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Molecular epidemiological surveillance of Africa and Asia imported malaria in Wuhan, Central China: comparison of diagnostic tools during 2011–2018

Yiting Xie
Hubei University of Medicine
Kai Wu
Wuhan Centers for Disease Prevention and Control
Weijia Cheng
Hubei University of Medicine
Tingting Jiang
Hubei University of Medicine
Yi Yao
Hubei University of Medicine
Mingxing Xu
Wuhan Centers for Disease Prevention and Control
Yan Yang
Wuhan Centers for Disease Prevention and Control
Huabing Tan
Wuhan Centers for Disease Prevention and Control
Jian Li (yxlijian@163.com)
https://orcid.org/0000-0001-9434-7803

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Abstract

Background Malaria remains a serious public health problem globally. Along with indigenous malaria elimination in China, imported malaria gradually became a major hazard. Well-timed and accurate diagnosis could support immediate therapeutic schedule, reveal the prevalence of imported malaria and avoid the disease transmission.

Method Blood samples were collected in Wuhan, China from August 2011 to December 2018. All patients first accepted microscopy and RDT examination. Subsequently, each of the positive or suspected positive cases was engaged in total four human Plasmodium species amplication by using 18S rRNA based nested PCR and Taqman probe based real-time PCR. Then performance of microscopy and two molecular diagnosis methods were analyzed. Importation origin was traced by country and prevalence of Plasmodium species was revealed by year.

Results Total 296 blood samples containing 288 microscopy and RDT positive, 7 RDT P. falciparum positive and 1 suspected cases were collected and reanalyzed. After two molecular methods and sequencing detection, 291 cases including 245 P. falciparum , 15 P. vivax , 20 P. ovale , 6 P. malariae and 5 mixed infection (3 P. falciparum + P. ovale , 2 P. vivax + P. ovale) were confirmed. These patients returned from Africa (95.53%) and Asia (4.47%). Although the prevalence displayed a small-scale fluctuation, the overall trend of the imported cases increased yearly.

Conclusions Results emphasized the necessity of combined utilization of the four tools for malaria diagnosis in clinic and field survey around potential risk regions worldwide including Wuhan.

Background

Malaria remains a serious threat to publics around the world with an estimated 219 million cases and 435,000 deaths in 2017 worldwide [1]. The mainly epidemic areas of malaria distribute in tropical and subtropical regions, especially in Sub-saharan Africa and Southeast Asia (SEA) [1]. World Health Organization hopes to eliminate malaria in at least 35 new countries (based on data from 2015) by 2030 [2]. In order to achieve the goal, China plans to eliminate the disease in 2020 [3]. In China, the occurrence and burden of indigenous malaria have rapidly shrunk attributes to employment of the integrated malaria control and elimination strategy since 2000 [4]. It is note that no indigenous malaria in 2017 was reported in the whole country. In accordance with the nationwide status, control of indigenous malaria in Hubei Province of China also achieved remarkable effect. Indigenous malaria case reported in Hubei was zero since 2013. However, imported malaria gradually became a major threat along with the process of prevention, control and elimination in endemic and non-endemic areas globally including China. Furthermore, it is worth mentioning that imported malaria cases from Africa region and SEA were gradually increasing yearly in China including the provincial capital of Hubei province, Wuhan [4, 5]. For efficient control and achieving malaria eradication before 2020 in China as the state plan [3], imported malaria patients in China, especially first-tier cities with high population density and mobility like Wuhan should be highly noticed.

For human being, there are five primary Plasmodium species causing malaria, including P. falciparum, P. vivax, P. ovale, P. malariae, and P. knowlesi. Different Plasmodium species infection caused dissimilar disease characteristics. Amongst them, P. falciparum is responsible for most of morbidity and mortality of humans [6]. P. vivax is generally the culprit for majority of malaria cases in areas with tropical and temperate climates and considered as one of the neglected tropic diseases [7, 8]. P. ovale seldom leads to severe malaria for people in endemic regions, but can arouse severe malaria disease for naive visitors [9, 10]. It is comprised of two subspecies, the classic type named as P. ovale curtisi and variant type named as P. ovale wallikeri [11]. P. ovale curtisi was demonstrated possessing significantly longer latency duration than that in P. ovale wallikeri [12, 13]. P. malariae usually causes the mildest infections, but may also account for splenomegaly or renal damage after chronic infection [14]. As the fifth species of Plasmodium causing malaria in humans, P. knowlesi is initially found from SEA in nature macaques [15]. Beyond that, simultaneous infections more than one human Plasmodium species commonly happen, especially in endemic regions [16]. However, influence of malaria caused by P. vivax, P. ovale and P. malariae as well as mixed infection was actually frequently underestimated [10, 13, 17, 18, 19]. Therefore, in order for timely and efficient treatment and preventing malaria transmission, early and accurate diagnosis of Plasmodium species infection was meaningful.

Due to inexpensive spends, relative high sensitivity in species recognition and even parasite density quantification, microscopic examination of thick and thin blood smear is traditionally regarded as the gold standard for malaria detection [20, 21]. However, the use of microscopic examination has been restricted because it requires well-trained laboratory personnel and shows less sensitive at low parasitaemia [22, 23, 24]. Actually, different observers may make two to three fold discrepancies in parasite quantification [25]. More importantly, mixed infections of different Plasmodium species were frequently missed [26]. Two subspecies of P. ovale (P. ovale curtisi and P. ovale wallikeri) are usually difficult to be distinguished by microscopy. Misidentification may also happen between different species [27]. All above may induce the unreasonable usage of antimalarial drugs and then hamper the parasite clearance and lead to the transmission of malaria, even antimalarial drug resistance. Rapid diagnosis test (RDT) as an immunologic method usually targets on Plasmodium specific antigens in blood samples such as histidine-rich protein 2 (HRP2) and lactate dehydrogenase (LDH) [14]. For RDT, it offers rapid diagnosis, even could be conducted by users without extensive training. And it often has comparable sensitivity as microscopy. However, RDT could only distinguish falciparum and non-falciparum Plasmodium species infection [28]. Futhermore, false negative results by RDT were also reported in previous studies [29, 30, 31]. Therefore, molecular tools such as nested PCR and real-time PCR that could not only detect Plasmodium infection, but also allow accurate species identification by primer design should be involved. Based on two rounds of amplification, nested PCR can evidently improve the detection sensitivity. Sequencing of products from nested PCR represents a further reliable guarantee for species identification just as in other studies [32, 33]. The major advantages of real-time PCR include direct result reading without downstream analysis and quantification of DNA copy number [34, 35].

Using suitable and effective methods to timely finding the imported malaria is the guarantee of successful completing the target assessment task for malaria elimination in China, Africa and SEA. In present study, Plasmodium species in all imported malaria cases in Wuhan were confirmed by using microscopy, RDT, nested PCR and real-time PCR and their performances in malaria diagnosis were assessed. Analysis of the diagnostic results also revealed the prevalence characteristics of imported malaria in Wuhan, China.

Methods

Collection of study specimens

Blood samples from clinically-suspected patients with manifestation or symptoms of malaria were accumulatively collected from Center for Disease Prevention and Control (CDC) of Wuhan, China, during August 2011 to December 2018. About 2–5 ml blood was drawn from each patient for microscopy and RDT testing and 400 μl of each sample was stored on 3 MM Whatman filter paper for molecular verification. Ethical approval for this study was obtained from the Medical Ethics Committee of the Hubei University of Medicine and Wuhan CDC. Informed consent was supplied by all participating individuals.

Microscopic examination and RDT assay

Blood samples were first subjected to One Step Malaria HRP2/pLDH (P.f/Pan) (Wondfo, Guangzhou, China) detection. Subsequently, thick and thin peripheral blood smears were made by the standard method [36]. Giemsa staining and microscopic examinations were conducted by professionals in Wuhan CDC. Parasitemia (parasites/μl) was determined according to our previous documents [37]. No asexual form of Plasmodium in 200 high-power fields in the thin blood films or on parasites even in each 1000 white blood cells were considered as negative [36, 38].

Genomic DNA extraction

Genomic DNA (gDNA) from the microscopic-positive or suspected positive blood samples was extracted using a TIANamp blood DNA kit (Tiangen Biotech Co., Ltd., Beijing, China). Briefly, approximately 130 μl peripheral blood (6 mm × 6 mm blood spot) was treated following manufacturer's instruction and finally dissolved in 50 μl elution buffer. After quantification with Gene 5, the purified gDNA was packed as aliquots and stored at -20 °C until further use.

Taqman probe based Real-time PCR

The parasite species including P. falciparum, P. vivax, P. ovale (P. ovale curtisi, P. ovale wallikeri) and P. malariae were designed to be distinguished with real-time PCR. The preparation of primers and Taqman probes was according to the previous documents [32, 39] (primers list in Table 1). Reaction was performed using the Premix EX Taq ^{TM} probe qPCR (Takara, Japan) following the manufacturer's instruction. Briefly, a reaction mixture was consist of 12.5 μl premix Ex Taq (2×), 0.5 μl each primer (final 0.2 μM), and about 1 μl fluorescence probe (final 0.1 ~ 0.5 μM), 2 μl DNA template in a final total volume of 25 μl supplemented with ultrapure water. All reaction was performed as the suggestion by manufacturer, including 1 cycle of 95 °C for 30 sec, 40 repeated cycles of 95 °C for 5sec and 60 °C for 30 sec, by using CFX96 real-time PCR machine (Bio-Rad, USA).

Table 1

Primers and probes for Plasmodium species detection.

Method	Species	Primer	Sequence (5'-3')	Length (bp)	Reference
Nested PCR	Plasmodium sp.	rPLU1	TCAAAGATTAAGCCATGCAAGTGA	1670	[40]
		rPLU5	CCTGTTGTTGCCTTAAACTTC		
	P. falciparum	rFAL1	TTAAACTGGTTTGGGAAAACCAAATATATT	205	
		rFAL2	ACACAATGAACTCAATCATGACTACCCGTC		
	P. vivax	rPVIV1	CGCTTCTAGCTTAATCCACATAACTGATAC	121	
		rPVIV2	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA		
	P. malariae	rMAL1	ATAACATAGTTGTACGTTAAGAATAACCGC	145	
		rMAL2	AAAATTCCCATGCATAAAAAATTATACAAA		
	P. ovale	rOVA1WC	TGTAGTATTCAAACGCAGT	659–662	
		rOVA2WC	TATGTACTTGTTAAGCCTTT		
	P. ovale curtisi	rOVA1	ATCTCTTTTGCTATTTTTTAGTATTGGAGA	800	[41]
		rOVA2	GGAAAAGGACACATTAATTGTATCCTAGTG		
	P. ovale wallikeri	rOVA1v	ATCTCCTTTACTTTTTGTACTGGAGA	780	
		rOVA2v	GGAAAAGGACACTATAATGTATCCTAATA		
Real-time PCR	Plasmodium sp.	Plasmo 1	GTTAAGGGAGTGAAGACGATCAGA		[32]
		Plasmo 2	AACCCAAAGACTTTGATTTCTCATAA		
	P. falciparum	P. fal-probe	FAM-AGCAATCTAAAAGTCACCTCGAAAGATGACT-TAMRA		
	P. vivax	P. v-probe	HEX-AGCAATCTAAGAATAAACTCCGAAGAGAAAATTCT-TAMRA		
	P. malariae	P. m-probe	FAM-CTATCTAAAAGAAACACTCAT-MGB		
	P. ovale	P. o-probe	HEX-CGAAAGGAATTTTCTTATT-MGB		
	P. ovale	POF	ATAAACTATGCCGACTAGGTT		[39]
		POR	ACTTTGATTTCTCATAAGGTACT		
	P. ovale curtisi	POC-probe	FAM-TTCCTTTCGGGGAAATTTCTTAGA-BHQ1		
	P. ovale wallikeri	POW-probe	HEX-AATTCCTTTTGGAAATTTCTTAGATTG-BHQ1		

Nested PCR assay

The above samples were further determined with nested PCR. For nested PCR, the classical primers targeting on Plasmodium 18S small subunit ribosomal RNA (18S ssrRNA) gene were synthesized and used as previously described [40, 41] (primers list in Table 1). The reaction system for the primary round contained 12.5 μl 2 × NovoStar Green PCR Mix (400 μM deoxynucleoside triphosphate [dNTP], 50 U/ml NovoStar Taq DNA polymerase, 4 mM Mg^{2+} and 2 × PCR buffer), 1 μl each primer (rPLU1 and rPLU5 for 18S Plasmodium species, 10 μM) and 1 μl DNA template in a final total volume of 25 μl

supplemented with ultrapure water. The amplification was carried out as following conditions: initial denaturation at 95 °C for 3 min, 30 repeated cycles at 95 °C for 30 sec, 55 °C for 1 min, 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. A 1,670 bp PCR product was obtained from each sample after the primary amplification, which was then used as the DNA template for the secondary amplification. The reaction system and condition in the second round were similar with that in the first round with minor modification. Briefly, the annealing temperature was adjusted to 56 °C and the extension time was changed to 30 sec considering the application products were shorter in the second reactions. All the products from secondary amplification were subjected to 1% agarose gel electrophoresis and judged by their stripe sizes. Products of nested PCR were used for sequencing once divergent results occurred among microscopy and two molecular methods.

Data analysis

To calculate the diagnostic sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and disease prevalence (DP) of four Plasmodium species, results from nested PCR were considered as the standard. Above parameters and 95% confidence interval (95% CI) were obtained from online calculator named vassarstats [42]. Flowchart and patients distribution map were drown in Microsoft Office Visio 2010. Other figures were finished in Graphpad Prism 5.0. The trend analysis was carried out by curve estimation in linear regression analysis of SPSS 17.0.

Results

Microscopy and RDT detection

Total 296 blood samples from malaria suspected patients were collected (Fig. 1). By using microscopy and RDT, 288 positive samples including 243 P. falciparum, 17 P. vivax, 21 P. ovale, 6 P. malariae and 1 coinfection (P. falciparum + P. ovale) were observed. Seven out of the remaining 8 cases (296 minus 288) were microscopy negative but RDT positive (Fig. 1). The last case involved was both microscopy and RDT negative, a suspected patient with clinical symptoms of malaria.

For the 243 microscopy positive P. falciparum cases (Fig. 2A), parasitaemia ranged from 100 to 500,000 parasite/μl and the mean parasite density was 88,879 parasite/μl (95% CI = 71,794 − 105,963). These cases were divided into six groups according to their densities: Very Low (≤ 100 parasite/μl), Low (101–500 parasite/μl), Low-middle (501-3,000 parasite/μl), Middle (3,001–10,000 parasite/μl), Middle-high (10,001-100,000 parasite/μl) and High (> 100,000 parasite/μl). From the very low group to the high group (see Fig. 2B and Table 2), case numbers of P. falciparum for each density interval were 7 (2.88%), 26 (10.70%), 34 (33 + 1, 13.99%), 30 (12.35%), 96 (39.51%) and 50 (20.58%), respectively. For P. vivax, parasitaemia ranged from 100 to 30,000 parasite/μl and the mean parasite density was 4,600 parasite/μl (95% CI = 989-8,211). For P. ovale, parasitaemia ranged from 500 to 10,000 parasite/μl and the mean parasite density was 2,610 parasite/μl (95% CI = 1,348-3,871). Of which, 23.53% (4/17) of P. vivax and 19.05% (4/21) of P. ovale had parasite densities ≤ 500 parasite/μl. All the 6 P. malariae cases had parasite densities from 800 to 4,000 parasite/μl and the one mixed infection was 50,000 parasite/μl (see Fig. 2A).

Table 2

Determination of Plasmodium species by microcopic examination and molecular diagnosis.

Microcopic	Parasitemia (No. of parasites /μl)			Nested PCR [a]		Real-time PCR	
	Species	Intensity (parasite/ul)	No.	Species	No.	Species	No.
Microcopy positive	P. falciparum	Very Low (≤ 100)	7	Pf	6	Pf	5
		Low (101–500)	26	Pf	25	Pf	21
		Low-middle (501-3,000)	33	Pf	32	Pf	32
		Middle (3,001–10,000)	30	Pf	30	Pf	30
		Middle-high (10,001-100,000)	96	Pf	96	Pf	96
		High (> 100,000)	50	Pf	50	Pf	50
		Error correction (3,000)	1	Pf + Po	1	Pf + Po	1
	P. vivax	From 100 to 10,000	15	Pv	15	Pv	15
		Error correction	2	Pv + Po	2	Pv + Po	2
	P. ovale	From 100 to 10,000	19	Po	19	Po	21
		Error correction	2	Pf [a] +Po	2	Pf + Po	0
	P. malariae	From 100 to 10,000	6	Pm	6	Pm	6
	P. falciparum + P. ovale	50,000	1	Po	1	Po	1
Microcopy negative	P. falciparum[b]		7	Pf	5	Pf	3
	Suspected case		1	Pf	1	Pf	1
Total			296		291		284

a,The nested PCR products of these cases have been confirmed by DNA sequencing. b, These cases were RDT positive for Pf.

Confirmation of parasite species with PCR based methods

These samples were confirmed via both nested PCR and real-time PCR. After reconfirmation by nested PCR (Primers were shown in Table 1), 1.04% (3/288) microscopy positive and 25% (2/8) microscopy negative cases failed to be detected out. While 2.78% (8/288) microscopy positive and 50% (4/8) microscopy negative subjects showed negative by real-time PCR (Fig. 1). Nested PCR products of the 7 cases (12 minus 5), which were P. falciparum positive by nested PCR but negative by real-time PCR, were sequenced and results proved they were all P. falciparum infection. As shown in Table 2, the total 5 negative cases by nested PCR were either distributed in the three groups with relative low P. falciparum density (Very low, Low, Low-middle) or in microscopy negative group. For the total 12 cases failed to be detected by real-time PCR, they were all related to P. falciparum. Their parasite density distributions were highly accordance with that in nested PCR detection, including 2 cases in Very low group, 5 cases in Low group, 1 case in Low-middle group and 4 cases in microscopy negative group. Additionally, both two molecular tools revealed one P. falciparum plus P. ovalis mixed infection case among the microscopy positive P. falciparum samples. And the one suspected case negative by both microscopy and RDT was proved to be P. falciparum infection.

Of the 17 microscopy positive P. vivax cases, two actually were proved to be P. vivax plus P. ovalis mixed infection by both nested and real-time PCR. All the 21 microscopy positive P. ovalis cases were diagnosed as positive by real-time PCR, but

actually, two of them were proved to be P. falciparum plus P. ovalis mixed infection by nested PCR and followed sequencing. Identification of 6 P. malariae cases showed the same results by three methods. There was also a case that was diagnosed as P. falciparum plus P. ovalis infection by microscopy. However, both nested PCR and real-time PCR only revealed the existence of P. ovalis but not P. falciparum (Table 2). Subspecies identification of the total 20 single P. ovalis positive cases showed that they were consist of 10 P. ovale curtisi, 9 P. ovale wallikeri and 1 mixture of the two subspecies according to nested PCR and sequencing. Real-time PCR revealed the similar results except for 3 P. ovale curtisi negative cases (data not shown).

Diagnostic profile of Plasmodium species by different methods

As showed in Fig. 1 and Table 3, amongst the 296 samples involved, there were 243 (82.09%) P. falciparum, 17 (5.74%) P. vivax, 21 (7.09%) P. ovalis, 6 (2.03%) P. malariae, 1 (0.34%) mixted infection (P. falciparum + P. ovalis) and 8 (2.70%) negative cases according to microscopic observation. Based on the nested PCR, 245 (82.77%), 15 (5.07%), 20 (6.76%) and 6 (2.03%) cases were identified as single infection of P. falciparum, P. vivax, P. ovalis and P. malariae, respectively, whereas dual species mixed infections included 3 (1.01%) cases of P. falciparum + P. ovalis, 2 (0.68%) cases of P. vivax + P. ovalis and 5 (1.69%) negative cases. Real-time PCR revealed small different results, including 238 (80.41%) P. falciparum, 15 (5.07%) P. vivax, 22 (7.43%) P. ovalis, 6 (2.03%) P. malariae, 1 (0.34%) mixed infections of P. falciparum + P. ovalis, 2 (0.68%) cases of P. vivax + P. ovalis and 12 (4.05%) negative cases. No triple even quadruple infection was detected in these samples.

Table 3

Comparison analysis of diagnostic tools for imported Plasmodium species infection in Wuhan, China.

		Nested PCR							
Microscopy	Species	P. falciparum	P. vivax	P. ovale	P. malariae	P. falciparum + P. ovale	P. vivax + P. ovale	Negative	Total (%)
	P. falciparum	239	0	0	0	1	0	3	243 (82.09)
	P. vivax	0	15	0	0	0	2	0	17 (5.74)
	P. ovale	0	0	19	0	2	0	0	21 (7.09)
	P. malariae	0	0	0	6	0	0	0	6 (2.03)
	P. falciparum + P. ovale	0	0	1	0	0	0	0	1 (0.34)
	Negative [a]	6	0	0	0	0	0	2	8 (2.70)
	Total	245 (82.77)	15 (5.07)	20 (6.76)	6 (2.03)	3 (1.01)	2 (0.68)	5 (1.69)	296 (100.00)
Real-time PCR	P. falciparum	238	0	0	0	0	0	0	238 (80.41)
	P. vivax	0	15	0	0	0	0	0	15 (5.07)
	P. ovale	0	0	20	0	2	0	0	22 (7.43)
	P. malariae	0	0	0	6	0	0	0	6 (2.03)
	P. falciparum + P. ovale	0	0	0	0	1	0	0	1 (0.34)
	P. vivax + P. ovale	0	0	0	0	0	2	0	2 (0.68)
	Negative	7	0	0	0	0	0	5	12 (4.05)
	Total	245 (82.77)	15 (5.07)	20 (6.76)	6 (2.03)	3 (1.01)	2 (0.68)	5 (1.69)	296 (100.00)

a, these included 7 RDT positive and 1 RDT negative but suspected cases

Diagnostic performance of microscopy and real-time PCR compared to nested PCR

Nested PCR was appointed as a reference for the diagnostic summarizing of Plasmodium species as well as the following performance assessment about the diagnostic methods. As shown in Table 4, for microscopy, the sensitivity in identification of P. falciparum, P. vivax, P. ovalis and P. malariae were 96.77%, 100%, 88.00% and 100.00%, respectively. Specificity for P. falciparum diagnosis was 91.67% and all 100.00% for other three species. The probabilities of identification for P. falciparum, P. vivax, P. ovalis and P. malariae by microscopy from the predicted positive (PPV) and not identification from predictive negative (NPV) samples were 98.36% and 84.62% (P. falciparum), 100.00% and 100.00% (P. vivax), 100.00% and 98.91% (P. ovalis), 100.00% and 100.00% (P. malariae), respectively. For the detection of P. falciparum by real-time PCR, sensitivity,

specificity, PPV and NPV were 96.37%, 100%, 100% and 84.21%, respectively. These four assessment indexes were all 100% in the other three species diagnosis by using real-time PCR.

Table 4

Performance of microscopy and real-time PCR compared to the reference nested PCR, including sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and disease prevalence (DP).

Nested PCR ™ as standard (Confirmed by sequencing)									
Methodological evaluation		Plasmodium species							
		P. falciparum		P. vivax		P. ovale		P. malariae	
Nested PCR VS Microscopy	Parameters	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
	Positive	240	4	17	0	22	0	6	0
	Negative	8	44	0	279	3	271	0	290
	Analyze	Percentage (95% CI)		Percentage (95% CI)		Percentage (95% CI)		Percentage (95% CI)	
	Sensitivity	96.77 (93.51–98.49)		100.00 (77.08–100.00)		88.00 (67.66–96.85)		100.00 (51.68–100.00)	
	Specificity	91.67 (79.13–97.30)		100.00 (98.31–100.00)		100.00 (98.26–100.00)		100.00 (98.37–100.00)	
	PPV	98.36 (95.58–99.47)		100.00 (77.08–100.00)		100.00 (81.50–100.00)		100.00 (51.68–100.00)	
	NPV	84.62 (71.37–92.66)		100.00 (98.31–100.00)		98.91 (96.57–99.72)		100.00 (98.37–100.00)	
	DP	83.78 (78.97–87.69)		5.74 (3.49–9.21)		8.45 (5.65–12.36)		2.03 (0.83–4.58)	
Nested PCR VS Real Time PCR	Parameters	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
	Positive	239	0	17	0	25	0	6	0
	Negative	9	48	0	279	0	271	0	290
	Analyze	Percentage (95% CI)		Percentage (95% CI)		Percentage (95% CI)		Percentage (95% CI)	
	Sensitivity	96.37 (92.99–98.22)		100.00 (77.08–100.00)		100.00 (83.42–100.00)		100.00 (51.68–100.00)	
	Specificity	100 (90.77–100)		100.00 (98.31–100.00)		100.00 (98.26–100.00)		100.00 (98.37–100.00)	
	PPV	100 (98.03–100)		100.00 (77.08–100.00)		100.00 (83.42–100.00)		100.00 (51.68–100.00)	
	NPV	84.21 (71.63–92.09)		100.00 (98.31–100.00)		100.00 (98.26–100.00)		100.00 (98.37–100.00)	
	DP	83.78 (78.97–87.69)		5.74 (3.49–9.21)		8.45 (5.65–12.36)		2.03 (0.83–4.58)	

The results also revealed that P. falciparum (83.78%) infection was the most prevalent, followed by P. ovalis (8.45%), P. vivax (5.74%) and P. malariae (2.03%) among the imported malaria cases in Wuhan, China. Due to RDT could only distinguish falciparum and non-falciparum Plasmodium species infection, RDT was not involved in methodological evaluation for these samples.

Origin and years distribution of patients

Tracing the origin of imported malaria patients demonstrated the patients returned from 28 countries of Africa and 4 countries of Asia (Fig. 3A). Of the total 291 patients confirmed by nested PCR, 112 (38.49%) malaria patients infected from West Africa, followed by Central Africa (66 cases, 22.68%), Southern Africa (62 cases, 21.31%), East Africa (38 cases, 13.06%), SEA (9 cases, 3.09%) and South Asia (4 cases, 1.37%) (Fig. 3B). Distribution of malaria patients caused by P. falciparum was highly accordance with above general tendency, including 102 cases from West Africa, 55 from Central Africa, 55 from Southern Africa, 27 from East Africa and 6 from SEA. Amongst the P. vivax patients, 8 returned from East Africa. The remainders all returned from SEA (3 cases) and South Asia (4 cases), respectively. The P. ovalis patients returned from West Africa (10/20), Central Africa (8/20) and Southern Africa (2/20). Only 6 P. malariae infected patients were involved, three returned from Southern Africa, two from Central Africa and one from East Africa. The five mixed infection patients also returned from the three regions as P. malariae infection group.

From 2011 to 2016 (Fig. 3B), the imported malaria patients have roughly gradually increased yearly. Luckily, only 20 imported malaria patients reported from the CDC and hospitals of Wuhan in 2017. However, clinical malaria patients in 2018 rose again and reached up to 44 cases. It is note that the predominant species was P. falciparum during the surveyed period. However, it is worth mentioning no considerably increasing trends in the prevalence of P. falciparum parasites infection ($F = 0.078$, $P = 0.790$) was detected yearly. Other Plasmodium species including P. vivax, P. ovalis and P. malariae single infection or mixed infection hadn't been found until 2014. In the next five years (2014–2018), patients infected with P. vivax (15 cases), P. ovalis (20 cases), P. malariae (6 cases) and mixed parasite species (5 cases) were accepted.

Discussion

In 2020, China hopes that national malaria elimination will become a real possibility based on effective control measures [4]. Although no indigenous malaria case has been reported in China since 2017, the imported malaria is becoming a serious obstacle for malaria elimination. If China plans to achieve the stated goals on schedule, continuous surveillance of the imported malaria is an essential measure. As an important city of Central China, Wuhan has a population of 11.08 million. Meanwhile, it faces similar problems: The imported malaria cases increased annually accompanied by the disappearance of indigenous malaria cases since 2013 [5]. Thus, it is very necessary to monitor the epidemic status of imported malaria in China including Wuhan. Furthermore, identification of the malaria parasite species with timely and effective method could provide valuable information for developing suitable clinical treatment.

In current study, of the 243 P. falciparum cases identified by microscopy, one case actually was proved to be infected with P. falciparum plus P. ovalis later by both molecular tests. Two microscopy positive P. vivax cases were also proved to actually infect with P. vivax plus P. ovalis. Likewise, two patients with single P. ovalis infection by microscopy were proved as P. ovalis plus P. falciparum infection by nested PCR. In the previous studies, P. ovale was also frequently reported involved in mixed infection with other Plasmodium species [17, 43, 44]. These results proved the limitation of microscopy in identification of mixed infection and the highly specialized requirement for observers [45]. The identification of P. ovale curtisi and P. ovale wallikeri in total 20 single P. ovalis infection cases, which were failed to be distinguished by microscopy, also evidenced the advantages of molecular tools in species identification. Their diagnosis will offer a clue for the precise treatment of malaria patients. Subsequently, nested PCR also proved 6 cases as P. falciparum infection, which were consist of 5 microscopy negative but RDT positive cases and even one double-negative case. This was probably because parasitaemia was too low to be observed for these cases. As reported previous studies [46, 47], microscopy has a sensitivity of 50 ~ 500 parasites/μl in Plasmodium detection, but the threshold, 50 parasites/μl was rarely obtained unless under optimum conditions. This means that, it is actually difficult for observer to accurately find out the species with relative low parasitemia, especially in mixed infection.

In the analysis of sensitivity, specificity, PPV and NPV, nested PCR-based detection was chosen as the reference standard. Actually, nested PCR was commonly selected as a reference method [48, 49]. By comparing to nested PCR, microscopy (96.77%) showed highly similar sensitivity with real-time PCR (96.37%) in P. falciparum identification. However, the specificity of microscopy was only 91.67%, lower than the 100% in rea1-time PCR detection. By designing complementary probe to target

gene sequence, Taqman probe is another guarantee besides primers to enhance the specificity and avoid the false positive results [35]. The NPV for P. falciparum detection was about 85% in both microscopy and real-time PCR detection, indicating relative high false negative rate about 15% appeared in these two methods. A false negative rate as high as 19.4% for microscopy assay was reported in other study [50], indicating the persistent limitation of microscopy. For P. ovalis, the sensitivity (88.00%) in microscopic identification was relative low and NPV for P. ovalis was 98.91%. Two of the 3 P. ovalis cases that failed to be detected out by microscopy were involved in P. ovalis plus P. vivax mixed infection. This may due to the similar morphology between P. ovale with P. vivax [10], as a result, the species with relative lower parasitemia may be hidden. Four indexes (Table 4) in real-time PCR detection were all 100% in P. vivax, P. malariae and P. ovalis detection, revealing the high consistency between two molecular tools in these three species identification. P. malariae could be accurately diagnosed by all the three methods. This may be attributed by only 6 P. malariae cases were involved in the study. The primers of real-time PCR used in this study have been used in several previous studies [32, 51]. But it is reported that different templates amplification simultaneous was difficult and the primers preferred to amplify species with higher parasitemia as result of competition for the shared primers (Plasmo1 and Plasmo2). In present data, 7 cases out of the total 9 nested PCR positive patients failed to be identified by real-time PCR. Actually, all had low parasitemia and the remained two were involved in mixed infection. Similar phenomena also reported in previous studies [52, 53]. As we know, false negative result means a person with Plasmodium infection failed to be diagnosed. This could cause misdiagnosis, delay treatment of malaria and even lead to a life-threatening problem, especially for P. falciparum infection. Beyond the individual damage, false negative patients may also spread malaria and produce resistance to antimalarial drugs without correct treatment guided by exact diagnosis [50].

Although nested PCR showed the highest sensitivity and real-time PCR has the best specificity, the role of microscopy and RDT is still not substituted. Because conduct of the two molecular methods is relied on DNA extraction of blood samples. Once errors happened during this program, the false negative would probably present. For example, in a previous study, there were 10 malaria cases failed to be identified by molecular methods due to the problems in nucleic acid extraction until reasons were found out by repeated experiments [54]. Therefore, the combining use of the four methods is quite necessary and highly recommended. Both microscopy and RDT can offer the preliminary result and suggest further diagnostic direction. Based on the valuable information, the Plasmodium species can be confirmed by molecular methods easily including nested PCR and real-time PCR.

The origin of patients was basically consistent with a previous report referring to epidemiologic features of imported malaria in the whole China [55]. In these epidemic regions of Africa and SEA, the natural environment, healthcare and situation of economic development may be primary influences on malaria infection. Chinese people overseas especially those didn't long-term inhabit in epidemic areas usually lacked adaptive immunity and related awareness to local Plasmodium infection [55]. Additionally, there were frequently asymptomatic patients existing [56]. These remind us that workers returned from these areas particularly West Africa, Central Africa, and Southern Africa should be recommended to accept malaria diagnosis. As the dominating malaria species, P. falciparum was similar to other study [50]. Therefore, prevention and treatment against P. falciparum should be highly considered in Wuhan. By the year elapsed, the Plasmodium species leading to imported malaria were becoming diversity just as the situation in another region of China, Shandong province [54]. The increase of imported P. vivax and P. ovalis cases may cause the re-introduction of malaria in those regions without cases [57, 58]. P. malariae could maintain in human for many years and keep infectivity to its vector at a really low parasitemia [59]. Thus, more attention should be payed on this increasing diversity. The sharp decrease of imported malaria cases in 2017 was a good sign indicating the performance of malaria control. However, the cases raised again in 2018, warning us the great challenging for complete elimination of Plasmodium infection worldwide, even in China.

Conclusions

Results in this study could assess the efficiency of 18S ssrRNA based nested PCR and real-time PCR in the differentiation of four Plasmodium species and two subspecies of P. ovalis. The results highlighted, once again, that PCR based detections are irreplaceable methods for exact species determination of imported malaria patients, especially for mixed infections. We also emphasized the combination use of the four methods involved in the present study. Meanwhile, the data as a very important

epidemiological resource, could supply guidance for the surveillance, prevention and timely treatment of imported malaria in Wuhan of China. The guiding effect is also significant to the epidemic areas as shown in the origin distribution survey or even to countries at potential high risk of imported malaria worldwide.

Abbreviations

Pf: P. falciparum; Pv: P. vivax; Pm: P. malariae; Po: P. ovalis; Poc: P. ovale curtisi; Pow: P. ovale wallikeri; RDT: rapid diagnosis test; PPV: Positive Predictive value; NPV: Negative Predictive value; DP: disease prevalence; CDC: Center for Disease Prevention and Control.

Declarations

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Authors' contributions

1. L. and K. W. conceived the study, and participated in its design and coordination. K.W., MX. X., and Y. Y. carried out sample collection. YT. X. and J. L. interpreted the data and wrote the manuscript. YT. X., TT. J. and Y.Y. carried out molecular assays and performed statistical analysis. YT. X., TT. J., K.W. and HB. T. participated in molecular assays and data analysis. YT. X. and J. L. reviewed the final manuscript. All authors read and approved the final version of the manuscript.

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Availability of data and materials

The datasets analyzed in this study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Current study was approved by the Medical Ethics Committees of the Hubei University of Medicine and Wuhan City Center for Disease Prevention and Control. The written informed consent was obtained from all participated individuals.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

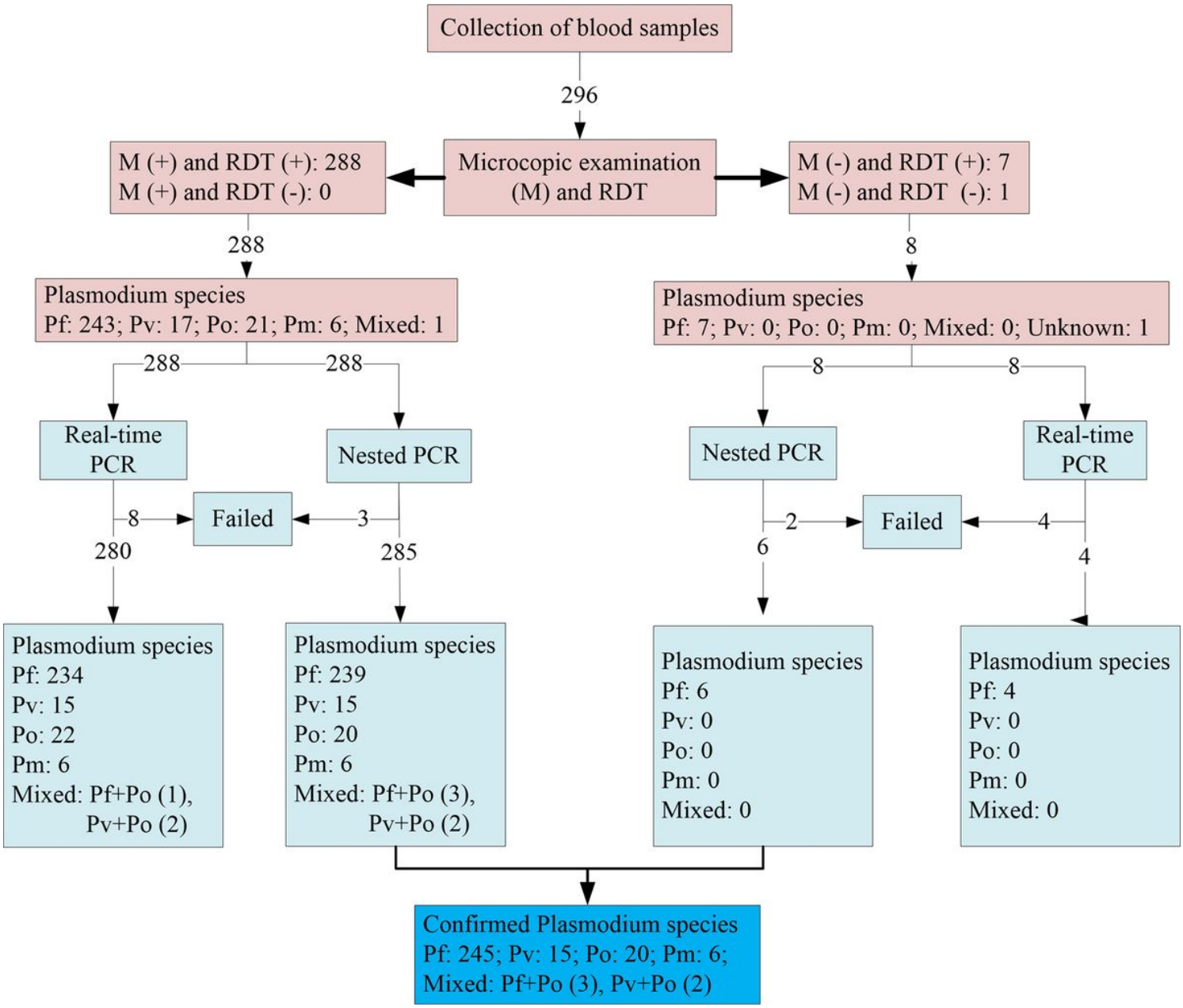

Figure 1

Flowchart detailing the study participation and compliance in Wuhan, China.

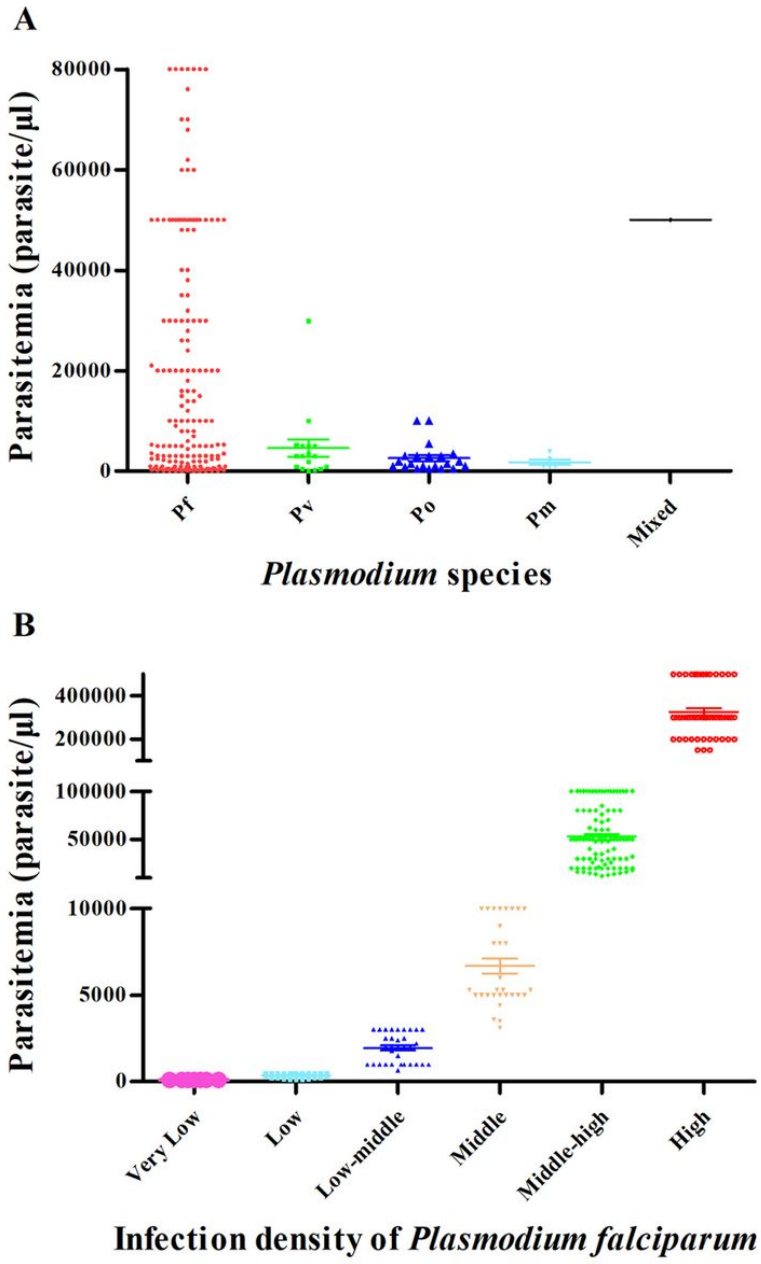

Figure 2

Parasitemia for Plasmodium species infection from imported malaria clinical cases in Wuhan, China. (A) Parasitemia for four Plasmodium species infection. (B) Infection density of Plasmodium falciparum.

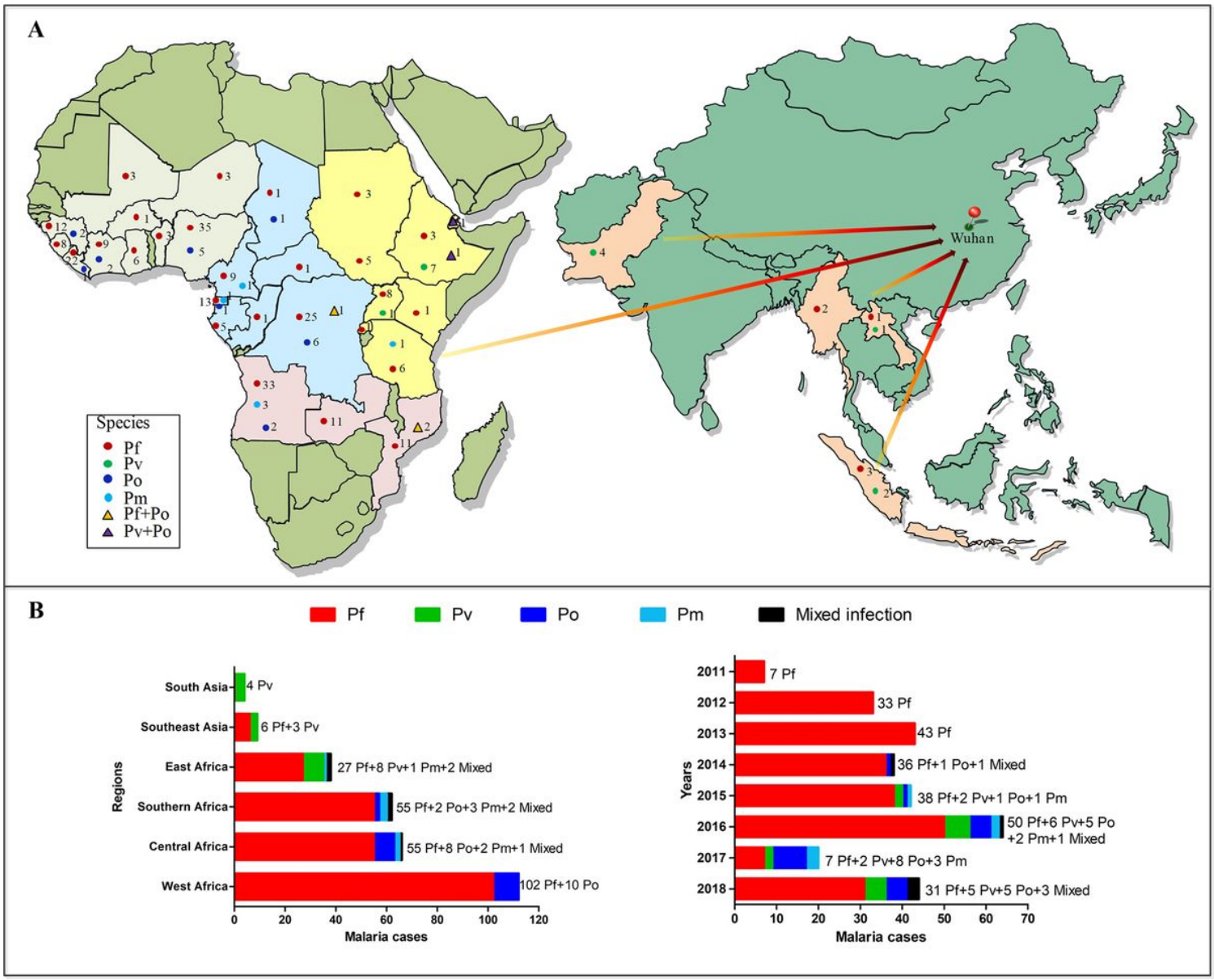

Figure 3

The importation origin and year distribution of imported malaria cases in Wuhan, China, from 2011 to 2018. (A) The map of import`ation origin and cases numbers for the four Plasmodium species by country. (B) The number counting of patients infected with each Plasmodium species by four importation regions in Africa, two in Asia and years. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.