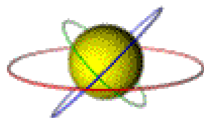

用乳酸脱氢酶为靶分子的免疫层析技术检测恶性疟原虫

Malaria remains one of the leading causes of morbidity and mortality in the developing countries in tropical and subtropical regions, killing 2.7 million people worldwide annually. One of the most pronoun- ced problems in controlling the malaria-related morbidity and mortality is limited access to effective diagnosis and treatment in malaria endemic areas . An ideal diagnostic test for malaria must be simple, rapid, sensitive, specific, cheap, and easy to interpret. The colloid gold-immunochromatography assay (GICA) based on monoclonal antibodies (mAbs), as the new antigen-capture technique, is capable of more rapid and sensitive parasite detection and has prompted the manufacturing of commercially available kit, such as Parasight-F (Becton Dickinson, Paramus, N.J.)[1], ICT Malaria Pf (ICT-Amrad, Sydney, Australia)[2], and OptiMAL (Flow Inc., Portland, Oreg.)[3], which include all the necessary reagents and do not require highly professionally trained personnel or sophisticated equipment. The parasite antigen used in Parasight-F and ICT is histidine-rich protein-2 (HRP-2). Though allowing rapid diagnosis of Plasmodium falciparum malaria, HRP-2-based GICA is of limited clinical application for several reasons. First, HRP-2 persists in the blood after resolution of the clinical symptoms of malaria and apparent parasite clearance from the host. In fact, HRP-2 has been found even in mummies, several thousand years post-infection[4]. Secondly, the monoclonal IgG against HRP-2 utilized in the ParaSight-F system may cross-react with rheumatoid factor and cause a false-positive response[5]. It was reported that the HRP-2-based ICT assay gave a false-positive reaction in 26% of the patients who had rheumatoid factors but were negative for malaria by microscopy[6].

In recent years, studies have shown that Plasmo- dium lactate dehydrogenase (LDHp) is significantly different from the host LDH in biochemical, immu- nological and enzymatic properties. One of the bioche- mical characteristics that distinguish LDHp from human LDH is the ability of LDHp to rapidly utilize 3-acetylpyridine adenine dinucleotide (APAD) as a coenzyme in the reaction leading to the formation of pyruvate from lactate. Human erythrocyte LDH could not make use of APAD[7][8]. This unique feature makes it feasible to differentiate these two types of LDHs either by spectrophotometry or by electrophoresis. As LDHp is only produced by live parasites and the degree of parasitemia is correlated with the activity of parasitic LDH, assay of LDHp activity may provide an pro- mising strategy to specifically detect a particular plasmodium clone[9] and to monitor the effectiveness of drug therapies[10][11].

In addition, it has been suggested that Plasmodium LDH possesses species- and genus-specific antigens, which are considered to be promising candidate targets for diagnostic

purpose[12][13]. LDHp-based assay, Opti- MAL test, has been evaluated under field conditions and proved to have sensitivities of 94% and 88% and specificities of 100% and 99%, respectively, in comparison with traditional blood smears for detecting P. vivax and P. falciparum malaria. OptiMAL test correctly identified P. falciparum malaria parasites in patients' blood samples more often than the other two commercially available diagnostic tests, ParaSight-F and ICT[3]. Unfortunately, these kits are currently too expensive to enable extensive application in the develo- ping countries. For example, the ICT Pf test costs 1.80 US dollars/test [14], the ParaSight-F needs 1.20/test in Uganda[14], and OptiMAL strip even amounts to 3.00/test. Patients who suffer from malaria normally live in remote areas where necessary facilities are scarcely accessible to go through such a diagnostic procedure. Hence a cheaper but accurate and rapid diagnostic kit is urgently required. In this study, BALB/c mice were immunized with purified recom- binant Plasmodium falciparum LDH (LDHpf), and the mAbs against LDHpf were prepared according to the protocol of hybridoma technique. The mAbs were cha- racterized by enzyme-linked immunosorbent assay (ELISA) and Western blot analyses. GICA was con- ducted for the diagnosis of Plasmodium using the prepared mAbs and its sensitivities and the specificities were evaluated in comparison with both the mi- croscopic examination of the blood smears and polymerase chain reaction (PCR) test.

MATERIALS AND METHODS

Patients

From each of the 89 patients with fever in Hainan Province of China, 5 ml venous blood was drawn into an EDTA-coated syringe. Aliquots of these 89 samples were frozen at -80 ℃ until use. A thick blood film was prepared for each patient during the blood collection process. After blood smears were stained with Giemsa, the species and the density of the Plasmodium parasites were determined and the parasite was quantified per 200 white blood cells (WBCs). The sample was determined to be negative when no parasites were found after examination of 200 microscopic fields at 1 000× magnification. The negative control sample was taken from an individual who had never been exposed to malaria.

PCR diagnosis

The frozen blood samples (20 μl) were mixed with 1 ml lysis buffer (50 mmol/L NaCl and 0.015% saponin). After centrifugation, the pellets were washed twice with TE buffer (containing 10 mmol/L Tris-HCl and 1mmol/L EDTA) before DNA extraction in 50 μl mixture containing 0.5 mol/L EDTA, 2% sodium dodecyl sulfate, and 2 mol/L NaOH at 100 ℃ for 10 min. After centrifugation at 10 000 g for 20 min, the supernatant was collected to be used as PCR template, using the primers designed based on the sequence of SSUrRNA of Plasmodium [15][19]. The sense primer of P. falciparum was 5'-TTAAACTGGTTTGGGAAAACCA AATATATT-3', and the antisense 5'-ACACAATGAAC TCAATCATGACTACCCGTC-3', with the amplifica- tion fragment of nucleotides 665-870. The sense primer of P. vivax was 5'-TTCGTATCGACTTTGTGCGCATT T-3', and the antisense 5'-ACTTCCAAGCCGAAGCA AAG AAAGTCCTTA-3', with the fragment for ampli- fication of nucleotides 630-749. One microliter of the DNA template was used for PCR amplification in a reaction volume of 50 μl containing 10 mmol/L Tris-HCl, 50 mmol/L KCl, 1 mmol/L MgCl2, 0.1% Triton X-100, 2.5 mmol/L of each dNTP, and 50 pmol of each primer.

After this mixture was heated at 93 ℃ for 3 min, Taq DNA polymerase (2 U) was added. PCR amplification was carried out for 30 cycles of dena- turation at 93 ℃ for 30 s, annealing at 58 ℃ for 45 s, and extension at 72 ℃ for 45 s. The size of the expected amplification product was 206 bp (P. falciparum) and 120 bp (P. vivax), confirmed by 2% agarose gel electro- phoresis and visualized under a UV transilluminator after ethidium bromide staining.

Preparation of mAbs

The recombinant LDHpf was expressed in Escherichia coli (TG1) using the vector PGEX-4T-1 (Pharmacia) containing the LDHpf open-reading frame from the FCC1/HN isolate of P. falciparum and purified by SDS-PAGE[20]. The mAbs to LDHpf were obtained from female BALB/c mice immunized with purified recombinant LDHpf. Splenocytes were fused to the myeloma cell line SP/20 and selected in hypoxanthine aminopterin thymidine (HAT) mediun according to the documented procedures. The mAbs against LDHpf were screened by ELISA and characterized by Western blot analyses. The hybridomas were injected into BABL/C for generating ascites which contained the antibody. The mAbs were purified by affinity chromatography on protein A-Sepharose (Pharmacia, Freiburg, Germany).

Preparation of GICA strip

The control line at the top of the test strip was coated with the recombinant LDHpf antigen, and the detection line at the bottom with the mAb 1G7. mAb 2A5 was labeled with colloidal gold about 20 nm in size. Fifty microliters of the lysis buffer was added to a test well of a 96 well plate, and 100 ml colloid conju- gate added to a second test well. Ten microliters of the blood sample was then added into the first test well and mixed gently. After the blood sample ran on the top of the test strip, the test strip was moved to the second test well for further reaction for about 8 min. The strip was then washed with washing buffer, and the test results interpreted after completion of washing. A negative control sample was included within each batch for testing. Positive results were defined by the presence of a control band with a test band, negative results by a control band, and invalid test by failure of appearance of the control band. Examples are shown in Fig.1. The stability of GICA strip stored at room temperature for 6 months was evaluated using FCC1/HN strain of P. falciparum cultured in vitro.

Data analysis

The sensitivity and specificity of GICA for detecting P. falciparum infection was evaluated with thick blood smear examination and PCR method as the standards. The sensitivity was calculated according to the formula: sensitivity = actual positive cases/(actual positive cases + false negative cases) and the specificity = actual negative cases/(actual negative cases + false positive cases). Statistical evaluation was done with Chi-square test.

RESULTS

Four mAbs against LDHpf antigen were obtained. The immunoglobulin classes/subclasses of the 4 mAbs designated as 2A5, 1H10, 1G7 and 2C12 were IgG_{2b}, IgG_{2b}, IgG_{2a}, IgG_{2a}, respectively, with titers of the 4 mAbs ranging from 1∶25 600 to 1∶12 800 in the ascites, and form 1∶512 to 1∶128 in the supernatant. The results of ELISA indicated that

the 4 mAbs reacted only with P. falciparum, but not with normal human red cell, P. vivax, Toxoplasma gondii, or Schistosoma japonicam. All the 4 mAbs recognized a 33-kD protein, identified as LDHpf by western blot analysis (Fig.1). These results indicated that 4 hybridoma cell lines secreting high-titer mAbs against LDHpf with high specificities were successfully established.

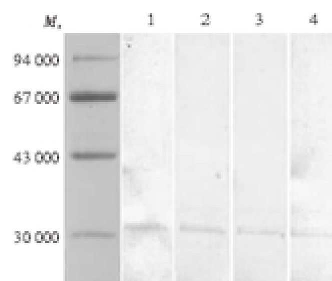

Fig.1 Western blot analysis of the 4 mAbs against Plasmodium falciparum
Lane 1:2A5;Lane 2:1H10;Lane 3:1G7;Lane 4:2C12

A total of 89 patients were screened for malaria parasites by microscopy of Giemsa-stained blood smears, and 61 were found positive for the parasites, including 35 infected with P. falciparum, 18 with P. vivax and 8 with mixed infection with P. falciparum and P. vivax. Parasitemia in the 35 patients infected with P. falci- parum ranged from 18 to 1986 parasites/ml and from 14 to 745 parasites/ml in 18 infected with P. vivax. Five blood samples were negative for microscopic diagnosis, but PCR detected one case of P. falciparum infection, 3 of P. vivax infection, and one of mixed (P. falciparum and P. vivax) infections. PCR detected malaria parasites in 66 patients, including 36 with P. falciparum infection, 21 with P. vivax infection, and 9 with mixed infection of P. falciparum and P. vivax. Of the 35 patients infected with P. falciparum identified by microscopy of the blood smears and PCR test, the infection was detected in 30 patients by GICA. Of the 18 patients with P. vivax infection, one was found with P. falciparum infection using GICA test. Twenty-eight cases of negative samples determined by microscopy, one case was infected with P. falciparum using GICA and PCR test. Eight patients with mixed infection by microscopy were found to have only P. falciparum infection by GICA. Compared with micros- copic examination of the blood smears and PCR test, GICA had the sensitivity of 88.37% and 86.67%, and specificity of 95.65% and 97.73%,respectively. The concordance rate of GICA with microscopic examination for P. falciparum detection was 91.55% (Tab.1).

Tab.1 Results of malaria parasites detected by GICA, PCR and blood smears

Blood smears		PCR		GICA	
Species	No. of patients	Positive	Negative	Positive	Negative
P. f.	35	35	0	30	5
P. v.	18	18	0	1	17
Mixed infection	8	8	0	8	0
Negative	28	5	23	1	27

The GICA and microscopyic results of parasitemia evaluation ranged from 18 to 986 parasites/ml level in 35 patients infected with P. falciparum, as shown in Tab.2. These results indicate that GICA test had a sensitivity of 100% for parasitemia at the levels above 200 parasites/ml, dropping to 77.27% for lower levels of parasitemia. The sensitivity decreased further to 66.67% for parasitemia below 100 parasites/ml ($\chi 2 = 5.41$, $P<0.05$ as compared with the sensitivity at the parasitemia level above 100 parasites/ml, Tab.2).

Tab.2 *P.falciparum* levels detected by GICA

No. of parasites/ml	No. of positive cases	
	Blood smears	GICA
<50	5	3
50-100	7	5
100-200	10	9
200-300	9	9
>300	4	4

After being stored at room temperature for 1, 10, 30, 60 and 90 days, the GICA strips were evaluated using FCC1/HN strain of P. falciparum cultured in vitro. All the strips yielded positive results (Fig.2), suggesting that the strips coated with the antigen and antibody can withstand a store at room temperature for more than 3 months.

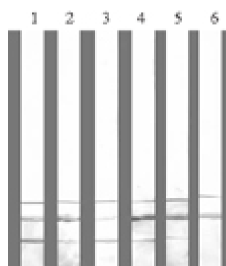

Fig.2 Stability evaluation of GICA strips
The GICA strips numbered 1 to 5 were stored at 4℃ for1,10,30,60 and 90 days, respectively. The number 6 strip is the negative control

DISCUSSION

In this study, 12 patients with P. falciparum and 10 with P. vivax infections had parasitemia at the level below 100 parasites/ml. Therefore, a PCR-based method was used as the standard due to its established sensitivity and specificity, considering also for its advantages over microscopy, particularly in cases with low-level parasitemia. Previous studies showed that PCR-based method had the ability to detect as few as only a single parasite[21]. Our results demonstrated that PCR told a totally different story of the 5

negative cases determined by microscopic examination: P. falciparum infection in 1 case, P. vivax infection in 3 cases, and mixed (P. falciparum and P. vivax) infection in 1 case.

GICA test is as sensitive as thick smear microscopy in the diagnosis of P. falciparum when the parasitemia level exceeds 200 parasites/ml, but its sensitivity dropped to 77.27% for a parasitemia level below 200 parasites/ml, and decreased further to 66.67% for a parasitemia level below 100 parasites/ml. Our results are consistent with that of a previous report describing the identification of 59% of the cases with a parasitemia level above 100 parasites/ml by LDHp OptiMAL assay[22]. In addition, compared with ParaSight-F based on HRP-2, LDHpf assay measures the enzyme level of intact viable parasites. Consequently, the decrease in parasite LDH levels can be used as the index for monitoring drug therapy.

In conclusion, GICA we established in this study provide an important tool in the diagnosis of P. falciparum infection and can be potentially useful in developing reagent kits for P. falciparum diagnosis with simplicity, rapidity and cost-effectiveness. This assay also has its limitations, such as the inability to provide information on the level of parasitemia and to discriminate mixed infections from exclusive P. vivax infection. Better diagnostic tools for both P. falciparum and P. Vivax are urgently required. As P. vivax can not be cultured in vitro, LDHpv gene from P. vivax should be investigated in order to overcome the current limitations in the diagnosis of P. vivax.

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