Renal Localization of *Plasmodium vivax* and Zoonotic Monkey Parasite *Plasmodium Knowlesi* in Malaria Associated Acute Kidney Injury

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ABSTRACT:

Acute kidney injury (AKI) is a frequent presentation in *Plasmodium vivax* (*P. vivax*) associated severe malaria. However, tissue presence of *P. vivax* has not been established. We show the renal tissue presence of *P. vivax* and *P. knowlesi* in cases of malaria associated AKI.

INTRODUCTION:

Acute kidney injury (AKI) is a frequent presentation in severe malaria due to both *Plasmodium falciparum* and *Plasmodium vivax*- the two most common causes of malaria in the world[1–3]. AKI has been shown to have a global prevalence in about 20-50% hospitalized malaria cases[4]. The mechanism of tissue specific pathogenesis in *P. falciparum* malaria is attributed to its cytoadherence to endothelial cells of tissue capillaries resulting in localized inflammation and tissue damage [5, 6]. On the other hand, cytoadherence of *P. vivax* is still under investigation and has not been linked to pathogenesis in humans [7]. AKI has been well documented in hospitalized cases of *P. vivax* infections [2,3,8,9]. Therefore, it is possible that *P. vivax* has host tissue specific interaction, and may be responsible for renal injury in malaria associated AKI. However, there is no evidence in literature to indicate *P. vivax* association with human tissues. Therefore, we investigated the presence of *P. vivax*, along with the other four *Plasmodium* species known to infect humans (*P. falciparum*, *P. knowlesi*, *P. malariae*, and *P. ovale*) in renal biopsies of malaria cases that presented with AKI at our center. We report our observations here.

METHODS:

Study design

This study was a cross-sectional retrospective study carried out at the All India Institute of Medical Sciences (AIIMS), New Delhi, as collaboration between the Departments of Biochemistry, Pathology, Pediatrics and Nephrology. The study has been cleared by the Institute Ethics Committee.

Collection of renal tissue samples: Archival formalin-fixed paraffin-embedded (FFPE) renal tissue blocks from 2011-2018 were retrieved from the Department of Pathology, AIIMS, New Delhi. The diagnosis of AKI was made using standard criteria, as defined in the Supplementary file. AKI cases were selected by the pathologists based on the presence of malaria parasites by peripheral smears, or patient symptoms. 16 AKI samples with confirmed or suspected malaria were selected as cases and 5 AKI samples without malaria were selected as controls. The investigators who carried out the PCR analysis were blinded to the identity of cases and controls until results of the PCR analysis were obtained.

Histological examination: Histological sections of all FFPE tissues were stained with H & E stain, picric acid solution, Prussian Blue stain and Jones methenamine silver stain. The glomeruli, tubules, interstitium, and blood vessels were examined in all the cores.

Detection of malaria parasite: Diagnosis of malaria was based on detection of different parasitic forms of *Plasmodium* species in the peripheral smear (thick and thin smears stained by Giemsa) or by quantitative buffy coat assay. Species identification was done on thin film microscopy. For detection of malaria parasite from kidney tissue, DNA was extracted using the QiagenQiAmp DNA FFPE tissue kit as per the manufacturer's protocol (Supplementary file). PCR of extracted DNA was carried out for all the five *Plasmodium* species (*P. falciparum, P. vivax, P. knowlesi, P. malariae* and *P. ovale*) were carried out as described in the Supplementary file. The PCR products obtained from the malaria positive reactions were subjected to Sanger Sequencing (Bencos Research Solutions Pvt. Ltd.) in order to confirm the species identity (Results in the Supplementary file).

RESULTS:

In the present study, 16 FFPE samples of AKI with confirmed or suspected malaria and 5 controls samples (AKI without clinical, laboratory, or histopathologic evidence of malaria, randomly selected) collected from 2011 to 2018 were included (Supplementary Table S1). Of the 16 cases, 12 presented with fever and associated oliguria, 1 presented with fever and associated hematuria; 1 as hematuria alone; 1 presented as AKI about one and half month after diagnosis as complicated *P. vivax* malaria (CM); and 1 had presented with AKI with subsequent placental abruption (Table 1; column 2). Peripheral blood smear revealed the presence of *P. vivax* in 7 samples while the remaining 9 were negative for malaria. However, in all the 16 cases, histological examination showed deposition of hemosiderin, and blackish brown pigment resembling hemozoin along with the presence of RBC cast in the tubular lumen and interstitial area of kidney whereas these features were absent in all 5 controls. Clinical features and histological observations of control samples are presented in Supplementary Table S2. Presence of hemozoin was confirmed using a combination of Prussian Blue stain and picric acid solution (malaria bleach) (Supplementary Figure S1).

The various histological diagnoses in these cases were – acute cortical necrosis (ACN), acute tubular injury / necrosis (ATI/ ATN), thrombotic microangipathy (TMA), and interstitial fibrosis and tubular atrophy (IFTA). Of the 16 cases, 14 had evidences of cortical necrosis,

TMA, or mesangiolysis, indicating vascular involvement (Table 1). The representative histopathology of cortical and tubular damage has been depicted in Supplementary Figure S1.

The peripheral blood smears of 8 cases were found to have infection of *P. vivax* alone, and 1 showed the presence of both *P. vivax* and *P. falciparum* trophozoites; 9 cases had no evidence of malaria from peripheral blood smears (Table 1). Of the 9 samples with no blood smear evidence of malaria parasites, 5 were clinically diagnosed as complicated malaria (CM), and 2 cases were not labeled as malaria infections (Table 1). Of the latter 2 cases, one presented with hematuria and one with placental abruption (Table 1).

All the 16 samples were subjected to PCR analysis of all the 5 *Plasmodium* spp. (*P. falciparum*, *P. vivax*, *P. knowlesi*, *P. malariae* and *P. ovale*). Eight cases had *P. vivax*, 1 had *P. knowlesi*, 1 had mixed infection of *P. vivax* and *P. falciparum*, 2 had mixed infections of *P. vivax* and *P. knowlesi*, 1 had mixed infection of *P. falciparum* and *P. knowlesi*, and 3 had mixed infections consisting of the three parasites *P. vivax*, *P. falciparum*, and *P. knowlesi* (Table 1). Overall, of 16 cases with malaria associated AKI, renal tissue of 14 had *P. vivax*, 7 had *P. knowlesi* and, 5 had *P. falciparum* infection. Interestingly, none of the 7 cases of malarial AKI having *P. knowlesi* by PCR had peripheral smear detection of the parasite (Table 1).

DISCUSSION:

It is well recognized now that *P. vivax* is capable of causing complicated malaria and is associated with several syndromes such as anemia, thrombocytopenia, impaired liver and renal function, and altered sensorium, among other features. However, its pathogenesis is unknown. In *P. falciparum*, pathogenesis of complicated malaria is linked to tissue cytoadhesion of the parasite. However, unlike *P. falciparum*, the cytoadhesion of *P. vivax* is still under investigation. It is not clear if it interacts with tissue capillaries or localizes within tissues. We have addressed the question of tissue localization of *P. vivax* in malaria associated acute kidney injury (AKI). The data presented here provide evidence for the renal localization of *P. vivax*. Surprisingly we also found *P. knowlesi*, the zoonotic parasite known to infect Rhesus macaques (*Macaca mulatta*), within the renal tissue of many malaria AKI cases.

Of the 16 malaria cases presenting with AKI, 14 (~88%) were found to have *P. vivax* infection. Although several studies show association of AKI with peripheral blood *P. vivax* infection [2,3,8,9]. This is the first study to demonstrate direct tissue presence of the parasite.

Interestingly, of the 16 cases, 9 harboring *Plasmodium* parasites in renal tissue had no evidence of the parasite from microscopic examination of peripheral blood smear, suggesting that microscopic examination alone may not be enough for detection and diagnosis of *P. knowlesi*.

In addition to PCR evidence of the presence of parasite DNA, we also find specific staining for iron containing hemozoin which is completely absent in all the control samples. Hemozoin pigment is known to be produced only by replicating parasites [10]. When in circulation, it is rapidly phagocytosed by macrophages and dendritic cells and therefore, has a short half-life [11]. Hemozoin pigment in renal tissue has been demonstrated in the case of *P. falciparum* associated AKI [12]. Therefore, presence of hemozoin staining specifically in renal tubules and interstitium suggests the presence of replicating parasites at the site, rather than non-specific accumulation of hemozoin.

This is the first study to demonstrate tissue localization of *P. knowlesi*. This is an important observation since the response of *P. knowlesi* to current anti-malarial therapy is not well understood [13,14]and may affect treatment outcomes in AKI cases that have co-infections of *P. vivax* and *P. knowlesi* within the renal tissue.

We find that of the 16 cases of malaria AKI, 14 have evidence of cortical necrosis, thrombotic microangipathy, and/or mesangiolysis indicating the involvement of the renal microvasculature. This suggests that *P. vivax*, as well as *P. knowlesi*, might have the ability bind tissue vasculature and exhibit cytoadherence.

It is interesting to note that many of the cases harboring renal *P. knowlesi* were from Delhi, and Uttar Pradesh where there has been a reported explosion of *Rhesus macaque* population in recent times (Supplementary Figure S2)[15]. This highlights the importance of malaria elimination not only in humans and mosquito vectors, but also in other hosts living in human proximity.

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Table 1. Summary of clinical presentation of AKI in patients with malaria infection, with PCR diagnosis of the five *Plasmodium* species infecting humans.

Sample Id	Age (Years)/ Sex	Clinical presentation	Laboratory investigations	Blood Smear evidence	Histology	PCR diagnosis of Renal tissue DNA
AIIMS K7167	6/F	Fever, oliguria	Thrombocytopenia, anemia, hyperkalemia, deranged RFT, LDH elevated urine: proteinuria (2+), 10-15 RBC/hpf	Nil	ACN (45-50%), viable area-ATI, Arteries and arterioles unremarkable	*Pk*
AIIMS K8299	23/F	Sudden onset placental abruption	Sudden rise of creatinine (4.2 mg/dl)	Nil	Patchy ACN	*Pv*+*Pk*
AIIMS K6691	22/M	Fever &edema 1month, hematuria, hypertensive retinopathy,CM	Low C3 level, LDH elevated, urine: RBCs full field	Nil	ATI and subacute TMA	*Pf* + *Pk*
AIIMS K1056	8/M	Fever, oliguria, CM	Creatinine-3.6 mg/dl, thrombocytopenia	Nil	ACN (70-75%), viable single artery-fibrin thrombus (TMA)	*Pk*+*Pv*
AIIMS K3873	30/M	Fever, oliguria-3	Creatinine-5.9mg/dl, proteinuria	Nil	Patchy cortical necrosis (scarring phase), acute	*Pv*+*Pk*+*Pf*

		months, CM	(2+), HD dependent		interstitial nephritis, vessels -UR	
AIIMS K0447	37/F	hematuria	Case of TMA on PLEX, on rituximab	Nil	Chronic TMA	*Pv* + *Pk* + *Pf*
AIIMS K4424	17/M	Fever, right-sided hemiplegia, oliguria-15 days,CM	Deranged RFT, LDH elevated	Nil	ATN, interstitial inflammation, IFTA (20%)	*Pv*+*Pk*+ *Pf*
AIIMS K0014	18/F	Fever, jaundice, oliguria	Creatinine-7.2 mg/dl, thrombocytopenia, proteinuria (2+), raised LDH, HD-dependent,	Nil	Multifocal cortical necrosis with scarring, ATI, chronic TMA	*Pv*+*Pf*
AIIMS K7559	20/F	Complicated malaria (one and half months back), Sepsis, anuria, myocarditis	Creatinine-4.1, thrombocytopenia, proteinuria (2+), HD dependent	*Pv*trophozoites	ACN (60%)	*Pv*
AIIMS K2788	22/F	Fever, oliguria, melena, jaundice, cardiac dysfunction	Creatinine-9.4, thrombocytopenia, proteinuria (1+)	*Pv*trophozoites	Patchy ACN	*Pv*

AIIMS K6701	11/F	Fever-10 days, oliguria-3 days, epistaxis, splenomegaly,	Creatinine-7.9 mg/dl, thrombocytopenia, urine: proteinuria (2+), 15-20 RBC/hpf, raised LDH	*Pv*trophozoites	Resolving ACN, AIN, mesangiolysis, arterioles-vacuolization & endothelial swelling	*Pv*
AIIMS K6683	12/M	Fever & oliguria-3 days, epistaxis, melena, splenomegaly	Creatinine-10.3 mg/dl, thrombocytopenia, urine: proteinuria (1+), 8-10RBC/hpf, raised LDH	*Pv*trophozoites	Resolving ATN	*Pv*
AIIMS K9006	10/F	Fever & oliguria-6 days, epistaxis, melena, splenomegaly	Creatinine-6.2 mg/dl, thrombocytopenia, urine: proteinuria (4+), 8-10 RBC/hpf, raised LDH	*Pv*trophozoites	ATN, TMA	*Pv*
AIIMS K7384	19/M	Fever & oliguria-3 days, epistaxis, melena, splenomegaly, CM	Creatinine-4 mg/dl, thrombocytopenia, urine: proteinuria (2+), 8-10 RBC/hpf, raised LDH	Nil	Focal ACN, TMA, blackish-brown pigments glomerulus, tubules &interstitium	*Pv*
AIIMS K3326	18/F	Fever-7 days, epistaxis, hypertension, coma, anemia,	Creatinine-9.8 mg/dl, thrombocytopenia, urine: proteinuria (4+), 15-20 RBC/hpf, raised LDH	*Pv*trophozoites	Resolving ATN with focal mesangiolysis	*Pv*

		oliguria-15 days				
AIIMS K6641	9/F	Fever-10 days, oliguria-2 days, splenomegaly	Creatinine-6.5 mg/dl, thrombocytopenia, proteinuria (1+), raised LDH	*Pv*trophozoites	Resolving ATN with AIN. Arteries and arterioles-endothelial swelling	*Pv*

Abbreviations: ACN; Acute Cortical Necrosis, ATI; Acute Tubular Injury, TMA; Thrombotic Microangiopathy, AIN: Acute Interstitial Nephrosis, IFTA; Interstitial Fibrosis Tubular Atrophy, RFT; Renal Function Test, HD; Hemodialysis;CM:Complicated Malaria, LDH: Lactate Dehydrogenase Pf: Plasmodium falciparum; Pv: Plasmodium vivax; Pk: Plasmodium knowlesi. Plasmodium ovale and Plasmodium malariae were not detected in these samples.