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Source: Journal of Parasitology, 99(5):923-926. 2013.
Published By: American Society of Parasitologists
DOI: http://dx.doi.org/10.1645/12-132.1
URL: http://www.bioone.org/doi/full/10.1645/12-132.1

J. Parasitol., 99(5), 2013, pp. 923–926

Amplification-Free Detection of *Cryptosporidium parvum* Nucleic Acids with the Use of DNA/RNA-Directed Gold Nanoparticle Assemblies

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ABSTRACT: This study describes the development and evaluation of an amplification-free molecular assay for detection of *Cryptosporidium parvum* oocysts. The assay employed a pair of oligonucleotide-functionalized gold nanoparticle (AuNP) probes that were complementary to adjacent sequences on *C. parvum* 18s rRNA. Hybridization of the probes to the target RNA resulted in the assembly of AuNPs into target-linked networks, which were detected both visibly and spectroscopically, by a redshift in the wavelength of light scattered by the gold nanoparticles. The limit of detection was between 4×10^5 and 4×10^6 copies of RNA per microliter reaction mix, when a short synthetic target or full-length in vitro transcribed target was employed. With total nucleic acids purified from *C. parvum* oocysts spiked into 100-mg stool, as few as 670 oocysts/μl reaction mix were detected. The ability to detect the nucleic acids of *C. parvum* oocysts in stool, without the need for complex amplification, offers unique advantages for such AuNP aggregation assays to be extended toward use in resource-limited settings where protozoan detection is needed most.

Protozoan parasites of the genus *Cryptosporidium*, primarily *Cryptosporidium parvum* and *Cryptosporidium hominis*, are increasingly recognized as common causes of persistent and chronic diarrhea in children, as well as immune-deficient adults worldwide (Lima and Guerrant, 1992; Xiao et al., 2001; Saksirisampant et al., 2009) contributing to the high diarrhea-related morbidity and mortality in these populations (White, 2010; Lozano et al., 2012). Current diagnostic methods include microscopic examination of stool for *Cryptosporidium* species oocysts with acid-fast stains (modified Ziehl-Neelsen, Auramine-O, or Kinyoun's), direct fluorescent-antibody (DFA) tests, antigen-based enzyme immunoassays (EIAs), PCR, and lateral-flow immunochromatographic "strip" tests (Johnston et al., 2003; White, 2010). The sensitivity of these tests range from 10^3 to 10^5 oocysts/g of stool, with PCR being the most sensitive and acid-fast microscopy the least, often requiring examination of multiple stool samples in order to report a negative test result (Weber et al., 1991; Huang and White, 2006; Parr et al., 2007; White, 2010; Calderaro et al., 2011; Centers for Disease Control, 2013). With the exception of lateral-flow tests, which provide the advantage of a simple qualitative result (Garcia et al., 2003), existing diagnostic techniques remain confined to clinical laboratories because of the high instrumentation and reagent costs, and the need for a highly skilled technician or pathologist.

Previously, we described a DNA-linked gold nanoparticle assay strategy to detect *C. parvum* heat-shock protein 70 (Javier et al., 2009) based upon the distance-dependent optical properties of gold (Mirkin et al., 1996; Storhoff et al., 2004). In the current study, we have broadened the selection of nucleic acid targets to encompass those that are expressed at high copy numbers, such as 18s ribosomal RNA transcript with approximately 350,000 copies per oocyst (Deere et al., 1998), in order to provide molecular-level detection without amplification. Performance of this *Cryptosporidium parvum* AuNP aggregation assay was evaluated with 3 key targets, including a short 42-base oligonucleotide, a full-length in vitro transcribed 18s RNA, and total nucleic acids purified from *C. parvum* oocysts in stool.

The overall assay sequence is illustrated in Figure 1. In this approach, formalin-fixed stool samples containing *C. parvum* oocysts (Iowa isolate from experimentally infected calves, Waterborne Inc.; New Orleans, Louisiana) were boiled in guanidinium thiocyanate-based lysis buffer for 20 min, releasing nucleic acids and other cellular contents into solution. Total nucleic acids were then purified with commercially available magnetic beads (50 μl elution volume, without optional DNA digestion; MagMAX™-96 Total RNA Isolation Kit; Ambion, Austin, Texas) yielding a mixture of DNA/RNA from the oocysts, as well as other stool components. *Cryptosporidium parvum*–specific sequences were detected in this mixture with the use of a pair of functionalized AuNP probes coated with either probe A or B DNA/RNA sequences (Table I) that recognized adjacent regions on the *C. parvum* 18s RNA transcript (NCBI GenBank AF108865.1, bases 444–485). AuNP-probes were prepared as previously described with the addition of an illustra NAP™-5 column (GE Lifesciences, Piscataway, New Jersey) to clean the thiol-reduced oligonucleotides prior to incubation with colloidal gold (Javier et al., 2009). Hybridization of the AuNP probes to the *C. parvum* target RNA was carried out in a thermal cycler at 70 C for 15 min, followed by 37 C for 1 hr, and then allowed to cool slowly to room temperature for an additional hour in hybridization buffer (16% dextran sulfate and 20% formamide). Optical light scattering detection of the target-linked AuNP aggregates was accomplished by spotting 1 μl of the reaction mix onto a side-illuminated glass slide as previously described (Javier et al., 2009). The reaction was considered positive when a visible redshift in the wavelength of light scattered by the nanoparticles occurred, from green (~550 nm) in the singlet state to red (>650 nm) in target-linked assemblies (Fig. 1).

A summary of the AuNP aggregation assay results obtained with targets of increasing complexity are shown in Table II. The detection limit with DNA-oligo–coated AuNP probes was between 4×10^6 and 4×10^7 copies of short and full-length RNA target, respectively. The use of RNA-oligo probes reduced the detection limit by 1 order of magnitude to 4×10^5 and 4×10^6 copies of these RNA targets. Improvement in the detection limit associated with RNA-coated AuNP probes was likely due to the increased stability of RNA duplexes over DNA/RNA hybrid duplexes (Lesnik and Freier, 1995). Light-scatter images and spectral data from a representative assay are shown in Figure 2. A visible transition from green–orange–red scattering occurred at increasing target concentrations (Fig. 2A) and was accompanied by the emergence of a spectral peak above 650 nm in the presence of as few as 4×10^5 target copies (Fig. 2B). Dual peaks in the AuNP spectra were indicative of a reaction containing both aggregated and unaggregated AuNP-probes when relatively few target RNAs were present. A standard curve of the scattering ratio at 665/555 nm versus copy number revealed a nearly 2-decade linear range over which the AuNP assay could be quantitative (Fig. 2C); although, for the purposes of *Cryptosporidium* spp. diagnostics a binary (present or not-present) determination would likely be sufficient. Assay reproducibility with these RNA standards was good (>75%; Table II) with differences in target and AuNP-probe preparation, sample mixing, and spotting all potential sources of interassay variation (Hurst et al., 2006).

When oocysts were spiked into stool (1×10^3 to 1×10^7 total oocysts in ~100 mg emulsified stool) or buffer control, *C. parvum* 18s rRNA was

DOI: 10.1645/12-132.1

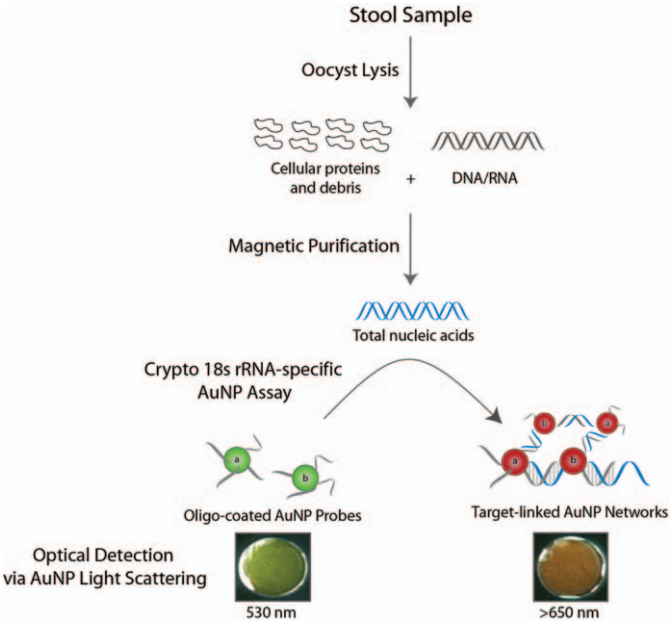

FIGURE 1. *Cryptosporidium* amplification-free AuNP assay schematic.

adequately detected in purified nucleic acids from as few as 670 oocysts per microliter of the AuNP reaction mixture, regardless of DNA/RNA-oligo probes used (Table II; Fig. 3). Results were reported per unit volume following nucleic acid purification and elution. When adjusted for the final reaction conditions, this 670 oocysts/μl corresponds to approximately 1×10^6 oocysts/g of stool, establishing the minimum threshold for detection of *C. parvum* oocysts with the use of the target-linked AuNP assay. Unfortunately, this was nearly 20-fold higher than anticipated based upon the literature value of 350,000 18s rRNA copies per oocyst (Deere et al., 1998). Theoretically, with an assay detection limit of 4×10^6 copies of full-length 18s rRNA target in 1 μl of reaction mixture, an initial target concentration of 1.2×10^7 copies/μl would be required (accounting for a 1:3 dilution in final reaction mix). Dividing this minimum target concentration needed (1.2×10^7 copies/μl) by the number of 18s rRNA copies present in each oocyst (3.5×10^5 copies/oocyst) suggests that as few as 34 oocysts/μl were theoretically detectable. A number of factors, alone or in combination, could account for these differences, including incomplete cell lysis, loss of RNA during purification, or RNA degradation. Regardless, the reported assay threshold of 1×10^6 oocysts/g does fall within the clinical range of oocyst shedding in patients with *Cryptosporidium*-related diarrhea (mean approximately 2×10^6 oocysts/ml; range 8×10^2–1×10^7 oocysts/ml; assuming roughly 1 g ≈ 1 ml for watery stool) (Bushen et al., 2007), albeit at the higher end of the range.

Specificity tests revealed little to no cross reactivity between the AuNP assay and *Giardia lambia* (ATCC; Manassas, Virginia) 2 pathogenic strains of *Escherichia coli* (O157:H7 and O127:H6), or commensal *E. coli* (K-12) (data not shown). However, the RNA-coated AuNP probes showed a very slight yellow/orange color shift in the presence of *Giardia* RNA, suggesting that the RNA-oligo probes may offer greater sensitivity, but with slightly less specificity than DNA-oligo probes. A combination of probes could ultimately be used to achieve the high sensitivity and high specificity needed for clinical assays. Other parasite genera found in stool, such as *Isospora*, *Cyclospora*, and *Entamoeba*, exhibited <50% sequence identity to the *C. parvum* 18s RNA transcript region recognized by the AuNP probes; therefore, no cross-reactivity would be expected with these organisms, either. However, the current assay would be expected to detect *C. hominis* because of the 99% sequence identity between *C. hominis* and *C. parvum* species in the targeted 18s RNA region.

The long-term goal of this research is to develop a point-of-care (POC) test for diarrhea-causing protozoan pathogens. The DNA/RNA-linked AuNP assay presented herein is well suited for point-of-care applications because it offers a straightforward readout (a color change that can be visually or quantitatively monitored) without the need for complex enzyme-based amplification, and utilizes relatively low-cost, robust reagents (Javier et al., 2009). In addition, the AuNP aggregation assay offers an advantage over existing molecular techniques, in that it can be performed on formalin-fixed stools. However, there are a number of additional steps needed in order to make the leap toward point of care, many of which are currently being explored including reagent lyophilization, microfluidics integration onto a custom POC platform, and use of a handheld optical analyzer. In addition, techniques to concentrate oocysts from a patient's stool will likely be needed in order to enhance sensitivity of the DNA/RNA-linked AuNP aggregation assay. Such techniques could

TABLE I. DNA/RNA probe and synthetic target sequences.

Oligonucleotide	Sequence
Probe A–DNA oligo	5′-thiol-PEG-A_{15}-ATTGTTATTTCTTGTCACTAC-3′
Probe A-RNA oligo	5′-thiol-PEG-A_{15}-AUUGUUAUUUCUUGUCACUAC-3′
Probe B–DNA oligo	5′-thiol-PEG-A_{15}-TACAAAACCAAAAAGTCCTGT-3′
Probe B–RNA oligo	5′-thiol-PEG-A_{15}-UACAAAACCAAAAAGUCCUGU-3′
42-nt RNA Target	5′-GUAGUGACAAGAAAUAACAAUACAGGACUUUUUGGUUUUGUA-3′

TABLE II. Summary of *Cryptosporidium parvum* AuNP assay performance.*

	Assay target				
Probe	42 nt. Synthetic (copies/μl)	Full length (copies/μl)	DNA/RNA from oocysts in PBS (oocysts/μl)	DNA/RNA from oocysts in stool (oocysts/μl)	Crude lysate (oocysts/μl)
DNA-oligo AuNP	4×10^6 (75%)†	4×10^7 (80%)‡	670	670	7,500
RNA-oligo AuNP	4×10^5	4×10^6	670	670	750

* Detection limits reported are the lowest concentration tested with a positive aggregation signal at >650 nm.
† Reproducibility across multiple assays; $n = 8$.
‡ $n = 5$.

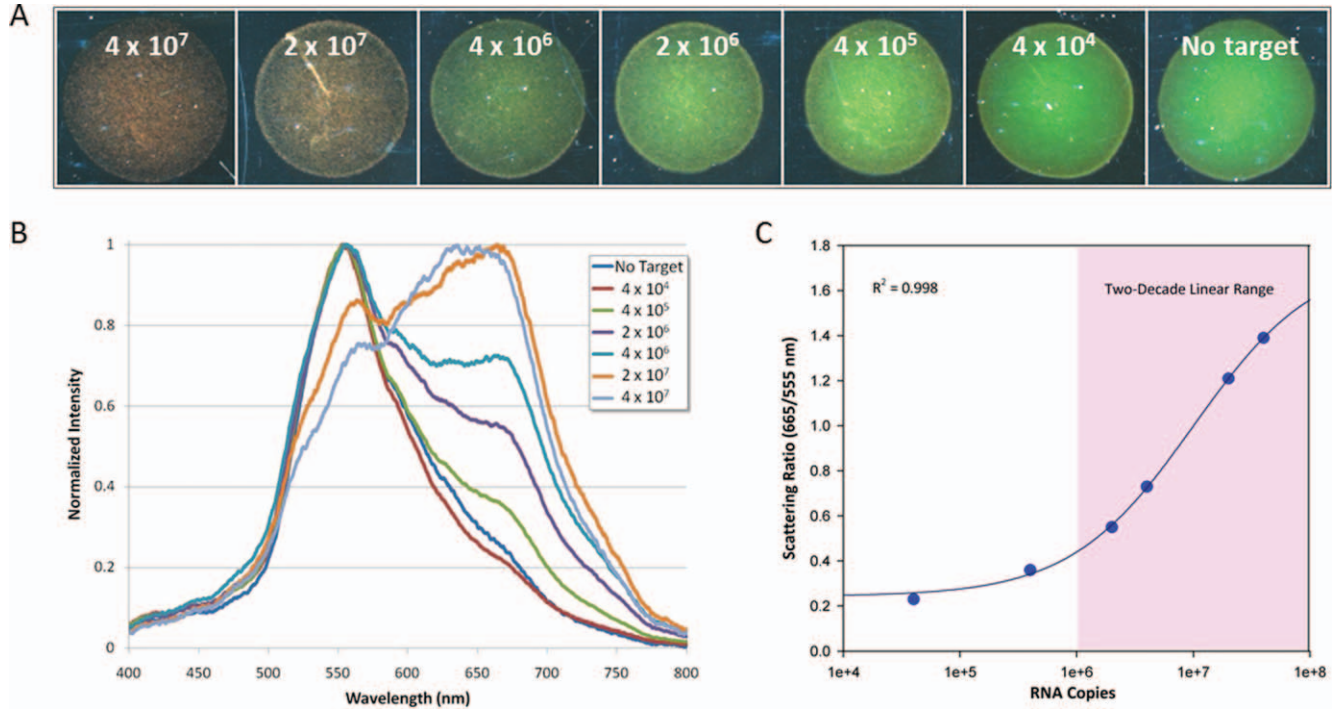

FIGURE 2. RNA target-linked AuNP aggregates detected via (**A**) light scatter imaging and (**B**) spectroscopy. (**C**) Standard curve of the light-scattering ratio at 665/555 nm versus copy number (fit to 4-point logistic curve; $r^2 = 0.998$).

potentially be integrated into the AuNP assay workflow and may include size-selective filtration or immunomagnetic separation. Use of a selective concentration method, in particular, could provide not only concentration of the *Cryptosporidium* oocysts, but isolation and purification from the extraneous stool matter/debris which is highly light scattering and interferes with AuNP aggregate detection. In the absence of these stool particulates, it is possible that nucleic acid purification would not be necessary and that crude cell lysates from isolated *Cryptosporidium* spp. oocysts could be directly interrogated with the DNA/RNA-oligo coated AuNP probes. We briefly explored the feasibility of this approach by boiling purified oocysts and harvesting the crude lysate as the starting material in the AuNP aggregation assay. The characteristic green-to-red color change and >650-nm spectral shift was detected in crude lysate samples containing less than 1,000 purified oocysts (Table II). Thus, additional functionality could be achieved without increasing the total number of steps in the overall assay scheme. It is anticipated that such preprocessing techniques will enhance the capability of the DNA/RNA-linked AuNP assay for rapid, amplification-free detection of diarrhea-causing intestinal protozoans with high sensitivity and specificity.

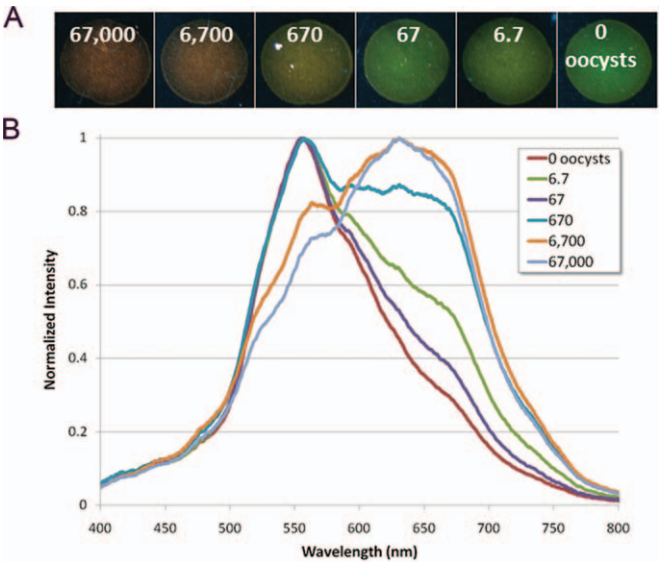

FIGURE 3. (**A**) Light scatter images and (**B**) spectra from a representative AuNP assay performed with total nucleic acids purified from *Cryptosporidium parvum* oocysts in stool.

The authors would like to thank Dr. Alfredo Torres and Dr. Douglas Botkin for providing *E. coli* bacteria and for funding from the NIH/NIAID supported Western Regional Center of Excellence for Biodefense and Emerging Infectious Diseases Research (Grant U54A1057156). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Allergy and Infectious Diseases or the National Institutes of Health. All human specimens were collected under a protocol (IRB 08-17X), which was reviewed and approved by the Rice University Institutional Review Board.

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