Research Note

An Oligonucleotide-Ligation Assay for the Differentiation between *Cyclospora* and *Eimeria* spp. Polymerase Chain Reaction Amplification Products

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ABSTRACT

An oligonucleotide-ligation assay (OLA) was developed and compared to a restriction fragment length polymorphism (RFLP) test for distinguishing between 294-bp polymerase chain reaction (PCR) amplification products of the 18S rRNA gene from *Cyclospora* and *Eimeria* spp. The PCR/OLA correctly distinguished between three *Cyclospora*, three *E. tenella*, and one *E. mitis* strains and the ratio of positive to negative spectrophotometric absorbance (A900) values for each strain ranged from 4.086 to 15.280 (median 9.5). PCR/OLA provides a rapid, reliable, spectrophotometric alternative to PCR/RFLP.

The coccidian parasite, *Cyclospora cayetanensis*, can cause prolonged diarrhea illness in immunocompromised and immunocompetent individuals (8, 9). More than 1,000 cases of cyclosporiasis were confirmed in 15 states, the District of Columbia, and Canada during the spring and summer of 1996, although only three outbreaks were reported previously in the United States (4).

A nested polymerase chain reaction (PCR) assay amplifies a *Cyclospora* 18S rRNA gene region but not that of human-associated *Babesia microti*, *Neospora*, *Cryptosporidium parvum*, *Toxoplasma gondii* or *Bordetella* (10, 12). However, the same molecular-sized product is amplified from the closely related genus *Eimeria*, and the amplified region exhibits 94 to 96% sequence similarity to *C. cayetanensis* (12).

*Eimeria* spp. may infect many nonhuman vertebrate animal hosts and be present in food and environmental samples. Therefore, from a public health perspective it is important to establish when using these primers, whether the amplification product is from the human pathogen *Cyclospora* or the member of the nonhuman pathogenic *Eimeria* genus.

A restriction fragment length polymorphism (RFLP) analysis using the restriction enzyme *MnlI* distinguishes between *Cyclospora* and *Eimeria* spp. based on the few nucleotide differences in the amplified region (5). In the present study we evaluated an oligonucleotide-ligation assay (OLA) test format to identify a single nucleotide difference and distinguish between amplification products from these two genera.

OLA is a technique that can specifically detect single base-pair differences (7) and uses two oligonucleotides, a capture probe (5’ biotinylated) and a reporter probe (3’ end-labeled with digoxigenin). The probes hybridize to adjacent positions on the target DNA. DNA ligase covalently joins the 5’ biotinylated capture probe to the 3’ digoxigenin reporter probe only if exact complementarity at the junction between the probes exists. Streptavidin immobilized on a solid support is used to capture the biotin-labeled oligonucleotide and the covalently bound, digoxigenin-labeled oligonucleotide is detected by standard colorimetric methods.

MATERIALS AND METHODS

Source of oocysts. Oocysts from three strains of *Cyclospora* were received in 2.5% potassium dichromate from Ynes Ortega (University of Arizona, Tuscon, Ariz.), Jeffery Bier, Food and Drug Administration (FDA) Center for Food Safety and Nutrition (CFSAN), Washington, D.C., and from Mike Arrowood and Mark Eberhard (Centers for Disease Control, Atlanta, Ga.) via Kathy Craven (FDA, Atlanta, Ga.) and stored at 4°C. Oocysts from three *Eimeria tenella* strains and one *E. mitis* strain in distilled water were received frozen from Patricia Allen (U.S. Department of Agriculture, Beltsville, Md.) and stored at −20°C.

PCR procedure. Template DNA was released from approximately 100 oocysts by a freeze–thaw method (6) for three *Cyclospora* strains, three *E. tenella* strains, and one *E. mitis* strain. Nested primers that amplify a region of the 18S rRNA described by Relman et al. (10) were synthesized without the restriction site leader (GenoSys, The Woodlands, Tex.): mCYC1F 5’TACCCATGAAAACAGTTT3; mCYC2R 5’CAGGAGAAAGCAGAGGTAGG3; mCYC3F 5’CTTCCGCGCTTCGCTGG3; and mCYC4R 5’CGTCTTAAACCCCCTACTG3. The second-round PCR amplification product has a predicted size of 294...
TABLE 1. Cyclospora OLA oligonucleotide sequences

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Function/label</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYCU</td>
<td>Capture probe/5’ biotin label</td>
<td>TTT GTT GGT TTC TAG GAC CG</td>
</tr>
<tr>
<td>EIMU</td>
<td>Capture probe/5’ biotin label</td>
<td>TTT GTT GGT TTC TAG GAC CA</td>
</tr>
<tr>
<td>CYCEIMD</td>
<td>Reporter probe/5’ phosphorylated; 3’ digoxigenin end label</td>
<td>AGG TAA TGA TTA ATA GGG AC</td>
</tr>
</tbody>
</table>

bp rather than 308 bp. The nested PCR was conducted as described previously (10) using DNA equivalent to 5 to 50 oocysts. Negative controls contained all reaction components except DNA template. Second-round PCR amplification products were detected by electrophoresis in a 2% agarose gel containing 0.4 µg/ml ethidium bromide.

RFLP analysis. The PCR products were digested with MnlI (Amersham Life Sciences Inc., Arlington Heights, Ill.) as previously described (5), electrophoresed in a 12.5% polyacrylamide gel (Owl Scientific, Inc., Woburn, Mass.) with Tris-borate-EDTA (TBE) buffer and poststained with ethidium bromide. Each sample lane was adjacent to at least one molecular size standard (Bio-Marker Low, BioVentures 101, Murfreesboro, Tenn.).

Images (*.tif bitmap) were acquired from UV-illuminated gels using the IS-1000 Digital Imaging System (Alpha Innotech Corp., San Leandro, Calif.) and analyzed using RFLPScan version 3.0 (Scanalytics Inc., Billerica, Mass.). Bands were automatically detected with the following parameters; lane width 50, band height 10, and smoothing factor 3. Sample lanes were calibrated by electrophoresis in a 2% agarose gel containing 0.4 µg/ml ethidium bromide.

OLA probe selection and procedure. GenBank Cyclospora and Eimeria 18S rRNA sequences U402061, U40262, U40263, U40264, U67118, U67121, U67119, U67115, U76748, U67120, U67116, U67117, U77084 (1, 10) were retrieved and aligned using GCG version 8.0 suite (Genetics Computer Group, Madison, Wis.) (3) on the Center for Food Safety and Analytical Nutrition VAX, Washington, D.C. Oligonucleotide probes were designed around position 864 of the consensus sequence, corresponding to position 832 of the GenBank sequence U40261 (Table 1).

The reporter probe (CYCEIMD) was 5’ phosphorylated, the capture probes (CYCU and EIMU, Table 1) were 5’ biotin labeled, and all probes were reverse-phase high-performance liquid chromatography-purified by the manufacturer (GENSET Corp., La-Jolla, Calif.). The reporter probe (CYCEIMD) was 3’ end-labeled with digoxigenin-11-UTP (Boehringer Mannheim, Indianapolis, Ind.) using the manufacturer’s protocol.

The OLA was conducted as described by Nickerson et al. (7), Stone et al. (11), and Delahunty et al. (2). Twenty microliters of test PCR product were diluted with 45 µl 0.1% Triton X-100. For each sample, one ligation reaction used oligonucleotides CYCU and CYCEIMD and the other contained EIMU and CYCEIMD. Each 20-µl ligation reaction contained 10 µl diluted amplification product, 40 mM Tris-HCl (pH 8.3), 50 mM KCl, 20 mM MgCl₂, 1 mM NAD, 0.02% Triton X-100, 167 fmol capture probe, 167 fmol reporter probe, and 0.1 unit Ampligase enzyme (Epicentre Technol., Madison, Wis.). Ligations were conducted on a DNA 9600 thermal cycler (Perkin-Elmer, Branchburg, N.J.) (10 cycles of 30 s at 93°C followed by 2 min at 58°C) and stopped with 10 µl 0.1 M EDTA in 0.1% Triton X-100. The entire volume was transferred to a prepared 96-well flat-bottomed microtiter plate well. To prepare microtiter plate wells, 50 µl streptavidin (25 µg/ml, Sigma) was allowed to incubate at 37°C for 1 h, then blocked with 200 µl 0.5% bovine serum albumin (Sigma) at 37°C, 20 min. Ligation products were captured at room temperature (25°C) for 30 min. Test wells were washed with 150 µl NaOH wash (0.01 M NaOH, 0.5% Tween 20) followed by two washes with 200 µl Tris wash (100 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% Tween 20). Forty microliters anti-digoxigenin-AP Fab fragments (Boehringer Mannheim) diluted 1:1,000 in phosphate-buffered saline were added and incubated at room temperature, 30 min. Test well contents were removed and wells washed six times with 200 µl Tris wash. Twenty-five microliters substrate (BRL enzyme-linked immunosorbent assay amplification system, Gibco-BRL, Gaithersburg, Md.) was added per test well and incubated 10 min at room temperature. Twenty-five microliters of enhancer (Gibco-BRL) was added and incubated 10 min at room temperature and stopped with 30 µl 0.3 M H₂SO₄. Spectrophotometric absorbance values were determined at A₄₉₀ and saved as *.txt files (Dynatech MR 5000 Microplate reader and BioLinx.

TABLE 2. Observed sizes of restriction fragments from MnlI digests of a PCR-amplified region of the 18S rRNA gene for three Cyclospora spp. strains, three E. tenella strains, and one E. mitis strain

<table>
<thead>
<tr>
<th>Observed fragment sizes (bp)</th>
<th>Cyclospora (U of A)</th>
<th>Cyclospora (CFSAN)</th>
<th>Cyclospora (CDC)</th>
<th>E. tenella #10</th>
<th>E. tenella #80</th>
<th>E. tenella Merck</th>
<th>E. mitis</th>
</tr>
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<tbody>
<tr>
<td>Fragment 1</td>
<td>142</td>
<td>146</td>
<td>143</td>
<td>132</td>
<td>133</td>
<td>129</td>
<td>130</td>
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<tr>
<td>Fragment 2</td>
<td>110</td>
<td>111</td>
<td>111</td>
<td>113</td>
<td>113</td>
<td>112</td>
<td>110</td>
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<tr>
<td>Fragment 3</td>
<td>47</td>
<td>48</td>
<td>48</td>
<td>66</td>
<td>66</td>
<td>64</td>
<td>64</td>
</tr>
</tbody>
</table>

*a Predicted MnlI RFLP fragment sizes for Cyclospora sp. are 140, 106, and 48 bp and for E. tenella and E. mitis strains 126, 106, and 62 bp.

*b Strain received from Ynes Ortega, University of Arizona (U of A), Tucson, Ariz.

*c Strain received from Jeff Bier, FDA Center for Food Safety and Nutrition (CFSAN), Washington, D.C.

*d Strain received from Mike Arrowood and Mark Eberhard, Centers for Disease Control and Prevention (CDC), Atlanta, Ga., via Kathy Craven, FDA, Atlanta, Ga.

*e Strain received from Patricia Allen, U.S. Department of Agriculture, Beltsville, Md.
TABLE 3. OLA mean readings (A 490) (n = 3) for both the CYCU/CYCEIMD and EIMU/CYCEIMD oligonucleotide pairs on three Cyclospora sp. strains, three E. tenella strains, and one E. mitis strain

<table>
<thead>
<tr>
<th>Probe pair</th>
<th>Cyclospora (U of A)</th>
<th>Cyclospora (CFSAN)</th>
<th>Cyclospora (CDC)</th>
<th>E. tenella #10</th>
<th>E. tenella #80</th>
<th>E. tenella Merck</th>
<th>E. mitis</th>
<th>Negative control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean A 490</td>
<td>CYCU/CED</td>
<td>0.447</td>
<td>0.462</td>
<td>0.307</td>
<td>0.057</td>
<td>0.069</td>
<td>0.073</td>
<td>0.059</td>
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<tr>
<td>SD</td>
<td></td>
<td>0.130</td>
<td>0.208</td>
<td>0.004</td>
<td>0.017</td>
<td>0.006</td>
<td>0.034</td>
<td>0.020</td>
</tr>
<tr>
<td>Mean A 490</td>
<td>EIMU/CED</td>
<td>0.042</td>
<td>0.052</td>
<td>0.065</td>
<td>0.385</td>
<td>0.777</td>
<td>0.472</td>
<td>0.732</td>
</tr>
<tr>
<td>SD</td>
<td></td>
<td>0.025</td>
<td>0.030</td>
<td>0.036</td>
<td>0.269</td>
<td>0.466</td>
<td>0.263</td>
<td>0.479</td>
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<tr>
<td>Mean ratio</td>
<td></td>
<td>10.846</td>
<td>8.638</td>
<td>4.712</td>
<td>6.724</td>
<td>11.100</td>
<td>7.003</td>
<td>12.093</td>
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<tr>
<td>SD</td>
<td></td>
<td>0.978</td>
<td>1.757</td>
<td>0.567</td>
<td>2.959</td>
<td>3.719</td>
<td>2.167</td>
<td>2.839</td>
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</table>

*a The average A 490 value from unused microtiter wells, 0.051, was subtracted from each of the test well values before calculating the mean. Spectrophotometric absorbance (A 490) ratios were calculated by comparing positive to negative sets of OLA probes for each strain and the mean (n = 3) ratios for each test strain presented. See Table 2 for strain information.

The detection of specific DNA sequences is becoming increasingly important in the diagnosis of human diseases and detection and epidemiology of disease-causing agents. Methods such as PCR allow for the rapid amplification of target DNA. In many situations there is a need to further study DNA sequence variants that are not required for the particular application. Oligonucleotide probes can be designed to target individual nucleotide locations and be used to detect sequence differences. Oligonucleotide probes can be used for the rapid amplification of disease-causing agents. Many sites were consistent with their respective predicted molecular sizes (Table 2). The mean (n = 3) A 490 values for each OLA test pair ranged from 4.086 to 15.820, and standard deviations were <0.15 for positive or negative ratios. A positive template was noted for each test strain (three Cyclospora spp. with the exceptions of E. bovis, E. nieschulzi, and E. mitis). The results in this study are presented in Table 3. The detection of specific DNA sequences is becoming increasingly important in the diagnosis of human diseases and detection and epidemiology of disease-causing agents. Methods such as PCR allow for the rapid amplification of target DNA. In many situations there is a need to further study DNA sequence variants that are not required for the particular application. Oligonucleotide probes can be designed to target individual nucleotide locations and be used to detect sequence differences. Oligonucleotide probes can be used for the rapid amplification of disease-causing agents. Many sites were consistent with their respective predicted molecular sizes (Table 2). The mean (n = 3) A 490 values for each OLA test pair ranged from 4.086 to 15.820, and standard deviations were <0.15 for positive or negative ratios. A positive template was noted for each test strain (three Cyclospora spp. with the exceptions of E. bovis, E. nieschulzi, and E. mitis). The results in this study are presented in Table 3.
describe an OLA application to diagnose genetic diseases such as sickle cell anemia and cystic fibrosis. The feasibility of an OLA-based system to type single nucleotide polymorphisms with a panel of 20 markers for analyzing human DNA samples was studied by Delahunty et al. (2). A PCR-OLA was developed by Stone et al. (11) to detect *Salmonella* serovars rapidly in clinical veterinary samples. Here, we have designed and applied an OLA method to provide a rapid, alternative approach to RFLP (5) for the confirmation of amplified product as being from *Cyclospora* or *Eimeria* spp.

ACKNOWLEDGMENTS

We are grateful to Dr. Ynés Ortega (University of Arizona, Tucson, Ariz.), Drs. Mike Arrowood and Mark Eberhard (Centers for Disease Control, Atlanta, Ga.) via Kathy Craven (FDA Atlanta, Ga.), and Dr. Jeff Bier (FDA/CFSAN, Washington, D.C.) for providing the three strains of *Cyclospora* and to Dr. Patricia Allen (U.S. Department of Agriculture, Beltsville, Md.) for providing the *Eimeria* species oocysts used in this study.

REFERENCES