Anthropophilic *Anopheles* species composition and malaria in Tierradentro, Córdoba, Colombia

David Joachim Schiemann[1,2]/+, Martha Lucía Quiñones Pinzón[2], Thomas Hankeln[1]

[1]Institute of Molecular Genetics, Biosafety Research and Consulting, Johannes Gutenberg University, Mainz, Germany
[2]Departamento de Salud Pública, Universidad Nacional de Colombia, Bogotá, Colombia

Malaria is still a primary health problem in Colombia. The locality of Tierradentro is situated in the municipality of Montelíbano, Córdoba, in the northwest of Colombia, and has one of the highest annual parasite index of malaria nationwide. However, the vectors involved in malaria transmission in this locality have not yet been identified. In this study, the local anthropophilic Anopheles *composition and natural infectivity with* Plasmodium *were investigated. In August 2009, 927 female* Anopheles *mosquitoes were collected in eight localities using the human landing catch method and identified based on their morphology. Cryptic species were determined by restriction fragment length polymorphism-internal transcribed spacer (ITS)2 molecular analysis. Eight species [*Anopheles nuneztovari s.l. *(92.8%),* Anopheles darlingi *(5.1%),* Anopheles triannulatus s.l. *(1.8%),* Anopheles pseudopunctipennis s.l. *(0.2%),* Anopheles punctimacula s.l. *(0.2%),* Anopheles apicimacula *(0.1%),* Anopheles albimanus *(0.1%) and* Anopheles rangeli *(0.1%)] were identified and species identity was confirmed by ITS2 sequencing. This is the first report of* An. albimanus, An. rangeli *and* An. apicimacula *in Tierradentro. Natural infectivity with* Plasmodium *was determined by ELISA. None of the mosquitoes was infectious for* Plasmodium. An. nuneztovari s.l. *was the predominant species and is considered the primary malaria vector;* An. darlingi *and* An. triannulatus s.l. *could serve as secondary vectors.*

Key words: malaria - *Anopheles* - *Plasmodium* - infectivity - Colombia

Colombia is among the 31 high-malaria-burden countries (Aregawi et al. 2009); although the total number of cases decreased by 45% from 2003 (125,064) to 2012 (56,175), malaria remains a major health problem. In 2012, the departments of Antioquia and Córdoba, situated in the northwest of the country, reported 53.1% of the total cases [National System for Public Health Surveillance (SIVIGILA)]. Recent studies of the anthropophilic *Anopheles* composition and natural mosquito infectivity with *Plasmodium* in the municipality of Montelíbano have led to the identification of four *Anopheles* species: *Anopheles nuneztovari s.l.*, *Anopheles darlingi*, *Anopheles oswaldoi s.l.* and *Anopheles punctimacula s.l.* Of those, *An. nuneztovari s.l.* (99.4%) was the most abundant species in the municipality. *An. nuneztovari s.l.* was also found to be infected with *Plasmodium vivax* [infectivity rate (IR) = 0.489%] and is therefore considered the main malaria vector in the municipality (Gutiérrez et al. 2009). Naranjo-Diaz et al. (2013) analysed species diversity in the neighbouring municipalities of Tierralta and Puerto Libertador and identified six species: *An. nuneztovari s.l.*, *An. darlingi*, *Anopheles triannulatus s.l.*, *Anopheles pseudopunctipennis s.l.*, *An. punctimacula s.l.* and *Anopheles argyritarsis*, of which *An. nuneztovari s.l.* (IR = 0.05-0.10) and *An. triannulatus s.l.* (IR = 1.52) were found to be infective for *P. vivax VK247* and *An. darlingi* (IR = 0.09) for *P. vivax VK210*. Tierradentro is one of the localities of Montelíbano that has a high malaria incidence. However, the previous entomological studies were performed in the urban sector only due to serious disturbances affecting law, order and security.

This study aimed to investigate urban and rural mosquito populations and their natural *Plasmodium* infectivity to determine malaria vectors in the locality of Tierradentro, Montelíbano. Eight species (complexes) of the subgenera *Nyssorhynchus* and *Anopheles* were identified: *An. nuneztovari s.l.*, *An. darlingi*, *An. triannulatus s.l.*, *An. pseudopunctipennis s.l.*, *An. punctimacula s.l.*, *Anopheles apicimacula*, *Anopheles albimanus* and *Anopheles rangeli*. Species identity was confirmed by restriction fragment length polymorphism-internal transcribed spacer (RFLP-ITS)2 analysis, ITS2 sequencing and National Center for Biotechnology Information (NCBI) BLAST search. None of the mosquitoes was found to be infectious for *Plasmodium* (IR < 0.1%).

The locality of Tierradentro is situated in the northwest of Colombia in the south of Córdoba in the municipality of Montelíbano (Supplementary data). The urban centre is located along the San Jorge River at 55 m above mean sea level (07º48'50''N 75º52'40''W). The tropical climate is characterised by a monthly mean temperature of 25.6-27.1ºC (SIPLAN 2008). The average annual precipitation is 2,386 mm and ranges from 350 mm in August to 20 mm in January (Hydrometrical Station Cuba Hda). The region is mountainous and the vegetation consists of natural forests, gallery forests, stubble, pastures and cropland.

doi: 10.1590/0074-0276130483
Financial support: DAAD
+ Corresponding author: daschiemann@gmail.com
Received 4 October 2013
Accepted 27 February 2014

The total population of Tierradentro is 6,447, with 77.75% inhabiting the urban sector and 22.25% the rural regions (SIPLAN 2008). From 2003-2009, Montelíbano reported 6.29% of the nationwide malaria cases (664,489); 42,039 cases were registered in the municipality, of which 77% were caused by *P. vivax*, 22% were caused by *Plasmodium falciparum* and 1% were caused by infection with both parasites. The annual parasite index for Montelíbano was estimated at 114/1,000 in 2007. The region is classified as endemic for malaria, with a high and constant transmission throughout the year (SIVIGILA).

In August 2009, during the rainy season, human landing catches were performed in the urban centre [Claret (CLA)] and seven rural localities of Tierradentro with distinct environmental conditions: Parcela Belén (BEL), San Antonio (SAN), Bocas de San Cipriano, Bocas de San Mateo (BSM), Vallecito (VAL) and Parque Paramillo, Venado (VEN) and Santa Isabel (ISA). The walking distance between the collection sites was 1-8 h. Female *Anopheles* mosquitoes were captured during one-five nights in each locality depending on the weather conditions, with one-three baits per site (inclusive the main investigator and local volunteers), indoors and peridomestically from 06:00-10:00 pm sacrificed hourly, individualised in perforated tubes and stored in plastic bags supplied with silica gel until they were transported to the laboratory where they were stored at 20ºC. The mosquitoes were identified based on their morphological characteristics (Carrejo & González 2007). However, *Anopheles* species belonging to the *Oswaldoi* Group may be misidentified due to intra and interspecific variations in morphology. Therefore, the identity of cryptic species (*An. nuneztovari s.l.*, *An. oswaldoi s.l.*, *An. rangeli*) was confirmed by polymerase chain reaction amplification of the ITS2 region directly from a mosquito leg and detection of an RFLP within ITS2 with Alu I restriction enzyme digestion (Zapata et al. 2007, Cienfuegos et al. 2008). The ITS2 amplicon from one-three specimens of each species was purified with ExoSAP-IT and sequenced by StarSEQ GmbH (Mainz, Germany); an NCBI GenBank database search using the BLAST algorithm was performed. All specimens were tested for *P. falciparum* and *P. vivax* infectivity by ELISA (Wirtz et al. 1987), as instructed in the ELISA kit protocol distributed by the Centers for Disease Control and Disease (Atlanta, GA, USA).

A total of 927 *Anopheles* mosquitoes were collected in Tierradentro in August 2009; 30.7% (285) in the urban centre (suburb CLA) and 69.3% in the rural sector (BSM: 182, BSC: 165, VAL: 163, BEL: 66, SAN: 40, VEN: 21, ISA: 11). Based on morphological characteristics and RFLP-ITS2 and ITS2 sequencing, eight *Anopheles* species belonging to the subgenera *Nyssorhynchus* and *Anopheles* were identified: *An. nuneztovari s.l.* (92.4%, KF436936), *An. darlingi* (5.1%, KF436940), *An. triannulatus s.l.* (1.8%, KF436938), *An. pseudopunctipennis s.l.* (0.2%, KF436937), *An. punctimacula s.l.* (0.2%, KF436941), *An. albimanus* (0.1%, KF436939), *An. apicimacula* (0.1%, KF436935) and *An. rangeli* (0.1%). The species identity of 43 specimens remained unclear. NCBI BLAST identification resulted in 99% sequence identity for *An. nuneztovari* B/C (Fritz et al. 1994, Sierra et al. 2004, Marrelli et al. 2005), *An. darlingi* (Marrelli et al. 2005), *An. pseudopunctipennis s.l.* (Miller et al. 1997, M Herrera et al., unpublished observations), *An. triannulatus* of the lineage NW (Rosero et al. 2012, Moreno et al. 2013), *An. punctimacula s.s.* [Cienfuegos & Correa (GU477275) (Loaiza et al. 2013)], *An. albimanus* (L78065) (Cienfuegos et al. 2011) and *An. apicimacula* (Loaiza et al. 2013). The species composition differed in each locality. *An. nuneztovari s.l.* was the most abundant species in all localities [CLA (198/201, 98.5%), BEL (64/67, 95.5%), BSC (131/164, 79.9%), BSM (171/183, 93.4%), SAN (36/40, 90%), VAL (144/160, 90%), VEN (19/21, 90.5%) and ISA (10/11, 90.9%)], due to its adaptability to variable larval habitats, particularly artificial ones (Tadei et al. 1998, Tadei & Thatcher 2000). In accordance with former studies, this species is considered the primary malaria vector (Gutiérrez et al. 2009). *An. darlingi* was largely collected in BSC (20/163, 12.3%) and VAL (14/160, 8.6%), with a few specimens from CLA (2/210, 1%), BEL (1/67, 1.5%), SAN (1/40, 2.5%) and BSM (3/183, 1.6%). BSC and VAL are located in partly deforested flat areas that constitute perfect breeding sites for this species (Hiwat & Bretas 2011). *An. triannulatus s.l.* showed no restriction to specific localities and was encountered in BSM (8/183, 4.4%), SAN (2/40, 5%), BSC (2/163, 1.2%), CLA (1/201, 0.5%), BEL (1/67, 1.5%) and VAL (1/160, 0.6%). As a habitat generalist, this species shows a wide distribution across Latin America, with no environmental constraints and is considered a regionally important malaria vector (McKeon et al. 2013). Although the *An. darlingi* and *An. triannulatus s.l.* density was low and locally restricted, these species could constitute regional secondary malaria vectors due to their high IR (> 1.5) (Gutiérrez et al. 2009). *An. apicimacula* (VEN: 1/21, 4.8%), *An. pseudopunctipennis s.l.* (VEN: 1/21, 4.8%; ISA: 1/11, 9.1%) and *An. punctimacula s.l.* (SAN: 2/40, 5%) were found in low numbers in dispersed villages and solitary farms in forested mountainous areas with little human activity and natural small water bodies. The latter two species have historically been collected in small numbers and have never been found to be infected with *Plasmodium*. Nevertheless, they are widely distributed and are considered secondary vectors of local importance in some regions. However, these species are not of epidemiological relevance in Colombia (Olano et al. 2001, Gutiérrez et al. 2008).

We for the first time registered specimens of *An. albimanus*, *An. rangeli* and *An. apicimacula* in Montelíbano. The increase in species diversity for the region may have resulted from the inclusion of urban and rural collection sites with different ecological conditions. *An. albimanus* is commonly associated with the low coastal regions of the Caribbean and Pacific in Colombia, where it is a primary malaria vector. *An. albimanus* may also be found at higher elevations (Montoya-Lerma et al. 2011) and has previously been reported for the neighbouring municipality of Tierralta (Gutiérrez et al. 2009). *An.*

rangeli has been considered to be a main local malaria vector in the department of Putumayo, in the south of Colombia (Quiñones et al. 2006), and recent studies provide evidence for *Anopheles benarrochi B* being mainly responsible for *Plasmodium* transmission in that region (Orjuela et al. 2013). Little is known about the distribution and taxonomic status of *An. apicimacula* and its epidemiologic importance for malaria transmission.

Constant epidemiological surveillance is essential because human activities, climate change and evolutionary processes might have an impact on the geographical and temporal distribution, susceptibility and efficiency of the vectors and the lifecycles of the pathogens (Gould & Higgs 2009). The high *Anopheles* density in the urban centre and the villages along roads is most likely the result of artificial water bodies (road ditches, fish breeding pools, drinking troughs), which provide adequate larval habitats for *Anopheles*, particularly *An. nuneztovari s.l.* (Rodríguez et al. 2010). Structural interventions for avoiding stagnant water near settlements will likely significantly reduce malaria transmission and cases.

Further studies should focus on breeding and crossing experiments to clarify the taxonomic status of the *Anopheles* species complexes. Contemporary studies have focused on applying molecular markers for species identification; however, in spite of their utility for analysing population structures, evolutionary events and speciation tendencies, such studies do not provide conclusive results regarding species differentiation. Furthermore, the presence of other arthropod-borne pathogens, e.g., arboviruses and possible synergies between different pathogen life cycles should be extensively studied to acquire comprehensive, integrated knowledge on the transmission cycles of infectious diseases.

ACKNOWLEDGEMENTS

To the community of Tierradentro, to Patricia Gutiérrez and Viviana Cerón, National Institute of Health, Bogotá, and Martha Irene Castro and John Jairo González, Departmental Health Secretariat of Córdoba, Montería, for their support during field work, to San Isidro Foundation, for access to the Zonal Planning System Community, Alto San Jorge, to Dr Adrian Luty, Radbound University, Nijmegen, Centre for Molecular Life Sciences, for providing *P. falciparum* infected *Anopheles*, and to all the collaborating members of the Medical Entomology Laboratory, National University of Colombia, and to the Institute of Molecular Genetics, Biosafety Research and Consulting, Johannes Gutenberg University Mainz.

REFERENCES

Aregawi M, Cibulskis RE, Otten M, Williams R 2009. World malaria report 2009. Available from: who.int/malaria/world_malaria_report_2009/en/.

Carrejo NS, González R 2007. Introducción al estudio taxonómico de *Anopheles* de Colombia, Universidad del Valle, Cali, 237 pp.

Cienfuegos AV, Gómez GF, Córdoba LA, Luckhart S, Conn JE, Correa MM 2008. Diseño y evaluación de metodologías basadas en PCR-RFLP de ITS2 para la identificación molecular de mosquitos *Anopheles* spp (Diptera: Culicidae) de la Costa Pacífica de Colombia. *Rev Biomed 19*: 35-44.

Cienfuegos AV, Rosero DA, Naranjo N, Luckhart S, Conn JE, Correa MM 2011. Evaluation of a PCR-RFLP-ITS2 assay for discrimination of *Anopheles* species in northern and western Colombia. *Acta Trop 118*: 128-135.

Fritz GN, Conn J, Cockburn A, Seawright J 1994. Sequence analysis of the ribosomal DNA internal transcribed spacer 2 from populations of *Anopheles nuneztovari* (Diptera: Culicidae). *Mol Biol Evol 11*: 406-416.

Gould EA, Higgs S 2009. Impact of climate change and other factors on emerging arbovirus diseases. *Trans R Soc Trop Med Hyg 103*: 109-121.

Gutiérrez LA, González JJ, Gómez GF, Castro MI, Rosero DA, Luckhart S 2009. Species composition and natural infectivity of anthropophilic *Anopheles* (Diptera: Culicidae) in the states of Córdoba and Antioquia, northwestern Colombia. *Mem Inst Oswaldo Cruz 104*: 1117-1124.

Gutiérrez LA, Naranjo N, Jaramillo LM, Muskus C, Luckhart S, Conn JE, Correa MM 2008. Natural infectivity of *Anopheles* species from the Pacific and Atlantic regions of Colombia. *Acta Trop 107*: 99-105.

Hiwat H, Bretas G 2011. Ecology of *Anopheles darlingi* Root with respect to vector importance: a review. *Parasit Vectors 4*: 177.

Loaiza JR, Scott ME, Bermingham E, Sanjur OI, Rovira JR, Dutari LC, Linton Y, Bickersmith S, Conn JE 2013. Novel genetic diversity within *Anopheles punctimacula s.l.*: phylogenetic discrepancy between the barcode cytochrome c oxidase I (COI) gene and the rDNA second internal transcribed spacer (ITS2). *Acta Trop 128*: 61-69.

Marrelli MT, Floeter-Winter LM, Malafronte RS, Tadei WP, Lourenço-de-Oliveira R, Flores-Mendoza C, Marinotti O 2005. Amazonian malaria vector anopheline relationships interpreted from ITS2 rDNA sequences. *Med Vet Entomol 19*: 208-218.

McKeon SN, Schlichting CD, Povoa MM, Conn JE 2013. Ecological suitability and spatial distribution of five *Anopheles* species in Amazonian Brazil. *Am J Trop Med Hyg 88*: 1079-1086.

Miller BR, Crabtree MB, Savage HM 1997. Phylogenetic relationships of the *Culicomorpha* inferred from 18S and 5.8S ribosomal DNA sequences (Diptera: Nematocera). *Insect Mol Biol 6*: 105-114.

Montoya-Lerma J, Solarte YA, Giraldo-Calderón GI, Quiñones ML, Ruiz-López F, Wilkerson RC, González R 2011. Malaria vector species in Colombia - A Review. *Mem Inst Oswaldo Cruz 106* (Suppl. I): 223-238.

Moreno M, Bickersmith S, Harlow W, Hildebrandt J, McKeon SN, Silva-do-Nascimento TF 2013. Phylogeography of the neotropical *Anopheles triannulatus* complex (Diptera: Culicidae) supports deep structure and complex patterns. *Parasit Vectors 6*: 47.

Naranjo-Diaz N, Rosero DA, Rua-Uribe G, Luckhart S, Correa MM 2013. Abundance, behavior and entomological inoculation rates of anthropophilic anophelines from a primary Colombian malaria endemic area. *Parasit Vectors 6*: 61.

Olano VA, Brochero L, Sáenz R, Quiñones ML, Molina JA 2001. Mapas preliminares de la distribución de especies de *Anopheles* vectores de malaria en Colombia. *Biomedica 21*: 402-408.

Orjuela LI, Herrera M, Erazo H, Quiñones ML 2013. Especies de *Anopheles* presentes en el departamento del Putumayo y su infección natural con *Plasmodium*. *Biomedica 33*: 42-52.

Quiñones ML, Ruiz F, Calle DA, Harbach RE, Erazo HF, Linton Y-M 2006. Incrimination of *Anopheles (Nyssorhynchus) rangeli* and *An. (Nys.) oswaldoi* as natural vectors of *Plasmodium vivax* in Southern Colombia. *Mem Inst Oswaldo Cruz 101*: 617-623.

Rodríguez VC, Carrascal DR, Jiménez MM, Gutiérrez P, Ahumada M, Quiñones ML 2010. Proyecto piloto nacional de adaptación al cambio clímatico (INAP) - Comonente D salud humana malaria - Informe. Available from: conservation.org.co/wp-content/themes/CI-Colombia/images/ci//2012/07/Anexo-25.-Sistema-de-Vigilancia-Malaria.pdf.

Rosero DA, Jaramillo LM, Gutiérrez LA, Conn JE, Correa MM 2012. Genetic diversity of *Anopheles triannulatus s.l.* (Diptera: Culicidae) from northwestern and southeastern Colombia. *Am J Trop Med Hyg 87*: 910-920.

Sierra DM, Velez ID, Linton Y 2004. Malaria vector *Anopheles (Nyssorhynchus) nuneztovari* comprises one genetic species in Colombia based on homogeneity of nuclear ITS2 rDNA. *J Med Entomol 41*: 302-307.

SIPLAN 2008. Sistema de Planeación Zonal Comunitario, Subregión Alto San Jorge, Departamento de Córdoba, Municipio de La Apartada, Montelíbano y Puerto Libertador. Fundación San Isidrio. Available from: fundacionsanisidro.org/, in construction.

Tadei WP, Thatcher BD 2000. Malaria vectors in the Brazilian Amazon: *Anopheles* of the subgenus *Nyssorhynchus*. *Rev Inst Med Trop Sao Paulo 42*: 87-94.

Tadei WP, Thatcher BD, Santos JM, Scarpassa VM, Rodrigues IB, Rafael MS 1998. Ecologic observations on anopheline vectors of malaria in the Brazilian Amazon. *Am J Trop Med Hyg 59*: 325-335.

Wirtz RA, Zavala F, Charoenvit Y, Campbell GH, Burkot TR, Schneider I, Esser KM, Beaudoin RL, Andre RG 1987. Comparative testing of monoclonal antibodies against *Plasmodium falciparum* sporozoites for ELISA development. *Bull World Health Organ 65*: 39-45.

Zapata MA, Cienfuegos AV, Quirós OI, Quiñones ML, Luckhart S, Correa MM 2007. Discrimination of seven *Anopheles* species from San Pedro de Uraba, Antioquia, Colombia, by polymerase chain reaction-restriction fragment length polymorphism analysis of its sequences. *Am J Trop Med Hyg 77*: 67-72.

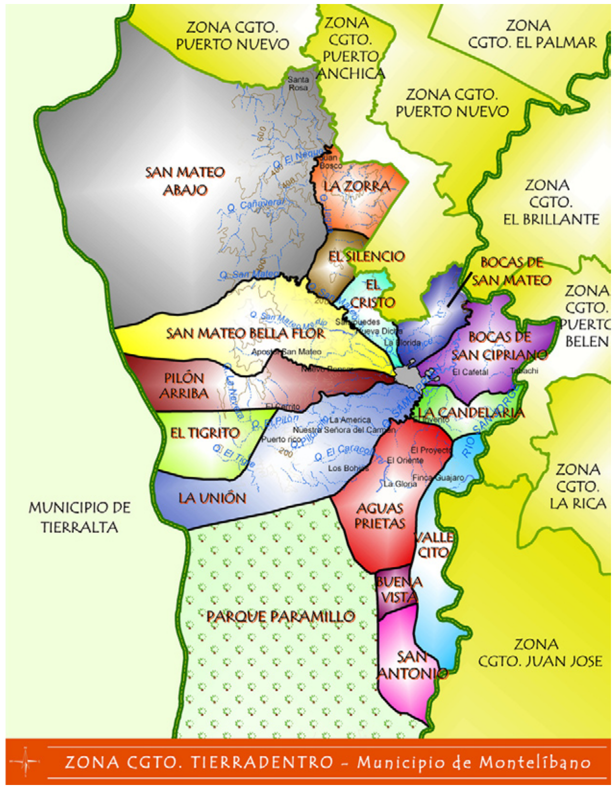

Fig. 1: map of the territorial division of Tierradentro. Tierradentro is a locality of the municipality of Montelíbano, department of Córdoba, located in the north-west of Colombia. The locality territory is 12,874.74 hectares. It consists of the urban centre and 14 rural settlements and shares borders with Tierralta and Puerto Libertador (SIPLAN 2008).

Fig. 2: city map of the urban centre of Tierradentro. The urban centre is located at the western bank of the San Jorge River at 55 m above mean sea level and consists of 12 suburbs. It has no water and sewage system, the health post is understaffed (SIPLAN 2008).