A chemiluminescent immunofocus assay (CIFA) for non-microscopic enumeration of Cryptosporidium parvum infectivity in cell culture

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Abstract As Cryptosporidium parvum continues to cause waterborne disease, despite extensive efforts by drinking water suppliers and regulators, it is important to have reliable and convenient methods for detection of this pathogen in wastewater discharges, environmental source waters and finished drinking water supplies. In order to better understand the health risks of this organism, it is necessary that detection methods be able to distinguish between infectious and non-infectious Cryptosporidium oocysts in these environmental samples. Cryptosporidium infectivity assay systems based on infections in mice and on in vitro infections in continuous mammalian cell lines are available. Currently, these methods are impractical for routine analysis of water samples because they are tedious, lengthy and costly. These methods rely on careful microscopic examination or further analysis by PCR and then characterisation of the amplified DNA. Practical and affordable non-microscopic methods to determine Cryptosporidium infectivity are much needed for environmental analysis. A cell culture infectivity detection system was developed for infectious Cryptosporidium oocysts that does not rely on microscopic examination of samples to score results, is applicable to a variety of samples and has the potential to be used for routine water monitoring and other environmental or biomedical analysis. Using a chemiluminescent immunoassay, the discrete foci of developmental stages of Cryptosporidium in cell cultures are clearly visible as discrete objects in an image of the entire cell culture layer in a dish or on a slide. These objects are directly countable with the unaided eye and their identity can be further confirmed or verified by microscopic examination.

Keywords Cell culture; chemiluminescent; Cryptosporidium parvum; immunofocus; infectivity

Introduction

The ability to detect and assay the infectivity of Cryptosporidium oocysts in conventional 2-dimensional mammalian cell culture systems has been reported since the early 1980s and it has been demonstrated that Cryptosporidium parvum will infect a wide range of continuous cell lines (Upton et al., 1994). Continuous cell lines most useful for supporting infections of Cryptosporidium parvum tend to be fast-growing, epithelial lines derived from kidney or intestinal cells. Examples of continuous cell lines commonly used for Cryptosporidium infectivity assays are human ileocaecal colorectal adenocarcinoma (HCT-8), Madin-Darby Canine Kidney (MDCK) and Madin-Darby Bovine Kidney (MDBK) cells. In vitro cell culture systems have compared favorably with mouse bioassays for quantifying infectious Cryptosporidium (Slifko et al., 1998).

In vitro cell culture infectivity assay systems for Cryptosporidium parvum have not been standardised and there are several different methods currently in use (Upton et al., 1994; Rochelle et al., 1996; Slifko et al., 1999). Few studies have applied infectivity detection methodologies for Cryptosporidium to environmental water samples (Rochelle et al., 1997, 1999). The current USEPA methods for detection of Cryptosporidium in water make no provisions for detection of infectious oocysts. However, it is important to have this capability in order to conduct reliable quantitative risk assessments and make informed risk management decisions for this waterborne pathogen. Current tissue culture infectivity systems for Cryptosporidium rely on either microscopic examination of cell monolayers...
for visualisation of infected cells or detection of Cryptosporidium nucleic acid by direct hybridisation or in vitro enzymatic amplification (PCR). Microscopic examination is labour intensive, time consuming, expensive and requires a trained analyst. Molecular methods for in situ detection of nucleic acid sequences associated with areas of infection are technically demanding and require expensive reagents and detectors (such as fluorometers) and/or microscopic examination of cell monolayers by a skilled analyst in order to score the molecular signal. Accordingly, because of these technological, time and cost barriers to routine sample evaluation, it is currently not practical to apply these methods for detection of infectious Cryptosporidium oocysts in environmental samples. A cell culture detection system is needed for detecting infectious Cryptosporidium oocysts in environmental water samples that is technically less complex, less labour intensive, less subjective, affordable and capable of processing large numbers of samples so as to be applicable for routine monitoring of environmental waters.

The focus of this report is the development of such a method, the chemiluminescence immunofocus assay (CIFA). For development of this assay system, two cell lines commonly used for detection of infectious Cryptosporidium oocysts, HCT-8 and MDCK cells were compared. Also, media supplements and excystation additives were tested in order to optimise cell growth and Cryptosporidium infection. The CIFA is antibody-based and uses a primary monoclonal antibody directed at Cryptosporidium parvum developmental stages coupled with a commercially available chemiluminescence kit for visualisation of the localised areas of infection (foci) in the cell monolayers. Several different primary monoclonal antibodies recognizing developmental stages of Cryptosporidium were tested for their affinity to target developmental stage antigens. Conditions were optimised for visualisation of the localised areas of infections or foci on the cell monolayers using the commercially available chemiluminescence detection kit. To overcome non-specific antibody binding to the mammalian cell monolayer, blocking reagents and conditions for chemiluminescence detection were optimised to reduce background signals and allow for visualisation of distinct focal areas of Cryptosporidium infection. A set of optimised conditions was developed for all key factors tested that allowed for direct visualisation and enumeration of foci produced by Cryptosporidium infections on cell monolayers without the use of microscopy. Additionally, the labeling system was such that the focal areas of infection visualised on the cell monolayer could be directly observed for confirmation by epifluorescence microscopy.

**Methods and materials**

*Cryptosporidium oocysts*

Cryptosporidium oocysts (Iowa strain) were produced in calves by Pat Mason, Pleasant Hill Farms, Troy, ID. The shed oocysts were collected and purified by ether extraction and sucrose gradient flotation. Approximately $4 \times 10^7$ oocysts/mL were resuspended in PBS (with 1,000 U/mL penicillin and 1,000 µg streptomycin/mL) and shipped with ice packs by overnight courier to the UNC laboratory. Oocysts were not exposed to dichromate. On arrival, oocysts were enumerated in a haemocytometer, examined for general quality (appearance, morphology) by differential interference contrast (DIC) microscopy and stored in the shipment buffer at 4°C until required. Production lots of Cryptosporidium parvum oocysts for these experiments were used for a period of time that did not exceed three months from the date of shedding from the host.

*Continuous mammalian cell lines*

HCT-8 cells (ATCC CCL-244) were obtained from the UNC cell culture facility (originally from the American Type Culture Collection). MDCK cells (ATCC CCL-34) were
obtained from M.J. Arrowood (Division of Parasitic Diseases, Centers for Disease Control and Prevention, Atlanta, GA). Cells were maintained by weekly serial passage in 75 cm² flasks and were produced for assay in 2-well chamber slides (Lab-Tek® II, Nalge Nunc Int., Naperville, IL). HCT-8 cells were maintained in RPMI 1640 medium with 1 mM sodium pyruvate, 10% v/v foetal bovine serum and antibiotics (50 µg/mL gentamicin sulphate, 250 µg/mL kanamycin and 15 µg/mL nystatin). In subsequent assays, to optimise in vitro conditions for mammalian cell growth and Cryptosporidium infection, medium supplements described previously were used to maintain cell monolayers following inoculation with oocysts (Upton et al., 1995). MDCK cells were maintained in a serum-free medium (UltraCulture, BioWhittaker, Walkersville, MD) supplemented with the same antibiotics described above.

Microscopic quantification of infected cell monolayers and estimation of infectivity concentrations

The microscopic method to quantify infected cell monolayers used a fluorescently-labelled monoclonal IgG₃ antibody (C₃C₃-FITC, courtesy of M.J. Arrowood, CDC, Atlanta, GA) specific for developmental stages of Cryptosporidium (Arrowood et al., 1996). After fixing and staining, cell layers were observed under epifluorescent microscopy by scanning the entire cell layer area in a systematic fashion. Apple-green fluorescing ovoid or spherical objects 3–6 µm in diameter were recorded as localised areas of infection. Observed microscopic fields were recorded as positive or negative for Cryptosporidium developmental life stages. Each microscopic field was treated as an individual sample and this quantification method assumed random distribution of infectious living stages on cell monolayers. Following enumeration of numbers of positive and negative fields, the Thomas Equation was used to generate an MPN estimate for the concentration of infectious Cryptosporidium parvum oocysts taking into consideration the assay dilution and inoculum volume per well. Estimated 95% CI for the MPN estimates were calculated by assuming conformity to a Poisson distribution and, therefore, equal variance and mean.

Chemiluminescent detection applied to in vitro cell culture

For in vitro cell culture, the C₃C₃ monoclonal antibody (labelled with FITC and coupled with a commercially available, antibody-based chemiluminescent detection system (Southern-Light Plus, Tropix, Inc., Bedford, MA)) was used for detection of Cryptosporidium focal areas of infection. The chemiluminescent detection kit used a streptavidin-alkaline phosphatase conjugate directed at FITC and a provided proprietary, ready-to-use substrate. Due to background fluorescence and consequent false-positive signals, alternative blocking solutions were tested in addition to the blocking solutions provided with the detection kit (data not shown). The chemiluminescence signal was visualised using dual emulsion scientific imaging film (Kodak Biomax ML, Eastman Kodak Company, Rochester, NY) developed using a Konica SRX-101A medical film processor (Konica Corporation, Taiwan).

Results and discussion

The first objective of these studies was to identify and optimise growing conditions for a continuous cell line that supported Cryptosporidium infections. Dilutions of Cryptosporidium oocysts were inoculated onto 90% confluent monolayers and allowed to adsorb (1 h, 37°C, 5% CO₂). Cultures were then supplemented with tissue culture medium and, after 48 h at 37°C and 5% CO₂, they were fixed with absolute methanol for 15 min. Cryptosporidium infections were quantified using the microscopic method previously described and results are shown in Table 1. Confidence intervals computed for MPN concentrations of infectivity in the two cell lines overlapped and were not considered signifi-
cantly different. Localised areas of infection, visualised by microscopy, showed similar morphology and appearance with each cell line tested. HCT-8 cells were chosen for subsequent infectivity assays because of their relative ease of maintenance and similarity to MDCK cells for sensitivity to *Cryptosporidium* infection.

As *in vitro* *Cryptosporidium* infections begin with excystation of oocysts, excystation conditions compatible with *in vitro* cell culture assay were tested and optimised. Treatment of oocysts with sodium taurocholate (NaT) had been previously reported to promote excystation and was chosen for use in preparing oocysts for inoculation into cell monolayers in infectivity assays. Excystation efficiency was (a) relatively high (57.5%) using 0.75% NaT in RPMI 1640 (60 min, 37°C) and (b) very low (6%) when the oocysts were suspended in only RPMI 1640 medium (60 min, 37°C). Although excystation efficiency was relatively high with 0.75% NaT, it was found that concentrations of NaT >0.5% were not compatible with this infectivity assay system because they destroyed the cell monolayers (data not shown). Therefore, the diluent used to suspend oocysts for these experiments was the supplemented RPMI 1640 medium previously described with NaT added to a final concentration of 0.5%.

An essential objective for development of the CIFA was to devise a chemiluminescence assay system that was compatible with detection of *in vitro* cell culture infections. The chemiluminescence detection kit chosen for application to *in vitro* cell cultures had FITC as the target antigen thus avoiding possible interference that biotin-based systems would have due to reaction with biotin contained within the cell monolayers. A second advantage of using the FITC-based antibody detection system was that the cell monolayers could be examined by epifluorescent microscopy following the CIFA for visual confirmation of positive areas of infection based on chemiluminescent foci. An additional treatment step in the protocol was to heat the cell monolayers to 75°C for 45 min following methanol fixation of the cell monolayers in order to avoid any background signal caused by phosphatase activity associated with the cell monolayers.

Other factors that were investigated for development and optimisation of this assay system were the selection of appropriate concentrations of primary antibody and substrate for the enzyme-mediated signal-generating system. The CIFA system for *Cryptosporidium* infectivity in cell cultures was developed from a chemiluminescence detection kit designed for Southern blot detection of labeled molecular products. As an outcome of this difference in assay format and target, the kit recommendations for reagent concentrations had to be adjusted for compatibility with the *in vitro* cell culture infectivity assay system. Experiments were done to test alternative blocking solutions, different concentrations of substrate and different concentrations of primary antibodies to develop optimised choices for these reagents and their use conditions (data not shown).

Table 2 compares enumeration methods for *Cryptosporidium* infections in 2-well chamber slides. The initial sample was enumerated by haemocytometer counts to be $9.6 \times 10^4$ oocysts after which 10-fold dilutions were made for cell culture inoculations. These results were obtained using the supplemented RPMI 1640 media with 0.5% NaT in the dilutions. The well slides were processed and enumerated by using the CIFA, after which the localised areas of infection were visually confirmed by epifluorescent microscopy.

### Table 1
Comparison of two cell lines by microscopic infectivity assay for sensitivity to *Cryptosporidium parvum* oocyst infection (inoculum = $10^7$/mL)

<table>
<thead>
<tr>
<th>Continuous cell line</th>
<th>MPN/mL</th>
<th>95% CI</th>
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<tbody>
<tr>
<td>MDCK (Madin-Darby canine kidney)</td>
<td>$1.1 \times 10^4$</td>
<td>± $5.3 \times 10^3$</td>
</tr>
<tr>
<td>HCT-8 (human ileocaecal colorectal adenocarcinoma)</td>
<td>$6.7 \times 10^3$</td>
<td>± $2.6 \times 10^3$</td>
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Additionally, the entire well slides were counted and positive fields were recorded for enumeration of *Cryptosporidium* developmental stages by the MPN method previously described.

Enumeration by CIFA yielded $1.4 \times 10^4$ IU/mL, visual observation by epifluorescence microscopy gave $1.3 \times 10^4$ IFU/mL and estimating the concentration by visually scoring microscope fields as positive or negative for infectious units and computing an MPN yielded $1.0 \times 10^4$ IU/mL.

Figure 1 shows the CIFA as used to compare the quantification of infectivity by these three different enumeration methods described in Table 2. When approximately $10^4$ oocysts (100 µL of $9.6 \times 10^4$ oocyst suspension) were initially applied, the infectious foci were too numerous to count. Dilutions (10-fold) with approximately $10^3$ and $10^2$ oocysts appeared to be countable for infectious foci in this experiment and were enumerated accordingly. Data from this comparison demonstrated that the foci detected visually by the CIFA were consistently confirmed by microscopic examination for fluorescently-labelled developmental stages. At an inoculum concentration of $10^3$ oocysts the foci were difficult to count visually as discrete objects because many of the areas of infection overlapped. This caused difficulty in counting the foci and in subsequent visual confirmation by epifluorescent microscopy. When 100 oocysts were applied to the cell monolayers, the foci were discrete and, therefore, easily distinguished and enumerated. At this oocyst concentration, the estimates of *Cryptosporidium* infectivity by the alternative enumeration methods were

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**Table 2** Comparison of enumeration methods for *Cryptosporidium* infectivity in cell cultures

<table>
<thead>
<tr>
<th>Enumeration method</th>
<th>Sample dilution</th>
<th>Titre</th>
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<tbody>
<tr>
<td></td>
<td>$10^{-1}$</td>
<td>$10^{-2}$</td>
</tr>
<tr>
<td>Oocyst count*</td>
<td>9,600</td>
<td>960</td>
</tr>
<tr>
<td>CIFA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNTC (n = 2)</td>
<td>109, 122</td>
<td>16, 13</td>
</tr>
<tr>
<td></td>
<td>135, 136</td>
<td>16, 18</td>
</tr>
<tr>
<td>Microscopy (focal areas of infection)</td>
<td>TNTC (n = 2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>94, 101</td>
<td>17, 12</td>
</tr>
<tr>
<td></td>
<td>147, 142</td>
<td>13, 15</td>
</tr>
<tr>
<td>Microscopic fields positive for infection (357 total fields/well)</td>
<td>ND</td>
<td>74, 69</td>
</tr>
<tr>
<td></td>
<td>103, 91</td>
<td>13, 15</td>
</tr>
</tbody>
</table>

*haemocytometer count – oocyst counts for subsequent 10-fold dilutions, 100 µL sample applied to each well; TNTC = too numerous to count; ND = not done; IFU = infectious focus units; IU = MPN infectious units

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**Figure 1** *Cryptosporidium parvum* infectivity detected as developmental stage foci using CIFA (number of oocysts/100 µL inoculum per well)
not significantly different (p >0.05). The similarities of counts based on the two infectivity detection methods demonstrated the quantitative nature of the CIFA and confirmed that this assay was as sensitive and reliable as the microscopic immunofluorescent MPN method for quantification of infectious Cryptosporidium.

The CIFA is a simple, practical, rapid and affordable assay method to enumerate Cryptosporidium infectivity in cell cultures. Furthermore, the method is compatible with and allows direct visual confirmation of chemiluminescent foci by microscopic examination for immunofluorescence detection and differential interference or phase contrast examination of the size, morphology and internal structures of the detected objects. In addition, the samples can be further examined by nucleic acid methods, such as hybridisation or PCR, in order to obtain molecular genetic confirmation and to perform phylogenetic analyses by nucleic acid sequencing.

Conclusions
A practical, convenient, affordable and reliable quantitative assay for infectious Cryptosporidium oocysts is needed in order to analyse water and other environmental samples for this enteric pathogen. The CIFA developed and evaluated in this report appeared to meet these criteria. Assay results were visualised directly as foci in the entire cell layer without microscopy, using an inexpensive commercially available chemiluminescence detection kit, within 60 h of initial sample inoculation of cell monolayers. The relative simplicity of the visual quantification approach of this assay system allows it to be directly applicable to many types of Cryptosporidium infectivity studies, including those directed at monitoring disinfection efficacy and environmental occurrence. Water samples can be quickly, and yet systematically, tested for the presence and concentrations of infectious Cryptosporidium oocysts, with presumptive positive samples initially identified and enumerated by focus detection and then confirmed by further examination of these infectious units using epifluorescent microscopy. The CIFA system should have immediate, widespread and practical applications in the water industry because of the utility of this assay system, as well as in other fields where Cryptosporidium infectivity assays are or can be usefully employed.

References

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