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Research paper

Aptamer-mediated *Plasmodium*-specific diagnosis of malaria

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ABSTRACT

There is a critical need for better malaria rapid diagnostic tests to discriminate *Plasmodium falciparum* and *Plasmodium vivax* infection given the recent observation of HRP2 deletions in *P. falciparum* parasites. We previously identified a DNA aptamer, 2008s, that targets *P. falciparum* lactate dehydrogenase (PfLDH) and developed a sensitive aptamer-tethered enzyme capture (APTEC) assay. Here, we characterise two different LDH-binding DNA aptamers in their species-specific activities, then integrate within biochemical diagnostic assays and test in clinical samples. An enzyme-linked oligonucleotide assay demonstrated that aptamer pL1 bound with high affinity to both PfLDH and *P. vivax* lactate dehydrogenase (PvLDH), whereas aptamer 2008s was specific to PfLDH. An aptamer-tethered enzyme capture (APTEC) assay confirmed the specificity of 2008s in binding and capturing the enzyme activity of PfLDH which could be observed colorimetrically. In malaria patient samples, the 2008s APTEC assay was specific for *P. falciparum* blood samples and could discriminate against *P. vivax* blood samples. An aptamer for specific detection of falciparum malaria holds promise as a new strategy for species-specific malaria diagnosis rather than the conventional HRP2 immuno-assay.

1. Introduction

Malaria is caused by infection with parasitic protozoans belonging to the genus *Plasmodium*. Among the five *Plasmodium* species which are known to infect humans, *P. falciparum* and *P. vivax* are the most prevalent species. *P. falciparum* accounts for 99% of deaths. Although malaria is curable, falciparum malaria may progress to severe illness that often leads to death if there is no appropriate treatment within 24 h. In most endemic regions, chloroquine is the first line of treatment for vivax malaria whereas artemisinin-based combination therapy is advised for *P. falciparum* infections [1,2]. As there are differences in treatment, the World Health Organization (WHO) advocates a policy of "test, treat and track" for the management of malaria [2,3]. WHO recommends all suspected cases of malaria should be confirmed by microscopy or rapid diagnostic tests (RDTs) before treatment. Therefore, rapid diagnostic methods for differentiating *P. falciparum* infection from malaria caused by other species are critical to guide choice of the appropriate therapy. Conventional RDTs that specifically detect *P. falciparum* infection mainly rely on the immuno-detection of histidine rich protein 2 (HRP2). However, *P. falciparum* which does not express HRP2 was first observed in Peru [4], and *pfhrp2* deleted mutants have since been observed in more than ten countries [2,5–8]. A new rapid diagnostic approach to differentiate *P. falciparum* malaria from *P. vivax* malaria which does not depend on HRP2 is critically needed.

Nucleic acid aptamers may provide an innovative alternative solution for the development of *Plasmodium*-specific RDTs for malaria. Since their original discovery [9,10], aptamers have been recognised as bio-recognition molecules with advantages including ease of manipulation, ease of synthesis, high stability, high sensitivity and tailored specificity [11–14]. Aptamer-based detection is a fast-growing area of biosensing, often incorporating 3-D printing [15], electrochemistry [16] or lateral flow assays [17]. DNA aptamers targeting one of the most important *Plasmodium* diagnostic markers, *Plasmodium* lactate dehydrogenase (pLDH), have been identified [18–20]. Previously the crystal structures of two aptamers, 2008s and pL1, were determined in complex with their

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targets by ourselves and others [18,20]. Although both aptamers bind to *P. falciparum* lactate dehydrogenase (PfLDH) with affinities in the nanomolar range, their sequences and structures are completely different [18,20]. Fig. S1 illustrates the secondary structures of 2008s and pL1 derived from their tertiary structures. 2008s shows a unique distorted hairpin structure in complex with PfLDH, which extensively interacts with the PfLDH substrate specificity loop that is absent in human LDH (Fig. 1a). pL1 folds into a hairpin-bulge and recognises *P. vivax* lactate dehydrogenase (PvLDH) via shape complementarity (Fig. 1b). 2008s has been incorporated into gold nanoparticles [18,21], DNA origami tiles [22], silver nanoclusters [23], DNA tweezers [24], whereas pL1 has been incorporated on gold surfaces [25,26] demonstrating a diversity of proven applications for aptamers in malaria diagnosis. When 2008s forms a complex with PfLDH, the substrate specificity loop of PfLDH remains in an open conformation. As enzymatic activity is maintained, an aptamer-tethered enzyme capture (APTEC) colorimetric assay for the detection of PfLDH was developed [27].

In the APTEC assay, PfLDH catalyses the interconversion of L-lactate to produce reduced nicotinamide adenine dinucleotide (NADH) by using oxidised nicotinamide adenine dinucleotide (NAD^+) as cofactor. The NADH produced from the enzymatic reaction then is coupled to the reduction of a tetrazolium blue dye for generating a colorimetric response [28]. While blood, serum, urine and saliva are the most common biological samples for diagnosis, the complexity of these samples can affect the accuracy of diagnosis by non-specific binding [29]. The viscosity and background colour of the biological samples also are factors to be considered when developing a new diagnostic approach. To achieve higher sensitivity and selectivity, 3-acetylpyridine adenine dinucleotide (APAD), an oxidised nicotinamide adenine dinucleotide (NAD^+) analogue, is used as a cofactor in the assay. pLDH is almost 300 times more active than human LDH in the oxidation of L-lactate when using APAD as the cofactor. This differential response enables the detection of malaria parasite from human biological samples. Similar to the common enzyme-linked immunosorbent assays (ELISA), APTEC assay only captures the target protein and the unbound species are washed away. Therefore, the pigments and other interferences will be excluded by the washing steps. Moreover, APTEC assay does not require additional enzymes or antibodies to facilitate detection. This allows minimising of the workload of the healthcare worker as well as shortening of the time required for diagnosis. Our previous study indicated APTEC successfully detected PfLDH in clinical whole blood samples from patients with sensitivity and specificity comparable to a commercialised malaria RDT [27]. APTEC has significant potential to be developed as a new approach to malaria diagnosis that avoids the use of antibodies entirely.

In this study, we investigate the abilities of the 2008s and pL1 aptamers to discriminate *P. falciparum* from *P. vivax* lactate dehydrogenases for the foundation of a new rapid diagnostic approach to distinguish the species. First, we evaluate the specificities of 2008s and pL1 in recognising PfLDH and PvLDH by using enzyme-linked oligonucleotide assay (ELONA). We then determine the sensitivity and specificity of the 2008s aptamer in the APTEC assay by using recombinant PfLDH and PvLDH. Finally, we investigate the feasibility of applying the APTEC assay as an RDT for malaria using clinical samples. Results show that the 2008s aptamer is highly specific to PfLDH and *P. falciparum* infected patient samples. This is the first report of an aptamer that can be applied for specific detection of falciparum malaria rather than the conventional HRP2 antibody-based RDTs.

2. Materials and methods

2.1. Chemicals, recombinant proteins and oligonucleotides

All chemicals were purchased from Sigma-Aldrich (U.S.A.) unless specified. PfLDH and human LDH were expressed and purified according to our previous study [18]. PvLDH was expressed in *Escherichia coli* BL21 (DE3) pLysS harbouring the pET28a-PvLDH plasmid and further purified by HisTrap chromatography (GE Biosciences). 2008s and biotinylated 2008s with the sequence 5′ – ctgggcggtagaaccatagtgacccagccgtctac – 3′ and biotinylated pL1 with the sequence 5′ – gttcgattggattgtgccggaagtgctggctcgaac – 3′ were synthesised by Integrated DNA Technologies (U.S.A.).

2.2. ELONA

Proteins were 2-fold serial diluted in phosphate buffered saline with 0.05% Tween 20 (PBST) at concentrations of 0, 1, 2, 4, 9, 18, 36 and 71 nM which is 0, 0.16, 0.31, 0.63, 1.25, 2.5, 5 and 10 μg/mL respectively. 200 μL of serial diluted proteins were added to Ni-NTA HisSorb Strips (QIAGEN) and incubated at room temperature for an hour. Subsequently, wells were washed 3 times with 200 μL PBST, 200 μL of 50 nM of aptamer in the corresponding binding buffer was added to the well and incubated at room temperature for an

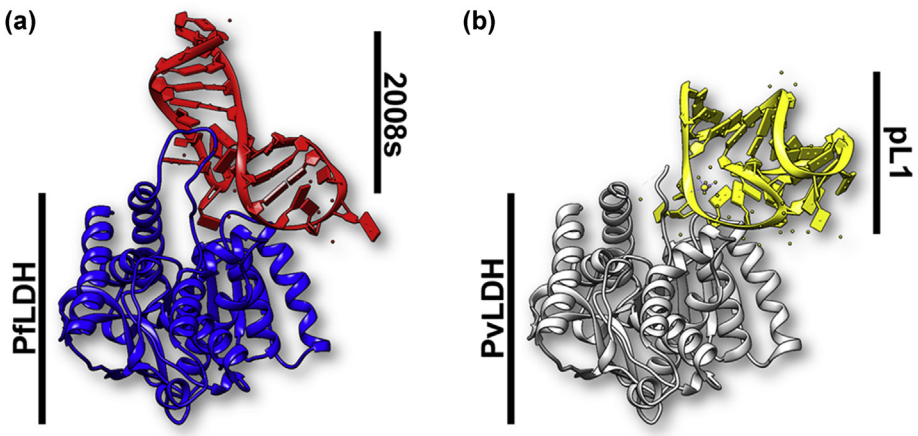

Fig. 1. Structure of aptamer-pLDH complexes. (a) 2008s-PfLDH complex (PDB ID Code: 3ZH2). 2008s (red) folds into a distorted hairpin and binds to PfLDH (blue) by extensively interacting with the substrate specificity loop. **(b)** pL1-PvLDH complex (PDB ID Code: 5HTO). pL1 (yellow) folds into a hairpin bulge in complex with PvLDH (white).

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