Acta Protozool. (2003) 42: 191 - 195

ACTA
PROTOZOOLOGICA

Antigenic Relationships Between *Aggregata octopiana* and *A. eberthi* Two Parasites of Cephalopods

Helena RODRÍGUEZ, Celia CRESPO, Montserrat SOTO, Cristina ARIAS, Raúl IGLESIAS and José M. GARCÍA-ESTÉVEZ

Cátedra de Parasitología, Laboratorio de Parasitología Marina, Facultad de Ciencias del Mar, Universidad de Vigo, Vigo, Spain

Summary. Proteins from soluble extracts of sporocysts of the two protozoan parasites of cephalopods *Aggregata octopiana* Schneider, 1875 and *A. eberthi* Labbé, 1895 (Apicomplexa: Aggregatidae) were compared by electrophoresis and immunoblotting using specific antisera and the immunologic cross-reactivity between both species analyzed. Both species showed a considerable cross-reactivity in ELISA and immunoblotting. However, each species showed a characteristic electrophoretic pattern and species-specific antigens which makes species differentiation possible in SDS-PAGE and immunoblotting.

Key words: *Aggregata octopiana*, *Aggregata eberthi*, antigens, Apicomplexa, cross-reactivity.

INTRODUCTION

The genus *Aggregata* has a two-hosts life cycle, with asexual stages in the intestines of crustaceans and sexual stages in the digestive tracts of cephalopods. In cephalopods, the merozoites migrate through the epithelium of the digestive tract causing degeneration and death of the parasitized cells and leading to detachment of necrotic fragments of the intestine (Hochberg 1990).

Since the first references to the genus *Aggregata* (Schneider 1875, Labbé 1899, Dobell 1925) were published until the present, one of the main controversies of the genus *Aggregata* has been its taxonomy. Problems related to synonymy have arisen due to the fact that the *Aggregata* species have traditionally been differentiated on the basis of the final host and morphological characters (Poynton *et al.* 1992, Estévez *et al*. 1996). For these reasons, several authors have suggested that most species of *Aggregata* in Europe require a new description to determine taxonomic validity (Sprague and Couch 1971; Levine 1985, 1988).

Because of the problems with *Aggregata* spp. systematics, we decided to undertake the biochemical and immunological characterization of two of the most common species of *Aggregata* in natural cephalopod populations in the Ria of Vigo: *A. octopiana* and *A. eberthi*, infecting *Octopus vulgaris* and *Sepia officinalis* with

Address for correspondence: José Manuel García Estévez, Cátedra de Parasitología, Laboratorio de Parasitología Marina, Facultad de Ciencias del Mar, Universidad de Vigo, 36200 Vigo, Spain; Fax: +34-986812565; E-mail: jestevez@uvigo.es

prevalences of 100% and 87% respectively (Pascual *et al*. 1996). We analyzed the protein patterns of both species and the possibility of a cross-reactivity between them.

MATERIALS AND METHODS

Parasites

Oocysts of *Aggregata octopiana* Schneider, 1875 and *A. eberthi* Labbé, 1895 were isolated from the digestive tracts of molluscan cephalopods *Octopus vulgaris* Cuvier, 1798 and *Sepia officinalis* L., 1758, respectively, naturally infected in the Ría de Vigo, NW of Spain.

Purification of sporocysts and sporocyst extracts

Sporocysts of both species *A. octopiana* and *A. eberthi* were purified from oocysts, as described previously Estévez *et al*. (1992). Briefly, the sporocysts were obtained by maceration of oocysts in phosphate-buffered saline (PBS). The resulting suspension was then filtered through increasingly fine meshes to remove tissue fragments. The filtrate was centrifuged at 2,000 *g* for 15 min; this filtration-centrifugation process was repeated various times until a pure sample of sporocysts was obtained. Sporocysts were washed twice in PBS, then resuspended in phosphate buffer containing 3% sodium dodecyl sulphate (SDS), and 1 mM ethylenediaminetetraacetic acid (EDTA; Sigma Chemical Co., St. Louis, USA), 10 mM iodacetamide (Merck, Darmstadt, Germany) and 50 µg/ml phenylmethylsulphonyl fluoride (PMSF; Sigma) as protease inhibitors. Sporocysts were lysed by sonication on ice (60 W in 1 min pulses for 45 min). The lysed sporocysts were centrifuged at 10,000 *g* for 30 min at 4°C and the supernatant, being used in all analyses, was exhaustively dialysed against PBS and stored at - 30°C until use (Leiro *et al*. 1993).

Immunization and sera

Ten BALB/c mice were immunized by subcutaneous injection of 0.2 ml mixture of Freund's complete adjuvant (Sigma) and PBS containing about 5 x 10^6 sporocysts of *A. octopiana* and re-immunized 30 days later by intraperitoneal (i.p.) injection of 0.2 ml of PBS without adjuvant containing the same number of sporocysts. On day 45, a third dose (without adjuvant) was administered by i.p. injection. Mice were bled 60 days after primary immunization through the retroorbital route. Serum (anti-*A. octopiana*) were separated by centrifugation at 2,000 *g* for 10 min, mixed 1:1 with glycerol, and stored at -30°C until use.

Another 10 mice were immunized with sporocysts of *A. eberthi* following the same protocol for obtaining anti-*A. eberthi* serum.

Enzyme-linked immunosorbent assay (ELISA)

Indirect ELISA was carried out as described previously by Estévez *et al*. (1994). The corresponding sporocyst extracts (1µg/well) were bound to PVC microtitre plates (Costar, Massachusetts) in 50 µl/well of carbonate/bicarbonate buffer, pH 9.6, overnight at 4°C. Plates were then washed 3 times with Tris-buffered saline (TBS: 50 mM Tris, 0.15 M NaCl; pH 7.4) and active sites remaining on the plates were blocked, for 2h at 37°C, with a solution of 5% non-fat dry milk in TBS containing 0.2% Tween 20 (TBS-T_1). Antigen-coated plates were incubated for 2h at 37°C with 50 µl of a dilution 1:20,000 of mouse anti-*A. octopiana* or anti-*A. eberthi* serum (both polyclonal sera showed similar titer in ELISA), in TBS-T_1 containing 1% non-fat dry milk, washed five times with TBS containing 0.05% Tween 20 (TBS-T_2) and incubated for 1h at 37°C with 50 µl peroxidase conjugated rabbit anti-mouse immunoglobulin polyclonal antibody (Dakopatts A/S, Glostrup, Denmark) diluted 1:2,000 in TBS-T_1 containing 1% non-fat dry milk and 3% polyethyleneglycol (PEG) 6,000. After 5 washes with TBS, 50 µl of substrate containing 0.04% *o*-phenylendiamine (Sigma) and 0.001% H_2O_2 in phosphate/citrate buffer (pH 5.0) were added to the wells. The reaction was stopped after 20 min by adding 25 µl of 3N H_2SO_4. Optical density at 492 nm was measured with a microtitre plate reader (Titertek Multiskan, Labsystems, Finland).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The components of soluble extracts of sporocysts were separated electophoretically by SDS-PAGE. Electrophoresis was carried out using linear gradient polyacrylamide gels (5-15% under non-reducing and 10-20% under reducing conditions), using a Mini-Protean II cell (BioRad, Richmond, USA), as described previously by Estévez *et al*. (1993). Reduction and denaturalization were carried out in 62 mM Tris-HCl buffer, pH 6.8, containing 2% SDS, 10% glycerol, 0.004% bromophenol blue and 0.02 M dithiothreitol (DTT), for 4 min at 95-100°C. Samples (8 µg/lane) were run for 45 min at 200 V in electrode buffer containing 25 mM Tris, 190 mM glycine and 1% SDS; pH 8.3. Bands were revealed with silver staining (Sambrook *et al*. 1989). Molecular weights were estimated using a calibration curve [log Mw vs log polyacrylamide concentration (%T) (Hames 1981)] constructed with the low molecular weight markers phosphorylase B (97.4 kDa), bovine serum albumine (66.2 kDa), ovoalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa) as standards (BioRad).

Immunoblotting analysis

Following electrophoresis as above, proteins were immunoblotted at 15 V for 45 min onto a 0.45 µm pore size nitrocellulose membrane using a Trans-Blot SD semi-dry transfer cell (BioRad) with electrode buffer recommended by the supplier (48 mM Tris, 39 mM glycine, 0.037% SDS and 20% methanol, pH 9.2). The membrane was washed with TBS and stained with Ponceau S to verify transfer and to locate molecular weight markers, then dried, and cut into strips. Each strip was incubated for 2 h at room temperature (RT) with TBS-T_1 containing 5% non-fat dry milk. Immunorecognition was performed by incubation for 2 h at RT with the corresponding antibodies (anti-*A. octopiana* or anti-*A. eberthi* serum) diluted 1:200 in TBS-T_1 containing 1% non-fat dry milk. Each strip was then washed five times with TBS-T_2 and incubated for 1h at RT with a 1:800 dilution of peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dakopatts) in TBS-T_1 containing 4% PEG-6,000. After washing five times with TBS-T_1 and other five with TBS, the recognized bands were stained by adding TBS containing 0.003% H_2O_2, 0.06% diaminobenzidine tetrahydrochloride (Sigma) and 0.03% $NiCl_2$.

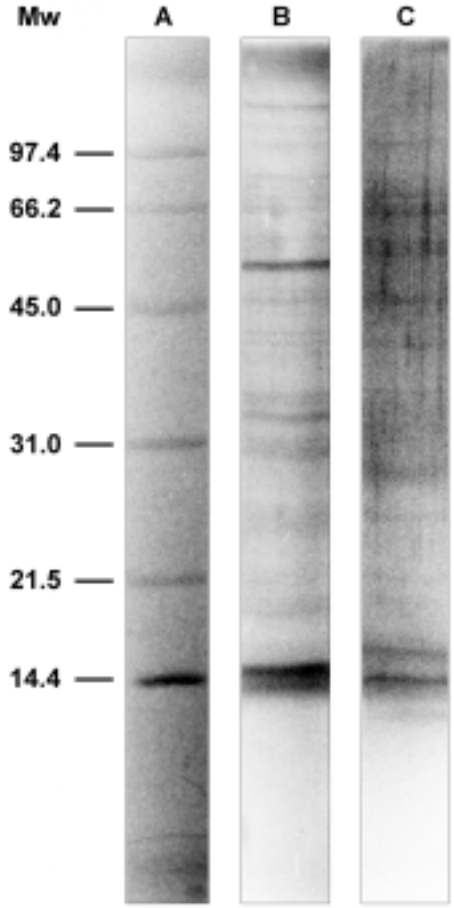

Fig. 1. SDS-PAGE of extracts of sporocysts of the two coccidian species under reducing conditions using a 10-20% linear gradient and silver staining. Lane **A:** Molecular weight markers (kDa). Lane **B:** Sporocysts extract of *Aggregata octopiana*. Lane **C:** Sporocysts extract of *A. eberthi*.

The reaction was stopped after approximately 3 min by exhaustive washing with TBS, and the stained lanes were recorded photographically (Estévez *et al.* 1993).

Protein determination

Protein concentration of all preparations were estimated using the Bio-Rad Protein Assay (BioRad) with bovine serum albumin (Sigma) as standard.

RESULTS

SDS-PAGE characterization of sporocyst extracts

Soluble extracts of sporocysts of *Aggregata* species, *A. octopiana* and *A. eberthi*, were characterized by SDS-PAGE under reducing conditions (Fig. 1). The banding patterns of the two extracts were completely different, noting the presence of four dominant bands of 13, 15, 34 and 57 kDa (Fig. 1, lane B) in the *A. octopiana* extract and the presence of six dominant bands of 13, 16, 45, 48, 60 and 66 kDa in the *A. eberthi* extract (Fig. 1, lane C).

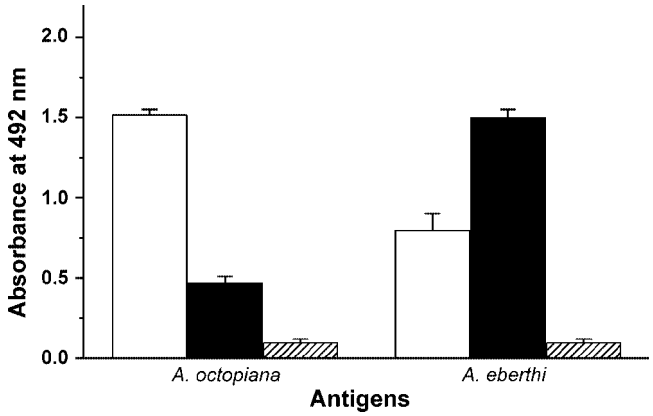

Fig. 2. Reactivity in ELISA of anti-*A. octopiana* mouse sera (white), anti-*A. eberthi* mouse sera (black) and normal mouse sera (ruled), with sporocysts extracts of *Aggregata octopiana* and *A. eberthi*. The values shown are the means ± standard error of assays of sera from ten mice.

Cross-reactivity between *Aggregata octopiana* and *A. eberthi*

In indirect ELISA, the polyclonal antibodies obtained in mice immunized with purified sporocysts of *A. eberthi* and *A. octopiana* showed cross-reactivity with heterologous antigenic extracts (Fig. 2). This cross-reactivity was greater when *A. eberthi* antigens were assayed.

Immunoblotting under reducing conditions (Fig. 3) confirmed the cross-reactivity between the two species observed in ELISA. However, a group of bands migrating between 28 and 34 kDa were specifically recognized by the anti-*A. octopiana* serum on the homologous antigenic extract. Some species-specific antigenic bands at the 21-24 kDa region were also detected on the *A. octopiana* extract in immunoblottings under non-reducing conditions (Fig. 4).

DISCUSSION

The results obtained showed that sporocyst extracts from *A. octopiana* and *A. eberthi* gave totally different profiles with SDS-PAGE, confirming the potential value of this technique for distinguishing between these two

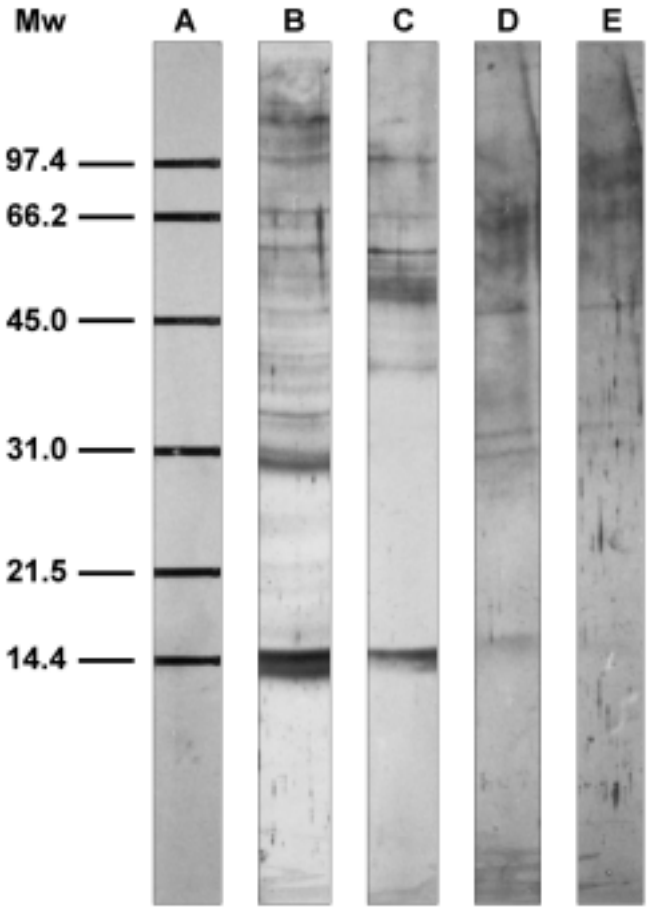

Fig. 3. Immunoblots of extracts of sporocysts of *Aggregata octopiana* and *A. eberthi* under reducing conditions using an 10-20% linear gradient, with anti-*A. octopiana* mouse serum or anti-*A. eberthi* mouse serum. Lane A: Molecular weigh markers (kDa). Lane B: *A. octopiana* sporocysts extract with anti-*A. octopiana* serum. Lane C: *A. octopiana* sporocysts extract with anti-*A. eberthi* serum. Lane D: *A. eberthi* sporocysts extract with anti-*A. eberthi* serum. Lane E: *A. eberthi* sporocysts extract with anti-*A. octopiana* serum.

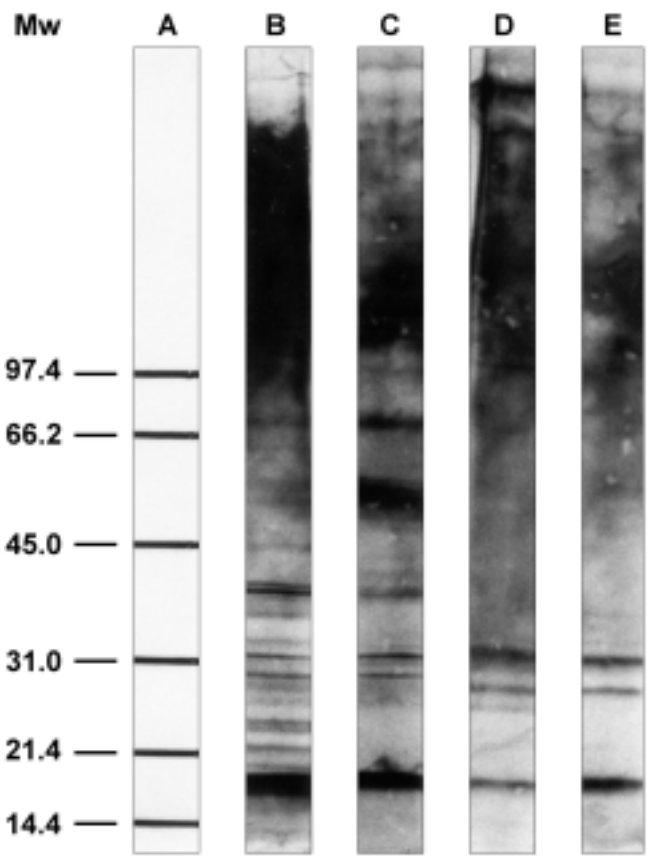

Fig. 4. Immunoblots of extracts of sporocysts of *Aggregata octopiana* and *A. eberthi* under non-reducing conditions using an 5-15% linear gradient, with anti-*A. octopiana* mouse serum or anti-*A. eberthi* mouse serum. Lane A: Molecular weigh markers (kDa). Lane B: *A. octopiana* sporocysts extract with anti-*A. octopiana* serum. Lane C: *A. octopiana* sporocysts extract with anti-*A. eberthi* serum. Lane D: *A. eberthi* sporocysts extract with anti-*A. eberthi* serum. Lane E: *A. eberthi* sporocysts extract with anti-*A. octopiana* serum.

species. The electrophoretic techniques have also been applied to distinguish between the spores of microsporidian species (Street and Briggs 1982, Leiro *et al*. 1994).

Our ELISA and immunoblotting studies revealed the existence of a considerable cross-reactivity between *A. octopiana* and *A. eberthi* species although some species-specific antigenic bands were detected in immunoblotting. Thus three low weight antigens migrating between 21 and 31 kDa were specifically stained on the *A. octopiana* extract. This result suggests that monoclonal antibodies raised against these antigens or other species-specific antigens may be helpful tools to differentiate species belonging to the genus *Aggregata*, where a great intraspecific morphologic variability exists (Hochberg 1990). On the other hand, in studies on other coccidian species, similar low weight bands have been identified as membrane or surface proteins (Jenkins and Dame 1987, Wisher and Rose 1987), some of which vary from one species to another in the same genus (Wisher 1986, Tomavo *et al*. 1989, Tilley and Upton 1990), and even between the different life cycle stages of the same species (Kawazoe *et al*. 1992). At the moment, we do not know if the low weight specific antigens detected in our study are expressed on the *A. octopiana* sporozoite surface but this possibility stimulates more exhaustive characterization studies focused on the exact location and functionality of these

antigens. Knowledge of the *A. octopiana* biology is very important because this coccidian parasite could become a serious octopus pathogen under culture conditions (at present, experiences on nutrition and reproduction of *Octopus vulgaris* under captivity are very advanced; Villanueva 1995, Iglesias *et al.* 2000).

Acknowledgements. This work was supported by the Secretaría Xeral de Investigación e Desenvolvemento of Xunta de Galicia, under Projects PGIDT99PX10501B and PGIDT01MAR30101PR.

REFERENCES

Dobell C. (1925) The life -history and chromosome cycle of *Aggregata eberthi* (Protozoa: Sporozoa: Coccidia). *Parasitology* **17:** 2-130

Estévez J., Iglesias R., Leiro J., Ubeira F. M., Sanmartín M. L. (1992) An unusual site of infection by a microsporean in the turbot *Scophthalmus maximus*. *Dis. Aquat. Org.* **13:** 9-142

Estévez J., Leiro J., Sanmartín M. L., Ubeira F. M. (1993) Isolation and partial characterization of turbot (*Scophthalmus maximus*) immunoglobulins. *Com. Biochem. Physiol.* **105A:** 275-281

Estévez J., Leiro J., Santamarina M. T., Domínguez J., Ubeira F. M. (1994) Monoclonal antibodies to turbot (*Scophthalmus maximus*) immunoglobulins: Characterization and applicability in immunoassays. *Vet. Immunol. Immunopathol.* **41:** 353-366

Estévez J., Pascual S., Gestal C., Soto M., Rodríguez H., Arias C. (1996) *Aggregata octopiana* Frenzel 1885 (Apicomplexa: Aggregatidae) from *Octopus vulgaris* (Cuvier, 1798) off NW Spain. *Dis. Aquat. Org.* **27:** 227-231

Hames B. D. (1981) An introduction to polyacrylamide gel electrophoresis. In: Gel Electrophoresis of Proteins. A Practical Approach, (Eds. B. D. Hames, D. Rickwood). IRL Press, Oxford, 1-91

Hochberg F. G. (1990) Diseases of Mollusca: Cephalopoda. Diseases caused by protistans and metazoans. In: Diseases of Marine Animals, (Ed. O. Kinne). Biologische Anstalt Helgoland, Hamburg, **3:** 47-202

Iglesias J., Sánchez F. J., Otero J. J., Moxica C. (2000) Culture of octopus (*Octopus vulgaris*, Cuvier): Present knowledge, problems and perspectives. *Cahiers Options Méditerranéennes* **47:** 313-321

Jenkins M. C., Dame J. B. (1987) Identification of immuno-dominant surface antigens of *Eimeria acervulina* sporozoites and merozoites. *Mol. Biochem. Parasitol.* **25:** 155-164

Kawazoe U., Tomley F. M., Frazier J. A. (1992). Fractionation and antigenic characterization of organelles of *Eimeria tenella* sporozoites. *Parasitology* **104:** 1-9

Labbé A. (1899) Sporozoa. In: Das Tierreich. Protozoa, (Ed. F. E. Schulze) R. Friendlander und Sohn, Berlin, **5:** 1-180

Leiro J., Estévez J., Santamarina M. T., Sanmartín M. L., Ubeira F. M. (1993) Humoral immune response of turbot, *Scophthalmus maximus* (L.), to antigens from *Tetramicra brevifilum* Matthews & Matthews, 1980 (Microspora). *J. Fish Dis.* **16:** 577-584

Leiro J., Estévez J., Ubeira F. M., Santamarina M. T., Sanmartín M. L. (1994) Serological relationships between two microsporidian parasites of fish. *Aquaculture* **125:** 1-9

Levine N. D. (1985) Phylum II. Apicomplexa Levine, 1970. In: An Illustrated Guide to the Protozoa, (Eds. J. J. Lee, S. H. Hutner, E. C. Bovee) Society of Protozoologists, Lawrence, Kansas, 322-374

Levine N. D. (1988) The Protozoan Phylum Apicomplexa. Vol. I. CRC Press, Boca Ratón, Florida

Pascual S., Gestal C., Estévez J., Rodríguez H., Soto M., Arias C. (1996) Parasites in commercially-exploited cephalopods (Mollusca: Cephalopoda) in Spain: an updated perspective. *Aquaculture* **142:** 1-10

Poynton S. L., Reinschuessel R., Stoskopf K. (1992) *Aggregata dobelli* n.sp. and *Aggregata millerorum* n.sp. (Apicomplexa: Aggregatidae) from two species of octopus (Mollusca: Octopidae) from the Eastern North Pacific Ocean. *J. Protozool.* **39:** 248-256

Sambrook J., Fritsch E. F., Maniatis T. (1989) Detection and analysis of proteins expressed from cloned genes. In: Molecular Cloning. A Laboratory Manual. Volume III, (Ed. C. Nolan). Cold Spring Harbour Laboratory Press, New York

Schneider A. (1875) Note sur la *Psorospermies oviformes* du Poulpe. *Arch. Zool. Exp. Gen.* **9:** 387-404

Sprague V., Couch J. (1971) An annotated list of protozoan parasites, hyperparasites and commensals of decapod crustacea. *J. Protozool.* **18:** 526-537

Street D. A., Briggs J. D. (1982) An evaluation of sodium dodecyl sulfate-polyacrylamide gel electrophoresis for identification of Microsporidia. *J. Invert. Pathol.* **40:** 159-165

Tilley M., Upton S. J. (1990) Identification of sporozoite surface proteins and antigens of *Eimeria nieschulzi* (Apicomplexa). *J. Protozool.* **37:** 86-90

Tomavo S., Dubremetz J. F., Entzeroth R. (1989) Characterization of a surface antigen of *Eimeria nieschulzi* (Apicomplexa: Eimeriidae) sporozoites. *Parasitol. Res.* **75:** 343-347

Villanueva R. (1995) Experimental rearing and growth of *Octopus vulgaris* from planktonic hatching to settlement. *Can. J. Fish Aquat. Sci.* **52:** 2639-2650

Wisher M. H. (1986) Identification of the sporozoite antigens of *Eimeria tenella*. *Mol. Biochem. Parasitol.* **21:** 7-15

Wisher M. H., Rose M. E. (1987) *Eimeria tenella* sporozoites: the method of excystation affects the surface membrane proteins. *Parasitology* **95:** 479-489

Received on 28th April, 2003; corrected version 11th June; accepted on 12th June, 2003