A Case Report of Simian Malaria, *Plasmodium knowlesi*, in a Taiwanese Traveler from Palawan Island, the Philippines

Ming-Chu Kuo[1], Ting-Yi Chiang[1], Chih-Wen Chan[1], Wen-Shih Tsai[2], Dar-Der Ji[1]

1. Research and Diagnostic Center, Centers for Disease Control, Taiwan
2. Puli Christian Hospital

From Chinese version, pp, 167-178

Abstract

In March 2005, a traveler came back to Taiwan from Palawan Island, Philippines, was reported as a simian malaria patient. The initial microscopic examination found that the mature trophozoites were ameba-form with few band-form, which was similar to the trophozoites of *Plasmodium malariae*. Compared to the published data, we used SSU rRNA and *csp* gene as typing markers to do polymerase chain reaction and gene sequence for the phylogenic relationship analysis. We confirmed that the malaria case was caused by *Plasmodium knowlesi* infection. This was the first human case that was infected naturally by monkey malaria. Since more and more human monkey malaria cases were reported in Southeast Asian

• Received : 5 September, 2008. • Accepted : 9 Octorber, 2008.
• Correspodence : Dar-Der Ji
• Address : No.161, Kun-Yang Street, Taipei, Taiwan, R.O.C.
• e-mail: jidarder@cdc.gov.tw

countries, and thus *Plasmodium knowlesi* has been suspected as the fifth malaria pathogen for human.

Keywords: SSU rRNA gene, csp gene, *Plasmodium knowlesi*

Background

The pathogens of malaria are *Plasmodium* spp. and mainly infect the red blood cells in vertebrate animals. Over 200 known *Plasmodium* spp. have been found worldwide [1]. There are four pathogens caused human malaria: *Plasmodium falciparum*, *P. malariae*, *P. ovale* and *P. vivax*. Other *Plasmodium* spp. infect early primitive reptile, birds, rodents and primate [1].

P. knowlesi was first found in the blood from a long-tailed Macaque (*Macaca fascicularis*) by Dr. Franchini et.al. in 1927, which was different to *P. inui* and *P. cynomolgi* [2]. Dr. Knowles and Dr. Das Gupta [3] described the morphology of *P. knowlesi* in 1932 and found that this pathogen could infect humans. Dr. Chin et. al. [4] confirmed that *P. knowlesi* could be transmitted from macaques to humans through *Anopheles balabacencis* mosquito biting. Dr. Balbir Singh et.al [5] found a large-scale natural infection of *P. knowlesi* in Borneo, Malaysia, in 2004 and this event had drawn the attention worldwide. Dr. Cox-Singh et. al. [5] further discovered that *P. knowlesi* infection was widely distributed in Sabah and Sarawak in Bornian, and Pahang in Malaysia in 2008. In addition, 4 *P. knowlesi*-infected death cases were also confirmed by Dr. Cox-Singh et. al. The age of these patients were between 39 to 69 years old and there were clinical signs, including abdominal pain, fever, high parasitemia, icterus and renal damage, similar to severe acute malaria,

which may indicate that P. knowlesi infection, could be fatal. [6]. At the same time, similar cases were reported in Thailand, Singapore, Philippines, Myanmar and China, and thus, *P. knowlesi* is now highly suspected as the fifth pathogen for human malaria [7-12].

It is very easy to misdiagnose due to the morphology of *P. knowlesi,* such as bi-nuclear ring-form small trophozoites are similar to *P. falciparum,* whereas band-form late trophozoites, schizonts and macro/micro-gametocytes are similar to *P. malariae* [5-7,10]. The life cycle of *P. malariae* is 72 hours and causes mild or even no clinical symptoms. The life cycle of *P. knowlesi* is 24 hours [6], which is shorter than other malaria pathogens and may proliferate very fast in the blood to a fatal parasitemia, if not be properly diagnosed. [12]. Many study groups re-examined the preserved samples retrospectively [5-6,9] to understand the real incidence of *P. knowlesi* in human and to understand the transmission mode: whether the human cases were transmitted from macaque or human were natural host.

This article described the morphology and molecular identification for the first imported simian malaria case.

Case Report

The patient was a 60 years-old retired teacher from Puli Town, Nantou County, Taiwan. The patient went to Southeastern Asia for bird watching and ecotourism for 2 weeks in February, 2005. He wore pants and long-sleeve shirt most of the time, however, changed to short pants due to hot and humid weather while in Palawan Island, Philippines, and felt been bitten by mosquitoes. When he came back to Taiwan, he occurred

high fever to 39.4°C, headache, intermittent cold shiver and sweat, and lasted for 4 days. He therefore consulted outpatient department of Puli Christian hospital and reported as a suspected malaria case later. The patient was originally diagnosed as *P. malariae* infection by Taiwan CDC staffs. Based on the WHO medical guideline of malaria [13], oral Chloroquine 1 gram (base 600mg) was provided initially and 500 mg (base 300mg) at 6 hours, 24 hours and 48 hours after the first dose. The patient was fully recovered.

Material and Method

1. Microscopic examination: Thick and thin blood smears were prepared and stained by 5% Giemsa solution (pH 7.0~7.2). The slides were rinsed 40 minutes later and air-dried. The slides were examined by 1000X oil immersion lens for 5 to 10 minutes: thick smear slide was fully examined, while thin smear slide was examined 300 fields minimum.
2. DNA isolation and Nested polymerase chain reaction: Total DNA was isolated from patient blood sample by QIAampTM BLood Kit (Qiagen) and used for PCR amplication based on the small subunit ribosomal RNA (SSU rRNA) and circusporozoite protein (csp) of four human malaria parasites. Positive result and malaria species were determined by the existence of amplicons and the size of gene fragments [14-16]. SSU rRNA primer and the size of gene fragments were listed in Table 1.

Table 1. The SSU rRNA primers and the size of gene fragments

Primer	Gene segment (5'→3')	Product
SSU rRNA Outer PCR		
genus–specific	rPLU 1：TCA AAG AAT AAG CCA TGC AAG TGA rPLU 2：TAC CCT GTT GTT GCC TTA AAC TCC	1.6 kb
SSU rRNA Inner PCR		
genus–specific	rPLU 3：TTT TTA TAA GGA TAACTA CGG AAA AGC rPLU 4：TAC CCG TCA TAG CCATGT TAG GCC AAT ACC	240-bp
P. falciparum	rFAL1：TTA AAC TGG TTT GGGAAA ACC AAA TAT ATT rFAL2：ACA CAA TGA ACT CAATCA TGA CTA CCC GTC	205-bp
P. vivax	rVIV1：CGC TTC TAG CTT AATCCA CAT AAC TGA TAC rVIV2：ACT TCC AAG CCG AAGCAA AGA AAG TCC TTA	117-bp
P. malariae	rMAL1：ATA ACA TAG TTG TACGTT AAG AAT AAC CGC rMAL2：AAA ATT CCC ATG CATAAA AAA TTA TAC AAA	144-bp
P. ovale	rOVA1 ATC TCT TTT GCT ATTTTT TAG TAT TGG AGA rOVA2 GGA AAA GGA CAC ATTAAT TGT ATC CTA GTG	787-bp
P. knowlesi	Pmkr8：GTT AGC GAG AGC CACAAA AAA GCG AAT Pmkr9：ACT CAA AGT AAC AAAATC TTC CGT A	153-bp
csp PCR *P. knowlesi*	PkCSP-F：TCCTCCACATACTTATATACAAGA PkCSP-R：GTACCGTGGGGGACGCCG	1.0 kb

3. Gene cloning and sequence analysis: Two PCR amplicons (1669 bp SSU rRNA and 1081 bp *csp*) were introduced into pCR4-TOPO (Invitrogen^{TM}) plasmid separately and transformed into *Escherichia*

coli DH5α for further sequencing. These two sequences were then compared with other Plasmmodium spp. in the GenBank using Genetics Computer Group (GCG) software. The phylogenetic relationship tree was performed by neighbor-joining method to analyze the relationship between these pathogens [17].

Result

Observed with microscope, we have found the ring-form trophozoites in the infected red blood cell was slightly larger than *P. falciparum.* The mature trophozoites were ameba-form with dense cytoplasm and some of them were more than half of the RBC in size. However, some of the mature trophozoites were band-form, similar to *P. malariae*. The mature trophozoites in this case contained 8 to 16 merozoites and dense dark-brownish cytochrome was found in mature trophozoites and merozoites. The gametocyte was round and occupied whole blood cell, which was very similar to *P. malariae* (Figure 1).

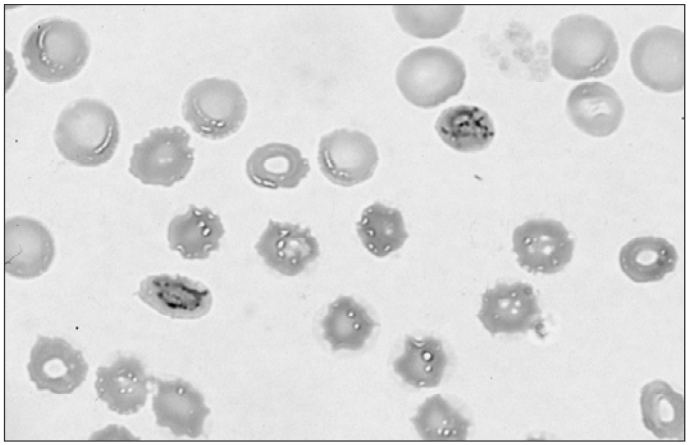

Figure 1. The morphology of *P. knowlesi* with Giemsa staining.

The evidence was not enough to make sure of this diagnosis by morphological examination only. Molecular analysis was therefore processed to confirm the species of this pathogen. *Plasmodium* genus -specific primers and species-specific primers to *P. falciparum, P. malariae, P. ovale* and *P. vivax* were used for the nested PCR based on SSU rRNA gene. The result indicated that the pathogen was *Plamodium* spp. but not the four human malaria parasites.

We further sequenced the SSU rRNA and *csp* sequencingene amplicons to compare the phylogenetic relationship, and the result revealed that the isolated pathogen was similar to a monkey malaria *P. knowlesi* (99% and 91% in similarity, respectively). It was obviously different to the other malaria parasites from human, primate, rodent and bird (Figure 2 and Figure 3).

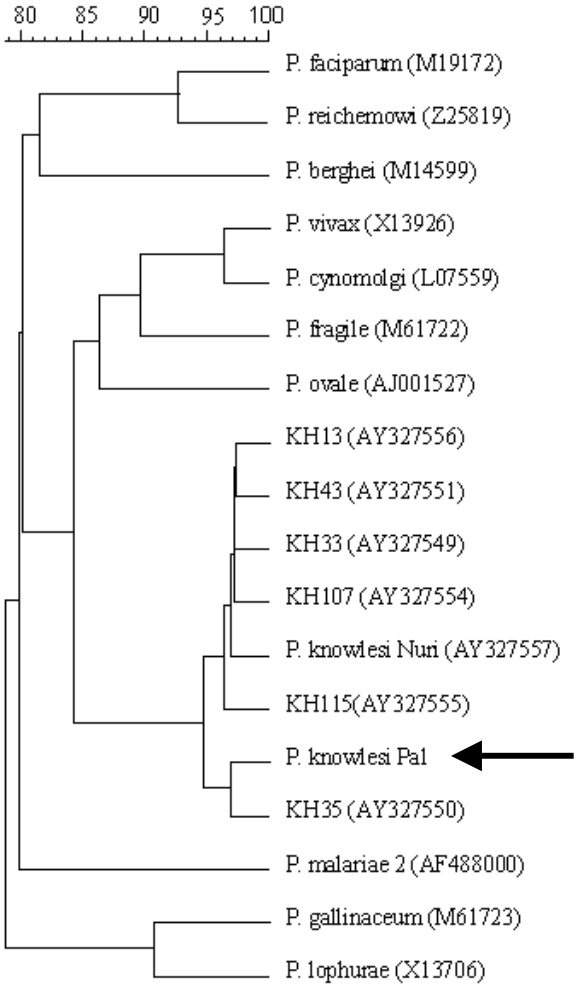

Figure 2. The phylogenetic tree of SSU rRNA gene from different *Plasmodium* spp. Arrow: the specimen from the isolated parasite.

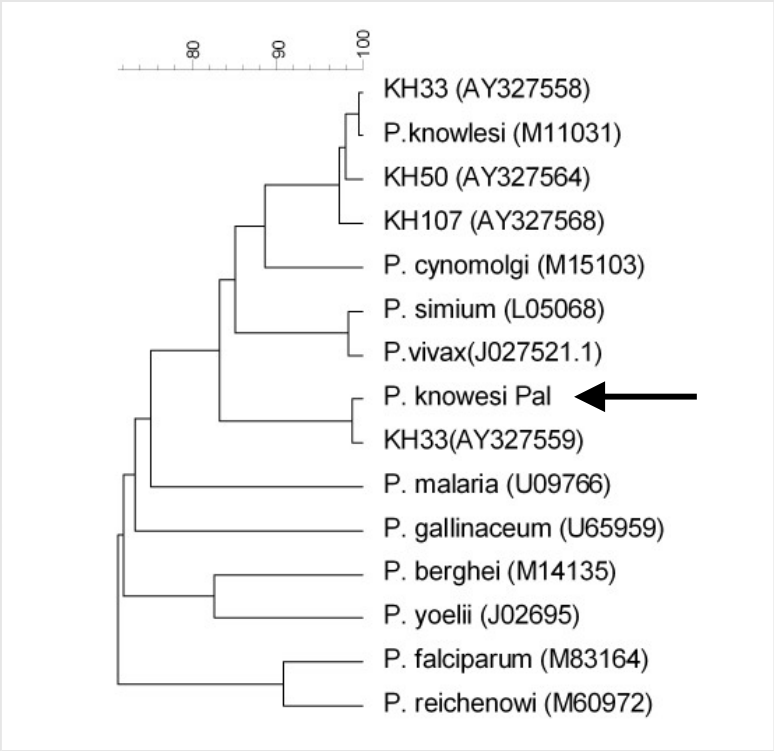

Figure 3. The *csp* phylogenetic tree (by the neighbor-joining method) from different *Plasmodium* spp. Arrow: the specimen from the isolated parasite.

A 153 bp of *P. knowlesi*-specific fragment was amplified by *P. knowlesi*-specific primers of SSU rRNA gene, whereas other malaria species not. This result confirmed that this patient was infected by *P. knowlesi.* The result of electrophoresis was shown in Figure 4.

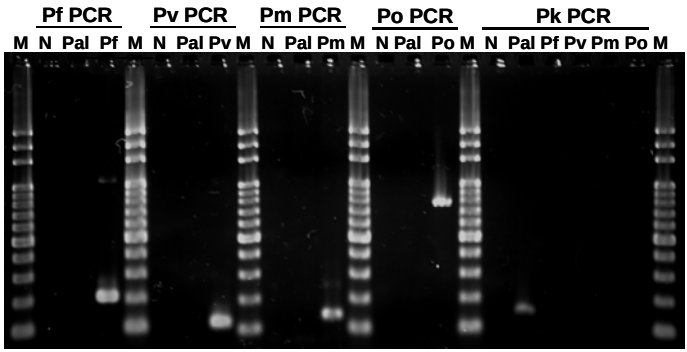

Figure 4. The result of electrophoresis using *P. knowlesi*-specific primers and four human *Plasmodium* species-specific primers. M：100 bp marker; Pf: *P. falciparum*，Pv: *P. vivax*; Pm: *P. malariae;* Po: *P. ovale;* Pal: specimen from the patient; N: negative control.

Discussion

The malaria diagnosis is normally based on the clinical signs of the patient, the morphology of the parasites and the morphological changes of the infected red blood cells. However, this is usually focused on the four human malaria parasites and the possibility of malaria infected by other *Plasmodium* spp. may be neglected. It is very easy to misdiagnose *P. knowlesi* as *P. malariae* due to morphological similarity. Although *P. malariae* was originally suspected as the pathogen of this patient, the morphology of the parasite was not typical to *P. malariae*. Furthermore, the result of *Plasmodium* genus-specific nested PCR was positive but the species-specific nested PCRs to four human *Plasmodium* were negative. These results indicated that the patient might infect by other non-human

malaria parasite. The amplicons of nested PCR based on SSU rRNA and csp was sequenced, and shown the 99% and 91% similarity to *P. knowlesi*, respectively. We compared the phylogenetic relationship between the isolate and other *P. knowlesi* strains from different areas, and the result indicated that this isolate was most similar to KH35 strain from Malaysia. Thus, we confirmed that the pathogen of this human malaria case was *P. knowlesi*, a malaria pathogen from the monkey.

Many pathogens can cause cross infection between animals and human. Among them, the wild primates are usually the potential resources of human diseases. Due to new road exploration, mining, lumbering, and cross-forest traveling, we increase more chances to contact with wild primates or animals and also to be infected by zoonotic diseases. *Anopheles balabacencis* is an important vector for malaria transmission in the forest areas of Malaysia, which is also the natural habitat for long-tailed monkey (*Macaca fascicularis*) and Pig-tailed monkey (*Macaca nemestrina*). Both primates are also natural hosts of *P. knowlesi* [18]. Thus, *P. knowlesi* may be naturally transmitted between wild primates and human by *Anopheles balabacencis.* In the past, the cases of *P. knowlesi* infection were rare and the infection areas are scattered to different locations. Therefore the importance of *P. knowlesi* to zoonotic disease was not respected, until the research data published by Balbir Singh et. al. in 2004 [5]. They found that there might be far more human malaria cases and more widely distributed in the world. Earlier studies indicated that *P. knowlesi* distributed from Malaysia to Philippines [19]. *P. knowlesi* was also found in Formosan macaque (*Macaca cyclops*) [18]. Although no human case of *P. knowlesi* infection was found in Taiwan, we must be

aware of the introduction of pathogenic *P. knowlesi* to Formosan macaque and the possibility of transmission between wild macaque and human.

Differential diagnosis is an important issue in the inter-species transmissible diseases. The life cycle of *P. knowlesi* is 24 hours, which indicates that rapid diagnosis and medical therapy are necessary. Based on the conserved gene sequence, the species-specific gene primers can be used to identify the pathogen and to help the diagnosis promptly. Luckily the mortality of *P. knowlesi* infection in human was low in present research and most malaria medicines are effective to the therapy. However, if a high parasitemia was abmornally shown in *P. malariae*-infected patient, it shall be aware *P. knowlesi* infection to prevent the mortality [12]. Balbir Singh et. al. also recommended that the travelers or residents in Southeastern Asia who was diagnosed of *P. malariae* infection with high parasitemia should be treated as *P. falciparum* infection to avoid delayed therapy [6].

This study established the molecular biological diagnosis methods for *P. knowlesi* infection in our laboratory. It is also the first clinical human case report of *P. knowlesi* infection in Taiwan.

References

1. Rich SM, Ayala FJ. Progress in malaria research: the case for phylogenetics. Adv Parasitol. 2003; 54: 255-80.
2. Franchini G. Plasmodio pigmentatodi unascimmia. Arch Ital Sci Med Colon Parasitol 1927; 8:187-90.
3. Knowles R, Das Gupta BM. A study of monkey-malaria and its experimental transmission to man. Ind Med Gaz. 1932; 67: 301–21.
4. Chin W, Contacos PG, Collins WE, et al. Experimental mosquito-transmission of *Plasmodium knowlesi* to man and monkey. Am J Trop Med Hyg. 1968; 17: 355–8.
5. Singh B, Kim Sung L, Matusop A, et al. A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. Lancet. 2004; 363: 1017-24.
6. Cox-Singh J, Davis TM, Lee KS, et al. *Plasmodium knowlesi* malaria in humans is widely distributed and potentially life threatening. Clin Infect Dis. 2008; 46:165-71.
7. Jongwutiwes S, Putaporntip C, Iwasaki T, et al. Naturally acquired *Plasmodium knowlesi* malaria in human, Thailand. Emerg Infect Dis. 2004; 10: 2211–3.
8. Ng OT, Ooi EE, Lee CC, et al. Naturally acquired human *Plasmodium knowlesi* infection, Singapore. Emerg Infect Dis. 2008; 14: 814-6.
9. Luchavez J, Espino F, Curameng P, et al . Human Infections with *Plasmodium knowlesi*, the Philippines. Emerg Infect Dis. 2008; 14: 811-3.
10. Zhu HM, Li J, Zheng H. Human natural infection of *Plasmodium knowlesi*. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi. 2006; 24:70–1.
11. Kawamoto F, Win TT, Mizuno S, et al . Unusual plasmodium malariae-like parasites in southeast Asia. J Parasitol. 2002; 88: 350-7.
12. White NJ. *Plasmodium knowlesi*: the fifth human malaria parasite. Clin Infect Dis. 2008 Jan 15; 46: 172-3.
13. World Health Organization. WHO Guidelines for the Treatment of Malaria. Geneva, Switzerland: WHO; 2006. Technical document WHO/HTM/MAL/ 2006.1108.
14. Singh B, Bobogare A, Cox-Singh J, et al. A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. Am J Trop Med Hyg. 1999; 60: 687–92.
15. Snounou G, Viriyakosol S, Zhu XP, et al. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Mol Biochem

Parasitol 1993; 61: 315-20.

16. Rougemont M, Van Saanen M, Sahli R, et al. Detection of four *Plasmodium* species in blood from humans by 18S rRNA gene subunit-based and species specific real-time PCR assays. J Clin Microbiol. 2004; 42: 5636–43.
17. McCutchan TF, Kissinger JC, Touray MG, et al. Comparison of circumsporozoite proteins from avian and mammalian malarias: biological and phylogenetic implications. Proc Natl Acad Sci U S A. 1996; 93: 11889–94.
18. Coatney GR, Collins WE, Warren M, et al. The primate malarias [CD-ROM; original book published 1971]. Version 1.0. Atlanta: Centers for Disease Control and Prevention; 2003.
19. Lambrecht FL, Dunn FL, Eyles DE. Isolation of *Plasmodium knowlesi* from Philippine macaques. Nature. 1961; 191: 1117-8.