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Pathological Indicators of Malarial Death
Forensic Aspect of Malaria

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PATHOLOGICAL INDICATORS OF MALARIAL DEATH
—Forensic Aspects of Malarial Pathology

BY

GUANG YU

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ABSTRACT

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—Forensic Aspects of Malarial Pathology

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Malarial deaths in non-endemic areas are increasing. Forensic examination is needed when forensic issues are involved including those in malarial areas. The pathological changes of malaria are fundamental evidence in the identification of a malarial death, but often relationships between such changes and the cause of death are obscured. A further study is needed to deepen the understanding of these relationships that will benefit the forensic identification of malarial death, especially when unnatural factors, such as trauma and poisoning, are suspected. In this study, 19 people dying of either malaria (14), or other reasons (5), were morphologically and quantitatively examined by histopathology, immunohistochemistry and electron microscopy. Based on a study of case history, pathological changes in the tissues and comparison of the cases dying of malaria with those from other reasons (e.g. viral infection, poisoning, and coma with unknown etiology), pathological indicators of malaria death are discussed. This data, together with a literature review, is used to provide a prospective consideration of the forensic identification of malarial death.

DEDICATION

I would like to dedicate this to my parents, Liehai Yu and Shaojie Peng, and to my wife, daughter and relatives for their constant encouragement and support.

I would also like to dedicate this to all of my friends here and in China, without them I really don't know how hard my time would be for so long separation from my family.

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INTRODUCTION

Malaria is "a mosquito-borne, hemolytic, febrile illness" caused by the microorganisms commonly referred to as malaria parasites. Killing more than 3 million persons per year [1], the World Health Organization recognizes malaria as causing more morbidity than any other infectious disease.

Malaria has essentially been eradicated from North America, Europe, Australia, Japan, and other developed countries, although it is highly prevalent in tropical and subtropical areas. With the increase of drug-resistant malaria species and more active international travelers, more and more imported malaria cases are being detected in non malarial regions [2, 3, 4, 5]; thus the issue regarding malaria being a possible cause of death is getting more attention [6, 7, 8]. To date, however, forensic cases involving malaria have not been widely reported. A paper on forensic aspects of fatal malaria was published in German in 1994. Of the 9 cases of malarial death, aside from a suspicion of the involvement of trauma in one case, misdiagnosis and improper treatment were the key issues [9]. In reality, forensic malaria-related autopsies are not unusual. Many sudden deaths in malaria are potential forensic issues and require a careful consideration of the differential causes of death [10, 11, 12, 13, 14]. The symptoms, such as vomiting, convulsions and coma, are easily confused with those of poisoning or trauma, as the cerebral complications -cerebral malaria is the most notorious form and often causes sudden death. Similarly, pulmonary, renal or heart failure, including

rupture of liver or spleen, can be a consequence of malaria infection and may contribute to a sudden death; these are all conditions that can be differential diagnosis in forensic cases.

In recent years, several reports suggest that malaria during pregnancy may result in fetal exposure to malaria when parasites are transmitted across the placenta, although congenital malaria was thought to be rare and the pathophysiology of transplacental transmission of Plasmodium is not well understood [15]. According to the study of Egwunyenga et al, of 656 near-term pregnant women, and of the cord and peripheral blood of newborns, transplacental passage of P. falciparum was confirmed by detection of parasitemia in the peripheral blood of 2.82% of newborns within 7 days of birth [16]. Serological investigation of sera of 284 newborns by Indirect Fluorescent Technique (IFA) with P. falciparum IgM specific conjugate indicated that 72 (25.35%) had IgM antibodies of P. falciparum in their blood. Serum IgG responses were found to be increased in cord blood in 97 Gambian women and their neonates when there was an active placental infection with malaria; IgM was not detected [17]. Thus in forensic cases involving neonates such as sudden infant death, it is prudent to consider malaria as a possibility when the necessary conditions, e.g. potential for infection, exist.

The diagnosis of malaria, especially in non-endemic areas, is often poor due to the variety of presenting symptoms possible in this disease, and the inadequacy of diagnostic techniques. According to an investigation published in 1998 from Canada, the

diagnosis of malaria was initially missed in 59% of cases [18]. The need to improve the technical aspects of malaria diagnosis is getting more attention in recent times and this will benefit the general treatment of malaria as well as the identification of malarial death in forensic situations.

Traditionally, diagnosis is based on the examination of Giemsa-stained thick and thin blood smears under a bright-field microscope. In 1995, Kong and Chung compared acridine orange (AO) and Giemsa stains for malaria diagnosis. It was believed that the AO staining method required less time and was more sensitive under lower magnification than the Giemsa staining method for the detection of malaria parasites [19]. Recently, more advanced malaria diagnostic techniques have come into use for not only research but also in the clinical setting. Compared with methods based on fluorescent microscopy, and the detection of nucleic acid (including PCR), diagnostic tests based on immuno-assays are believed to be most useful [1].

In addition to the detection of the malaria parasite itself, the presence of malarial pigment as evidence of malaria infection is also a focus as a target test for malaria diagnosis. Malarial pigment (or hemozoin) is known to be an end product of hemoglobin digestion by the malaria parasite. Because it needs careful differentiation from other pigments, many efforts are being made to improve its detection. In 1995, a molecular-based magnet test for malaria was introduced and was believed to be more sensitive than the thin blood film test [20]. This new test however still requires the detection of the characteristic birefringence demonstrated by polarized light to

differentiate malarial pigment.

To interpret the clinical meaning of the malarial pigment test, Amodu et al[21] studied the distribution of intraleucocyte malarial pigment in a group of 92 children -consisting of 32 children with asymptomatic malaria, 32 children with mild or uncomplicated malaria and 28 children without malaria. Over 90% of children in each of the three groups had pigment-containing monocytes and the numbers of pigment-containing monocytes were not significantly different between the three groups. While over 90% of children in both the asymptomatic malaria and the uncomplicated malaria groups had pigment-containing neutrophils, 71.4% of the non-malaria group had such neutrophils. The numbers of these pigment-containing neutrophils was highest in the uncomplicated malaria group, followed by the asymptomatic malaria group with the non-malaria group having the least numbers; the pigmented neutrophil:monocyte ratio followed the same pattern. It was concluded that the number of pigment-containing neutrophils, and also the pigmented neutrophil:monocyte ratio, may be a marker of the severity of malarial infection when comparing non-malaria, asymptomatic malaria and mild malaria. Later in 1998, these researchers again tested this hypothesis (i.e. that intraleucocytic malarial pigment is a good measure of disease severity in malaria) by studying 146 children aged 6 months to 14 years in 4 categories -cerebral malaria, mild malaria, asymptomatic malaria and 'no malaria'- in Ibadan, Nigeria. The proportion of pigment-containing neutrophils clearly rose across the spectrum from no malaria, asymptomatic malaria, mild malaria and cerebral malaria

(median values 2.0%, 6.5%, 9.0% and 27.0%, respectively; $P < 0.0001$). The proportion of pigment-containing monocytes did not differ significantly between the mild malaria, asymptomatic malaria and the non-malaria groups but the cerebral malaria group had a higher median value than the other 3 groups. The ratio of pigment-containing neutrophils to pigment-containing monocytes showed the same trend across the groups of subjects as was observed with the number of pigment-containing neutrophils. It was concluded that the pigment-containing neutrophil count is a simple marker of disease severity in childhood malaria to be used in addition to the parasite count [22]. Thus there is an additional goal for pathology studies in malaria —the exploration of pathological markers for the forensic identification of malaria deaths.

Pathological evidence of malarial damage is necessary and critical to the forensic identification of a malarial death. The presence of parasite or malarial pigment only means there is a "malaria infection", but not necessary death from malaria. The clinical symptoms can not usually be used as definitive evidence in forensic work. Practically, it is those cases that are without typical symptoms and are lacking in dependable clinical diagnoses that dominate the forensic malaria cases.

The typical pathology of malaria infection is well described in the book, "Bruce-Chwatt's Essential Malariology", by H.M. Gilles and D.A. Warrell in 1993 [23]. From then on, many researchers have focused on the pathogenesis of malaria and have improved the understanding of malaria pathology. Recently, more and more attention has been paid to the study of malaria-

associated immunomolecules, such as TNF-alpha [24], ICAM-1 and VCAM-1 [25]. Although the pathological significance of the presence of monocytes in malarial damage is still controversial [26, 27], many research results suggested that monocytes do play an important role in malaria damage [28, 29]. Medana, et al [24], examined brain sections from uninfected and FMCM mice for the presence of cytokine mRNA and protein by in situ hybridization and immunohistochemistry. Tumor necrosis factor (TNF)-alpha mRNA and protein were associated with microglia and astrocytes, monocytes, as well as the cerebral vascular endothelium in FMCM mice but not in uninfected animals. To study the importance of cytokines in malarial nephritis, Sinniah-Raja et al investigated the expression of tumour necrosis factor-alpha (TNF-alpha), interleukin-1 alpha (IL-1alpha), IL-6, IL-10 and granulocyte macrophage-colony stimulating factor (GM-CSF) in kidneys C57BL/6 J mice acutely infected with the murine malaria parasite Plasmodium berghei ANKA. Elevated levels of messenger RNA (mRNA) specific for these cytokines were seen in infected kidneys after day 5 of infection as demonstrated by reverse transcription-polymerase chain reaction (RT-PCR) analysis. Kidney sections stained with specific antibodies against TNF-alpha, IL-1alpha, IL-6, IL-10 and GM-CSF by immunohistochemistry showed these cytokines present in the glomeruli from day 10 of infection, and thereafter increasing progressively, mainly in the infiltrating macrophages and the glomerular mesangium [30].

Besides the malaria-associated immunomolecules, factors commonly found in DIC (disseminated intravascular coagulation)

have been actively pursued. In early 1970's, a coagulation and fibrinolytic study of malaria cases was reported by Sucharit, et al [31]. In their study, the levels of serum fibrin degradation products in 18 severe falciparum malaria patients, including those with parasitaemia above 5% and with pernicious manifestations such as coma, jaundice, anuria, pulmonary edema, and bleeding tendency, were examined. Marked changes in blood coagulograms and high levels of serum fibrin degradation products appeared only in cases with very severe cerebral involvement as well as in cases with only very high parasitaemia. Another study of coagulation factors and serum fibrin degradation products (FDP) was published in 1975 [32]. In this study, 31 cases of acute falciparum malaria had severe complications, 13 cases were without severe complications, and there were 6 cases of vivax malaria. Seven of 13 cases of acute falciparum infection with severe complications died and the coagulation parameters in these were markedly changed and there were high levels of serum FDP. The degree of change in coagulation and serum FDP varied with the severity of the symptoms and signs and there were virtually no changes in the cases of vivax malaria. It was speculated that these changes might be the result rather than the cause of the pathogenic processes in falciparum malaria.

In 1989, the activation of the coagulation cascade in falciparum malaria was investigated by Pukrittayakamee et al [33]. The incidence and progression of coagulation abnormalities were studied in 52 patients with acute falciparum malaria. These patients were prospectively divided into 3 groups: severe

(parasitemia greater than or equal to 5% or with vital organ dysfunction), 12 patients; moderate (parasitemia 1%-5% without complications), 16 patients; and mild (parasitemia less than 1%), 24 patients. Non of these cases died or developed clinical evidence of disseminated intravascular coagulation. Conventional indices of coagulation (prothrombin time, partial thromboplastin time, fibrinogen, and fibrin degradation products) were within the normal range but reduced plasma concentrations of antithrombin III (AT-III) levels were noted in all groups, and the incidence was significantly higher in patients with severe and moderate malaria (83% and 81%) compared with the mild group (37%; P less than 0.005). Depletion of AT-III was associated with thrombocytopenia, decreased AT-III activity and elevated plasma concentrations of thrombin-antithrombin III complexes (P less than 0.01), confirming activation of the coagulation cascade and increased clotting factor consumption. AT-III levels returned to normal coincident with clinical improvement. Thus, "activation of coagulation is a common and sensitive measure of disease activity in acute falciparum malaria", although "it is not a specific feature, nor is there evidence to suggest it has a primary pathological role in severe infection". In 1990, the serum of patients with falciparum malaria (14 cases) and vivax malaria (34 cases) were evaluated by Tanabe et al [34], as to whether DIC was a complication or not. Serum concentration of fibrin-degradation products (FDP) was elevated in 8 cases (57%) of falciparum malaria and 3 cases (9%) of vivax malaria. Thrombocytopenia was found in 12 cases (88%) of falciparum malaria and in 30 cases (86%) of vivax malaria. Prothrombin time

was prolonged in 4 cases (8%) and plasma concentration of fibrinogen decreased in 3 cases (17%). Only 4 of the patients included, all of them infected with falciparum malaria and three with cerebral malaria, met the criteria of the diagnosis of DIC complication; one case of vivax malaria was suspected of the DIC. The authors believed that "abnormality grades in FDP concentration have the closest association with DIC among the coagulation tests, therefore the FDP test is indispensable for checking complication of DIC in malaria cases". In 1992, to determine whether or not DIC occurs in patients with uncomplicated malaria, Chek at al, conducted a study on fibrinogen and its degradation products, euglobulin lysis time and parasite counts in 30 cases of uncomplicated malaria. By a spectrophotometric method, plasma fibrinogen in patients with uncomplicated malaria was found to be normal compared to normal healthy adults, although "DIC is an important intermediate mechanism in the pathophysiology of severe and complicated malaria such as cerebral malaria" [35].

Recently, a histological, immunohistochemical, and quantitative study of placentas was reported by Ismail et al [36]. To characterize the histological changes in malarial placentas and their relationship with parity and maternal and cord parasitemias, immunohistochemical and quantitative studies for CD45, fibrin, and villous area were performed. Four hundred fifteen placentas (35.2%) showed parasites (active infections); in 303 of them, parasites co-existed with pigment covered by fibrin (chronic infections) and in 112 only parasites were detected (acute infections). Four hundred seventy-five cases

(40.3%) showed hemozoin deposition without parasites (past infections). Of women with parasitized placentas, 46.3% did not show parasites in the peripheral blood. Basal membrane thickening, fibrinoid necrosis and prominence of syncytial knots were associated with active malarial infection, however, no quantitative differences in perivillous fibrin deposition in the villous area were found. The most significant association with active malarial infection was seen with the intervillous infiltration by mononuclear inflammatory cells ($P < 0.001$). Chronic infections were associated with the most severe changes, particularly intervillous mononuclear inflammation ($P < 0.001$). Only minimal differences were found between the noninfected placentas and those with past infections. Primiparas showed chronic infections more frequently than multiparas (52% v 15%, $P < 0.001$). They also showed significantly higher placental parasitemias and intervillous inflammatory infiltrate. In conclusion, placental histology is more sensitive than peripheral blood examination in detecting malarial infection during pregnancy. Most malarial infections recover during pregnancy, leaving few residual changes in the placenta. Intervillous inflammation is the most frequent finding associated with malaria and is especially severe in primiparas, suggesting that mechanisms other than immunosuppression are responsible for the high susceptibility of this group.

Although remarkable progress has been made in the understanding of the pathogeneses of malarial damage, no detailed investigation into the pathological indicators of malaria death for forensic identification purposes has been

published yet, and thus new differential criteria for diagnosis are still needed. "The discovery of parasitemia provides an explanation for symptoms in non-immune patients, but in those who are immune, parasitemia may be an incidental finding with no diagnostic relevance to the patient's current illness" [27]. Vice versa, as Frydl indicated, a negative parasitic result does not exclude malaria as a cause of death [37].

There are several factors that may hamper the forensic identification of malaria death. A significant factor is the lack of details of the subject's symptoms, which are hard to get in many forensic cases. Much of the information available is usually unscientific and often from non-medical individuals personally familiar with the dead. These blurry, indistinct impressions can lead to difficulties in determining the clinical characteristics and features that can contribute to the determination of the pathogenesis. Moreover, the postmortem changes can obscure or destroy some evidence of the pathogenesis, especially those useful for molecular and biological techniques. Additionally, in some cases the contributing causes of death may be complex, i.e. poisoning or trauma associated with natural disease, and this makes the interpretation of pathological changes difficult. The progress that has been achieved in malaria research, such as the monocyte-related pathogenesis, show that there is hope for new findings through further study that will improve this situation.

To deepen the understanding of the forensic implications of malaria pathology and expand the range of forensic approaches to studying the pathogeneses of malaria, tissues from 19

children dying of malaria and of other reasons from Malawi were morphologically and quantitatively studied by histopathology, immunohistochemistry and transmission electron microscope (EM) methods. Based on clinical records, those dying of malaria were divided into two groups, cerebral malaria and non-cerebral malaria. Pathological differences between these groups have been sought and the forensic implications of the findings are discussed. Combined with a literature review, this work has led to a prospective consideration of the forensic identification of malaria deaths.

MATERIALS AND METHODS

I. MATERIALS

The materials used for this study are part of a NIH funded malaria research project based in Malawai (NIH No: 63698). There were total of 19 cases, 14 were clinically diagnosed as malaria death and 5 were non-malaria (i.e. dying of coma of other causes). Of the 14 malaria cases, 6 were non-cerebral malaria (NCM) and 8 were cerebral malaria (CM) according to their clinical characteristics, respectively. In addition to tissue samples, personal and clinical records, including blood parasitized red blood cells (PaRBC), packed cell volume (PCV) on admission and time of death after admission, and autopsy report data, such as time of postmortem autopsy (postmortem interval) and gross autopsy findings were gathered for each case.

The personal, clinical and autopsy data of the 19 cases are listed in table 1.

The tissue samples were formalin fixed and paraffin embedded. The sections of the tissue samples and their labels are listed in table 2.

Table 1. Personal, clinical and autopsy data of the 19 cases

Case No.	Age (m)	Clinical diagnosis	PaRBC On adm*.	PCV on adm.	Time of death (hr)	PMI* (hr)	Clinical feature	Gross findings
962	19	Anemia	no	9%	On adm.	5	Congestive cardiac failure	Very pale, petechiae at cerebral WM, severe pulmonary edema, no cerebral edema
963	19	Anemia	+++ on thick film	7%	On adm.	4	No signs of congestive cardiac failure (CCF) or gross neurological deficits.	Pale. Cerebral (WM) and cerebellar (GM) patechiae, but no edema.
964	20	Anemia	19%	8%	5	12	Malnourished, hypoglycemic, lungs clear.	Pale, no cerebral edema, moderate pulmonary edema
965	14	CM	3.5%	21%	18	2	Opisthotonic, decerebrate rigidity, vertical nystagmus	Pale, mild cerebral edema, state gray of brain surface.
966	17	Anemia + CM	6%	15%	On adm.	4	Hypoglycemic and respiratory arrest, intractable convulsion 19 hours, no CCF	Pale, mild cerebral and pulmonary edema, state gray of brain surface, Cerebral and cerebellar hemorrhages.
967	29	Anemia	++++ on thick film	4%	On adm.	2	Alert until 1 hour prior to admission, unconscious on admission, no focal deficit, no creps. hypoglycemic	Pale, no cerebral or pulmonary edema, no hemorrhages.

Table 1. (cont'd)

ase No.	Age (M)	Clinical diagnosis	PaRBC On adm.	PCV on adm.	Time of death (hr)	PMI (hr)	Clinical feature	Gross findings
968	41	Coma (unknown etiology)	–	30%	8	5	HIV +, labored breathing, a few convulsions, sudden death (respiratory arrest)	Minimal cerebral edema, prominent mesenteric nodes.
979	16	CM	635/mm^3	23%	23	4	Convulsion prior to admission, decorticate posturing, worsening fundus fundings, persistent extensor hypertonicity.	Diffuse petechial hemorrhages in cerebrum, cerebellum, marked pulmonary congestion.
9710	66	Organic phosphate toxicity	330/mm^3	37%	4	14	Responded to atropine q 30 min, died with dyspnea and "pink forth".	Marked edema and congestion of lungs
9711	29	Anemia Pneumonia Septicemia CM	29.5%	12%	27	13	?CHF*, diuresis, repeated convulsions, post-RX hypoglycemia, 2nd blood culture grew *H. flu*	Marked cerebral edema, petechial hemorrhages, upward herniation of cerebellum, mild lung edema.
9713	22	CM	31.3%	20%	On adm.	4	In extremis on admission, deep breathing, conjugate gaze deviation	Pulmonary congestion, hemorrhagic gastritis, gray brain, scanty petechiae in brain.

Table 1. (cont'd)

Case No.	Age (M)	Clinical diagnosis	PaRBC On adm.	PCV on adm.	Time of death (hr)	PMI (hr)	Clinical feature	Gross findings
9614	9	Anemia Septicemia	– Gameto-cytes +	7%	6.5	14	Revived after BTF and begin sucking, developed crackles in both lungs, *E. coli* from blood culture, dying of respiratory arrest.	Pulmonary edema.
9615	8	CM Edema	11,300/ mm^3	10%	10	6	BTF* and tolerated well, episodes of extensor hypertonicity. Dying of respiratory arrest.	Gray brain, cerebral edema and petechial hemorrhages
9716	51	CM	125,950 /mm^3	27%	4.5	3	Few transient episodes of twitching, died suddenly	Moderate cerebral edema, petechial hemorrhages in cerebellum, gray liver.
9717	51	Reye's syndrome	-	36%	9	14	Recurrent hypoglycemia, increased ammonia, liver non-palpable, deep breathing	Gastric erosions; Pale yellow/green liver; Dark spleen; Distended bladder; ? Pulmonary edema.
9718	7	Meningitis	-	27%	14	8.5	Dramatic opisthotonos, conjugate gaze deviation, Cheyne-Stokes respirations.	Purulent meningitis.

Table 1. (cont'd)

Case No.	Age (M)	Clinical diagnosis	PaRBC On adm.	PCV on adm.	Time of death (hrs)	PMI (hr)	Clinical feature	Gross findings
9719	28	Anemia	"+"	7%	4	12	No blood available for transfusion, remained alert till gasped and died.	Pale, diffuse darkening spleen and liver
9720	20	Viral encephalo-pathy	1-2/800 WBCs	49%	22	6.5	CSF* WBS increased, conjugate gaze deviation, decerebrate rigidity, urinary retention, unilaterally dilated pupils just prior to death.	Hemorrhagic nodules, lower lobes of lungs, meningitis, multiple small pale lesions throughout brain parenchyma.
9621	25	CM	189/200 WBCs	29%	12	8	Recurrent twitching/fits, dramatic fundi (hems ++, increasing macular edema)	Plum colored brain, moderate swelling. No hemorrhages.

* adm.= admission; PMI = postmortem interval, CHF = congestive heart failure; BTF = blood transfusion; CSF = cerebrospinal fluid.

Table 2. Sections of tissue samples and their labels

Site	Label
Pituitary	A1
Frontal lobe	B1
Parietal lobe	B2
Temporal lobe	B3
Occipital calcarine fissure	B4
Hippocampus	B5
Basal ganglia(caudate)	B6
Thalamus	B7
Midbrain	B8
Pons	B9
Medulla	B10
Cerebellum (peripheral)	B11
Cerebellum (dentate nucleus)	B12
Spinal cord	B13
Optic nerve	C1
Lung: Right upper lobe	D1
Right lower lobe	D2
Left upper lobe	D3
Left lower lobe	D4
Heart: Right atrium + Aorta +Pulmonary artery	E1
Heart: Right ventricle	E2
Left ventricle	E3
Submandibular + Esophagus	F1
Stomach + Ileum	F2
Jejunum + Right colon	F3
Liver	F4
Gall bladder + Pancreas	F5
Right kidney	G1
Left kidney	G2
Urinary bladder + Parathyroids	G3

II. METHODS

The paraffin embedded blocks tissues were sectioned and stained with hematoxylin and eosin (H.E.)[38].

The samples from parietal cerebral cortex (B2), white matter of the cerebellum (B12, WM) and gray matter of the cerebellum (B12, GM) were examined under a light microscope. For each slide, the pigmented and non-pigmented monocytes (Mono), lymphocytes (Lympho), neutrophils (Neutro), eosinophils (Eosino), uncertain white blood cells (UWBC), pigmented red blood cells (PRBC), endothelial cells (EC) and fibrin clots (FC), as well as the ring hemorrhages were counted per 100 vessels. The total white blood cells (TWBC), total pigmented white blood cells (TpWBC), and the ratio of Mono to EC, Mono to TWBC, pigmented monocyte (PMono) to TWBC, TWBC to EC, PRBC to EC and the FC to EC were calculated.

To verify the H.E. results, macrophage marker 3A5 (Novocastra, #210501) that recognizes macrophage and monocyte, and the antibody against human fibrin (American Diagnostica Inc. ADI #350), were used for immunohistochemistry staining of parietal sections (B2), following the standard procedures being used in Histopathological Laboratory at the Clinical Center, Michigan State University (Appendix I and II).

With those cases lacking pathological changes that identify them as malaria death and those needing a differential diagnosis, the H.E. stained sections of other organs were also thoroughly examined to confirm or exclude any clue of malarial

death. The suspected malarial pigments in sections were observed under polarized microscope and compared with the malarial pigments seen in the typical cases. Samples of lungs from each patient, which were glutaraldehyde fixed, washed in cacodylate buffer and stored at 4^{0} C before plastic embedding, were examined with transmission electron microscope. Between six and twelve electron micrographs of alveolar walls were taken to demonstrate the location of parasitized or pigmented red blood cells and white blood cells, as well as any other morphological damages.

The cases were separated based on clinical diagnoses into cerebral malaria (CM), non-cerebral malaria (NCM) and coma of other causes (COC) three groups, and the numerical data obtained from H.E. stained sections (B2, B12GM and B12WM) were compared between groups by Students' t test. The monocyte and fibrin clot counting results of the 3A5 and fibrin immunohistochemically stained sections (B2) were graphically compared with that of the H.E. and the differences between each groups were also analyzed by Students' t test.

RESULTS

The numerical results from the H.E. stained sections of parietal lobe (B2), white matter of the cerebellum (B12WM) and gray matter of the cerebellum (B12GM) are listed in table 3, 4, and 5*. The Students' t test results of these data are shown in table 6, 7 and 8*, respectively.

With 3A5 immunohistostaining, monocytes were well recognized by the yellow-brown colored cytoplasm. The picture of 3A5 immunostained section B2 is shown in figure 9. The monocyte count data in H.E. and immunostained stained brain section B2 are listed in table 9, and the comparison of monocyte count data in H.E. and immunostained (3A5) brain section B2 is shown in figure 7.

The presence of fibrin clots was demonstrated by orange colored anti-fibrin immunohistochemistry staining. The picture of Fibrin immunostained section B2 is shown in figure 10. The fibrin clot count data in H.E. and Fibrin immunostained brain section B2 are listed in table 10, and the comparison of fibrin clot count data in H.E. and immunostained (Fibrin) brain section B2 is shown in Figure 8.

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*In these tables, WM = white matter, Mono = monocyte, P = pigmented, T = total, Lymph(o) = lymphocyte, Neutro = neutrophil, Eosino = eosinophil, Uncert = uncertain WBC, PU = Pigmented Uncertain WBC, EC = endothelial cell, FC = Fibrin Clot, Hemo = hemorrhage.

Table 3. Numerical Results of B2 (H.E.)

B2	962	963	964	967	9714	9719	965	966	979	9711	9713	9715	9716	9721	968	9710	9717	9718	9720
Mono	20	17	36	25	2	0	2	48	19	46	27	36	4	12	0	6	2	3	0
PMono	9	2	20	2	0	0	2	31	8	26	21	22	1	5	0	0	1	0	0
Lympho	74	18	52	80	3	17	5	49	27	19	16	33	5	2	6	11	13	4	3
PLymph	1?	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0
Neutro	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1
PNeutro	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Eosino	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2?	0	0
PEosino	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Uncert	25	2	31	66	2	5	9	55	37	40	26	24	11	18	6	12	4	13	5
PU	1	2	2	0	0	0	3	1	7	2	6	2	4	1	0	0	0	0	0
TWBC	119	37	119	171	7	23	16	152	83	105	69	93	20	32	12	29	19	20	9
TpWBC	11	4	22	2	0	0	5	32	16	28	28	24	5	6	0	0	1	0	0
Mono/TWBC	0.17	0.46	0.30	0.15	0.29	0.00	0.13	0.32	0.23	0.44	0.39	0.39	0.20	0.38	0.00	0.21	0.11	0.15	0.00
PMono/TWBC	0.08	0.05	0.17	0.01	0.00	0.00	0.13	0.20	0.10	0.25	0.30	0.24	0.05	0.16	0.00	0.00	0.05	0.00	0.00
TpWBC/TWBC	0.09	0.11	0.18	0.01	0.00	0.00	0.31	0.21	0.19	0.27	0.41	0.26	0.25	0.19	0.00	0.00	0.05	0.00	0.00
EC	162	115	148	219	158	120	121	112	182	103	86	89	96	95	197	210	191	152	140
TWBC/EC	0.73	0.32	0.80	0.78	0.04	0.19	0.13	1.36	0.46	1.02	0.80	1.04	0.21	0.34	0.06	0.14	0.10	0.13	0.06
TpWBC/EC	0.07	0.03	0.15	0.01	0.00	0.00	0.04	0.29	0.09	0.27	0.33	0.27	0.05	0.06	0.00	0.00	0.01	0.00	0.00
PRBC	40	380	235	7	9	0	1120	1026	360	650	970	635	400	1130	4	6	6	0	0
PRBC/EC	0.25	3.30	1.59	0.03	0.06	0.00	9.26	9.16	1.98	6.31	11.28	7.13	4.17	11.89	0.02	0.03	0.03	0.00	0.00
FC	0	0	0	0	0	0	1	0	0	18	0	13	0	0	0	0	0	0	0
Hemo	0	5	0	0	0	0	3	6	23	9	0	23	0	0	0	0	1	0	0
Group	non cerebral malaria (NCM)						cerebral malaria (CM)								coma other cause (COC)				

Table 4. Numerical results of B12GM (H.E.)

B12 GM	962	963	964	967	9714	9719	965	966	9711
Mono	20	13	8	8	1	1	1	59	6
PMono	9	8	2	0	0	1	0	50	5
Lympho	56	21	13	43	3	2	4	23	19
PLymph	0	2	0	0	0	0	0	3	0
Neutro	1	0	0	0	0	1	0	0	0
PNeutro	0	0	0	0	0	0	0	0	0
Eosino	3	10	0	0	0	1	0	0	0
PEosino	2	0	0	0	0	0	0	0	0
Uncert	54	24	44	18	8	10	8	65	52
PU	3	2	17	0	1	0	6	43	33
TWBC	134	68	65	69	12	15	13	147	77
TpWBC	14	12	19	0	1	1	6	96	38
Mono/TWBC	0.15	0.19	0.12	0.12	0.08	0.07	0.08	0.40	0.08
PMono/TWBC	0.07	0.12	0.03	0.00	0.00	0.07	0.00	0.34	0.06
TPWBC/TWBC	0.10	0.18	0.29	0.00	0.08	0.07	0.46	0.65	0.49
EC	348	132	123	148	89	162	142	124	86
TWBC/EC	0.39	0.52	0.53	0.47	0.13	0.09	0.09	1.19	0.90
TpWBC/EC	0.04	0.09	0.15	0.00	0.01	0.01	0.04	0.77	0.44
PRBC	17	361	239	0	5	0	1186	1156	1255
PRBC/EC	0.05	2.73	1.94	0.00	0.06	0.00	8.35	9.32	12.27
FC	0	0	0	0	0	0	2	1	5
Hemo	0	0	0	0	0	0	3	12	8
Group	non cerebral malaria (NCM)								malaria

)	9711	9713	9715	9716	9721	968	9710	9717	9718	9720
	6	5	16	2	1	0	0	0	0	0
	5	5	10	1	0	0	0	0	0	0
	19	5	17	5	1	2	6	3	3	8
	0	0	3	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0
	52	23	46	26	4	0	7	6	8	6
	33	11	29	12	2	0	0	0	0	0
	77	33	79	33	6	2	13	9	11	14
	38	16	42	13	2	0	0	0	0	0
1	0.08	0.15	0.20	0.06	0.17	0.00	0.00	0.00	0.00	0.00
1	0.06	0.15	0.13	0.03	0.00	0.00	0.00	0.00	0.00	0.00
5	0.49	0.48	0.53	0.39	0.33	0.00	0.00	0.00	0.00	0.00
5	86	101	133	102	99	135	162	125	149	77
0	0.90	0.33	0.59	0.32	0.06	0.01	0.08	0.07	0.07	0.18
5	0.44	0.16	0.32	0.13	0.02	0.00	0.00	0.00	0.00	0.00
5	1055	720	750	259	1065	1	1	0	0	0
3	12.27	7.13	5.64	2.54	10.76	0.01	0.01	0.00	0.00	0.00
	5	0	0	0	0	0	0	0	0	0
	8	0	6	0	0	0	0	0	0	0
ɔral malaria (CM)						coma other cause (COC)				

Table 5. Numerical results of B12WM (H.E.)

B12 WM	962	963	964	967	9714	9719	965	966	9711	97
Mono	7	7	3	29	2	1	3	16	25	1
PMono	2	2	2	0	0	0	0	15	17	1
Lympho	16	6	3	82	5	16	3	16	20	
PLympho	0	0	0	0	0	0	0	0	0	
Neutro	0	0	0	0	0	0	0	0	0	
PNeutro	0	0	0	0	0	0	0	0	0	
Eosino	4	0	0	0	0	1	0	0	0	
PEosino	0	0	0	0	0	0	0	0	0	
Uncert	14	13	21	75	10	21	9	54	44	5
PU	8	6	15	0	0	0	5	24	30	1
TWBC	41	26	27	186	17	39	15	86	89	8
Mono/TWBC	0.1707	0.269	0.1111	0.156	0.1176	0.026	0.2	0.18	0.2809	0.2
PMono/TWBC	0.0488	0.077	0.0741	0	0	0	0	0.174	0.191	0.
TpWBC	10	8	17	0	0	0	5	39	47	3
TpWBC/TWBC	0.2439	0.308	0.6296	0	0	0	0.3333	0.453	0.5281	0.3
EC	127	79	114	371	106	222	89	641	109	1
TWBC/EC	0.32	0.33	0.24	0.50	0.16	0.18	0.17	1.34	0.82	0.
TpWBC/EC	0.08	0.10	0.15	0.00	0.00	0.00	0.06	0.61	0.43	0.
PRBC	11	486	205	0	1	0	1443	1021	1015	7
PRBC/EC	0.09	6.15	1.80	0.00	0.01	0.00	16.21	15.9	9.31	4
FC	0	1	0	0	0	0	0	0	2	
Hemo	0	3	0	0	0	0	1	30	4	
Group	non cerebral malaria (NCM)								malaria	

9	9711	9713	9715	9716	9721	968	9710	9717	9718	9720
	25	19	49	9	3	0	0	2	2	0
	17	14	26	7	2	0	0	0	2	0
5	20	8	19	15	0	2	16	7	8	1
	0	0	0	0	0	0	0	0	0	0
	0	0	1	2	0	2	0	0	0	0
	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	1	0	1
	0	0	0	0	0	0	0	0	0	0
1	44	53	58	38	4	5	16	9	25	7
	30	17	27	12	0	0	0	0	0	0
0	89	80	127	64	7	9	32	19	35	9
04	0.2809	0.2375	0.386	0.141	0.4286	0	0	0.105	0.057	0
01	0.191	0.175	0.205	0.109	0.2857	0	0	0	0.057	0
	47	31	53	19	2	0	0	0	2	0
09	0.5281	0.3875	0.417	0.297	0.2857	0	0	0	0.057	0
4	109	157	133	231	103	161	161	270	253	118
54	0.82	0.51	0.95	0.28	0.07	0.06	0.20	0.07	0.14	0.08
05	0.43	0.20	0.40	0.08	0.02	0.00	0.00	0.00	0.01	0.00
5	1015	775	822	251	860	0	0	1	0	0
73	9.31	4.94	6.18	1.09	8.35	0.00	0.00	0.00	0.00	0.00
	2	1	4	0	0	0	0	0	4	0
	4	1	10	0	0	0	1	0	0	0
ebral malaria (CM)						coma other cause (COC)				

Table 6. 't' test of count data in section B2

B2	NCM Vs CM	NCM Vs COC	CM Vs COC
PRBC	0.0003	0.155	0.0002
PRBC/EC	0.0005	0.1782	0.0004
Mono/TWBC	0.3144	0.1164	0.0037
PMONO/TWBC	0.0087	0.1963	0.0006
TpWBC	0.0532	0.1335	0.0032
TpWBC/TWBC	0.0003	0.2668	<0.0001
TWBC/EC	0.3816	0.3816	0.0087
FC	0.1628	1	0.1616
Hemo	0.0852	0.4899	0.0591

Table 7. 't' test of count data in section B12GM

B12, GM	NCM Vs CM	NCM Vs COC	CM Vs COC
PRBC	0.0002	0.1681	0.0001
PRBC/EC	0.0003	0.1717	0.0003
Mono/TWBC	0.6484	0.0012	0.0124
PMONO/TWBC	0.3575	0.0532	0.0631
TpWBC	0.0764	0.0659	0.0246
TpWBC/TWBC	0.0001	0.0337	<0.0001
TWBC/EC	0.3172	0.0173	0.0164
FC	0.1546	1	0.1546
Hemo	0.0241	1	0.0241

Table 8. 't' test of count data in section B12WM

B12, WM	NCM Vs CM	NCM Vs COC	CM Vs COC
PRBC	0.0025	0.2075	0.0012
PRBC/EC	0.0190	0.2398	0.0072
Mono/TWBC	0.1113	0.0227	0.0022
PMONO/TWBC	0.0165	0.2849	0.0063
TpWBC	0.0270	0.1185	0.0011
TpWBC/TWBC	0.2202	0.1321	0.0001
TWBC/EC	0.1001	0.0164	0.0167
FC	0.2630	0.5113	0.4862
Hemo	0.1466	0.4636	0.2155

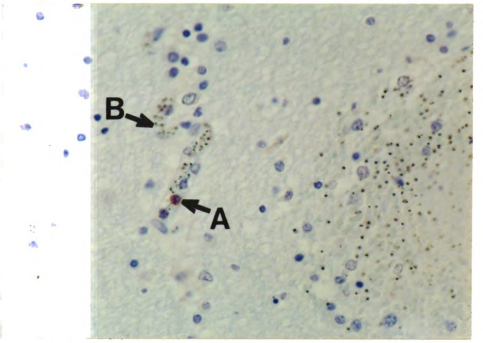


Figure 9. Picture of 3A5 immunstained section B2. The monocytes are well recognized by the yellow-brown colored cytoplasm (A); Pigmented RBCs are present (B).

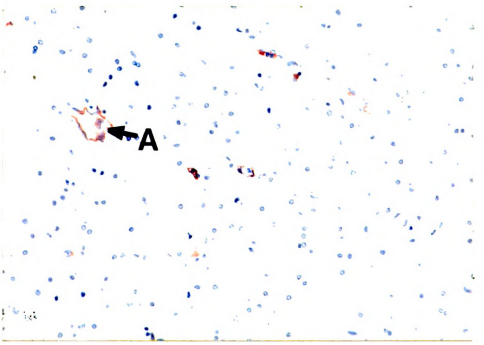


Figure 10. Picture of Fibrin immunstained section B2. The presence of fibrin clot is demonstrated by the orange colored immunohistochemistry staining (A).

Table 9. Monocyte count data in H.E. and immunostained sectio

B2	962	963	964	967	9714	9719	965	966
Mono(H.E)	20	17	36	25	2	0	2	48
Mono(3A5)	23	15	27	38	3	7	4	55

non cerebral (NCM)

T Test:

H.E: NCM v CM: P=0.625>0.05; NCM v COM: P=0.031<0.

3A5: NCM v CM: P=0.149>0.05; NCM v COM: P=0.025<0.

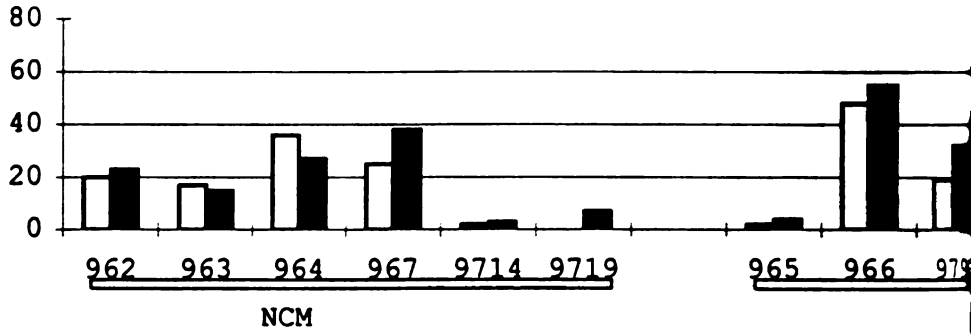


Fig. 7. Comparison of monocyte count data in H.E. and immunos

Table 10. Fibrin clot count data in H.E. and immunostained s

B2	962	963	964	967	9714	9719	965	966
FC (H.E.)	0	0	0	0	0	0	1	0
FC(fibrin)	46	6	2	25	5	8	40	49

Non Cerebral (NCM)

T Test:

H.E: NCM v CM: P=0.162>0.05; NCM v COC:-; CM v COC:

Fibrin: NCM v CM: P=0.156>0.05; NCM v COC: P=0.368>0.

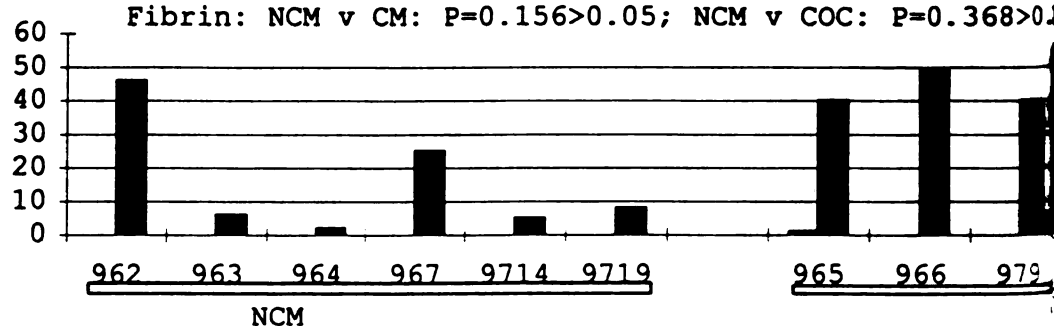
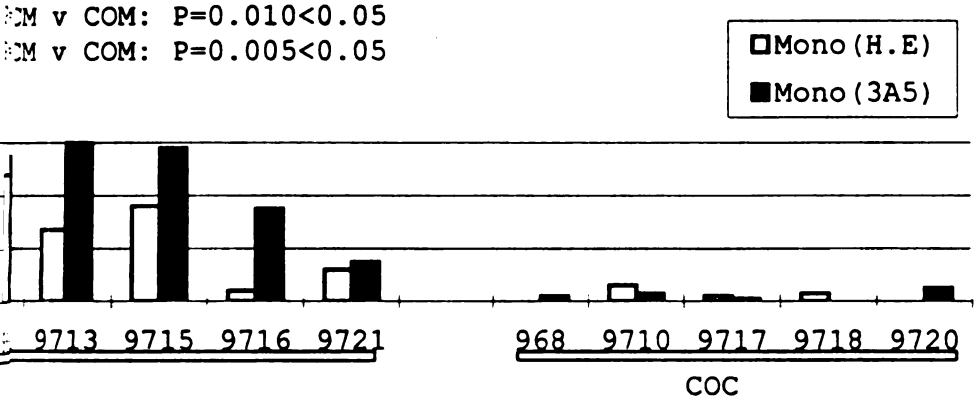


Fig. 8. Comparison of fibrin clot conut data in H.E. and imm

9711	9713	9715	9716	9721	968	9710	9717	9718	9720
46	27	36	4	12	0	6	2	3	0
10	60	58	35	15	2	3	1	0	5

cerebral (CM) | coma other causes (COC)

CM v COM: P=0.010<0.05
CM v COM: P=0.005<0.05



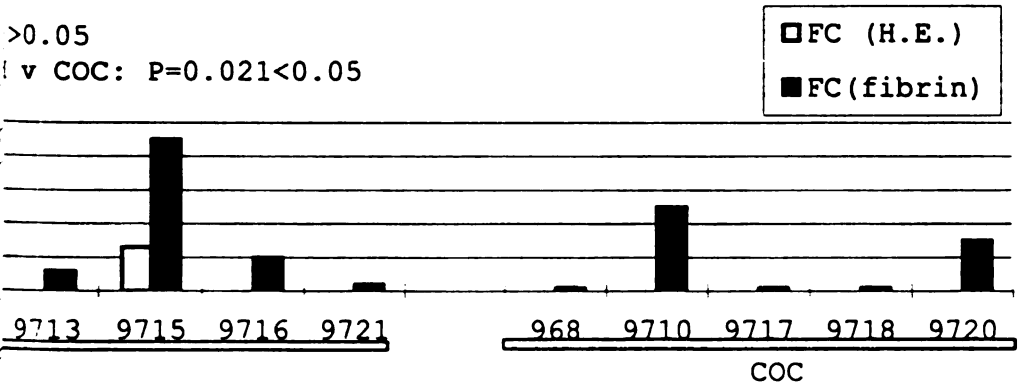
l (3A5) section B2.

B2

9711	9713	9715	9716	9721	968	9710	9717	9718	9720
18	0	13	0	0	0	0	0	0	0
55	6	45	10	2	1	25	1	1	15

ral Malaria (CM) | Coma Other Causes (COC)

>0.05
v COC: P=0.021<0.05



ned (Fibrin) section B2.

All the tissues sections associated with cases number 967, 9714, 9719, 968, 9710 and 9717 were examined under a light microscope. The main findings are as follows:

Case 967:

Brain: Most of capillaries and small vessels were filled with WBC and hemoglobin (Hb), WBC thrombi with fibrin and pigments were seen in several small vessels. The increased WBC was seen to be lymphocytes and monocytes. Pigmented RBC was not clearly detected. No hemorrhages were found.

Heart: Most of the myocardial capillaries were empty, some were filled with Hb, lymphocytes, monocytes and fibrin. No myocardial necrosis was detected, nor pigmented RBC.

Lung: The alveolar walls were thickened and the capillaries were filled with WBC, mainly lymphocytes and monocytes. Pigments and pigment-laden macrophages could be seen, plasma cells. There were Hb, RBC, and WBC in alveoli and small airway spaces.

Liver: There were remarkable autolysis (possibly necrosis) of hepatocytes, sinusoidal dilation and congestion with Kupffer cells, as well as the phagocytosis of infected and non-infected erythrocytes by Kupffer cells and sinusoidal macrophages which are increased in number. There was wide spread pigment deposition, mostly in Kupffer cells, but some in RBC. The infiltration of WBC into some portal areas was significant. Some pigmented cells similar to hepatocytes were seen in hepatic vein at portal area.

Spleen: Significant autolysis had occured. The cords and sinuses were filled with monocytes and macrophages containing pigment,

and RBC. Pigmented RBC was not remarkable.

Kidney: Autolysis was significant. Pigment could be found but no PRBC.

Other tissues: No remarkable clue of malaria infection.

Case 968:

Brain: Cerebral edema. Most of capillaries and small vessels were filled with RBC and Hb, some with WBC. There was no focal ring hemorrhages, nor PRBC. A sub-arachnoid hemorrhage was seen in B10 (medulla).

Heart: Most of the myocardial capillaries were filled with RBC and Hb, some with packed RBC and pink-colored debris. No myocardial necrosis and no pigmented RBC were seen. In E3 (left ventricle), thromboses were seen in the chamber and some blood vessels.

Lungs: Congestion and significant edema, with focal hemorrhages. Packed RBC and WBC filled the small vessels, and many of these cells were pigmented. Some alveoli were congested with pigment-laden macrophages, plasma cells, lymphocytes and erythrocytes. No parasitized or pigmented RBC was seen.

Liver: There was noticeable necrosis of hepatocytes. The infiltration of WBC in portal area was significant. Pink-colored debris could be seen in sinusoids and some small vessels. No pigment was seen.

Spleen: Autolyzed. There were monocytes and macrophages in the cords and sinuses accompanied by lighter colored hemosiderin. No pigmented RBC was seen.

Kidneys: Congestion. There were significant RBC and Hb in the

tubules. No pigmented WBC or RBC was seen.

Other tissues: No remarkable clue of malaria infection.

Case 9710:

Brain: Edema. Leucocytes and packed RBC could be seen in capillaries and small vessels. Pigmented RBC was not remarkable. No hemorrhages were detected.

Heart: Edema. Most of the myocardial capillaries were filled with RBC and Hb, some with WBC and dark-pink-colored granules. There was no myocardial necrosis and no pigmented RBC.

Lungs: Congestion and significant edema, the alveolar walls were thickened and the capillaries were filled with WBC and packed RBC. Some alveoli were congested with pigment-laden macrophages, lymphocytes, Hb and erythrocytes. Pigmented RBCs were uncertain.

Liver: Slight autolysis of hepatocytes. Sinusoid dilated and congested with Kupffer cells and RBC. There was wide spread pigment deposition but PRBC were hardly seen. The infiltration of WBC in portal tract was significant. In hepatic vein at portal area, there were WBC, Hb, RBC and pink-colored debris.

Spleen: Autolyzed. The cords and sinuses were expanded by monocytes, lymphocytes and some macrophages containing pigment. The red pulp was filled with RBC and some pigment deposition.

Kidneys: Congestion with some uncertain pigmentation.

Other tissues: No remarkable evidence of malaria infection.

Case 9714:

Brain: Congestion and edema. In B9, the vessels were packed with RBC, WBC (mainly monocytes and lymphocytes), and "host" RBC in small vessels; Petechial hemorrhages could also be seen.

Heart: Edema. Most of the myocardial capillaries were filled with RBC and Hb. No myocardial necrosis or pigmented RBC was present. In E3, increased monocytes and lymphocytes could be seen in small vessels.

Lungs: Congestion and edema, the capillaries were filled with packed RBC, Hb and WBC. Alveoli were congested with, lymphocytes, Hb and erythrocytes and some pigment-laden macrophages. No pigmented RBC was seen.

Liver: There were cellular ballooning hepatocytes containing lipid vacuoles. Bile canaliculi distended with inspissated bile, Kupffer cells contained phagocytized bile and some uncertain pigments. No PRBC could be found.

Spleen: Very significant autolysis. The fibrous connective tissue increase and there are monocytes and macrophages containing pigment. The red pulp is filled with RBC, WBC, pigment and some pigmented RBC.

Kidneys: Congestion. No pigment was seen.

Other tissues: No remarkable clue of malaria infection.

Case 9717:

Brain: congestion, edema and autolysis. In capillaries and small vessels, there were packed RBC and Hb. In B10, WBC, mainly monocytes and lymphocytes, and packed RBC could be seen in small vessels. No pigmented RBC was present.

Heart: Edema. Most of the myocardial capillaries were filled with RBC and Hb. Some monocytes and lymphocytes could be seen in small vessels. No pigmented RBC.

Lungs: Congestion and edema, the alveolar walls were thicken and

some capillaries were filled with WBC, mainly lymphocyte. There were lymphocytes, Hb, erythrocytes and some pigment-laden macrophages in the alveoli. Some focal hemorrhages and no pigmented RBC were detected.

Liver: Significant cellular ballooning hepatocytes with vacuoles and autolysis. In portal area, WBC, mainly lymphocyte, infiltration could be found. Some pigments in Kuffer similar to that of malaria. No significant Kupffer cell proliferation. Lack RBC, No PRBC.

Spleen: Significant autolysis. The cords and sinuses were expanded by lymphocytes, monocytes and some macrophages containing pigment. The red pulp was full filled by RBC. There was some pigment accumulation, although the pigmented RBC were not remarkable in numbers.

Kidneys: Autolysis. No pigment.

Other tissues: No remarkable clue of malaria infection.

Case 9719:

Brain: Edema. Most of capillaries lacked RBC but some were packed with RBC. Hb and WBC could be seen in some capillaries and small vessels in white matter. No hemorrhages, no PRBC.

Heart: Edema. Most of the myocardial capillaries were filled with RBC and Hb. WBC, mainly monocytes and fibrin, thrombosis could be seen in right atrium. Interstitial WBC, mainly lymphocyte, infiltration was found in left ventricle. No myocardial necrosis or pigmented RBC was detected.

Lungs: Congestion and significant edema. There were focal hemorrhages or WBC infiltration. No pigmented RBC present.

Liver: Slight autolysis of hepatocytes. Congested bile, Kupffer cells and RBC in dilated sinusoids was significant. There were wide spread pigment deposition and remarkable monocytes and lymphocytes infiltration in portal areas, but PRBC were hardly seen. In addition to WBC, Hb, RBC, pink-colored debris, some pigmented cells similar to hepatocytes were present in hepatic vein.

Spleen: Remarkable autolysis and congestion. Pigment was significantly present. The cords and sinuses were filled with RBC, monocytes, and pigmented macrophages. RBC and WBC expanded the red pulp.

Kidneys: Congestion with some uncertain pigments.

Other tissues: No remarkable clue of malaria infection.

Under the polarized microscope brilliantly birefringent granules of the pigment and the parasites were detected in all liver samples except in case number 968 and 9720. The signal in case number 966, 967 and 9713 are very strong, and it is weaker in case number 962, 963, 964, 965, 979, 9711, 9715, 9717, 9719 and 9721. In case number 9710, 9712 and 9714 the signal are weak, and in 9718, it is very weak. A polarized light picture of H.E. stained liver section is shown in Figure 11.

The transmission electron microscope (EM) examination revealed the parasitized, pigmented red blood cells and white blood cells, as well as fibrin in case number 9710 and all malarial cases except case number 9719. In some cases it is hard to tell the morphological details because of the autolysis. An EM picture from lung section (case 9710) is shown is Figure 12.

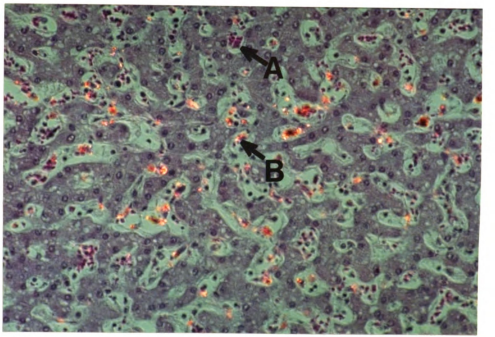


Figure 11. Polarized light picture of H.E. stained liver section. Under the polarized microscope the paratisized RBC (A) and brilliantly birefringent granules of the pigment (B) are detected.

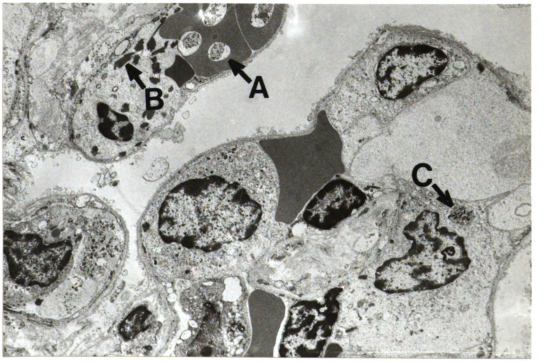


Figure 12. Ttransmission electron microscope picture from lung section (case 9710). Paracite in RBC (A), fibrin in blood vessel (B) and pigment in WBC (C) are seen. 2500 X 2.7.

DISCUSSION

According to the numerical data obtained from the H.E stained brain sections (Table 3, 4, and 5), the most marked differences between CM group and the other two lies in the numbers of pigmented RBC, monocyte, total pigmented WBC and hemorrhages. In evaluating the difference of these markers between each group, a "t" test was used (Table 6, 7 and 8). It is known that an increasing of total WBC in blood is not specifically relate to malaria infection and that total counting area per 100 vessels may vary with the difference of vessel lumen. Thus to reduce the effects of total WBC number and counting area in the comparisons, the ratio of monocyte to total WBC (Mono/TWBC), total pigmented WBC to total WBC (TpWBC/TWBC), and pigmented RBC to endothelial cells (PRBC/EC), were taken into account. As it is shown in table 6, 7, and 8, the PRBC/EC ratio is significantly different between CM and NCM or COC groups ($p < 0.05$). This suggests that the PRBC in capillaries could possibly be used as markers for the identification of cerebral malaria damage. However, in the NCM group there are two cases that have high PRBC numbers overlap with the CM group (Figure 1). This observation may imply that some malaria patients do not suffer a cerebral consequence even though they have significantly increased pigmented PRBC in their cerebral capillaries. Considering that in one of these two cases (case number 963) the patient died on admission, and that the gross autopsy and histological examination revealed significant brain

edema and petechae or ring hemorrhages (Table 1, 3 and 5), it is possible that the patient did in fact have a cerebral event. While the other case (number 964) does suggest that the PRBC count can not be used as a definitive marker for the diagnosis of cerebral malaria.

Although the "t" test of the numerical data with section B2 and B12WM did not give a statistically significant difference between each group (Table 6 and 8), the numbers of ring hemorrhages found in the cerebral malaria cases are still remarkable (Figure 2). As mentioned above, in NCM group the only case with ring hemorrhages is possible a CM case, and it is this case that considerably affects the P value (Table 7). Morphologically, the malarial ring hemorrhage is so easily distinguished that its diagnostic value should not be minimized. At worst ring hemorrhages suggest a malaria death, and combined with the presence of a noticeable level of pigmented RBC in cerebral capillaries, cerebral malaria is more likely to be defined.

Besides pigmented RBC and ring hemorrhages, the other remarkable difference between CM and the other groups is the ratio of TpWBC/WBC in B2 and B12 gray matter ($p<0.0003$). This ratio is not only significantly higher in the CM group, but also has small overlap with other groups (Figure 3 and 4). Although the ratio of TpWBC/WBC could be another valid marker for the use in the identification of malaria deaths, it should be emphasized that there are some CM cases where the capillaries are occupied by pigmented RBC and there are only a few PWBC to be found (Table 3 and 4). To these cases, the ratio of TpWBC/EC may

overlap some of those in NCM group (Figure 5), so that the diagnostic value of PWBC numbers is reduced. Even so, combining the PWBC/WBC ratio with the PRBC number and with ring hemorrhage presence, all CM can be identified and thus prevents misdiagnosis for all cases from the other groups in this study.

The increase of monocytes is closely related to the outcome of malarial infection (table 6, 7 and 8), especially when adding with the counts made with the immunohistochemical stains (Table 9 and Figure 7). But, as the range of cases included in the COC group is limited and does not cover all non-malarial deaths, its practical diagnostic value still needs validation. The large overlap between the CM and NCM group shows strong evidence that monocytes are actively involved in malaria infection (Figure 6 and 7). As to the other WBC species (i.e. neutrophils, lymphocytes and eosinophils), no special difference could be drawn from this study, although some researchers have reported an increase in pigmented neutrophils in CM cases [39].

The presence of fibrin was not significantly evident in the H.E. stained slides, but with immunostaining a remarkable difference between the CM and COC group ($P < 0.05$) could be detected. Because there is considerable overlap among the cases with this criterion, especially those between NCM and COC group (Table 10, figure 8.), its specificity and significance needs further evaluation.

It should be noticed that case number 9719 in NCM group did not show sufficient evidence for the positive identification of malaria death using the techniques of this study. Besides, the pathological changes in case number 9710 strongly support a

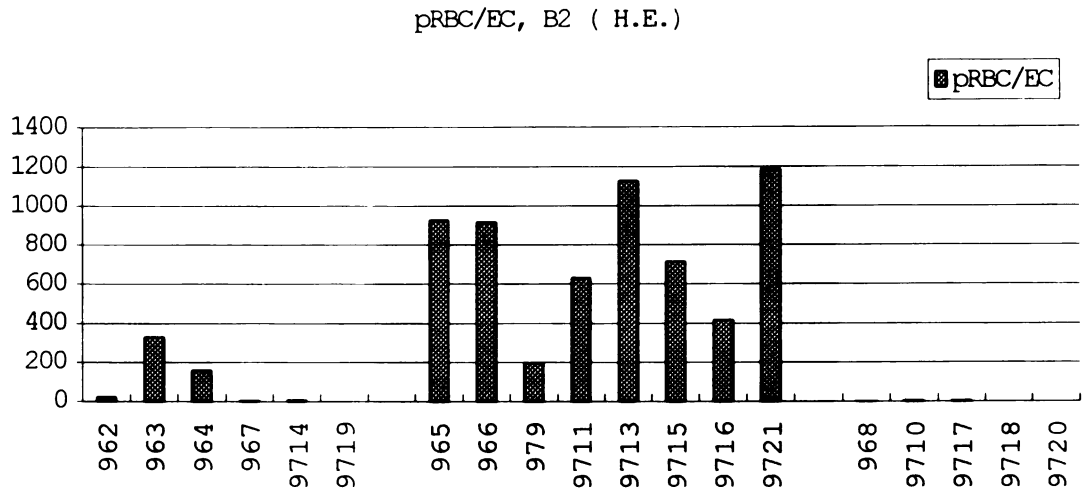


Fig.1. The ratio of pigmented RBC to endothelial cells in section B2. The numbers on category axis indicate the case numbers of the three groups.

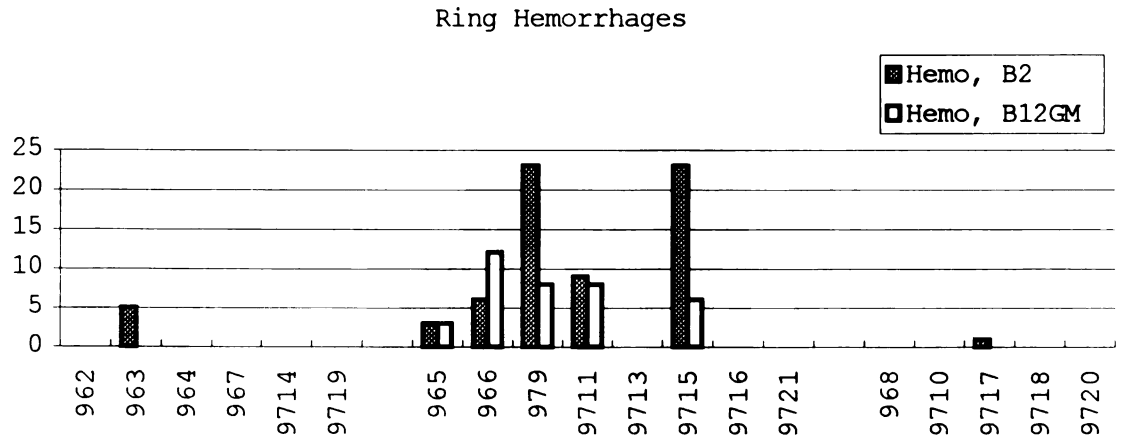


Fig.2. The numbers of ring hemorrhages in section B2 and B12GM. The numbers on category axis indicate the case numbers of the three groups.

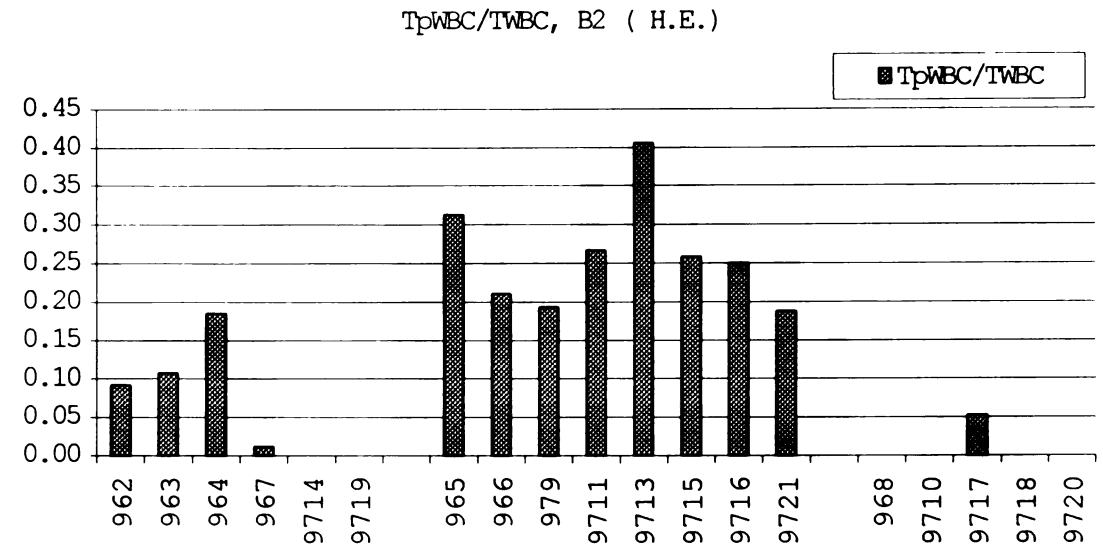


Fig.3. The ratio of total pigmented WBC to total WBC in section B2. The numbers on category axis indicate the case numbers of the three groups.

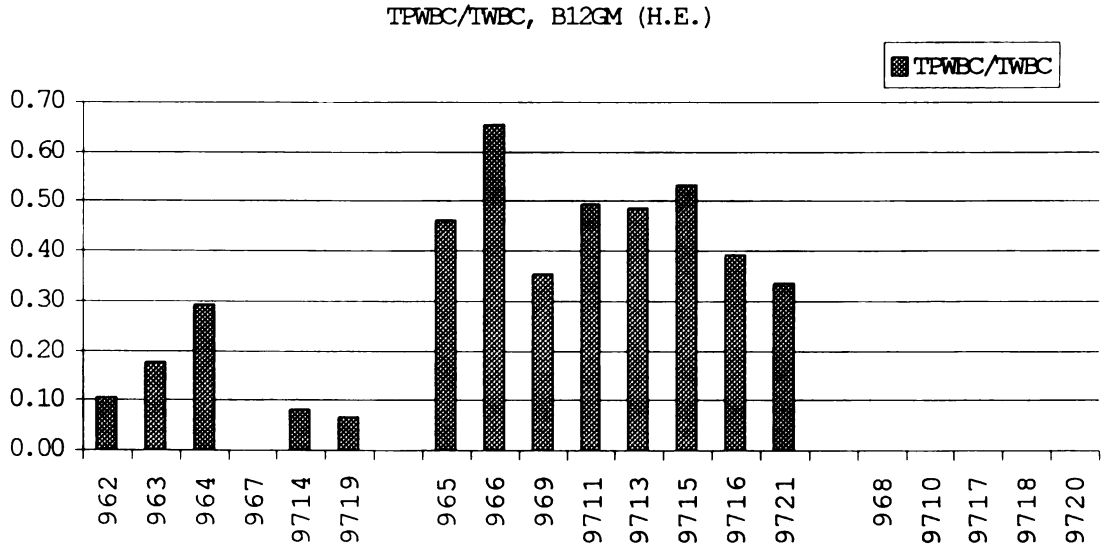


Fig.4. The ratio of total pigmented WBC to total WBC in section B12GM. The numbers on category axis indicate the case numbers of the three groups.

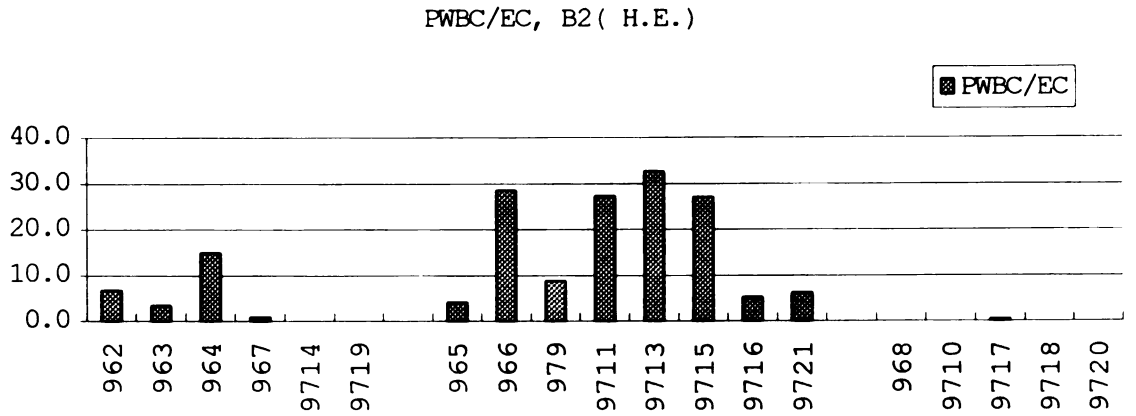


Fig.5. The ratio of pigmented WBC to endothelial cells in section B2. The numbers on category axis indicate the case numbers of the three groups.

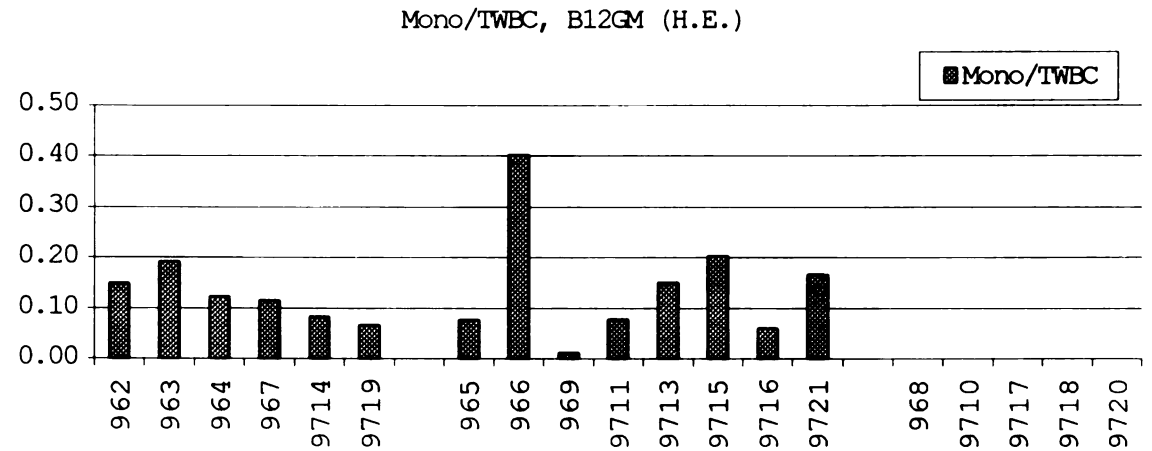


Fig.6. The ratio of monocyte to total WBC in section B12GM. The numbers on category axis indicate the case numbers of the three groups.

malarial infection, which makes it difficult to differentiate this case with cases 9714 and 9719. On reviewing the clinical data, the pre-mortem blood transfusion might explain the smaller numbers of pigmented cells detected, but the morphological character of cases 9710 and 9719 make it hard to identify the actual cause of death. Although the parasitized or pigmented RBC, fibrin, pigment and pigmented phagocytes can be demonstrated under EM, this technique has its limitation in forensic use, not only because of the detrimental effect of postmortem autolysis on morphological analysis, but also the sampling problems it presents when screening in forensic cases. A polarized microscope is helpful in locating the pigment, but, as it is shown in case 9717 (Figure 11), a positive result is not necessarily specific and the differences of the pigment in livers between each group is hard to evaluate based on this observation. However, with thorough examination of the slides, it is found that there are some hepatocyte-like cells with pigment in the portal vein in those cases of malaria death. This may be usable, as a marker for the diagnosis of active malaria infection, however further study is needed. The possibility of a pathogenesis involving lethal anemia or hyperimmune response, in the diagnosis of case 9719, is outside of the morphological territory, and molecular markers for the detection of malarial RBC damage and malaria related immunomolecules might be needed.

All of the cases in this current study are children, and it is believed the pathophysiology of severe malaria is different in children and adults [40], however, the findings in this study are similar to those reported in both published and

unpublished adult malaria deaths in the forensic literatures[9,41,42].

CONCLUSIONS

1. Pigmented RBC packed in the cerebral capillaries, ring hemorrhages and the increased TpWBC/TWBC ratio are important markers in the identification of a cerebral malaria death.
2. The increased Mono/WBC ratio, the presence of fibrin, as well as pigmented monocytes have a close relationship to the extent of malaria cerebral damage and can be used for reference in the diagnosis of malarial or cerebral malarial death.
3. The identification of non-cerebral malaria death requires not only a thorough autopsy and histological examination, but also good personal and clinical data. Some malaria deaths may not demonstrate enough significant morphological evidence for complete identification; thus further study on markers of malarial damage is needed.

RECOMMENDATIONS

As mentioned above, the indicators of malarial death having been explored and evaluated in this study are insufficient in identifying any malarial death. The increasing of the practical value of these indicators in forensic identification of malarial death can be achieved by studying with more samples, especially that in CM and COC groups. Using immunohistochemical and molecular biological techniques, the origin of the hepatocytes like cells in portal veins and their relationship with malarial death might be better interpreted, and the detection of molecular markers of malarial RBC damages and other malaria-related immunomolecules that have been used for clinical and research purposes are as well as considerable. Since a forensic examination may be delayed in some cases, the effect of postmortem changes must be taken into account. Thus, a proper sample control is needed for the evaluation of these markers before they are used in forensic practices.

APPENDICES

Immunostain Procedures

APPENDIX I

3A5* immunostain procedure

1. Deparaffinize for 10 minutes in 2 changes of Xylene.
2. Hydrate to distilled water through graded ethanol.
3. Block in 3% Hydrogen Peroxide for 10 minutes.
4. Rinse slides in running tap water for 5 minutes.
5. Place slides in Tris Buffered Saline (TBS) for 5 minutes.
6. Place slides into staining dish with preheated Biogenex Citra Antigen Retrieval (diluted 1:9), cover with lid and place dish into steamer incubating for 40 minutes.
7. Remove dish and set on counter with the lid off for 10 minutes.
8. Rinse in 3 changes of distilled water.
9. Place in TBS+ Tween 20 for 5minutes.
10. Put slide on stainer with the following protocol:

Super block-5minutes,
Monoclonal Macrophage 3A5 at 1:80 for 60 min, RT
Biotinylated Horse anti-Mouse at 1:136 for 30 min, RT
Vector R.T.U. ABC Comlex-30 min, RT
Vector Nova Red-15 min. RT
Remove from stainer, then,
Counterstain in Lerner 2 Hematoxylin for 1 ½ minutes
2 quick dips in 1% Glacial Acetic Water
Rinse in running tap water for 2 min
Dehydrate through several changes of graded ethanol
Clear through several changes of Xylene
Coverslip with a synthetic mounting media

* Novocastra Laboratoryies Ltd, #210501

APPENDIX II

Anti-Fibrin immunostain procedure

1. Deparaffinize for 10 minutes in 2 changes of Xylene.
2. Hydrate to distilled water through graded ethanol.
3. Block in 3% Hydrogen Peroxide for 10 minutes.
4. Rinse slides in running tap water for 5 minutes.
5. Place slides in Tris Buffered Saline (TBS) for 5 minutes.
6. 0.03 % Protease (Pronase E) 10 min at 37^0 C.
7. Rinse in running tap water for 5 minutes.
8. Place in TBS+ Tween 20 for 5 minutes.
9. Put slide on stainer with the following protocol:

   Super block-5minutes,
   Monoclonal Fibrin at 1:100 for 90 min, RT
   Biotinylated Horse anti-Mouse at 1:150 for 30 min, RT
   Vector R.T.U. ABC Comlex-30 min, RT
   Vector Nova Red-15 min. RT
   Remove from stainer, then,
   Counterstain in Lerner 2 Hematoxylin for 1 ½ minutes
   2 quick dips in 1% Glacial Acetic Water
   Rinse in running tap water for several dips
   Blue in ammonia water for 30 seconds
   Rinse well in running tap water.
   Dehydrate through several changes of graded ethanol
   Clear through several changes of Xylene
   Coverslip with a synthetic mounting media.

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