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Fertilization state of *Ascaris suum* determined by electrorotation

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Abstract

Electrorotation is a non-invasive technique that is capable of detecting changes in the morphology and physicochemical properties of microorganisms. The first detailed electrorotation study of the egg (ovum) of a parasitic nematode, namely *Ascaris suum* is described to show that electrorotation can rapidly differentiate between fertilized and non-fertilized eggs. Support for this conclusion is by optical microscopy of egg morphology, and also from modelling of the electrorotational response. Modelling was used to determine differences in the dielectric properties of the unfertilized and fertilized eggs, and also to investigate specific differences in the spectra of fertilized eggs only, potentially reflecting embryogenesis. The potential of electrorotation as an investigative tool is shown, as undamaged eggs can be subjected to further non-destructive and destructive techniques, which could provide further insight into parasite biology and epidemiology.

Introduction

Electrorotation is a versatile technique that has been used to investigate a variety of disparate particles including protozoa (Goater *et al.*, 1997; Dalton *et al.*, 2001a, 2004), bacteria (Zhou *et al.*, 1996; Hodgson & Pethig, 1998), viruses (Gimsa *et al.*, 1989), mammalian cells (Gascoyne *et al.*, 1997), liposomes (Chan *et al.*, 1997), algae (Gimsa *et al.*, 1991), cell lines (Cen *et al.*, 2004) and latex beads (Arnold *et al.*, 1987). A particular advantage in investigations of microorganisms is its non-destructive nature, being sensitive to the morphology and integrity of living organisms, so that organisms analysed by electrorotation can be subjected to further investigations. The technique is rapid, versatile and simple, and can be used as part of a sequential set of tests to fully characterize an organism. As such, electrotation has important diagnostic and healthcare implications.

During electrorotation, particles are subjected to a uniform rotating electric field that causes them to rotate (Arnold & Zimmermann, 1982; Mischel *et al.*, 1982). The induced rotation of the particle is a sensitive function of the particle's dielectric properties, namely the conductivity and permittivity of the organism's constituent components. These components include the wall (if present), the plasma membrane and the cytoplasm. The rotation is also a function of the conductivity and permittivity of the suspending medium. Electrorotation has been successfully used to investigate the viability of oocysts of the protozoans *Cryptosporidium parvum* (Goater *et al.*, 1997) and *Cyclospora cayetanensis* (Dalton *et al.*, 2001a) and cysts of the flagellate, *Giardia intestinalis* (Dalton *et al.*, 2001b). In the present work, the first full electrorotation investigation of a metazoan parasite within the economically important phylum, Nematoda, is reported.

In May 2001, the World Health Assembly passed a resolution affirming that the control of gastrointestinal helminth infections should be considered a public health priority (WHA, 2001). Infection with gastrointestinal helminths has a pronounced impact on nutrition (Stephenson *et al.*, 1993a), growth (Hadju *et al.*, 1996), physical fitness (Stephenson *et al.*, 1993b), cognitive functions (Simeon *et al.*, 1995; Watkins & Pollitt, 1997) and anaemia

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(Dossa *et al.*, 2001; Gilgen *et al.*, 2001; Torlesse & Hodges, 2001) in infants, school-age children and adults. Around 20 species are considered to cause disease, of which six together affect half the world population (Horton, 2003). The large parasitic roundworm of swine (*Ascaris suum*, Nematoda) and man (*A. lumbricoides*) is one of the six, and has considerable economic impact. In swine, the impact is apparent in suppressed weight gain and feed efficiency, increased morbidity and mortality, condemnation of organs at slaughter and predisposition to secondary pathogens through T-cell (T_H1:T_H2) imbalance (Hoover, 1997). Apart from swine, *A. suum* infects various animal species including ruminants, rodents and humans, but in non-porcine hosts, the parasite does not normally complete its life cycle, infection resulting in granulomatous lesions of the liver and an asthma-like syndrome. *Ascaris lumbricoides* infects approximately 1.5 billion people globally (Crompton, 1999), up from 1 billion in 1988 (Crompton, 1988), and the disease, ascariasis, is particularly prevalent in developing countries (Meeking *et al.*, 1996). In the USA, the Centers for Disease Control estimate that at any one time, 4 million people in the USA have ascariasis. An adult female *Ascaris* worm can lay >200,000 fertilized eggs into the environment, daily. These eggs can survive for 6–12 months in soils (Gaspard *et al.*, 1997) and have been reported to survive in sub-arctic regions (Embil *et al.*, 1984) for up to 14 years (Krasnosos, 1978). Heavy intestinal *A. lumbricoides* infections can prevent up to 25% of ingested calories being utilized (Latham *et al.*, 1977) and have been strongly linked to retarded growth in children (Hlaing, 1993; Ananthakrishnan *et al.*, 1997). Furthermore, high intensity infections can affect mental performance (Tarr & Fairbairn, 1973b). As for *A. suum*, *A. lumbricoides* infections also induce T-cell (T_H1:T_H2) imbalance.

Ascariasis control is dependent upon chemotherapy and/or improving sanitation. Since ascariasis is particularly predominant in developing countries (Meeking *et al.*, 1996), where anthelmintics are expensive and not readily available, improvements in sanitation (Crompton, 1985, 1999; Carneiro *et al.*, 2002) and reducing environmental contamination are more effective options in the long term. Therefore, identifying eggs of *Ascaris* spp. in the environment, and whether they are fertilized or not, is of importance in determining their potential to cause infection.

Distinct differences in the electrorotational response between fertilized and unfertilized eggs of *A. suum* are presented, which can be used for identification purposes in mixed populations. Variations within the electrorotational spectra of fertilized eggs are also reported and such variability may be due to structural changes during embryonation. Finally, the dielectrical properties of eggs are determined by analysing their electrorotational response using the dielectric multi-shell model, described by Chan *et al.* (1997) and Zhou *et al.* (1998).

Materials and methods

Adult female *A. suum* were collected from the intestines of slaughtered pigs at a local abattoir (Cig Arfon, Caernafron, UK) and stored intact in 5% formalin solution at 4°C. Eggs were collected from female worms by dissection of the uterus according to Smith (1989), decorticated by adding an equal volume of 12% sodium hypochlorite to the suspension, incubated at 37°C for 30 min, following which they were stored in ultra-pure water at 4°C. Unfertilized and fertilized eggs at the zygote (single cell, uncleaved) stage were analysed.

To obtain reproducible electrorotation data, eggs were initially washed and resuspended in a solution of well defined chemical composition and conductivity. For this work, phosphate buffered saline solution (PBS, pH 7.4; 10 mM phosphate buffer, 27 mM KCl, 137 mM NaCl, Sigma Chemical Co.), diluted to a conductivity of $0.65 \pm 0.1\,\mathrm{mS\,m^{-1}}$, was used as the suspending medium to achieve readily measurable rotation rates for modest applied voltages. To reduce electrical heating effects, the magnitude of the rotating field should also be as low as possible. The washing procedure (repeated three times) consisted of diluting a 100 μl aliquot of suspended eggs in ultra-pure water (conductivity $0.1\,\mathrm{mS\,m^{-1}}$) to 1.5 ml, vortexing for 30 s, microcentrifuging for 1 min (3300 *g*), then aspirating the supernatant to 100 μl. After the third wash, the sample was resuspended in PBS solution, previously diluted with ultra-pure water to give a conductivity of $0.65 \pm 0.1\,\mathrm{mS\,m^{-1}}$. To ensure that the addition of decorticated *A. suum* eggs did not modify the conductivity of the suspending medium, a sample of suspended eggs was centrifuged, and 200 μl of supernatant was removed and the conductivity measured using a Hanna Instruments Pure Water Tester™. Individual decorticated eggs were studied from samples with particle densities $\leq 5 \times 10^4\,\mathrm{eggs\,ml^{-1}}$.

Descriptions of the basic theory and experimental procedures of electrorotation are provided elsewhere (Goater & Pethig, 1998; Jones, 2003; Morgan & Green, 2003). In brief, 20 μl of suspended eggs were pipetted into a chamber surrounded by four planar gold electrodes. The electrodes were manufactured by photolithography in a class 10,000 clean room on a glass microscope slide with a 5 nm chromium adhesion layer and a 70 nm gold top layer. The so-named '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 centre of the chamber (fig. 1, Dalton *et al.*, 2001a). The shape and magnitude of the spectra obtained are known to be influenced by the particle position in the chamber and by the presence of debris on the particle surface (Dalton *et al.*, 1999). Therefore, eggs which (i) were positioned outside the middle third of the chamber, (ii) drifted more than their own diameter during the recording or (iii) possessed debris on their surface were excluded from analysis. Using these carefully selected criteria, coupled with an electrode design that minimized particle drift, enabled us to maximize the number of eggs investigated.

Identification of eggs and their direction of motion in the electric field were undertaken using phase contrast microscopy at a total magnification of ×100 and recorded by videotape for later analysis and timing by stopwatch (Nikon Optiphot-2 microscope with JVC model TK-1280E colour video camera attachment). A minimum of 20 s of behaviour was recorded at each

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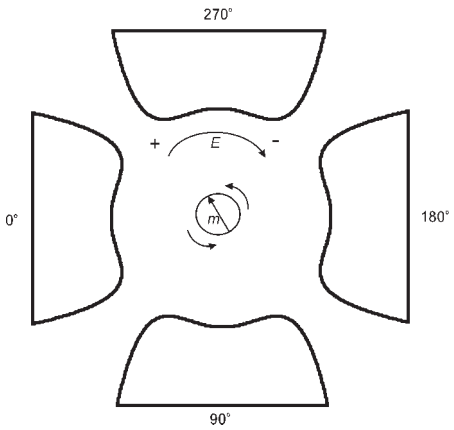

Fig. 1. Rotating field *E* generated by applying sinusoidal voltages to four 'bone' electrodes, with phases spaced 90° apart. The rotational torque acting on the particle, *p*, will be either co- or anti-field, depending on the angle of the induced dipole moment, which is dependent on the dielectric properties of the particle and the electrical frequency. In the present case, the particle is rotating counter to the clockwise rotating field. The distance between opposing electrode faces is 2 mm.

of 20, approximately equidistant, applied frequency points on a log scale over the range 100 Hz to 10 MHz. After each aliquot had been examined and the spectra recorded, eggs were removed from the chamber by washing under pressure, with ultra-pure water from a wash bottle, and the chamber dried under a stream of nitrogen gas.

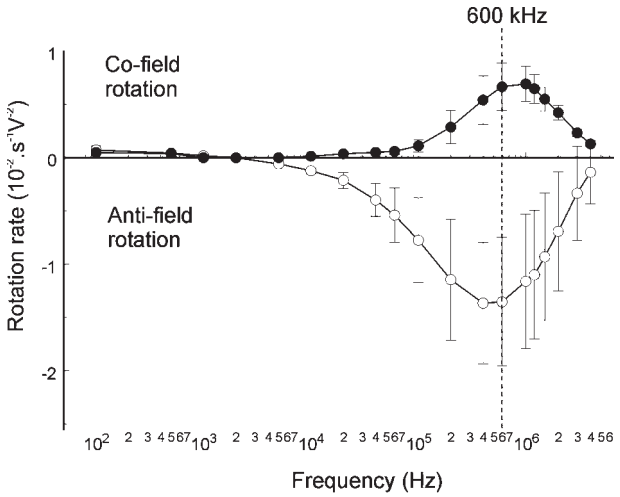

Fig. 2. Electrorotation spectra of *Ascaris suum* eggs comparing 14 unfertilized (●) and 104 fertilized (○) eggs. Error bars represent the mean rotation rate and one standard deviation. Solid lines show the best fit from the multi-shell model using values listed in table 1. Unfertilized eggs rotate in the co-field direction while fertilized eggs rotate in the anti-field direction (medium conductivity $= 0.65 \pm 0.1$ mS m^{-1}).

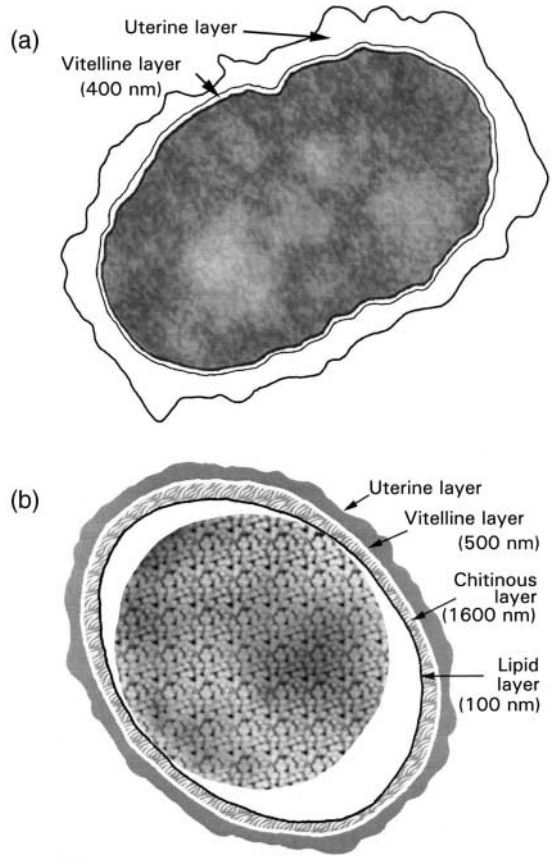

Fig. 3. Artist impression of (a) an unfertilized and (b) a fertilized *Ascaris suum* egg showing the egg shell structure.

Results and Discussion

The electrorotation spectra obtained for 118 *A. suum* eggs are shown in fig. 2. Unfertilized eggs of *A. suum* have the dimensions $74 \pm 4 \times 37 \pm 2\,\mu$m. Following fertilization, dimensions of eggs change to $60 \pm 5 \times 45 \pm 5\,\mu$m, enabling confirmation of the fertilization state by microscopy. From fig. 2, 104 eggs were identified as fertilized (one cell stage) and 14 as unfertilized. When subjected to electrorotation, fertilized eggs rotated more rapidly and in the opposite direction compared with unfertilized eggs, for most of the applied frequency range. At 600 kHz, the peak rotation rate of fertilized eggs is at a maximum in the anti-field region, as defined by the minimum error bars. Unfertilized eggs have a maximum peak rotation rate at a frequency of 800 kHz in the co-field region. Importantly, by applying a field at approx. 600 kHz, the distinction between fertilization states due to direction of rotation is at a maximum (fig. 2). This process can be automated readily (Zhou *et al.*, 1996; DeGasperis *et al.*, 1998) and used to rapidly assess large numbers of eggs in mixed populations, without the need for accurate size measurements.

Significant developmental differences between unfertilized and fertilized eggs are the thickening of the vitelline

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Table 1. Values used in the multi-shell model to fit the fertilized and unfertilized *Ascaris suum* egg spectra shown in fig. 2.

	Ascaris suum		
	Unfertilized	Fertilized	Reference
Egg dimensions (μm)	74 × 38	60 × 44	Foor, 1967
Vitelline layer thickness (nm)	400	500	Darben, 2002
Chitinous layer thickness (nm)	–	1600	Darben, 2002
Lipid membrane thickness (nm)	–	100	Darben, 2002
Relative permittivity (ε_r)			
Vitelline layer	60	60	Hoover, 1997
Chitinous layer	–	60	Hoover, 1997
Lipid membrane	–	6	Hoover, 1997
Cytoplasm	50	50	Hoover, 1997
Conductivity (S m^{-1})			
Vitelline layer	0.05	0.05	Asami *et al.*, 1976
Chitinous layer	–	0.05	Asami *et al.*, 1976
Lipid membrane	–	1×10^{-7}	Gimsa *et al.*, 1991
Cytoplasm	0.01	0.8	Tarr & Fairbairn, 1973a

layer and the formation of the chitinous and lipid membrane layers upon fertilization (fig. 3) (Foor, 1967; Darben, 2002). Modelling experimental data using the ellipsoidal multi-shell model (Kakutani *et al.*, 1993; Zhou *et al.*, 1996) with values taken from the relevant literature (table 1) indicates that the model is in good agreement with experimental data for the fertilized spectra (fig. 2). However, above 2 MHz, the model does not fit the unfertilized spectra well. This variation from experimental data above 2 MHz for unfertilized eggs can be attributed to known limitations of the model (Chan *et al.*, 1997). To obtain the best fit of the model to the unfertilized spectra data, a value of 0.01 S m^{-1} for cytoplasm conductivity was required, which is a value typically associated with a non-viable organism (Huang *et al.*, 1992; Zhou *et al.*, 1996). This suggests that either viable, unfertilized eggs are more permeable to passive ion flow than fertilized eggs or die *in utero*, rapidly losing membrane integrity and becoming leaky. Selective permeability to polar and non-polar substances in fertilized eggs is a function of the lipid membrane layer.

There is a large degree of variance in the fertilized spectra data as indicated by the large error bars in fig. 2. Closer examination of spectra for fertilized eggs indicated three likely trends in the anti-field region, with rotation rate peaks at 200 kHz, 600 kHz and 1.5 MHz respectively (fig. 4). The only factor that appears to correlate with these three trends of spectra for fertilized eggs is the observed microscopical distribution of their internal contents (table 2). At the peak rotation rate frequency of 1.5 MHz, 78% of eggs contain developing embryos which appeared in apposition to the chitinous layer of the eggshell. Corresponding figures at 600 kHz and 200 kHz are 29% and 8% respectively. This is likely to be due to developmental changes in eggs associated with embryogenesis, particularly changes in the lipid layer, which is composed of 75% 'free' ascarosides (glycosides of ascaryl alcohol) and 25% protein (Fairbairn, 1957). The presence of amino acids in the lipid layer suggests the presence of a lipoprotein structure (Jaskoski, 1962), and while almost all ascarosides secreted in the ovaries of *A. suum* occur as esters (Tarr & Fairbairn, 1973a), the levels of ascaroside esters steadily decrease as eggs develop, accompanied by a subsequent increase in the levels of 'free' ascarosides (Tarr & Fairbairn, 1973b). By varying the modelling data to obtain the best fit to the experimental data for the three trends at 200 kHz, 600 kHz and 1.5 MHz (table 3), changes in the properties of the egg surrounding the developing embryo, particularly those associated with the lipid membrane and cytoplasm, can explain the differences between the spectra obtained. It is important to note that the egg of *A. suum* is the most complex biological structure to which this model has been applied, and further investigation using multicellular organisms, including fully embryonated, infective eggs, should be undertaken.

In conclusion, this is the first detailed electrorotation study of nematode eggs, specifically *Ascaris suum*.

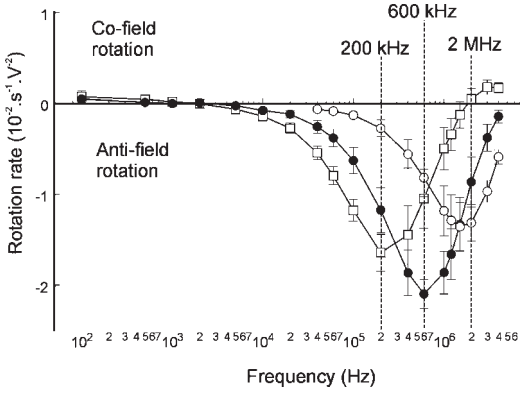

Fig. 4. Electrorotation spectra for 75 fertilized *Ascaris suum* eggs showing three distinct areas of rotation at the peak rotation frequencies of 200 kHz (□, $n = 30$), 600 kHz (●, $n = 26$) and 2 MHz. (○, $n = 19$). The absence of experimental data below 60 kHz for '○' is due to the torque exerted by the field being insufficient to overcome frictional forces between the egg and the substrate. Error bars represent the mean rotation rate and one standard deviation. Solid lines show the best fit from the multi-shell model using values listed in tables 1 and 3 (medium conductivity $= 0.65 \pm 0.1$ mS m^{-1}.

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Table 2. Analysis of data producing the three peak anti-field rotation frequency sets observed in fig. 4.

	Peak anti-field rotation frequency		
	1.5 MHz ($n = 14$)	600 kHz ($n = 21$)	200 kHz ($n = 37$)
Contents touching chitinous layer (%)	78	29	8
Contents not touching chitinous layer (%)	22	71	92

Table 3. Changes to the model parameters given in table 1 to obtain the best fit to the three anti-field sets shown in fig. 4.

		Changes to model parameters for fertilized spectra		
	Initial value from table 1	New value 200 kHz peak	New value 600 kHz peak	New value 1.5 MHz peak
Lipid membrane thickness (nm)	100	–	–	200
Lipid membrane conductivity (S m^{-1})	1×10^{-7}	5×10^{-6}	1×10^{-6}	5×10^{-6}
Lipid membrane permittivity (ε_r)	6	15	4	3
Chitinous layer conductivity (S m^{-1})	0.05	0.09	0.04	0.09
Cytoplasmic conductivity (S m^{-1})	0.8	0.5	–	–

Electrorotation readily distinguishes between fertilized and unfertilized decorticated eggs, as confirmed using standard microscopical techniques. Spectra for fertilized eggs follow three trends, potentially reflecting embryogenesis. Automated electrorotation systems based on image processing techniques have been described elsewhere (DeGasperis *et al.*, 1998; Zhou *et al.*, 1998) and provide a rapid and reproducible method for determining the contents of a mixed population. As electrorotation is a non-invasive technique, undamaged eggs can be subjected to further non-destructive and destructive techniques which could provide further insight into parasite biology and epidemiology.

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