al., 1996; Vasuki et al., 2001; Yameogo et al., 1999) and trypanosomes (Aransay et al., 2000; Silber et al., 1997), among others. DNA extraction and PCR amplification have been used to detect *Plasmodium* parasites in blood samples from patients (Snounou, 1996), but it still remains problematic in detecting these parasites in whole mosquitoes because of the inhibition of the PCR (Schriefer et al., 1991; Snounou et al., 1993b) by inhibitors present in mosquito tissues and in the hard exoskeleton of the head and the thorax (Arez et al., 2000). Because PCR primer sets to identify and distinguish between human malaria parasites are available, the key factor in entomological studies is finding an optimal protocol to extract *Plasmodium* DNA from whole bodies of mosquitoes.

The present study describes a protocol for optimal DNA extraction and a semi-nested PCR to identify the four human malaria parasites in single individuals and pools of *Anopheles* without entomological dissection, and thus can be used in epidemiological studies where large numbers of mosquitoes need to be processed. The technique was carried out on wild-caught *An. pseudopunctipennis* and *An. argyritarsis* from a village in the central Andean region of Bolivia, where *P. vivax* transmission occurs.

2. Materials and methods

For all PCR reactions, an Indy Air Thermocycler model 500 (Idaho Technology Inc., Salt Lake City, UT, USA) was used. Chemicals for reactions came from Promega (Charbonnières-les-Bains, France) or Sigma-Aldrich (Lyon, France), primers and dNTPs from Eurogentec (Angers, France) and *Taq* DNA polymerase from Qiagen (Courtaboeuf, France). Mosquitoes were homogenized with an ultrasonic homogenizer (CC30 sonificator, Bioblock, Illkirch, France).

2.1. Search for an optimal *Plasmodium* DNA extraction protocol

We evaluated eight DNA extraction protocols followed by PCR amplification.

2.1.1. Extraction with chelex 100 and phenol-chloroform (i.e. the selected protocol)

Mosquito heads and thoraces were homogenized in saline solution (NaCl 0.9%). The volume of saline solution depended on the number of mosquitoes pooled (Table 1). Chelex 100 (a cationic exchange resin) at 5% or 10% (w/v) was added and vortexed. Products were incubated at 100 °C for 10 min and then centrifuged at 13 000 rpm for 5 min. The supernatant was mixed in 1:1 volume with phenol-chloroform and centrifuged at 10 000 rpm for 5 min three times. Then the supernatant was mixed with a 1:1 volume of 70% ethanol and centrifuged at 14 000 rpm for 20 min. The pellet was dried at 37 °C, suspended in 100 μl nuclease-free H_2O and kept at 4 °C until processed by PCR.

2.1.2. Extraction with chelex 100

Mosquitoes were homogenized in 50 μl saline solution (NaCl 0.9%). A volume of 240 μl chelex 100 at 5% (w/v) was added and vortexed. The mixture was heated to 100 °C for 10 min in an oil bath, then centrifuged at 13 000 rpm for 5 min, and the supernatant was used for PCR.

2.1.3. Extraction with CTAB

Mosquitoes were homogenized in 200 μl lysis CTAB solution (100 mmol/l Tris HCl pH 8.0; 10 mmol/l EDTA pH 8.0; 1.4 mol/l NaCl and CTAB 2%). Incubation was carried out at 70 °C for 5 min; the resulting extract was washed with a 1:1 volume of chloroform:isoamylalcohol (24:1), and centrifuged for 5 min at 12 000 rpm. The DNA from the supernatant was precipitated in an equal volume of cold isopropanol and centrifuged again at 12 000 rpm for 15 min. The pellet was washed with 70% ethanol, centrifuged at 12 000 rpm for 5 min, dried at 37 °C and suspended in 100 μl nuclease-free H_2O.

2.1.4. Extraction with proteinase K

Mosquitoes were homogenized in 200 μl buffer solution (10 mmol/l Tris HCl pH 7.8; 5 mmol/l EDTA; 0.5% SDS) and proteinase K was added to a final concentration of 50 μg/ml. The mixture was incubated at 55 °C for 1 h and the proteinase K was inactivated at 95 °C for 10 min. Centrifugation was carried out at 10 000 rpm for 5 min and the supernatant was washed with 200 μl chloroform and centrifuged again at

Table 1 Concentration of chelex 100 (%) and volumes (μl) of saline solution and of chelex 100 used in the preparation of the DNA template in accordance with the size of the pool of mosquitoes

Mosquito pool size (no. mosquitoes processed)	Chelex 100 concentration (%)	Volume (μl) of saline solution (NaCl 0.9%)	Volume (μl) of chelex 100
2–10	5	50	240
20	5	100	480
30	5	150	750
40	10	200	800
50	10	250	800
60	10	300	900
70	10	350	900
80	10	400	1000
90	10	450	1000
100	10	500	1000

12 000 rpm for 5 min. The DNA in the supernatant was precipitated in 1:1 volume cold isopropanol and kept at −20 °C for 20 min, centrifuged at 12 000 rpm for 15 min, and washed with 70% ethanol and centrifuged again at 12 000 rpm for 5 min. The pellet was dried at 37 °C and suspended in 100 μl nuclease-free H_2O.

2.1.5. Extraction with sarcosil

Mosquitoes were homogenized in 200 μl extraction solution [10 mmol/l Tris HCl pH 7.8; 100 mmol/l NaCl; 100 mmol/l EDTA pH 8.0; 1% (w/v) sarcosil], incubated at 70 °C for 5 min and washed with 1:1 volume chloroform:isoamylalcohol (24:1). The mixture was centrifuged at 12 000 rpm for 5 min and the DNA in the aqueous phase was precipitated in 1:1 volume cold isopropanol. The microtube was kept in vertical position at −20 °C and then centrifuged at 12 000 rpm for 15 min. The pellet was washed with 70% ethanol and centrifuged at 12 000 rpm for 5 min. It was then dried at 37 °C and suspended in 100 μl nuclease-free H_2O.

2.1.6. Extraction with sarcosil and proteinase K

After homogenizing the mosquitoes in the extraction solution with sarcosil, proteinase K was added to a final concentration of 100 μg/ml. The microtube was incubated at 55 °C for 1 h. Then the proteinase K was inactivated at 95 °C for 10 min. After that, the previous protocol was used, beginning with the washing in chloroform:isoamylalcohol.

2.1.7. Extraction with CTAB, mercaptoethanol and polyvinylpolypyrrolidone

Mosquitoes were homogenized in 200 μl of the following extraction solution: 200 μl lysing CTAB solution (100 mmol/l Tris HCl pH 8.0; 10 mmol/l EDTA pH 8.0; 1.4 mol/l NaCl and CTAB 2%), 0.08 mg polyvinylpolypyrrolidone (PVPP) and 1 μl mercaptoethanol. The tubes were incubated at 55 °C for 1 h and then 200 μl 1:1 volume chloroform:isoamylalcohol (24:1) was added. The mixture was centrifuged at 14 000 rpm for 10 min and 0.08 volume of ammonium acetate 7.5 mol/l and 0.54 volume of isopropanol were added to the supernatant. The mixture was kept cold on ice for 15 min and centrifuged at 12 000 rpm for 3 min. The pellet was washed with 200 μl 70% ethanol and was centrifuged at 12 000 rpm for 5 min. The DNA was dried at 37 °C for approximately 1 h and was resuspended in 100 μl nuclease-free H_2O.

2.1.8. Extraction with CTAB and mercaptoethanol

Mosquitoes were homogenized in 1:4 CTAB:mercaptoethanol solution (CTAB solution: 100 mmol/l Tris HCl pH 8.0; 10 mmol/l EDTA pH 8.0; 1.4 mol/l NaCl and CTAB 2%) and incubated for 1 h at 65 °C. Then 200 μl nuclease-free H_2O and 200 μl 1:1 volume chloroform:isoamylalcohol (24:1) were added. The mixture was centrifuged at 12 000 rpm for 5 min and 200 μl isopropanol was added to the supernatant. The microtube was incubated at −20 °C for 10 min and centrifuged at 12 000 rpm for 15 min. The pellet was washed with 70% ethanol and then centrifuged at 12 000 rpm for 5 min. The pellet was dried at 37 °C and then suspended in 100 μl nuclease-free H_2O.

Infective *An. pseudopunctipennis* were obtained by feeding uninfected laboratory-reared individuals on the forearm of one of the authors (RT), who contracted *P. vivax* malaria while working in the field in Guayaramerin (north of Bolivia). Infected mosquitoes were kept in the insectary at 27 °C for 2 weeks to guarantee the presence of sporozoites in the salivary glands and were regularly dissected to verify this. Infective mosquitoes were preserved at −20 °C until processed.

The amplification of *Plasmodium* DNA was carried out using the semi-nested multiplex PCR protocol described below. The internal transcribed spacer 2 (ITS2) region of *Anopheles* was used as an internal control for DNA extraction and PCR amplification. It was amplified using the primers from Porter and Collins (1991) with a slightly modified PCR protocol from Manguin et al. (1999). PCR inhibition tests were then validated with both *Plasmodium* spp. amplifications and ITS2. The most efficient DNA extraction protocol among those tested was then selected following visualization of PCR products under UV light, after electrophoresis on 2% agarose gels and staining with ethidium bromide.

2.2. Identification of *Plasmodium* spp. in wild-caught *Anopheles*

Wild-caught *An. pseudopunctipennis* and *An. argyritarsis* were obtained from human landing catches in Mataral

(S 18.6024, W 65.1444, altitude 1500 m), a small village characteristic of those encountered in the dry valleys of the Bolivian Andes, where human malaria occurs. It is situated in the centre of Bolivia, about 100 km north of the constitutional capital Sucre. Approximately 1000 inhabitants live there in around 80 grouped houses. Mosquitoes were caught inside and outside houses on four consecutive nights on a monthly basis during 2003. Mosquitoes were identified and classified as parous or nulliparous following examination of the ovaries. Mosquitoes were pooled by 'capture', which consisted of insects from one house, location within the house (inside or outside), night and hour of the night. Pools were labeled and kept at −20 °C until processed. These mosquitoes are part of a larger study on malaria transmission dynamics and only infection results for May 2003 are presented here as an application of the DNA extraction and PCR protocol. The *An. pseudopunctipennis* sample consisted of 567 females (264 parous, 277 nulliparous and 26 not classified), which were separated into 168 pools of 1—18 individuals. The mean number of *An. pseudopunctipennis* bites per person per hour was 2.0. At the same time, and in the same conditions, *An. argyritarsis* was captured and the sample consisted of 101 females (48 parous, 47 nulliparous and 6 not classified), grouped in 13 pools of 3—21 individuals.

Mosquito DNA was extracted from the pools using the chelex 100 + phenol-chloroform protocol, which proved to be the most efficient (see the results). DNA from these pools was used in the semi-nested multiplex PCR protocol described below, and prevalence of infection in the mosquito samples was computed using PoolScreen 2.0, an algorithm that takes into account the pooling of insects (Katholi et al., 1995).

2.3. Semi-nested multiplex PCR amplification and sensitivity

The detection and identification of *Plasmodium* species in mosquitoes were simultaneously performed with a sequence of two semi-nested multiplex PCR based on Rubio et al. (1999a, 1999b, 2002) with the following modifications.

The PCR mix for the first reaction consisted of 1× buffer PCR; 1.5 mmol/l $MgCl_2$; 0.5 mmol/l dNTPs; 0.05 μmol/l of each primer: UNR (Universal reverse) and PLF (*Plasmodium* spp.); 0.075 U/μl *Taq* DNA polymerase and 10 μl DNA template for a final volume of 20 μl. This first reaction should result in amplified DNA fragments of 783—821 bp, depending on the *Plasmodium* species present (Table 2).

The PCR mix for the second reaction consisted of 1× buffer PCR; 1.5 mmol/l $MgCl_2$; 0.5 mmol/l dNTPs; 0.08 μmol/l PLF primer; 0.04 μmol/l of each reverse primer VIR (*P. vivax*); FAR (*P. falciparum*); MAR (*P. malariae*); 0.075 U/μl *Taq* DNA polymerase and 2 μl of the amplicon from the first PCR, for a final volume of 20 μl. The primer OVR (*P. ovale*) (Rubio et al., 2002) could also be added at the same concentration as the others, but was not used because the parasite is absent from South America.

For both PCRs, 15 μl mineral oil was added before the amplification. The first PCR began with 5 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, 1 min at 60 °C and 90 s at 72 °C. The second PCR began with 5 min at 94 °C, and 35 cycles of 30 s at 94 °C, 30 s at 62 °C and 1 min at 72 °C. The final cycle was followed by an extension time of 10 min at 72 °C.

The sizes of the PCR products were estimated after electrophoresis on 1.5% agarose gel and stained with ethidium bromide. Positive samples were confirmed on an 8% polyacrylamide gel, staining with 0.2% silver nitrate and revealed with a 2:1 volume of 30 g/l sodium carbonate:0.02% formaldehyde.

The first PCR included a universal reverse primer with a forward primer specific for human *Plasmodium*, and this product was used as a DNA template for the second PCR, in which specific reverse primers (Rubio et al., 2002) identified the *Plasmodium* species (Table 2).

Several controls were used: a mosquito negative control (a non-infected mosquito from the insectary), a mosquito positive control (an infected *P. vivax* mosquito from the laboratory) and blood infected with *P. vivax* and *P. falciparum*. Positive controls helped to verify that the PCR reaction worked and that there was no contamination, and made it easier to localize bands from positive wild mosquitoes on the agarose gels.

The sensitivity of the semi-nested multiplex PCR protocol was investigated using pools of mosquitoes consisting of 1, 4, 9, 19, 29, 49 and 99 uninfected individuals from the insectary in which one infective mosquito was added.

3. Results

3.1. Optimal *Plasmodium* DNA extraction protocol and PCR amplification

The eight extraction protocols did not yield large amounts of DNA, as indicated by weak bands on 1.5% agarose gels,

Table 2 Primer names, sequence targets and size of the PCR product (in bp) from Rubio et al. (2002) and used in the present study

Primer	Sequence (5′—3′)	Specificity	Size of PCR product (bp)
UNR	GACGGTATCTGATCGTCTTC	Universal	
PLF	AGTGTGTATCAATCGAGTTTC	*Plasmodium*	*P. malariae* = 821 *P. falciparum* = 787 *P. vivax* = 783
FAR	AGTTCCCCTAGAATAGTTACA	*P. falciparum*	395
MAR	GCCCTCCAATTGCCTTCTG	*P. malariae*	269
VIR	AGGACTTCCAAGCCGAAGC	*P. vivax*	499

Table 3 Results of PCR amplification of extracted DNA from *Anopheles pseudopunctipennis* with various protocols[a]

DNA extraction protocol	Extracted DNA dilutions							
	0.1	0.02	0.01	0.002	0.001	0.0005	0.0001	0.00002
Chelex 100	+	+	+	+	+	+	+	+
Chelex 100 + phenol-chloroform	+	+	+	+	+			
Proteinase K	+	+/−	+	+	−	−	−	−
CTAB	+	+	+/−	+	−	−	−	−
Sarcosil	−	−	−	+	−	−	−	−
Sarcosil + proteinase K	−	−	−	+	−	−	−	−
CTAB + PVPP + mercaptoethanol	−	−	−	−	−	−	−	−
CTAB + mercaptoethanol	+	+	+	+	+	+	+	+

[a] PCRs were carried out at various dilutions of extracted DNA (range 10^{-1} to 2×10^{-5}): '+' indicates a clear DNA amplification; '+/−' indicates a weak amplification; and '−' indicates no amplification.

although the extracted DNA was not degraded. Regardless, there were clear differences among the protocols in the yield of DNA (Table 3). PCR amplification using chelex or CTAB (except with PVPP) was found to be the most efficient, whereas Proteinase K- and sarcosil-based protocols were the least efficient. Chelex and CTAB + mercaptoethanol protocols presented amplification bands at higher dilutions of the DNA extract. The amplification of *Plasmodium* species DNA by PCR indicated that there was little or no inhibition of the PCR reaction with the chelex, chelex + phenol-chloroform and CTAB + mercaptoethanol protocols. Thus, the chelex + phenol-chloroform protocol was selected because it yielded stronger bands, seemed to be the most efficient, and was cheaper than the CTAB-based protocol.

This extraction protocol, and the semi-nested multiplex PCR used to analyze field-caught mosquitoes, enabled the detection of one infected mosquito in pools of uninfected mosquitoes, including pools of 99 uninfected individuals (Figure 1). To increase the sensitivity of detection of positive samples, the systematic use of polyacrylamide gels in place of agarose for the electrophoretic migration of PCR products is recommended.

3.2. Detection of *Plasmodium* species in wild-caught *Anopheles*

For *An. pseudopunctipennis*, five pools were found positive for *P. vivax* on agarose gels (Figure 1) and confirmed on polyacrylamide gel (Figure 2). These pools consisted of 10, 6, 3 and 2 mosquitoes.

The prevalence of infection of the whole sample from Mataral was 0.9% (95% CI 0.3—2.1). When only the parous females were taken into account, the prevalence of infection was 1.9% (95% CI 0.6—4.4). Neither *P. malariae*- nor *P. falciparum*-infected mosquitoes were detected. For *An. argyritarsis*, no mosquito pool was found positive for any of the three *Plasmodium* species tested.

4. Discussion

In accordance with other studies (Arez et al., 2000; Schriefer et al., 1991; Snounou et al., 1993b), our experiments showed that the main barrier in identifying *Plasmodium* parasites in mosquitoes is the inhibition of the PCR reaction by

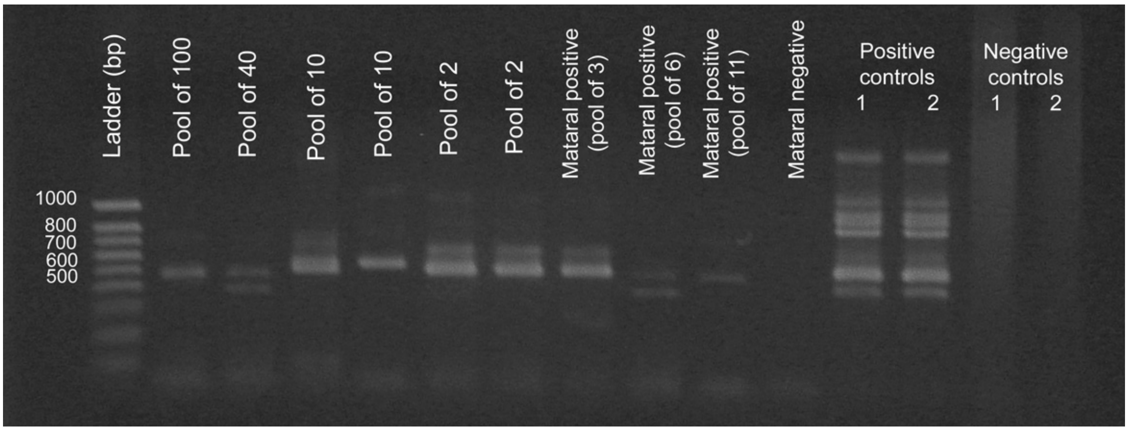

Figure 1 Electrophoretic migration on agarose gel showing (from left to right): the Smart Ladder (1000, 800, 700, 600 bp, etc.); a *Plasmodium vivax*-positive mosquito in a pool of 99 non-infected mosquitoes from the laboratory; a *P. vivax*-positive mosquito in a pool of 39 negative ones; two results of a *P. vivax*-positive mosquito in a pool of nine negative ones; two results of a *P. vivax*-positive mosquito with a negative one; three positive samples from Mataral (pools of 3, 6 and 11 wild mosquitoes, respectively); and the controls [two positive controls from infected blood (mix of *P. vivax* + *P. falciparum*) and two negative controls (uninfected mosquitoes)].

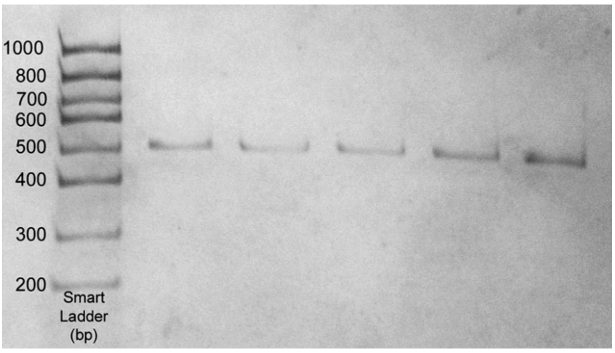

Figure 2 Confirmation with electrophoretic migration on polyacrylamide gel of the five *Plasmodium vivax*-positive pools of *Anopheles pseudopunctipennis* from Mataral (499 bp bands). Pool sizes were (from left to right): 2, 6, 11, 3 and 2 mosquitoes.

inhibitors still present after DNA extraction. To overcome this problem, many authors dissect the midgut and/or the salivary glands of each mosquito before PCR processing. This approach may be adequate for specific laboratory experiments, but not for epidemiological studies in which large numbers of mosquitoes are processed. Unlike other protocols (Snounou et al., 1993a, among others), the chelex treatment of samples before PCR presented here seems to overcome the PCR inhibition phenomenon. Chelex-based extraction enhances PCR amplification of gene sequences (Schriefer et al., 1991; Singer-Sam et al., 1989), and has been considered by Arez et al. (2000) as one of the most efficient techniques. They also indicated that chelex-based protocols may be among the most powerful to extract small amounts of DNA and as such to identify lightly parasitized mosquitoes.

Furthermore, the semi-nested PCR technique used here enhances the sensitivity of DNA detection and thus may detect as few as three sporozoites (or 0.06 pg DNA) (Li et al., 2001). Distinguishing among the human malaria parasites is based on features of the small subunit nuclear rRNA gene, a multicopy gene that possesses both highly conserved domains and highly specific domains characteristic of the four human malaria parasites. As such, the semi-nested multiplex PCR technique is very specific (Rubio et al., 2002).

To improve the PCR protocol and distinguish uninfective mosquitoes from simple PCR failures, an internal positive control such as a specific universal reverse primer of *Anopheles* may be added to the universal reverse primer and the specific *Plasmodium* forward primer in the first PCR reaction. This approach was suggested by Rubio et al. (2002), who used a specific mammalian primer to work on human blood (not on mosquitoes).

The chelex-based DNA extraction protocol and the semi-nested multiplex PCR presented here may be one of the most efficient methods to extract and identify malaria parasite DNA from whole mosquitoes. In countries such as Bolivia, where more than one malaria species may circulate in the same area, the semi-nested multiplex PCR presented here can distinguish between the various species of *Plasmodium* and can easily process large amount of mosquitoes grouped in batches. This makes the technique a powerful tool for epidemiological studies or to incriminate particular *Anopheles* species as vectors. Because of the high sensitivity of the technique, one needs to be careful of contamination in assays.

When the prevalence of infection in mosquitoes is thought to be low, as in most countries of South America (Mouchet et al., 2004), larger numbers of mosquitoes must be examined to compute a statistically relevant prevalence index. PCR permits the processing of large numbers of mosquitoes with less human error than that which can occur with the dissection technique. Moreover, it does not need to be carried out in the field with fresh mosquitoes. The sensibility of the PCR also enables the detection of lightly parasitized mosquitoes with few parasites in the salivary glands, which is more difficult with the dissection technique. PCR also has several advantages over the ELISA technique, which may not be feasible after storage of mosquito samples at high temperatures, as this deteriorates the CS protein. Moreover, ELISA cannot differentiate between the actual surface of the sporozoite and CS protein that may be deposited on the vector tissue (Golenda et al., 1990), which can falsify results. The technique may also miss positive mosquitoes because it lacks sensitivity (Povoa et al., 2000) and thus is not suitable for processing large batches of mosquitoes. Hybridization-based methods may be unattractive in large-scale experiments because radioactive materials are required, and the detection threshold of 500—1000 sporozoites/mosquito is not sensitive enough to detect low parasite burdens and may exhibit non-specific hybridization. The PCR approach overcomes all these problems.

The semi-nested multiplex PCR technique used in this study enables the processing of pools of up to 100 *Anophele*s at a time, whereas with ELISA the maximum pool size is generally about 10. However, care must be taken not to identify false positives as infective mosquitoes. The malaria parasite may develop up to the oocyst stage in many *Anopheles* species (the mosquito is infected, but no sporozoites develop in the salivary glands), but may mature to the sporozoite stage in only a few. In the field, some species may then carry dead *Plasmodium* in their midgut, and others may harbor degenerated parasite oocysts but never sporozoites because they are not competent vectors. As PCR cannot distinguish among the developmental stages of the parasite, only mosquito heads and thoraces should be used to ensure the analysis of the salivary glands and not the midgut.

In some South American countries, including Bolivia, only indirect evidence of malaria transmission has been reported, such as the presence of certain *Anopheles* species in high densities, their presence in high proportion preceding the highest point of transmission, or their marked anthropophily in some malaria-endemic areas. Depending on the year, Bolivia reports between 10 and 70 000 annual cases of malaria, of which about 15% are *P. falciparum*. All others cases are *P. vivax*, and *P. malariae* is virtually absent. *Anopheles pseudopunctipennis* is likely to be the main malaria vector in the Andean region (Hackett, 1945), whereas *An. darlingi* is the main vector in the Amazonian region. However, for Bolivia, there is no direct proof that *Plasmodium* sporozoites occur in these species. ELISA has been used to confirm the presence of natural infection of *An. pseudopunctipennis* with *P. vivax* in Mexico (Fernandez-Salas et al., 1994) and Peru (Hayes et al., 1987). Malaria parasites have also been observed by dissection of this species in many countries in Latin America, but never in Bolivia. Thus the discovery of infected *An. pseudopunctipennis* in Mataral by PCR represents the first direct evidence of *P. vivax* transmission by *An. pseudopunctipennis* in this country. The prevalence of infection of 0.9% in the Mataral sample is similar to results obtained in Mexico (Fernandez-Salas et al., 1994). It is lower than values from Hayes et al. (1987) in Peru or Loyola et al. (1991) in Mexico, who found a prevalence of infection of 2.6 and 3.1%, respectively. However, depending on the season, the prevalence of infection may vary. Further results from Mataral will specify the monthly variations of this parameter.

In Bolivia, *An. pseudopunctipennis* may also be encountered in association with other species, such as *An. oswaldoi*, *An. argyritarsis*, *An. triannulatus*, *An. strodei*, *An. benarocchi* or *An. Marajoara*, which have also been incriminated as malaria vectors in other American countries (Hayes et al., 1987; Mouchet et al., 2004). As such, the status of *An. pseudopunctipennis* as the only malaria vector in the Andean region of Bolivia may be questioned, even if the other species are less abundant. In Mataral, the only other *Anopheles* species present is *An. argyritarsis*. The preliminary results obtained in Mataral (no infective individuals found) agree with other studies that this species is not a malaria vector in Latin America (Rubio-Palis, 1993). Again, further results from Mataral will specify the vector status of this species in Bolivia, where it seems to be more anthropophilic than in Venezuela (Rubio-Palis and Curtis, 1992).

The experiment in Mataral permitted the first use of PCR for identification of *Plasmodium* in *Anopheles* in South America, the first pooling of *Anopheles* for *Plasmodium* identification, and one of the first uses of PCR in field-caught non-dissected mosquitoes.

In conclusion, the method presented here, to extract malaria parasite DNA from *Anopheles* and identify the malaria species by PCR, is simple, efficient, sensitive and specific. It may be recommended for field surveys of *Anopheles* to rapidly identify malaria vectors, to compute prevalence of infection in mosquito populations and to identify even lightly infective individuals and mixed infections. As such, it may be the method of choice to be used by malaria control programs in assessing the impact of control measures. The study permitted the certification of *An. pseudopunctipennis* as a *P. vivax* vector in the Andean region of Bolivia, and is likely to be the main vector in this region.

Authors' contributions: FL designed the study, oriented the choice of primers and protocol studies, and designed the *Anopheles pseudopunctipennis* experiments and captures in Mataral; TC facilitated the laboratory work and helped to organize the fieldwork; FL, RUB and TC captured wild *An. pseudopunctipennis* in Mataral; CL and RUB helped to design the laboratory components of the study and standardized the initial PCR conditions; RT tested the DNA extractions and PCR amplification protocols in the laboratory and standardized the final protocol of *Plasmodium* species identification in pools of mosquitoes by PCR; RT processed the Mataral mosquito samples with help from CA who confirmed the mosquito infections with polyacrylamide gels; FL and RT analyzed the results; FL wrote the manuscript and CL and RUB revised it critically for intellectual content. All authors read and approved the final manuscript. RT and FL are guarantors of the paper.

Acknowledgements: The authors would like to thank people from Mataral who helped in capturing wild mosquitoes, along with B. Bouchité (IRD), and J. Ericsson for reviewing the manuscript.

Funding: This research was supported by a French Ministry of Research PAL+ grant. The participation of RUB in this study was possible thanks to the Association of Universities and Colleges of Canada (AUCC) and its Canada-Latin America and the Caribbean Research Exchange Grants. CL was funded by grants from NSERC (261940), CIHR (69558), the Canada Research Chair program and an MSFHR scholar award.

Conflicts of interest: None declared.

Ethical approval: The field surveys received approval from the Bolivian Ministry of Health.

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