Loop-mediated isothermal amplification (LAMP) is a method for enzymatically replicating DNA that has great utility for clinical diagnosis at the point of care (POC), given its high sensitivity, specificity, speed, and technical requirements (isothermal conditions). Here, we adapted LAMP for measuring protein analytes by creating a protein-DNA fusion (referred to here as a “LAMPole”) that attaches oligonucleotides (LAMP templates) to IgG antibodies. This fusion consists of a DNA element covalently bonded to an IgG-binding polypeptide (protein L/G domain). In our platform, LAMP is expected to provide the most suitable means for amplifying LAMPoles for clinical diagnosis at the POC, while quantitative PCR is more suitable for laboratory-based quantification of antigen-specific IgG abundance. As proof of concept, we measured serological responses to a protozoan parasite by quantifying changes in solution turbidity in real time. We observed a >6-log fold difference in signal between sera from vaccinated versus control mice and in a clinical patient sample versus a control. We assert that LAMPoles will be useful for increasing the sensitivity of measuring proteins, whether it be in a clinical laboratory or in a field setting, thereby improving acute diagnosis of a variety of infections.

Numerous applications require sensitive measurement of biological analytes to provide actionable information in real time. In considering biomarker measurement, no other molecule is as easily or sensitively quantified as nucleic acid because of facile means to enzymatically replicate sequence-specific templates in vitro. The detection limit of specific DNA templates has been reported to be at or below 10 molecules when measured by real-time PCR (quantitative PCR [qPCR]) (1, 2). This level of sensitivity is useful in the context of a laboratory, but there is need for diagnostics that can be used at the point of care (POC) without thermocycling equipment. An alternative method for amplifying DNA is loop-mediated isothermal amplification (LAMP), which requires four different oligonucleotides to promote Bst polymerase-catalyzed DNA synthesis at a constant 65°C. The end products of cycling equipment. An alternative method for amplifying DNA is loop-mediated isothermal amplification (LAMP), which requires four different oligonucleotides to promote Bst polymerase-catalyzed DNA synthesis at a constant 65°C. The end products of LAMP (pyrophosphate and high-molecular weight DNA) can be monitored by measuring changes in turbidity or color with the addition of Mg²⁺ (3) or dyes (4), respectively.

Highly sensitive measurement of non-DNA analytes (e.g., proteins, toxins, lipids, and carbohydrates) at the POC can be achieved by combining the molecular recognition of immunogens with the signal amplification of LAMP. We previously developed means to label protein and small-molecule ligands with unique oligonucleotides measurable by PCR (5). We labeled molecular targets using a protein-DNA fusion known as a “Tadpole,” which binds ligand with high specificity so as to attach an identifying oligonucleotide. When we labeled antibodies or antigen with oligonucleotides, we created toxin and biomarker assays that were hundreds of times more sensitive than matched enzyme-linked immunosorbent assay (ELISA) (5–7). Compared to PCR, however, LAMP is more amenable to POC use because of reaction simplicity and product detection by visual readouts (8–11). In this regard, we previously advocated (12) that LAMP and Tadpoles be combined such that the DNA element of a Tadpole is amplified by LAMP instead of PCR. To investigate this concept, we created a protein-DNA fusion consisting of a polypeptide that binds mammalian IgG and a DNA element encompassing 1 PCR and 2 LAMP amplicons. During the construction of this fusion, we explored the activities of 10 different primer pair combinations for conducting LAMP and several of chemistries for conjugating protein and DNA. We further investigated the specificity of the LAMPole fusion for recognizing human IgG and, as a benchmark of this approach, applied it to measuring antiparasite antibodies in humans and mice. Our assay was inspired by an ELISA for detecting host antibodies to Trypanosoma brucei gambiense variable surface glycoproteins (VSGs), which detected titers of 1:30,000 and 1:40 from serum and saliva, respectively (13). This work indicated that detection of relatively low titers of antibodies in noninvasive specimen types is possible provided the method is sufficiently sensitive. Here, we present our findings. Rapid, accurate diagnosis of other proteins, such as antigen and IgM antibody, may enable...
sensitive and specific diagnosis of the etiology of acute illness at the POC.

MATERIALS AND METHODS

To construct the LAMPole fusion, we needed to design (i) the sequence of the DNA element and primer sets that specifically recognize this template, even in the presence of contaminating human or pathogen DNA, and (ii) a conjugation chemistry that bonds the DNA element to the polypeptide without inhibiting IgG-binding activity or interfering with the ability to promote LAMP. We designed 10 LAMP primer sets (4 different primers in each set) predicted to hybridize with elements in the *Arabidopsis thaliana* chalcone synthase (CHS)-coding sequence (GenBank accession number M20308.1). GC content (G/C rate), 5′ and 3′ gene positions, free energy (5′dG/3′dG), and melting temperatures (Tm) are indicated. Also shown are the forward and reverse primer sequences (5′ to 3′), along with the fluorescent hybridization probe sequence, used for real-time PCR.

LAMP / PCR Primers and Probes

Chalcone Synthase LAMP Primers (5′ to 3′ sequence)

| Label | CHS_ID:18 | CHS_ID:25 |
|-------|------------|------------|
| F3    | ACATGTGTCCCTATACCTGCT    | CACCAGACCCAGACCATCCTG    |
| B3    | GAAGCACTCTTGGTCTAAGC    | GGTGTGACATCATGAGAAACCC    |
| FIP   | TTGATGCTCCTTGCCTGCA    | CTGCCCAGCTTTAGTGAGTC    |
| BIP   | TTCTGCACTATCCCTGGGCTG    | GTCTCTGCACTACCTCCGC    |

**FIG 1** Primers sets developed for sequence-specific LAMP and real-time PCR. The LAMP primer set ID:18 and ID:25 sequences are shown going from 5′ to 3′ with the F3, B3, FIP, and BIP nomenclature derived from Notomi et al. (14). The annealing positions corresponding to the *A. thaliana* chalcone synthase (CHS) complete coding sequence (GenBank accession number M20308.1), GC content (G/C rate), 5′ and 3′ gene positions, free energy (5′dG/3′dG), and melting temperatures (Tm) are indicated. Also shown are the forward and reverse primer sequences (5′ to 3′), along with the fluorescent hybridization probe sequence, used for real-time PCR.

PCR Primers and Probe

Forward PCR primer: 5′-TGTTGCAAGCTTCCATAGC-3′

Reverse PCR primer: 5′-GATCTTCTGACTGGCGTGG-3′

PCR hybridization probe: 5′-CCTG+TCA(C|G)+TCTT([+N])LNA residue-3′

**FIG 2** The 5′-to-3′ sequence of the LAMP template. The annealing sites for the ID:18 F3 and B3 primers are underlined, while the annealing sites for the ID:25 F3 and B3 primers are shaded. The predicted melting temperature (Tm) for the F3 and B3 primers was fixed close to 60°C in each set to ensure CHS plasmid equally well.

DNA (18) with Picl and Nael to liberate an element with a single 3′ recessed terminus (Picl) and a single blunt end terminus (Nael). We incorporate an amino-allyl dUTP nucleotide at the 3′ recessed terminus using the 3′-to-5′ exon-Klenow fragment (New England Biolabs) for 30 min according to the manufacturer’s instructions. We converted this amino residue to an azide using 10:1 excess N-hydroxysuccinimide-azide (Pierce) in phosphate-buffered saline (PBS) for 1 h at 25°C and purified this element using a NAP-5 column (Life Technologies). To create our protein L/G chimera, we directed expression of the L/G mammalian antibody-binding polypeptide (19) fused to polyhistidine and the Methanobacterium thermotogae RIR1 intein (20) Rosetta-gami-B (DE3) pLysS (Novagen) as previously described (21). Briefly, we selected transformants in Luria-Bertani broth to an optical density (OD) of 0.6 at 600 nm, induced L/G expression with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h, and collected cells at 3,000 × g for 10 min at 4°C. We purified full-length L/G-intein fusion protein by immobilized metal affinity chromatography from cell-free lysates and cleaved the intein domain from L/G using 1 mM 2-mercaptoethanesulfonic acid *in vitro* (21). We labeled the purified L/G protein with phosphinic-N-hydroxysuccinimide (NHS) at a 1:5 protein/phosphinic stoichiometry in 0.1 M HEPES (pH 8.5) for 2 h at 25°C and buffer exchanged the phosphinic-labeled L/G protein into phosphate-buffered saline. We conjugated azide-modified CHS dsDNA to phosphinic-modified L/G protein for 6 h at 25°C to yield an L/G protein-DNA fusion (LAMPole) and purified fusions by cation exchange (Q-Sepharose) and hydrophobic chromatography (pHENYL-Sepharose) as described previously (5, 21).

Human African trypanosomiasis (HAT) is caused by *Trypanosoma brucei gambiense* (22). Our prototype LAMPole assay is based on a published ELISA that detects host antibodies against *T. b. gambiense* LiTat 1.3, 1.5, and 1.6 variable surface glycoproteins (VSGs) (13). To create the solid-phase substrate for use in our assays, we conjugated 2,8-μm tosyl-activated beads (InVitrogen) with either purified LiTat 1.3 VSG (23) or purified mouse total IgG (Jackson Immunologicals) in 0.1 M borate (pH 9.5)–1 M (NH4)2SO4–0.1 M NaCl for 16 h at 37°C. We capped unreacted tosyl residues with 0.1 M ethanolicamine for 2 h at 37°C and blocked unoccupied surface area with PBS containing 0.1% dephosphorylated casein and 0.05% Tween 20 (blocking buffer). The mouse sera used to optimize assay conditions were prepared from animals vaccinated with 20 μg purified VSG once a week for 3 weeks. No adjuvant was used. Sham (naive) controls received vehicle alone (PBS). For proof of concept, we also used archived serum obtained from a patient with stage 2 *T. b. gambiense* HAT confirmed by a positive card agglutination test for trypanosomiasis (CATT) and with trypanosomes identified on blood and cerebrospinal fluid (CSF) smears.

For our serological assays, we serially diluted sera from test samples and negative controls. We incubated 10° VSG-coated beads with 50-μl
serial dilutions of blocking buffer for 1 h at 25°C, washed the beads 3 times in 1 ml of casein-Tween-PBS by vortexing for 10 s, and then resuspended the beads with 50 μl of protein L/G LAMPole. After incubating for 1 h, we washed the beads 3 times with vortexing (as described above), resuspended the beads in 10 μl 10 mM Tris-HCl (pH 8.0)–0.05% Tween 20, and used 1 μl as the template for amplifying CHS templates by LAMP (25-μl total volume) at 63°C. Here, we monitored the production of insoluble Mg²⁺-pyrophosphate in real time using an LA-320C turbidimeter according to the manufacturer’s instructions. For instances in which we used real-time PCR to quantify the amount of LAMPole bound to beads (affinity measurements), we used a fluorescent hydrolysis probe containing locked nucleic acid (LNA) nucleotides (5′–CCT|+T|[CA]+C|TTCT, where [+]N is a LNA residue). For these measurements, we thermocycled bead-bound templates for 15 s at 95°C, 20 s at 60°C, and 60 s at 72°C. The quantities of LAMPole present in these samples were calibrated by measurement of known numbers of CHS template with an r² value of 0.9988 over a 6-log range in concentration. We used the four-parameter logistic equation in the software package Prism v4.0 (GraphPad Software, Inc.) to fit a curve describing LAMPole activity to calculate the binding affinity constant according to previously published methods (5, 21).

RESULTS

We designed primer sets that specifically annealed and amplified a 253-nucleotide (nt) element of the A. thaliana chalcone synthase (CHS) gene (i) even in the presence of contaminating human or pathogen DNA and (ii) conjugated to the protein L/G polypeptide without inhibiting IgG-binding activity or interfering with the ability to promote LAMP. We identified 2 primer sets out of 10 tested against A. thaliana chalcone isomerase (CHI) plasmid and genomic DNAs from several protozoan parasites as negative controls, including Babesia microti, Plasmodium falciparum, Toxoplasma gondii, E. coli, Rickettsia parkeri, and bacteremic human patients, and normal human blood and assessed LAMP by monitoring turbidity in real-time. We used primer set ID:18 to direct LAMP in these reactions.

We previously used this “L/G” domain to construct a probe with PCR readout and quantified as few as 100 fg pET32(a)CHS as positive control (roughly 12,000 template molecules), ID:25 generated a more rapid LAMP reaction. We confirmed the primers to be 100% specific for A. thaliana CHS when tested against A. thaliana chalcone isomerase (CHI) plasmid and genomic DNAs from several protozoan parasites as negative controls, including Babesia microti, Plasmodium falciparum, African trypanosomes (T. b. brucei, T. b. rhodesiense, and T. b. gambiense) (Fig. 3), and Toxoplasma gondii (not shown). DNA from Rickettsia prowazekii, normal human blood (Fig. 3), and Escherichia coli and blood from bacteremic patients (not shown) and also gave negative results. We considered precipitate occurring after 60 min to be an artifact. Importantly, the amount of time needed to develop a visual turbidity of ≥0.1 OD unit in these experiments was inversely proportional to the amount of pET32(a)CHS template, demonstrating that the signal is quantitatively proportional to the abundance of template (Fig. 4). Fortuitously, the ID:18 and ID:25 amplicons overlap in sequence, which allowed us to finalize our LAMPole DNA design to include both overlapping amplicons and an internal PCR amplicon containing a hybridization site for a locked nucleic acid (LNA) (24) hydrolysis probe (see Materials and Methods).

To construct the antibody-binding protein domain, we used a recombinant protein originally created from the immunoglobulin-binding domains of Streptococcus sp. protein G (which binds mammalian immunoglobulin G [IgG] Fc chains) and Peptostreptococcus sp. protein L (which binds mammalian immunoglobulin κ and λ light chains) (19). We previously used this “L/G” domain to construct a probe with PCR readout and quantified as few as
To visualize a specific antigen-specific antibodies, we measured levels of IgG that recognize targets in serological assays for HAT (13, 22). For all these assays, patient sera were compared to those from naive controls (n = 2) are shown.

We initially formulated assay conditions using sera from naive and VSG-vaccinated mice. We incubated 10⁶ VSG-coated magnetic beads with mouse sera and measured the amount of bound antibody using the ID:25 primers (Fig. 1) to amplify the LAMPole DNA element. When tested neat, only sera from VSG-vaccinated mice (n = 4) yielded LAMP-dependent turbidity, in contrast to naive controls (n = 2). We also serially diluted sera from one immune mouse and one nonimmune mouse (in 1:10 steps) to 1:10⁻⁶ and found no loss of anti-VSG antibody signal at maximal dilution (Table 1). However, after further dilution, anti-VSG antibodies were detected in sera diluted to at least 1:10⁻⁶. Based on these findings, we incubated VSG-coated beads with the sera of a patient with HAT and a healthy control (Fig. 6). We detected anti-VSG antibodies in maximally diluted (1:10⁻⁷) serum from a patient with stage 2 HAT but not in serum from the healthy control. Thus, we observed a >6- to 7-log fold difference in signal between sera from vaccinated versus control mice and from a stage 2 HAT patient versus a control.

As proof of concept that our LAMPole could be used to assay antigen-specific antibodies, we measured levels of IgG that recognize a specific T. b. gambiense antigen, variable surface glycoprotein (VSG), in the sera of vaccinated mice and a stage 2 HAT patient. Various VSGs have traditionally been selected as antigenic targets in serological assays for HAT (13, 22). For all these assays, we scavenged antiparasite IgG from sera using LiTat 1.3 VSG-coated magnetic beads and detected bound IgG by forming a sandwich with our anti-IgG LAMPole (Fig. 5). Using spike and recovery assays, we previously confirmed that no substances in human or mouse serum interfered with the use of L/G-DNA fusions to measure IgG (21), and we found the same to be true in these experiments. We accounted for cross-reactive binding of IgG to the beads and nonspecific binding of the LAMPole by subtracting the baseline signal obtained in replicates with neither IgG nor antigen, and we confirmed equivalently low levels of non-specific signal, even in negative-control serum. We formulated assays with 0.576 mg/ml LAMPole (10-fold Kₐ) to ensure that at least 90% of available epitopes are occupied during measurement. We initially formulated assay conditions using sera from naive and VSG-vaccinated mice. We incubated 10⁶ VSG-coated magnetic beads with mouse sera and measured the amount of bound antibody using the ID:25 primers (Fig. 1) to amplify the LAMPole DNA element. When tested neat, only sera from VSG-vaccinated mice (n = 4) yielded LAMP-dependent turbidity, in contrast to naive controls (n = 2). We also serially diluted sera from one immune mouse and one nonimmune mouse (in 1:10 steps) to 1:10⁻⁶ and found no loss of anti-VSG antibody signal at maximal dilution (Table 1). However, after further dilution, anti-VSG antibodies were detected in sera diluted to at least 1:10⁻⁶. Based on these findings, we incubated VSG-coated beads with the sera of a patient with HAT and a healthy control (Fig. 6). We detected anti-VSG antibodies in maximally diluted (1:10⁻⁷) serum from a patient with stage 2 HAT but not in serum from the healthy control. Thus, we observed a >6- to 7-log fold difference in signal between sera from vaccinated versus control mice and from a stage 2 HAT patient versus a control.

**DISCUSSION**

Our strategy to measure protein analytes (e.g., pathogen-specific host antibodies) by LAMP is to use an adaptor probe that attaches DNA oligonucleotides to the protein analyte to enable sensitive and specific molecular detection of the protein-DNA LAMPole. We found that we could measure protein analytes using LAMP as a signal amplifier. Importantly, our goal was not to formulate a better test for HAT, per se, but to demonstrate the LAMPole concept as a technological advance applicable to improving the measurement of a wide variety of protein analytes or pathogens with high sensitivity and low cost. This is the first instance that we are aware of that LAMP is used for protein detection.

**FIG 4** Quantitative LAMP of CHS templates. Based on data in Fig. 1, the amount of time necessary to accumulate turbidity above the threshold of detection (0.1 OD unit) is inversely proportional to the amount (100 fg to 1 ng) of starting A. thaliana CHS plasmid DNA template. The individual data points (n = 2) are shown.

**FIG 5** LAMPole-based measurement of antigen-specific IgG. Measuring antigen-specific serology is a four-step process. (1) Capture. Antigen-coated beads (LiTat 1.3 VSG) are incubated with serum samples containing anti-VSG IgG (Ab). (2) Detect. After nonspecific IgG is washed away, bound IgG is detected by adding LAMPoles to create a molecular sandwich. (3) LAMP. The LAMPole sandwich is subjected to Bst polymerase-catalyzed LAMP to yield amplified high-molecular-weight DNA and insoluble pyrophosphate in complex with Mg²⁺. Here, the abundance of LAMPole retained on the bead surface is proportional to the amount of specific anti-VSG IgG. (4) Visualize signal. Insoluble precipitate may be monitored in real time, or DNA may be directly visualized using intercalating dyes or gel electrophoresis.
aware of in which a protein-DNA fusion has been used to combine LAMP with immunoassays to measure protein analytes.

In its fully developed form, we expect that this technology may be used in field situations without electricity, but for the sake of this benchmark, we chose to detect turbidity using a turbidimeter for increased precision. We envisioned the “field-deployed” assay to be a two-step process in which a relevant biomarker of interest (e.g., antigen) present in a patient’s sample (e.g., blood, serum, saliva, urine, etc.) is captured onto magnetic beads coated with a specific capture agent (e.g., capture antibody). The bound biomarker is then detected by addition of LAMPole to the beads, and a visual signal is developed by supplementation with primers and Bst polymerase. The inclusion of overlapping amplicons in the DNA element of the LAMPole enables the use of two separate LAMP primer sets to generate signal output and may be confirmed using an orthogonal amplification processes such as qPCR for more precise quantification when needed. However, our LAMPole fusion may also be embedded into existing and future microfluidic platforms to provide signal amplification in situ.

While a colorimetric or turbidity output visible to the naked eye could be used instead of microtiter plate readers in resource-poor settings, this visual readout could also be useful in developed

### TABLE 1 LAMPole detection of VSG antibodies in immune mouse serum

| Expt\(^a\) | Serum source | LAMP primer set | Result\(^b\) with serum dilution: |
|----------|-------------|----------------|------------------|
|          |             | CHS_ID:25      | Neat  | 10\(^{-1}\) | 10\(^{-2}\) | 10\(^{-3}\) | 10\(^{-4}\) | 10\(^{-5}\) | 10\(^{-6}\) | 10\(^{-7}\) |
| A        | Vaccinated mouse | CHS_ID:25      | +/-   | +/-      | +/-     | +/+     | +/-     | +/-     | +/-     | ND      |
|          | Control mouse  |               | -/-   | -/-      | -/-     | -/-     | -/-     | -/-     | -/-     | ND      |
| B        | Vaccinated mouse | CHS_ID:25      | +/-   | +/-      | +/-     | +/-     | +/-     | +/-     | +/-     | -/-     |
|          | Control mouse  |               | -/-   | -/-      | -/-     | -/-     | -/-     | -/-     | ND      | ND      |

\(\text{ND}\) stands for not done.

\(^a\) The same serum samples were used in experiments A and B.

\(^b\) Duplicate test samples were used. -/-, negative result; +/-, positive result; ND, not done.

![FIG 6](http://cvi.asm.org/ on July 20, 2018 by guest)
countries for POC diagnosis. When quantitative analysis is important, real-time LAMP is also available.

The cost of LAMPole synthesis makes it possible to create a wide array of probes for detecting all antigenic varieties expressed by infectious agents that undergo antigenic variation, such as the trypanosome parasites that cause HAT. Using our originally published method for making Tadpoles that employed chemically synthesized DNA elements (5), we estimate a reagent cost of about $1.00 per assay. However, using the Staudinger ligation as outlined here, we estimate a reagent cost of about $0.0075 per assay. Although this cost consideration is less important in developed countries that can afford higher-priced methods, this issue is significant in resource-limited countries where cost alone may prevent test availability. Moreover, the modular nature of LAMPole synthesis allows new probes to be easily reconfigured, which may accommodate a multitude of antigenic variants corresponding to geographic location.

Several multiplex LAMP methods are available (29, 30), which may be applied to amplifying mixtures of our LAMPole probes and to simultaneously measure different target biomarkers in a clinical sample. Using technologies that we describe here, the creation of antibody LAMPoles for antigen detection should now also be possible. Together with intelligent LAMPole tail and LAMP primer design, the creation of a single diagnostic LAMP-based test for detection of host antipathogen antibodies (i.e., protein L/G LAMPoles), pathogen antigens (antibody LAMPoles), and pathogen DNA or RNA (LAMP or RT-LAMP) in a single assay format should now be possible to increase the likelihood of identifying the cause of the acute infection (increase sensitivity). For example, an ideal LAMPole diagnostic test for acute dengue might detect NS1 antigen, viral RNA, and IgM antibody; if it also detected IgG, it could rapidly distinguish secondary from primary dengue, which could have prognostic implications (higher risk of severe dengue).

By merging LAMP and Tadpoles, we created a method that may improve pathogen detection at the POC (7, 21). We previously showed that the usefulness of traditional LAMP assays that recognize multicycle gene targets for pathogen DNA detection can be dramatically enhanced by sample pretreatment to lyse or solubilize the pathogen(s) and facilitate release of its DNA prior to assay, especially when the sample size is limited (31). Similar modifications could also be applied to the LAMPole-based assays described here. Furthermore, we predict that LAMPoles will be a suitable platform for the development of noninvasive saliva- and potentially lachrymal (tear) fluid-based assays (32, 33), with sensitivities equivalent to those found with blood or CSF. Therefore, the use of LAMPoles could provide cost-effective, ultrasensitive antigen/antibody diagnostic assays with low technical requirements, which could improve acute diagnosis of neglected tropical and other infections that plague the developing world and thereby related clinical outcomes.

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D.J.G. conceived of the project. I.E.B., S.T.P., S.M., and D.J.G. discussed and designed the experiments. D.J.G., O.V.N., K.Y., S.T.P., M.S., and I.E.B. performed the experiments. I.E.B., K.Y., O.V.N., S.T.P., M.S., S.B., M.E.R., and D.J.G. analyzed the data and made comments on the manuscript. I.E.B. and D.J.G. wrote the manuscript.

We declare no competing financial interests.

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