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First molecular detection of *Plasmodium relictum* in *Anopheles sinensis* and *Armigeres subalbatus*

Jilei Zhang[1,2], Guangwu Lu[1], Patrick Kelly[3], Jing Li[1], Min Li[1], Jiawei Wang[1], Ke Huang[1], Haixiang Qiu[1], Jinfeng You[1], Rong Zhang[1], Yaoyao Wang[1], Yuanyuan Zhang[1] and Chengming Wang[1,4*]

[1]*College of Veterinary Medicine, Yangzhou University, Yangzhou 225009, China*
[2]*Division of Gastroenterology and Hepatology, Department of Medicine, University of Illinois at Chicago, Chicago, IL 60607, USA*
[3]*Ross University School of Veterinary Medicine, Basseterre, St. Kitts & Nevis, West Indies*
[4]*College of Veterinary Medicine, Auburn University, Auburn, AL 36849, USA*

Abstract

Background: *Plasmodium relictum* is one of the most important avian malaria species, which is mainly seen in wild birds, with infections reported in more than 70 different species and at high prevalence.

Aim: The aim of this study was to determine the molecular prevalence of *Plasmodium* spp. in mosquitoes collected in China.

Method: A *Plasmodium* -specific fluorescence resonance energy transfer (FRET) polymerase chain reaction (PCR) was established in this study to analyze five species of mosquitoes (1,620 *Culex pipiens* pallens, 806 *Aedes albopictus*, 377 *Armigeres subalbatus*, 168 *Anopheles sinensis*, and 80 *Culex tritaeniorhynchus*) collected in hand nets from homes in 25 provinces of China.

Results: Only females originated from six provinces were determined to be positive (0.6%, 10/1,809). *Plasmodium* species were detected in three mosquito species, such as *C. pipiens* pallens (0.5%, 8/1,620), *A. sinensis* (0.6%, 1/168), and *A. subalbatus* (0.3%, 1/377). Of the three mosquito species positive for *P. relictum*, only *C. pipiens* pallens is known to feed on birds and is recognized as the natural vector of *P. relictum*.

Conclusion: This is the first time that *P. relictum* has been detected in *A. sinensis* and *A. subalbatus*. *P. relictum*, the agent of avian malaria, was present in mosquitoes in China, including mosquito species not previously thought to be the vectors.

Keywords: *Anopheles sinensis*, *Armigeres subalbatus*, China, Mosquito, *Plasmodium relictum*.

Introduction

Plasmodium, the mosquito-borne agent of malaria, belongs to the phylum *Apicomplexa* which is a taxonomic group of single-celled parasites with characteristic secretory organelles (de Koning-Ward *et al*., 2016). The genus *Plasmodium* contains over 200 species which can be divided into 14 subgenera based on morphology and host range (Martinsen and Perkins, 2013; Perkins, 2014). *Plasmodium* parasites are the most common in tropical areas, such as India, Australia, and Southeast Asia (Schoener *et al*., 2017), and have been described in a broad array of vertebrate hosts. In particular, over 150 *Plasmodium* species can be found infecting a variety of birds (Sylvie *et al*., 2008) with *Plasmodium relictum*, *Plasmodium elongatum*, *Plasmodium vaughani*, and *Plasmodium* sp. lineage LINN1 being the most common in birds and mosquitoes in Europe (Schoener *et al*., 2017). With its size and multiple geographies, China has a wide diversity of mosquito species (Guo *et al*., 2018), many of which have been reported to transmit *Plasmodium* species elsewhere in the world. Four human malarial species have been reported in China, such as *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium ovale* (Li *et al*., 2016), but the cases of human malaria have decreased dramatically recently, from the more than 30 million cases a year reported in the 1940s to 1,380 cases in 2018 (Zhang *et al*., 2018; Zhou *et al*., 2008). Mosquito control programs have resulted in near elimination of the historically most important mosquito vectors of malaria in people in China, *Anopheles lesteri* (synonym: *An. anthropophagus*) and *Anopheles dirus* s.l. This, however, has led to other species such as *Anopheles sinensis* now becoming important vectors (Zhang *et al*., 2017b). There are only limited data on bird malaria in China with infection rates of up to 8% in wild species (Zhang *et al*., 2014b), and a variety of species, such as *P. relictum* and *Plasmodium homonucleophilum*, and their lineages were described in wild and captive birds (Huang *et al*., 2015; Jia *et al*., 2018). To the best of our knowledge, there are no data on the mosquitoes carrying *Plasmodium* species infecting birds in China. In this report, we describe the development of a *Plasmodium*-specific FRET-qPCR and its use in detecting *Plasmodium* species in mosquitoes collected from across China.

***Corresponding Author:** Chengming Wang. College of Veterinary Medicine, Auburn University, Auburn, AL 60607, USA.
Email: *wangche@auburn.edu*

Materials and Methods

To establish the *Plasmodium*-specific FRET-qPCR, we obtained 18S rRNA sequences for representative *Plasmodium* species from GenBank: *P. falciparum* (M19172, CP016997, JQ627152, U07367), *P. malariae* (AF487999, AF488000, M54897), *Plasmodium inui* (FN256230, FN430725, XR_606809), *Plasmodium cynomolgi* (L08241, AB287289), *P. ovale* (KF018655, L48987), *P. vivax* (X13926, JQ627154), *Plasmodium knowlesi* (U83876, DQ350263), *Plasmodium berghei* (AJ243513), *Plasmodium vinckei* (XR_552296), *Plasmodium cathemerium* (AY625607), *Plasmodium gallinaceum* (M61723), *Plasmodium lophurae* (X13706), *Plasmodium juxtanucleare* (AF460507), *Plasmodium reichenowi* (Z25819), *Plasmodium gaboni* (LVLB01000008), *Plasmodium brasilianum* (AF130735), *Plasmodium gonderi* (AB287270), *Plasmodium fieldi* (AB287284), and *Plasmodium fragile* (M61722). The Clustal multiple alignment program was used to identify a conserved region of the 18S rRNA common to all the species. Primers and probes were selected to amplify a 234-242 bp target (forward: 5′-TAAGGATAACTACGGAAAAGCTGTA-3′; reverse: 5′-CGTTACCCGTCATAGCCATGT-3′; FAM-probe: 5′-TAGGCCAATACCCTAACATCAAAAG-6-FAM-3′; and LCRed640-probe: 5′-LCred640-TGATAGGTCAGAAACTCGATTGATACAC-phos-3′) and synthesized by Integrated DNA Technologies (Coralville, IA).

The *Plasmodium*-specific FRET-qPCR reaction and high-resolution melting curve analysis were performed on the LightCycler 480II Real-time PCR platform (Roche, Basal, Switzerland) under previously described conditions (Zhang *et al*., 2013), except that the hybridization temperature was 53°C. The specificity and sensitivity of the FRET-qPCR were determined using DNA of four plasmids manufactured with the pUC57 cloning vector (GenScript, Nanjing, Jiangsu, China) containing an appropriate portion of the 18S rRNA gene of *P. falciparum*, *P. malariae*, *P. ovale,* and *P. vivax*. Specificity was also tested, with DNA of *Babesia canis*, *Hepatozoon americanum*, *Theileria equi*, *Hepatocystis kochi,* and *Dirofilaria immitis* obtained as described before (Zhang *et al*., 2014a, 2015). The *Plasmodium*-specific FRET-qPCR proved to be highly sensitive detecting two copies of the *Plasmodium* 18S rRNA per 20 µl reaction system. Further, it was highly specific, not detecting the closely related organisms which were included in the study.

The validated *Plasmodium*-specific FRET-qPCR was used to analyze five species of mosquitoes [*Culex pipiens* pallens (*n* = 1,620), *Aedes albopictus* (806), *Armigeres subalbatus* (377), *A. sinensis* (168), and *Culex tritaeniorhynchus* (80)] which were collected in hand nets from homes in 25 provinces and identified as described previously (Zhang *et al*., 2019). Briefly, between July and September 2014, student volunteers from Yangzhou University used hand nets to collect convenience samples of mosquitoes in their primary homes located in 26 cities in 25 provinces or municipalities in China. Mosquitoes were placed individually in sterile tubes containing 400-µl DNA/RNA Stabilization Reagent (Roche Molecular Biochemicals, Indianapolis, IN, USA). Then, the samples were transported to the Yangzhou University College of Veterinary Medicine at room temperature.

Results and Discussion

Only females were positive (0.6%, 10/1,809) for *Plasmodium*, and these originated from six provinces, such as Zhejiang (4.2%, 1/24), Gansu (2.4%, 2/82), Shandong (5.4%, 3/56), Jilin (1.7%, 2/115), Guizhou (5.6%, 1/18), and Jiangsu (2.0%, 1/51). *Plasmodium* species were only detected in three mosquito species, such as *C. pipiens* pallens (0.5%, 8/1,620), *A. sinensis* (0.6%, 1/168), and *A. subalbatus* (0.3%, 1/377) (Table 1). The 18S rRNA sequences of all the positive samples were identical to one another (GenBank accession number: MK061746) and to that of a reference sequence of *P. relictum* (LN835296) from GenBank (Fig. 1). As far as we know, this is the first time that *P. relictum* has been detected in *A. sinensis* and *A. subalbatus*.

Of the three mosquito species we found positive for *P. relictum*, only *C. pipiens* pallens is known to feed on birds (Wang *et al*., 2012) and recognized as the natural vector of *P. relictum* (Zele *et al*., 2014). *A. sinensis*

Table 1. Data on mosquitoes positive for *P. relictum* identified with a *Plasmodium*-specific FRET-qPCR.

Province	City	Mosquito Species	Gender	*Plasmodium* Positivity
Zhejiang	Wenzhou	*A. sinensis*	F	1/24, 4.2%
Gansu	Jingyuan	*Culex p. pallens*	F	2/82, 2.4%
Shandong	Heze	*Culex p. pallens*	F	1/6, 16.7%
Shandong	Liaocheng	*Culex p. pallens*	F	2/50, 4.0%
Jilin	Changchun	*Culex p. pallens*	F	2/115, 1.7%
Guizhou	Liupanshui	*A. subalbatus*	F	1/18, 5.6%
Jiangsu	Yangzhou	*Culex p. pallens*	F	1/51, 2.0%
Total				10/346, 2.9%

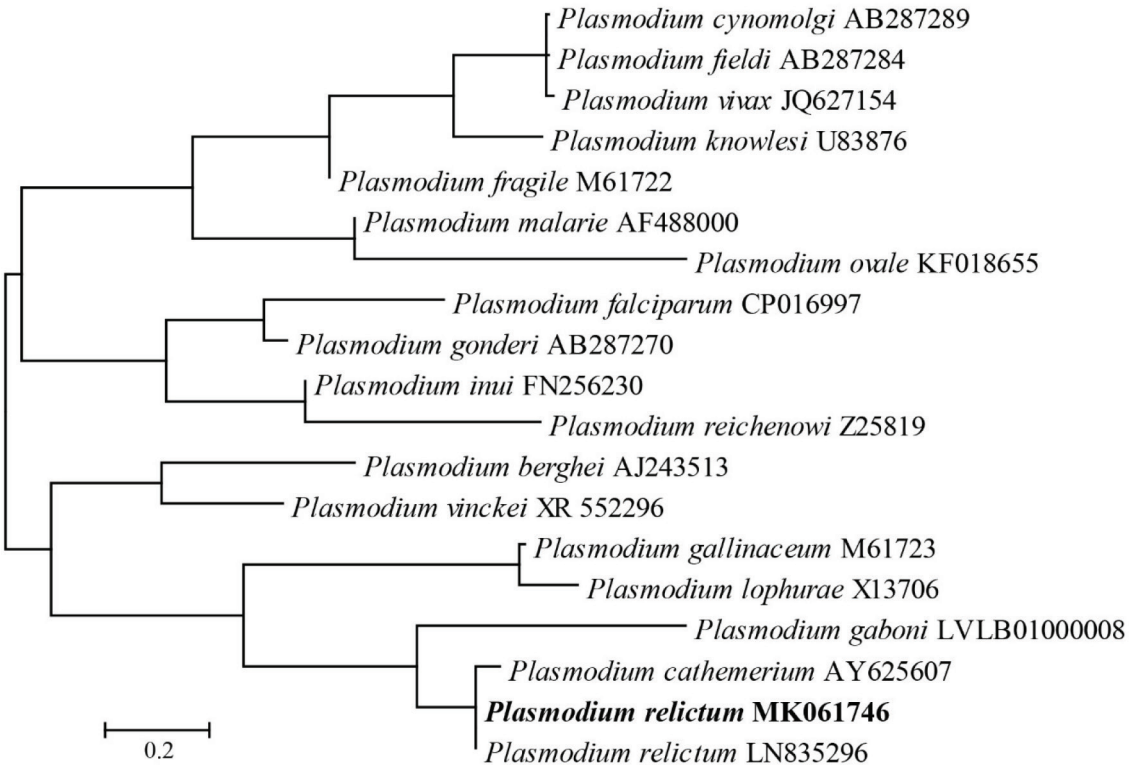

Fig. 1. Phylogenetic analysis of *Plasmodium* spp. detected in this study. Distances and groupings of *Plasmodium* detected from the mosquitoes (bold font) were determined by applying the neighbor-joining method to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach with MEGA version 6 software based on 18S rRNA gene (242 bp). Scale bar indicates a genetic distance of 0.2-nt substitution per position.

and *A. subalbatus* are found widely in China and other countries in Southeast Asia (Chaves *et al*., 2015; Guo *et al*., 2018) and feed mainly on cattle (Ramesh *et al*., 2015; Zhang *et al*., 2017a). From the data showing very low infection rates with *P. relictum*, it seems that they might also infrequently feed on birds. It is of note that all three species which are positive for *P. relictum* feed on people raising the possibility that humans are exposed to infection. Although it has been reported that host shifts appear to have a common occurrence in the evolution of the genus *Plasmodium* among avian and reptilian malaria parasites (Rich and Ayala, 2003), what would now be impermissible and unethical experiments have shown potential infections are unlikely in human being (McLendon, 1943).

P. relictum is one of the most important avian malaria species, which is mainly seen in wild birds, with infections reported in more than 70 different species (Garcia-Longoria *et al*., 2014) and at high prevalence. The organism has been reported in birds in China previously, such as Beijing (Jia *et al*., 2018) and Gansu Province (Jia *et al*., 2018; Zehtindjiev *et al*., 2013), and we now report its presence in mosquitoes in five further provinces, indicating that the organism is present in China, consistent with the findings in Europe (Schoener *et al*., 2017). To date, avian *Plasmodium* parasites have been found in *Aedes vexans*, *C. pipiens* complex, *Culex modestus*, *Culex hortensis*, *Culiseta annulata*, *Ochlerotatus caspius,* and *A. albopictus* in Central Europe and *Culex torrentium* in Australia (Schoener *et al*., 2017). The findings of *P. relictum* in *A. sinensis* and *A. subalbatus* (Table 1) expand the possible vector range for the parasite.

Conclusion

In conclusion, this study describes the establishment of a sensitive and specific *Plasmodium*-specific FRET-qPCR. With this validated PCR, we found that *P. relictum*, the agent of avian malaria, was present in mosquitoes in China, including species not previously thought to be the vectors.

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Conflicts of interest

The authors have no conflicts of interest to declare.

Author's contribution

Jilei Zhang and Chengming Wang designed this study. Jilei Zhang, Guangwu Lu, Jing Li, Min Li, Jiawei

Wang, Ke Huang, Haixiang Qiu, Jinfeng You, Yaoyao Wang, and Yuanyuan Zhang collected the mosquitoes and performed DNA extraction and PCR. Jilei Zhang, Patrick Kelly, and Chengming Wang wrote the manuscript.

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