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LACTATE DEHYDROGENASE AS A MARKER OF *PLASMODIUM* INFECTION IN MALARIA VECTOR *ANOPHELES*

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ABSTRACT. Lactate dehydrogenase (*Ldh*) electrophoresis showed the presence of *Plasmodium yoelii yoelii* in *Anopheles stephensi* and *An. gambiae.* The *Ldh* appeared as an additional band (p*Ldh*) whose activity was more intense with 3-acetyl pyridine adenine dinucleotide as coenzyme than with β nicotinamide adenine dinucleotide. Several allelic forms occurred both in the vector and the host. The isoelectric point of *Ldh,* similar in the vector and host, differed from those of *Ldh* from mosquito and mouse. The presence of p*Ldh* was detected from the 2nd to the 28th day of infection. The p*Ldh* appeared to be proportional to the number of sporozoites present in infected salivary glands. However, p*Ldh* was not found in salivary glands or midguts, but it was detected in the rest of the corresponding mosquito. The origin and use of p*Ldh* as a marker of *Plasmodium* in its vector is discussed.

INTRODUCTION

Throughout the world, about 2 million people die of malaria annually (Gentilini 1991), with 120 million clinical cases/year and 300 million people carrying the parasite (World Health Organization 1992). Different strategies are used to fight this disease and one involves control of the *Anopheles* vectors. A rapid method to diagnose malaria-infected and malaria-infective mosquitoes is needed.

Plasmodium can be detected in human blood using Giemsa-stained smears, whereas in mosquitoes, microscopic examination of midguts and/or salivary glands is necessary. This latter method is labor intensive and time consuming and many mosquitoes have to be dissected to determine sporozoite rates. Other techniques have thus been developed, including the enzyme-linked immunosorbent assay (ELISA) (Wirtz et al. 1985) and DNA probes (Delves et al. 1989, Ponglikitmongkol et al. 1994).

Analysis of *Plasmodium* enzymes (Broun 1961; Sherman 1961; Carter 1973; Walliker et al. 1971, 1973) showed that host- and parasite-specific enzymes could be distinguished. In *Plasmodium falciparum,* the coenzyme for lactate dehydrogenase (*Ldh*) is preferably 3-acetyl pyridine AD (APAD), whereas the activity of human *Ldh* requires β nicotinamide adenine dinucleotide (NAD). This property permits measurement of parasite *Ldh* and malarial diagnosis in man (Makler and Hinrichs 1993). Our goal was to adapt this method to reveal p*Ldh* of *Plasmodium yoelii yoelii* in our model vector *Anopheles stephensi* Liston in order to develop a rapid and reliable technique to detect the parasite in mosquitoes.

MATERIAL AND METHODS

Parasite: Plasmodium y. yoelii (Killick-Kendrick 1974), strain 17X, a rodent malaria parasite, was obtained from the lab of Biologie Parasitaire, Helminthologie et Protistologie du Museum National d'Histoire Naturelle, Paris (I. Landau). It was stored at −80°C in mouse blood with 5% glycerol.

Vectors: The *An. stephensi* strain used in this study was collected in Iraq in 1973 and reared at 20–24°C, 67 ± 2% RH, and a 12/12 photoperiod. Three strains of *Anopheles gambiae* Giles were included in the study: "Bobo," originating from Burkina Faso, was obtained from Institut Pierre Richet (IPR) at Bouaké (Côte d'Ivoire), and 2 strains obtained from the lab of Ecologie des Systèmes Vectoriels (Institut Pasteur, Paris). The latter two strains included a strain from The Gambia that was sensitive to *Plasmodium* (G3) and a second strain (Blue) that was selected from G3 for its refractoriness to *Plasmodium cynomolgi* (Collins et al. 1986, 1991; Vernick and Collins 1989).

Mosquito samples: Infected mice were caged overnight with *An. stephensi,* resulting in an 87% infection rate. Bloodfed mosquitoes were collected the following day. Every 2 days from day 2 to day 28 following the blood meal, 10 mosquitoes were frozen at −80°C and stored until electrophoresis analysis. The presence of oocysts in midguts (from day 6) and sporozoites in salivary glands (from day 14) was checked by dissection and microscopic examination.

Sporozoite purification: After checking positive for the presence of sporozoites (day 17), 200 mosquitoes were ground in RPMI medium

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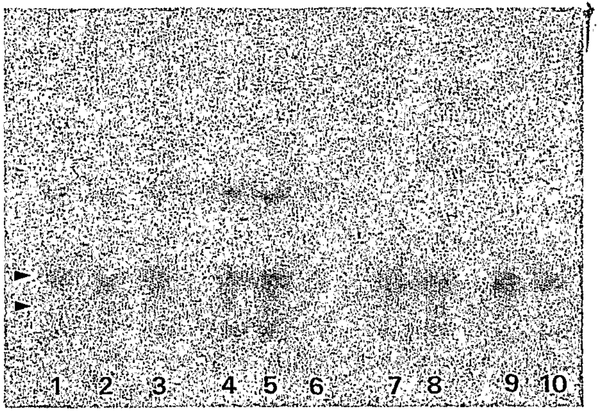

Fig. 1. The *Ldh* of mouse blood. Lanes 1 to 6: infected mice. Lanes 7 to 10: control mice. Migration goes from bottom to top. Bands with ▶ are due to hemoglobin. The lower band is mouse *Ldh,* the upper band is parasite *Plasmodium yoelii yoelii Ldh.*

and the triturate centrifuged for 10 min at 1,000 × *g*. The resulting supernatant was centrifuged for 15 min at 10,000 × *g*. The pellet was resuspended and sporozoites were counted with a Malassez cell (Williams 1973).

Extraction and electrophoresis of Ldh*:* Mosquitoes frozen at −80°C were used to analyze the parasitic cycle; otherwise uninfected mosquitoes were first killed at −20°C. In all cases, the insects were subsequently ground either individually or in pools in a small amount of water (3 μl/mosquito). The mouse blood was diluted by half with water. Whatman no. 1 filter papers were soaked with samples or diluted blood and placed on 12% starch gel at pH 8 (Selander et al. 1971) as previously described (Riandey 1993). The *Ldh* bands were revealed according to Second and Trouslot (1980) by replacing NAD with APAD. The isoelectric point of the enzymes was determined by isoelectric focusing (IEF). The pH gradient ranged from 3.5 to 10 in 0.8% Isogel agarose gel.

RESULTS

Detection with NAD and APAD: With NAD, uninfected and infected mouse blood showed numerous *Ldh* bands and there was very little detectable difference between them. In mosquito, 2 bands were observed when the parasite was present and only one in its absence. When NAD was replaced by APAD, the zymogram was much easier to read (Figs. 1 and 2). Only one mouse-specific *Ldh* band was noted in uninfected mouse blood, whereas 2 bands were observed with infected mouse blood. These results are in agreement with those of Mackler and Hinrichs (1993). In the vector, the specific parasite *Ldh* (p*Ldh*) was much more intense with APAD than with NAD and its activity appeared before mosquito *Ldh* (m*Ldh*), which was still always present.

Isoenzymatic variability: Repeated blood analyses and analyses of single infected female mosquitoes led us to observe some variability in p*Ldh*. It did not always migrate at the same place in the host as in the vector. In each case, more than one level was observed (Fig. 3). These different electrophoretic patterns for the same enzyme had already been observed with several other markers (Carter 1973, Walliker et al. 1975, Babiker et al. 1991).

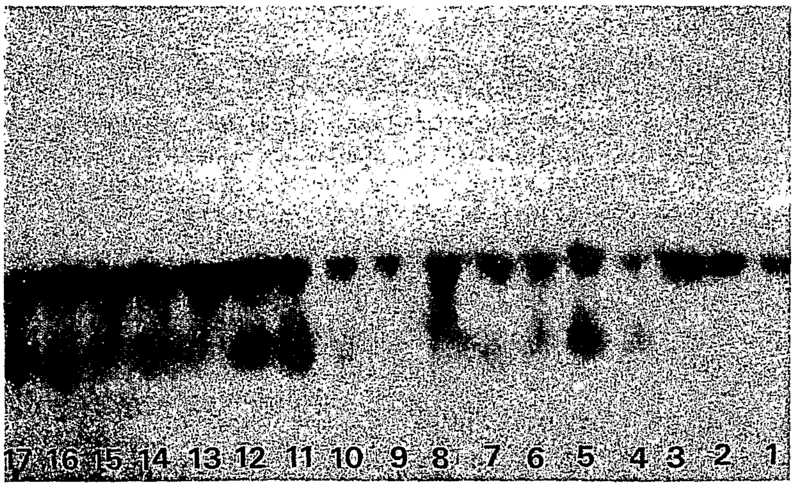

Fig. 2. The *Ldh* of mosquitoes (*Anopheles stephensi*) during the *Plasmodium yoelii yoelii* parasitic cycle. From right to left: Lane 1: nonbloodfed control female. Lane 2: bloodfed control female. Lane 3: infected female at day 24, which allowed infection of new mice. Lanes 4 to 17: infected females at different periods of the cycle every 2 days from 2 to 28 days. Lower band is parasite *Ldh,* upper band is mosquito *Ldh.*

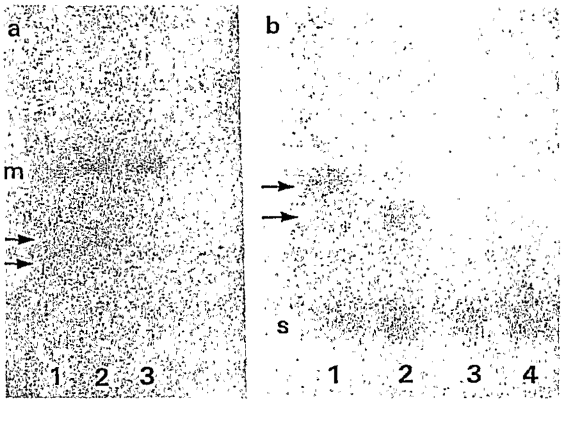

Fig. 3. Isoenzymatic variability of p*Ldh* in *Anopheles stephensi* and in rodent, infected with *Plasmodium yoelii yoelii.* a. *Anopheles*: Lane 1 and 2: infected females at day 15. Lane 3: control female. b. Mouse blood: Lane 1 and 2: infected mice no. 53-4 and 53-1. Lane 3 and 4: control mice no. 53-4 and 53-1. →: parasite *Ldh,* m: mosquito *Ldh,* s: mouse *Ldh.*

Isoelectric point: An electrotitration curve was plotted with mouse blood diluted by half and with a pool of 15 infected females. The resulting isoelectric points (pI) ranged from 5.39 to 5.67 for mosquitoes and from 6.0 to 6.9 for rodents. These values were much lower than the p*Ldh* values, which were 8.27 in mouse and 8.26 in mosquito, respectively.

Parasite growth in Anopheles*:* The infected mosquitoes were tested individually for p*Ldh* as soon as the infected blood meal was taken and up to day 28 of the cycle. Several parasite cycles were analyzed. The presence of the parasite was checked by dissection from day 6 for evidence of infection. From day 2, all traces of hemoglobin had disappeared on the zymogram and the result was as follows (Fig. 2): p*Ldh* appeared at every stage of growth from day 2 to day 28, and was never found in the control fed with uninfected blood or only with a glucose solution. Eight of 16 (50%) mosquitoes that were allowed to infect healthy mice on day 19 and tested on day 24 showed faint p*Ldh* intensity. To explain this result, as suggested by Rosenberg et al. (1990), only part of the sporozoites might have been ejected during the infectious blood meal.

Some assays with the same parasite but other *Anopheles* strains are in agreement with the results on *An. stephensi*: some *An. gambiae* from Burkina Faso analyzed on days 8, 15, and 17 following an infected blood meal (9 samples) showed p*Ldh* activity. Following the same scheme, *An. gambiae* "G3" and "Blue" were tested at day 17 (23 and 17 samples, respectively): the strain sensitive to *Plasmodium* (G3) showed 6 positive faint p*Ldh* bands (26%) but the refractory strain (Blue) was fully negative. These results are consistent with those of Collins et al. (1986) who showed that refractoriness can be observed between 16 and 24 h after ingestion of an infective blood meal. We observed brown bodies in the midgut cells corresponding to the encapsulated parasite in the 2 strains from day 6 onward.

Localization of pLdh*:* To determine the limits of this detection method, p*Ldh* was checked on purified sporozoites. The extract was obtained from 200 infected mosquitoes at day 17 after an infected blood meal and 160 sporozoites/μl were counted. No p*Ldh* activity was detected on the zymogram. A further experiment was carried out to check the location of p*Ldh*: infected mosquitoes were dissected into 3 parts on days 13 and 14 after the blood meal (salivary glands, guts, and mosquito debris). Electrophoresis was then performed separately on the 3 parts, both on pools of 5 insects and on individual dissected mosquitoes. Parasite *Ldh* activity was found in debris and never in salivary glands and/or guts. To certify that enzyme activity was not lost during dissection, another enzyme, esterase (*Est*), was revealed on the second part of the gel. This control showed *Est* activity both in debris and guts but not in salivary glands. This agrees with former studies on localization of *Est* in *An. stephensi* (Riandey et al., unpublished data).

Relation between pLdh *and parasite quantities:* Dissection of salivary glands allowed evaluation of sporozoite quantities in terms of 0 (none), 1+ (1–10), 2+ (11–100), and 3+ (>101). Following dissection of salivary glands and sporozoite counts, debris of the corresponding mosquitoes was analyzed. The result was given by the densitometric recording of the *Ldh* intensity (Fig. 4). On the 550-nm optic density (OD) scale, the different intensities of bands observed on the starch gel could be represented, but not the quantitative *Ldh* values. A very high quantity of sporozoites (3+) led to very strong activity on the zymogram; however, the lowest sporozoite quantity observed with the microscope still gave a positive response on the electrophoretic support (1+). In blood, "p*Ldh*" activity could be detected until a dilution corresponding to 0.03% parasitemia, which is close to the 0.02% parasitemia in human blood detected by Makler and Hinrichs (1993) through *Ldh* measurement.

DISCUSSION

Starch electrophoresis allowed detection of *P. y. yoelii* in its host or vector. In *An. stephensi*, p*Ldh* was observed from day 2 to day 28 of the parasitic cycle, whether or not infections were high. The p*Ldh* assay did not enable distinctions

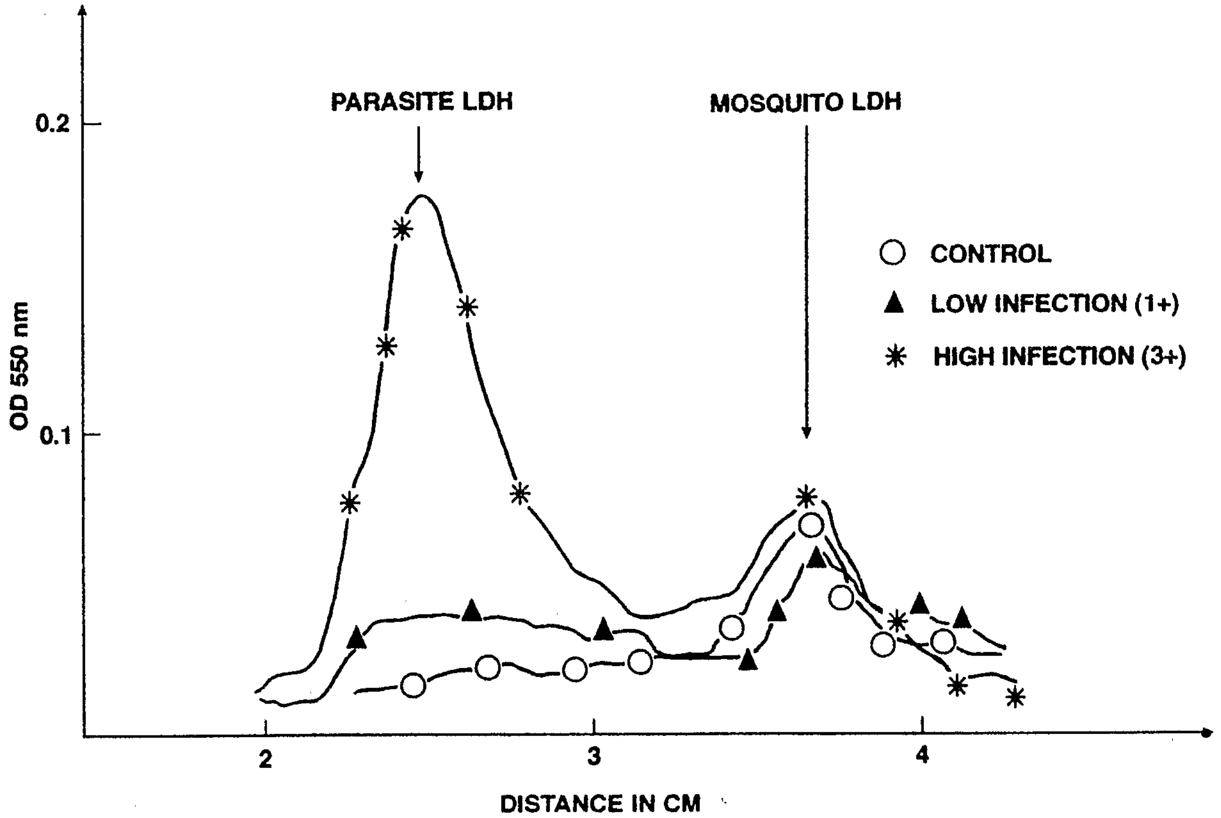

Fig. 4. Lactate dehydrogenase zymogram of *Anopheles stephensi* debris. Densitometric recording of the *Ldh* intensity.

between infected and infectious mosquitoes because the infection stage could not be determined; however, there was good correlation between the cause (parasite) and the effect (p*Ldh*). Therefore, p*Ldh* is a good marker for the *Plasmodium* parasite. The technique is rapid; a single person can test 100 mosquitoes daily. It is not expensive as only small quantities of APAD, the most expensive chemical, are needed (50 mg/100 mosquitoes). The ELISA and DNA probe require specialized equipment, more expensive chemicals, and several long successive processes. Furthermore, the ELISA has to be calibrated to avoid false-positive or -negative responses. This electrophoretic method is suitable for field use. Nevertheless, further work is required to extend this method to other species of mosquitoes infected by other *Plasmodium* species.

A number of questions were raised by the fact that p*Ldh* was only detected in the vector, not in the parasite. Is this enzyme really synthetized by the parasite or the mosquito as a response to the parasite infection? If so, what is the origin of the "p*Ldh*" present in the blood of infected mice? If these "p" bands are synthetized either by the host or the vector, does this only occur as a response to the presence of *Plasmodium* or also as a response to other aggressions? Sherman (1962) showed that another parasite (*Babesia rodhaini*) did not cause this phenomenon in the mouse, but given the size of the parasite, he suggested that the detection technique is perhaps limited. In some diseases, additional *Ldh* is considered as evidence of disease (Yamaoka and Kameya 1972, Beyer et al. 1990).

However, p*Ldh* activity was more intense with the APAD coenzyme than with NAD and the pI level was the same in mouse and mosquito. These results are in agreement with the supposed synthesis of this enzyme by the parasite. The localization of p*Ldh* outside *Plasmodium* is comparable to another type of enzymatic induction: lipolysis in host tissues, which provides lipidic acids for intraerythrocytic growth of the parasite under control of a lipase originating from the parasite (Vial and Ancelin 1992). In the same way, p*Ldh* seems to be active outside of the parasite, allowing ATP production by the host for the glycolytic pathway of *Plasmodium* (Döbeli et al. 1990).

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