Viability of *Giardia intestinalis* Cysts and Viability and Sporulation State of *Cyclospora cayetanensis* Oocysts Determined by Electrorotation

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Electrorotation is a noninvasive technique that is capable of detecting changes in the morphology and physicochemical properties of microorganisms. Electrorotation studies are reported for two intestinal parasites, *Giardia intestinalis* and *Cyclospora cayetanensis*. It is concluded that viable and nonviable *G. intestinalis* cysts can be differentiated by this technique, and support for this conclusion was obtained using a fluorogenic vital dye assay and morphological indicators. The viability of *C. cayetanensis* oocysts (for which no vital dye assay is currently available) can also be determined by electrorotation, as can their sporulation state. Modeling of the electrorotational response of these organisms was used to determine their dielectric properties and to gain an insight into the changes occurring within them. Electrorotation offers a new, simple, and rapid method for determining the viability of parasites in potable water and food products and as such has important healthcare implications.
there are often distinct differences between laboratory and field data (33). No successful viability assays using vital dyes (10) or a suitable animal model have yet been reported for C. cayetanensis oocysts, and while the excystation method provides a means of viability determination, this typically takes between 1 and 2 weeks (21, 22), as the oocysts have to undergo sporulation first.

As the oocysts of C. cayetanensis are resistant to current water treatment procedures, including chlorination (25), and the infective dose is low, probably between 10 and 100 oocysts (2), there is a need for more-rapid viability assays that work at the single-organism level.

Based on our previous C. parvum study (12) and the current G. intestinalis data, we show strong evidence that, in the absence of a vital dye technique, the viability of C. cayetanensis oocysts can be determined by the electrorotation method. We also show that electrorotation can determine the sporulation state of an oocyst, which is important for assessing the potential risk of water contaminated with C. cayetanensis. Finally, we have analyzed the electrorotation response of the particles to determine their dielectric properties using the so-called dielectric multishell model, described elsewhere (18, 38).

MATERIALS AND METHODS

Cysts of G. intestinalis were obtained from human diarrheic samples provided by the public health laboratories of Gwynedd Hospital, Gwynedd, Wales, United Kingdom. Samples were purified by a water-ether sedimentation protocol followed by a sucrose flotation method (6). Cysts were then immediately washed in King mole solution, pH 7.4 (10 mM phosphate buffer, 27 mM KCl, 137 mM NaCl; Sigma Chemical Co.), for 30 min in a water bath at 37°C. Purified C. cayetanensis oocysts were supplied by the Scottish Parasite Diagnostic Laboratory (SPDL) (Glasgow, United Kingdom). Samples were collected from three infected humans (two male, one female) in the Glasgow area who had recently traveled abroad. The samples were purified as for G. intestinalis. Oocysts were then washed in deionized water and stored at 4°C for use within 1 month.

To obtain reproducible electrorotation data, the particles are first washed and resuspended in a solution of well-defined chemical composition and conductivity. To reduce electrical heating effects, the magnitude of the rotating field should be as low as possible. For the work reported here, PBS of conductivity 1 mS m⁻¹ was used as the suspending medium to achieve easily measurable rotation rates for modest applied voltages in the convenient frequency range of 100 Hz to 10 MHz. The washing procedure (repeated three times) consisted of diluting a 100-μl aliquot of particle suspension in ultrapure water (conductivity, 0.1 mS m⁻¹) to 1.5 ml, vortexing for 30 s, microcentrifuging for 1 min (3,300 × g), and then aspirating to 100 μl. After the final wash, the sample was resuspended in PBS solution which had been diluted with ultrapure water to give a conductivity of 1 mS m⁻¹. The suspending medium conductivity was then checked by testing 200 μl of supernatant following centrifugation using a calibrated Hanna Instruments Pure Water Tester, modified to reduce the volume of the sample chamber. Working particle density concentrations were 3 × 10⁴ cysts ml⁻¹ for G. intestinalis and 5 × 10⁰ oocysts ml⁻¹ for C. cayetanensis.

Descriptions of the basic theory and experimental procedures of electrorotation are given elsewhere (13, 16). In brief, 20 μl of (oo)cyst suspension was pipetted into a chamber surrounded by four planar gold electrodes, as shown in Fig. 1. The so-called “bone” electrode design is described by a 4th-order polynomial and is optimized to create a uniform rotating electric field over as large an area as possible in the center of the chamber. The shape and magnitude of the spectra obtained are known to be affected by the particle position in the chamber and by the presence of debris on the particle surface (8). Particles were therefore excluded from analysis if they were positioned outside the middle third of the chamber, drifted more than three times their own diameter during the recording, or possessed debris on their surface. These careful selection criteria, coupled with an electrode design that minimizes particle drift, improved the proportion of oocysts from which data could be obtained.

Particle identification and motion in the electric field were visualized using phase-contrast microscopy with a total magnification of ×400 (Nikon Optiphot-2 microscope with JVC model TK-1280E color video camera attachment) and recorded by video for later analysis and timing by stopwatch. A minimum of 10 s of behavior was recorded at each of 20, approximately equidistant, applied frequency points on a log scale over the range of 100 Hz to 10 MHz. After each aliquot had been examined and spectra had been recorded, the chamber was washed under pressure with ultrapure water from a wash bottle and dried under a stream of nitrogen gas.

RESULTS AND DISCUSSION

The electrorotation spectra obtained from 27 G. intestinalis oocysts, identified as possessing intact cyst walls with no debris adhering to the surface, are shown in Fig. 2. The spectra are grouped according to viability. It is clear that viable and nonviable cysts exhibit markedly different electrorotation behavior.
Spectrum profiles are in good agreement to those previously reported for the viability of isolated animal cells (14), biocide-treated yeast cells (*Saccharomyces cerevisiae* RXII) (37), and *C. parvum* oocysts (12).

Within the applied frequency range there is a frequency window, centered around 400 kHz, within which viable and nonviable cysts rotate in opposite directions. The terms “cofield” and “antifield” denote the sense of rotation of the particles relative to that of the applied field. At 400 kHz, cofield rotation thus characterizes nonviable cysts while antifield rotation characterizes viable cysts. The rotation direction, observed through a light microscope, is easily determined after a few seconds of observation and is aided by the ellipsoidal shape of the cysts. An alternative method of distinguishing the particle viability through electrorotation is by noting the significant difference in the magnitude of the antifield rotation rate and 1 standard deviation. The open symbols show the rotation rates of the n = 19 oocysts with the second distinct type. Similarities between these spectra and those shown in Fig. 2 for *G. intestinalis* cysts indicate the possibility that the viable cysts as it cannot traverse intact biological membranes (17).

Initial electrorotation spectra for a second particle type of similar size, namely, the spherical oocysts of *C. cayetanensis*, are shown in Fig. 3. All of the n = 19 oocysts were in an unsporulated state and were morphologically indistinguishable. The spectra recorded, however, were of two distinct types. Similarities between these spectra and those shown in Fig. 2 for *G. intestinalis* cysts indicate the possibility that the

![FIG. 3. Electrorotation spectra of unsporulated C. cayetanensis oocysts at a suspending medium conductivity of 1 mS m⁻¹. The spectra obtained from the n = 19 intact unsporulated oocysts were one of two distinct types. The first type, that characteristic of n = 17 oocysts, is summarized by the solid symbols and error bars representing the mean rotation rate and 1 standard deviation. The open symbols show the rotation rates of the n = 2 oocysts with the second distinct spectra type. Also shown is the electrorotation spectrum for an oocyst without internal contents (+). Solid lines show the best fit from the multishell model, using values listed in Table 1.](http://aem.asm.org/)

### TABLE 1. Particle dimensions and dielectric parameter values used in the modeling of electrorotation spectra

<table>
<thead>
<tr>
<th>Parameter</th>
<th><em>Giardia</em></th>
<th></th>
<th><em>Cyclospora</em></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Viable</td>
<td>Nonviable</td>
<td>Viable</td>
<td>Nonviable</td>
<td>Viable</td>
</tr>
<tr>
<td>Dimensions (µm)</td>
<td>9 x 6⁴</td>
<td>9 x 6⁴</td>
<td>9³</td>
<td>9³</td>
<td>9³</td>
</tr>
<tr>
<td>Wall thickness (nm)</td>
<td>300³</td>
<td>300³</td>
<td>113³</td>
<td>113³</td>
<td>113³</td>
</tr>
<tr>
<td>Mean membrane thickness (midpoint ± range)(nm)</td>
<td>6 ± 0.5</td>
<td>6 ± 0.5</td>
<td>9 ± 0.5</td>
<td>9 ± 0.5</td>
<td>9 ± 0.5</td>
</tr>
<tr>
<td>Permittivity (ε)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interior</td>
<td>60³</td>
<td>60³</td>
<td>50³</td>
<td>50³</td>
<td>50³</td>
</tr>
<tr>
<td>Membrane (midpoint ± range)</td>
<td>6 ± 0.5</td>
<td>5 ± 0.5</td>
<td>4 ± 0.5</td>
<td>4 ± 0.5</td>
<td>4 ± 0.5</td>
</tr>
<tr>
<td>Wall</td>
<td>60³</td>
<td>60³</td>
<td>60³</td>
<td>60³</td>
<td>60³</td>
</tr>
<tr>
<td>Mean conductivity (midpoint ± range) (S m⁻¹)</td>
<td>0.8 ± 0.2</td>
<td>0.02 ± 0.002</td>
<td>0.8 ± 0.1</td>
<td>0.2 ± 0.03</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Membrane</td>
<td>2 x 10⁻⁶ ± 0.8</td>
<td>1 x 10⁻⁵ ± 0.2</td>
<td>5.5 x 10⁻⁶ ± 0.3</td>
<td>1 x 10⁻⁵ ± 0.1</td>
<td>8 x 10⁻⁶ ± 0.5</td>
</tr>
<tr>
<td>Wall</td>
<td>0.032 ± 0.002</td>
<td>0.05 ± 0.005</td>
<td>0.09 ± 0.01</td>
<td>0.01 ± 0.04</td>
<td>0.09 ± 0.01</td>
</tr>
</tbody>
</table>

* a As determined by references 29 and 34.
* b Measured values.
* c From reference 15.
* d —, none observed.
* e From reference 31.
* f From reference 21.
* g From reference 16.
two types of spectra shown in Fig. 3 are those of viable and nonviable oocysts. Discrimination of nonviable and viable C. cayetanensis oocysts by rotation in opposite directions at a specific frequency is not possible, due to overlap between the anti- and cofield crossover points and the very low rates of cofield rotation observed above 2 MHz.

The distinct n = 2 unsporulated intact-walled oocysts have a spectrum comparable to that of an oocyst that had no observable structural contents (Fig. 3) and which was considered nonviable. These spectra are similar over most of the frequency range, indicating that they are of a similar physiological state. The previous report on C. cayetanensis assumed that this type of response was due to a change in viability (8), but this could not be verified. Minor differences between spectra can be attributed to differing states of deterioration following oocyst death. Estimations of the electrical parameters of the different oocyst components for the viable and nonviable best model fits shown in Fig. 3 are listed in Table 1. Trends similar to those of the G. intestinalis cysts are found for the C. cayetanensis oocysts, namely, an increase in membrane conductivity with a corresponding decrease in the interior conductivity, providing further evidence that the change in shape of the spectra is indeed due to differences in physiological state.

Oocysts of C. cayetanensis were stored at room temperature (approximately 20°C) to induce sporulation. After 14 days, 30% were found to be sporulated. Exsolation studies (21) on these sporulated oocysts performed at the SPDL showed that 75% excysted. An electrorotational comparison between unsporulated and sporulated oocysts of C. cayetanensis is shown in Fig. 4. The sporulation state was determined by the presence or absence of two sporozoites with the oocyst wall as identified using phase-contrast microscopy. Significant differences in the spectra are found in the frequency window from 20 to 200 kHz, with a much-reduced rotational velocity for the sporulated oocysts in this range. The previous report, for a single isolate of C. cayetanensis, indicated that at a frequency of 1 MHz, unsporulated and sporulated oocysts rotate in opposite directions (8). Although this previous conclusion was valid for that isolate, this distinction is not possible with mixed isolates. Importantly, however, the distinction based on the rate of rotation in the frequency window mentioned is confirmed. From the multishell model, the change in the spectra upon sporulation can be considered to be primarily associated with a slight increase in oocyst membrane conductivity. It must be noted, however, that whereas the multishell model enables accurate determinations to be made of the dielectric properties of the outermost membrane and wall of the oocysts, at best it can only provide a rough indication of any changes occurring in the properties or level of complexity of internal structures (7).

In conclusion, we have demonstrated that the electrorotation technique can differentiate between viable and nonviable cysts of G. intestinalis. Two simple methods were identified for the rapid determination of cyst viability, the first of which involves only momentary observation of the particle to decide the direction of rotation. The second method involves determining the rotational velocity at a frequency close to the antifield maximum and may be more useful in environmental samples, as this feature is less sensitive to changes in the suspending medium conductivity. In using morphological inspection in conjunction with PI inclusion (36), we consider that our method for determining viability was sufficiently reliable in terms of deciphering the two distinct categories obtained in the electrorotation data.

In the absence of current viability surrogates for small numbers of organisms, we have also demonstrated that electrorotation velocity at the antifield maximum can be used to determine C. cayetanensis oocyst viability. The data presented in this paper for G. intestinalis cysts and from previously published electrorotation data from other protozoans (12), fungi (16), and animal cells (14) support this hypothesis. Indeed, where there is a need to develop viability assays for new pathogens or cells, by probing membrane integrity, electrorotation may provide a simple and rapid solution. Electrorotation also overcomes the problems associated with vital dyes, such as toxicity and the requirement for specialized storage.

The ability to determine the sporulation state of C. cayetanensis oocysts was also demonstrated with electrorotation. This is of importance as only following sporulation are the oocysts potentially infectious. Determining oocyst viability and sporulation state is therefore important in assessing the risk associated with potentially contaminated water. Currently only trained laboratory workers can identify the degree of sporulation, as oocysts of C. cayetanensis do not change their physical size upon sporulation.

Within the known limitations of the multishell model for...
characterizing bioparticles of complex structure (7), the data (Table 1) indicate that the membrane conductivity of nonviable (oo)cysts is significantly greater than that of viable ones. The corresponding decrease in the internal conductivity of the (oo)cysts confirms that this is associated with a physical degradation of the membrane and the loss of its ability to act as a barrier to passive ion flow. It is also of interest to note from Table 1 that the best fit of the multishell model suggests that the C. cayetanensis membrane is thicker that than for G. intestinalis (9 and 6 nm, respectively). On the assumption that the chemical composition of the membrane remains constant, then the reduced reduction in the relative permittivity of the G. intestinalis membrane from a value of 6 ± 0.5 to 5 ± 0.5 e, also suggests that the effective surface area of the membrane is reduced on transition from the viable to nonviable state. A reduction in membrane surface area could correspond to a reduction in the complexity of the surface morphology, such as, for example, a reduction in membrane folding. Alternatively, the membrane polarizability could be reduced through a loss of protein function, for example, by the formation of protein complexes within the lipid bilayer (24).

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REFERENCES