A chemiluminescence immunoassay for evaluation of *Cryptosporidium parvum* growth in vitro

Xiangdong You a, Michael J. Arrowood c, Marisa Lejkowski a, Longti Xie c, Raymond F. Schinazi ab, Jan R. Mead ab *

a Department of Pediatrics, Emory University School of Medicine, Atlanta, GA 30322, USA  
b Georgia VA Research Center on AIDS and HIV Infection Veterans Affairs Medical Center, 1670 Clairmont Road, Decatur, GA 30033, USA  
c Division of Parasitic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Public Health Service, US Department of Health and Human Services, Atlanta, GA 30341, USA

Received 15 November 1995; revised 13 December 1995; accepted 13 December 1995

Abstract

A chemiluminescence immunoassay (CLIA) was developed to detect *Cryptosporidium parvum* growth in Madin-Darby canine kidney (MDCK) cell cultures. Optimal results were obtained when MDCK cells were plated at a density of $1 \times 10^4$ cells/well (96-well plate) and maintained as a monolayer for 4 days prior to infection with $2 \times 10^4$ parasites/well. Two compounds (paromomycin and maduramicin) were evaluated and shown to have selective activity against *C. parvum* in a dose-dependent manner. There was excellent correlation between CLIA and immunofluorescence assay when assessing anti-*C. parvum* agents in MDCK cells. CLIA offers advantages over conventional enzyme-linked immunosorbent assay and immunofluorescence assay methods in that it is more sensitive and efficient. The combination of CLIA and MDCK culture provides an efficient tool for evaluating potential anti-cryptosporidial compounds prior to testing in animal models.

Keywords: *Cryptosporidium parvum*; Chemiluminescence immunoassay; Cell culture; In vitro; Drug evaluation

1. Introduction

*Cryptosporidium parvum* is an intracellular protozoan parasite that infects epithelial cells of many animal species, including humans, and is the causative agent of cryptosporidiosis. Cryptosporidial diarrhea is normally self-limiting in immunocompetent individuals, but is severe, irreversible and life-threatening in the immunocompromised, especially HIV-infected individuals. Moreover, no effective therapeutic compounds are currently available for clinical use against *C. parvum* infection [1]. The need for a more efficient, reproducible in vitro culture technique and a sensitive, quantitative detection method for *C. parvum* growth illustrates the obstacles in developing drug evaluation systems for potential therapies.

Several cell lines have been reported to support the development of *C. parvum* in vitro [2–7]. The most promising include Caco-2, MDCK and HCT-8 cells. Immunofluorescence assay (IFA) and enzyme-
linked immunosorbent assay (ELISA) methods have recently been described for assessing *C. parvum* growth and screening potential anti-cryptosporidial compounds [5–7]. Because current methods can be subjective, time-consuming or insensitive, the development of more efficient techniques for evaluating growth of *C. parvum* in cell culture is warranted.

Chemiluminescence immunoassay (CLIA) is a highly sensitive, rapid and simple technology that has been used in many biomedical fields [8]. The application of CLIA to detect and quantify *C. parvum* in vitro has not been reported previously. In the present study, we describe a luminol-enhanced CLIA for quantifying parasite loads in MDCK cells.

2. Materials and methods

2.1. Production of *C. parvum* oocysts

The IOWA bovine isolate of *C. parvum* was generated in newborn Holstein bull calves. Oocysts were collected and purified through discontinuous sucrose gradients as previously described [9]. Purified oocysts were stored at 4°C in 2.5% aqueous K$_2$Cr$_2$O$_7$ solution and were ≤ 6 months old when used in vitro.

2.2. Host cells and compounds

MDCK cells (ATCC CCL 34) were initially maintained in Eagle’s MEM medium with 10% fetal bovine serum and then subsequently passaged in serum free Ultraculture™ medium (BioWhittaker Inc., Walkersville, MD). Cells were seeded into 2-well Lab-Tek Chamber Slide™ (Nunc Inc., Naperville, IL) or 96-well Microlite™ plates (Dynatech Lab., Chantilly, VA). Paromomycin sulfate (Sigma, St. Louis, MO) was dissolved directly in culture media. Maduramicin (American Cyanamid Corp., Princeton, NJ) was dissolved in 100% DMSO before addition to culture media. The final DMSO concentration in cultures was less than 0.01%. Ultraculture medium with 0.75% sodium taurocholate and incubated for 10 min at 37°C [5]. The excystation mixture was diluted with Ultraculture medium and promptly dispensed in plates containing confluent MDCK cells maintained in Ultraculture medium for 4 days. The oocyst inoculum was incubated with the cells for 3 h before being washed with PBS and replaced with fresh Ultraculture medium, with or without test compounds. Plates were incubated at 37°C, in a 5% CO$_2$ air atmosphere for 48 h. Cultures were washed with PBS, and then fixed with Bouin’s solution.

2.3. Preparation of anti-*C. parvum* monoclonal and polyclonal antibodies

Monoclonal antibody C3C3, an IgG$_3$ antibody directed against *C. parvum* cytoplasmic antigens found in sporozoite, meront and gamont life cycle stages, was prepared in a Cell-Pharm™ mini bioreactor system (UniSyn Fibertec Corp., San Diego, CA). The antibody was labelled with Fluorolink Cy3™ reactive dye (Research Organics Inc., Cleveland, OH) for IFA use. Polyclonal antibodies were prepared in rabbits against lysed whole oocyst antigens. The titer of the rabbit anti-*C. parvum* sera was 1:12,500 as determined by IFA.

2.4. Chemiluminescence immunoassay (CLIA)

In preparation for inoculation on cell cultures, oocysts were washed free of K$_2$Cr$_2$O$_7$ by centrifugation (16,000 × g, 3 min) and suspended in 0.1 M sodium acetate-buffered saline (ABS, pH 5.5). Oocysts were treated with sodium periodate (10 mM in ABS) for 20 min at 4°C and washed with ABS. After centrifugation, the pellets were resuspended in DMEM base with 0.75% sodium taurocholate and incubated for 10 min at 37°C [5]. The excystation mixture was diluted with Ultraculture medium and promptly dispensed in plates containing confluent MDCK cells maintained in Ultraculture medium for 4 days. The oocyst inoculum was incubated with the cells for 3 h before being washed with PBS and replaced with fresh Ultraculture medium, with or without test compounds. Plates were incubated at 37°C, in a 5% CO$_2$ air atmosphere for 48 h. Cultures were washed with PBS, and then fixed with Bouin’s solution.

The fixed plates were decolorized with 70% ethanol, washed 20 mM Tris·HCl (pH 7.5), 150 mM NaCl, 0.05% Tween-20 (TBST) and then blocked with 1% BSA-TBST for 30 min at room temperature (RT) with shaking. Rabbit anti-*C. parvum* sera (1:200 dilution) was applied to the plates and incubated for 1 h. After washing with TBST, the samples were sequentially incubated with biotin-labelled goat anti-rabbit IgG and horseradish peroxidase-labelled streptavidin (working dilution 1:1000, Kirkegaard and Perry Laboratories (KPL Inc., Gaithersburg, MD). Enhanced luminol
(Luminol, 4-iodophenol, and hydrogen peroxide, Aldrich Chemical Co. Inc., Milwaukee, WI) was used as substrate. The plates were read with an ML3000 Luminometer (Dynatech Lab., Chantilly, VA) and the relative light units (RLU) were determined. Means of RLU were calculated from four replicate wells and all experiments were repeated at least twice.

2.5. Immunofluorescence assay (IFA)

MDCK cell monolayers were grown in two-well chamber slides. The cells were infected and treated as specified above and as previously described [5]. Bouin’s-fixed chambers were decolorized with 70% ethanol, washed with PBS, and blocked with 0.1% BSA-PBS for 30 min at RT. Cy3™-conjugated C3C3 monoclonal antibody was applied to the chambers and incubated for 1 h. After washing with PBS, coverslips were mounted on the labelled cultures and the fluorescing life cycle stages were quantified with a microscope-based image analysis system employing Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). For compound evaluation, each concentration was tested in triplicate and 12 images were analyzed for every chamber.

2.6. Data analysis

The 50% inhibition concentration (IC_{50}) was calculated by using ComboStat software [10]. Statistical analyses included analysis of variance (ANOVA), correlation and regression.

3. Results

Initial experiments determined optimal culture conditions supporting C. parvum growth. These included seeding 96-well culture plates with $1 \times 10^4$ MDCK cells/well and maintaining the monolayer for 4 days prior to infection. Using a lower initial cell density or shortening the pre-infection culture period (less than 4 days) resulted in decreased parasite growth (data not shown). Experiments were performed to demonstrate a correlation between parasite growth and the signal generated by CLIA. As shown in Fig. 1, higher concentrations of oocyst inoculum resulted in higher RLU production. The signal generated was markedly reduced when the oocyst inoculum was less than $2 \times 10^3$/well. The best performance was achieved with inocula between $2$ and $20 \times 10^4$/well. This parallels the results observed in the microscopy-based assay after correcting for area differences between the 96-well plate and culture chamber slides. At concentrations of $2 \times 10^5$ oocysts/well or higher, moderate cell toxicity was observed using the CellTiter 96™AQueous assay (Table 1) and confirmed by microscopic examination.

The CLIA-generated signal was specific and was not due to non-specific binding of the reagents. A
Table 1
Toxicity of different concentrations of oocysts and compounds to MDCK cells as measured by an CellTiter 96™AQ™ assay

<table>
<thead>
<tr>
<th>Treatment</th>
<th>OD±S.D.</th>
<th>Toxicity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 × 10⁶ oocysts/well</td>
<td>1.11±0.05</td>
<td>36.8</td>
</tr>
<tr>
<td>2 × 10⁵ oocysts/well</td>
<td>1.41±0.06</td>
<td>20.2</td>
</tr>
<tr>
<td>2 × 10⁴ oocysts/well</td>
<td>1.71±0.05</td>
<td>2.9</td>
</tr>
<tr>
<td>2 × 10³ oocysts/well</td>
<td>1.80±0.02</td>
<td>0</td>
</tr>
<tr>
<td>Paromomycin (2.4 mM)</td>
<td>1.79±0.05</td>
<td>0</td>
</tr>
<tr>
<td>Maduramicin (1 μM)</td>
<td>1.79±0.07</td>
<td>0</td>
</tr>
<tr>
<td>Ultraculture control</td>
<td>1.76±0.04</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as means of OD₄₅₀ from three wells ± standard deviations. Toxicity (%) is relative to uninfected control.

The signal-to-noise ratio (RLU infected/RLU non-infected) was improved significantly by using Microlite™ plates compared with standard Corning 96-well plates. The signal generated using the Microlite™ plate was approximately three-fold higher than the Corning plate, greatly increasing the dynamic range of the assay. The signal-to-noise (S/N) ratio was also reduced by using the C3C3 monoclonal antibody instead of using polyclonal antisera. However, the maximal signal generated using the monoclonal antibody was approximately 50% lower than that achieved using the polyclonal antisera.

Representative dose–response curves for paromomycin and maduramicin against C. parvum are illustrated in Fig. 2. Inhibition of growth by paromomycin was observed at concentrations ranging from 10 to 250 μM. The activity of this compound never exceeded 90% even when the dose was increased beyond 250 μM. The IC₅₀ for paromomycin was calculated to be 133.5 μM in the CLIA. This correlated well with the calculated IC₅₀ (92.3 μM) for paromomycin when the compound was evaluated using IFA (Table 2). Maduramicin was active at a concentration approximately 10-fold lower than paromomycin and approached 100% inhibition at concentrations as low as 1 μM. The IC₅₀ was determined to be 0.32 μM when evaluated using the

![Graph of Paromomycin inhibition](image1.png)

![Graph of Maduramicin inhibition](image2.png)

Fig. 2. Potency of paromomycin and maduramicin against C. parvum in MDCK cells as determined by CLIA. Paromomycin (A) and maduramicin (B) at different concentrations. Data are expressed as percent inhibition. Error bars represent 95% CL.
Table 2
The 50% inhibition concentration (IC₅₀) of compounds determined by IFA and CLIA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IC₅₀ IFA (μM)</th>
<th>IC₅₀ CLIA (μM)</th>
<th>Correlation coefficient (r²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paromomycin</td>
<td>92.3</td>
<td>133.6</td>
<td>0.95</td>
</tr>
<tr>
<td>Maduramicin</td>
<td>0.29</td>
<td>0.32</td>
<td>0.98</td>
</tr>
</tbody>
</table>

Linear regression of comparative inhibition of *C. parvum* growth in MDCK cells for IFA vs. CLIA.

CLIA method and 0.29 μM by IFA (Table 2). The correlation coefficients, when comparing the two methods (IFA and CLIA) for the inhibition of *C. parvum* growth, were r² = 0.95 for paromomycin and r² = 0.98 for maduramicin (Table 2). No significant toxicity to host cells was observed at paromomycin and maduramicin concentrations exhibiting maximal activity against *C. parvum*, nor was there toxicity associated with Ultraculture medium supplemented with 0.01% DMSO (drug vehicle control).

4. Discussion

Chemiluminescent immunoassays have been developed over the years for a number of applications as a safe alternative to isotopic assays. Luminol-based chemiluminescence assays have been improved significantly by introduction of phenolic enhancers in combination with horseradish peroxidase, which increases the sensitivity and produces a more prolonged signal. The level of light produced in this enhanced chemiluminescence reaction is about 100-fold higher than non-enhanced reaction [11] and is even superior to radioimmunoassay (RIA) [12]. CLIA has several distinct advantages over microscopic methods. It employs a convenient 96-well format that allows for high throughput of samples. Secondly, since quantitation is automated, one is not dependent on manual enumeration that is both time-consuming and subject to variable interpretation depending on the expertise of the microscopist.

CLIA may also offer advantages over a conventional ELISA method [7]. CLIA measures light emitted from the top of the plate using a photomultiplier tube (PMT) in the luminometer. There is no interference from the cell monolayer that can occur with ‘through the well’ absorption analyses in conventional ELISA readers. In our experience, CLIA has been more sensitive with a greater dynamic range than conventional ELISA. The highest OD reading that we were able to achieve in ELISA was 0.3–0.4 OD with a S/N ratio of 3–4. In contrast, the CLIA had a range of between 0 and 800 RLU with a S/N ratio of 10–15.

In the current study, CLIA was found to be specific and sensitive as demonstrated by the S/N ratio. There was no significant difference between RLU in cultures inoculated with heat-inactivated oocysts (at dose 2 × 10⁴/well or below) and non-infected cultures. Non-specific binding was minimal as demonstrated by the low signal produced when primary antibody, secondary antibody, enzyme conjugate, and substrate were omitted respectively in the CLIA procedure. These results suggest that signals obtained by CLIA specifically represent *C. parvum* growth in the MDCK culture system rather than residual antigenic components from oocyst inoculum or non-specific binding of the reagents.

The S/N ratio was lower when monoclonal antibodies were used instead of polyclonal antibodies, but the relative overall signal was reduced by 50%. Mixtures of several monoclonal antibodies may improve the sensitivity of the assay. The greatest improvement in signal and decrease in background was achieved with the use of Microlite™ plates. The advantage of Microlite™ plates is that their opacity prevents light scattering between wells and reflects the chemiluminescent light into the PMT to increase sensitivity. Using a microplate washer and robots may further improve the efficiency of CLIA, especially for the purpose of large-scale compound evaluation.

Paromomycin, an aminoglycoside, and maduramicin, a polyether ionophore, are two compounds that have shown activity against *C. parvum* in vitro and in vivo [5,7,13,14]. We compared the activities of these two compounds with results obtained in our microscopy-based assay. As demonstrated in both systems, maduramicin was more potent than paromomycin (Table 2). Strong correlation was observed between IFA and CLIA in evaluating the activities of these compounds as shown by the agreement in the IC₅₀ and by the correlation coefficient. Although CLIA has many advantages over a microscopic as-
say, one can gain useful information about the growth of the parasite at different life cycle stages that can not be obtained from a non-microscopic assay. Together, these two systems compliment each other. Compounds can be evaluated initially by CLIA and then selective agents can be confirmed by the microscopic based IFA. The IFA would not only allow for confirmation regarding the compounds’ activity but might provide additionally insight regarding life cycle stage-specific inhibition.

In summary, enhanced CLIA is a highly sensitive, simple, rapid, quantitative, and reproducible technique for detection of C. parvum loads in MDCK cells. CLIA combined with the MDCK culture system provides an efficient and rapid system for screening large numbers of potentially useful therapeutic agents directed against C. parvum.

Acknowledgements

This work was supported by contract #NO1-AI-25144 from the National Institutes of Health and the Georgia VA Research Center on AIDS and HIV Infection.

References