

## IMMUNOLocalIZATION OF AN ENTEROTOXIC GLYCOPROTEIN EXOANTIGEN ON THE SECRETORY ORGANELLES OF *CRYPTOSPORIDIUM PARVUM* SPOROZOITES

EL-SHEWY K.A.\* & EID R.A.\*\*

### Summary:

In this study, the fine ultrastructures of the secretory organelles of *C. parvum* sporozoites were demonstrated using transmission electron microscopy (TEM). Meanwhile, a previously identified enterotoxigenic 18-20 kDa copro-antigen (18-20 kDa CCA), associated with cryptosporidiosis in both human and calves, was isolated and immunolocalized on *C. parvum* sporozoites. Using immunoelectron microscopy and anti-18-20 kDa monospecific antibody demonstrated marked existence of the 18-20 kDa CCA on the apical organelles and at the trilaminar pellicles. An anterior extrusion of this protein was demonstrated around the excysted and released sporozoites. However, non excysted sporozoites did not show this protein. Affinity blotting, with biotinylated jacalin, demonstrated the O-linked oligosaccharide moiety of this protein. The potential role of this protein in the host cell invasion and/or gliding motility remains unelucidated. However, its enterotoxigenicity, location and secretory nature suggest that it may be a target for neutralization or invasion inhibition of *Cryptosporidium*.

**KEY WORDS :** *Cryptosporidium parvum*, coproantigen, 18-20 kDa glycoprotein, exoantigen, immunolocalization.

### Résumé :

IMMUNOLocalISATION D'UN EXOANTIGÈNE GLYCOPROTÉIQUE ENTÉROTOXIQUE DANS LES ORGANITES SÉCRÉTOIRES DE SPOROZOÏTES DE *CRYPTOSPORIDIUM PARVUM*

Dans cette étude, l'ultrastructure fine des organites sécrétoires de sporozoïtes de *C. Parvum* a été étudiée en microscopie électronique à transmission (TEM), alors qu'un coproantigène entérotaxigène de sporozoïte (18 kDa CCA), préalablement identifié et associé avec la cryptosporidiose de l'homme ou des bovins, était localisé à l'aide d'anticorps. L'anticorps monospécifique anti-antigène (18-20 kDa) révèle la présence de l'antigène dans les organites apicaux et dans la pellicule trilaminaire. Une extrusion antérieure de cette protéine est repérable autour du sporozoïte relâché par dékystement, alors qu'elle n'existe pas autour des sporozoïtes non dékystés. Le transfert d'affinité réalisé avec de la jacaline biotinylée a révélé une partie oligosaccharidique O liée sur cette protéine. Le rôle potentiel de cette protéine dans l'invasion de la cellule et dans le glissement du sporozoïte n'a pas été tiré au clair. Cependant, son entérotaxigénité, sa localisation et sa nature sécrétoire suggèrent qu'elle pourrait être la cible d'une neutralisation ou d'une inhibition de l'invasion de *Cryptosporidium*.

**MOTS CLÉS :** *Cryptosporidium parvum*, coproantigène, glycoprotéine de 18-20 kDa, exoantigène, immunolocalisation.

*Cryptosporidium parvum* (*C. parvum*) is an apicomplexan protozoan parasite that is increasingly recognized as a major cause of diarrhea and gastroenteritis in both human and animals (Laurent *et al.*, 1999). In the past few years the parasite received a great deal of attention because of the persistent fatal diarrhea in the immunocompromized patients, and because of the public health threat of water borne outbreak in otherwise healthy individuals (Mackenzie *et al.*, 1995). So far, no approved effective drug or immunotherapy for controlling or prevention

of cryptosporidiosis is currently available (Fayer & Ungar, 1986). The invasive stages of apicomplexan parasites characteristically possess sets of secretory organelles. These organelles proved to be involved in the production and storage of secretory materials that assist in gliding motility, cells adhesion and host cell invasion (Lingelbach & Joiner, 1998; Langer & Riggs, 1999; Preiser *et al.*, 2000). Many workers refer to the importance of such secretory products in developing vaccines against apicomplexan parasites as *Plasmodia*, *Toxoplasma* and *Eimeria* (Schwartzman 1986; Whitmire *et al.*, 1988; Perkins, 1992).

In a previous study we have identified and isolated an 18-20 kDa *Cryptosporidium* copro-antigen (18-20 kDa CCA), which demonstrated an enterotoxigenic activity (El-Shewy *et al.*, 1994; El-Shewy *et al.*, 1999). In the present study we have attempted to demonstrate the ultrastructures of the sporozoites secretory organelles and to localize this protein using a monospecific anti-18-20 kDa antibody.

\* Microbiology and Parasitology Department, Faculty of Medicine, Suez Canal University, Ismailia, Egypt.

\*\* Electron Microscopy Center, Faculty of Science, Zagazig University, Egypt.

Correspondence: Dr Khalid A. El-Shewy, Microbiology Department, College of Medicine and Medical Sciences, King Khalid University, PO Box 641, Abha, Kingdom of Saudi Arabia. Fax: 00966 72247570. E-mail: Khel666@hotmail.com

## MATERIALS AND METHODS

### STOOL SAMPLES COLLECTION AND ANTIGEN PREPARATION

Positive stool samples of *C. parvum* (as proved by microscopic examinations) were collected from naturally infected dairy calves. Stool samples were used in purification and isolation of *C. parvum* oocysts and as a source for extraction of the previously detected 18-20 kDa protein (El-Shewy *et al.*, 1994). Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electroelution techniques were used to analyze and extract the protein band of interest (18-20 kDa) according to Laemmli (1970) and Jacobs & Clad (1986) respectively.

### OOCYSTS PURIFICATION AND EXCYSTATION

*C. parvum* oocysts were concentrated and purified from stool samples using Sheather's floatation and cesium chloride (CsCl) gradient methods (Ma & Soave, 1983; Kilani & Sekla, 1985). The concentrated purified *C. parvum* oocysts were excysted with the method of Current & Haynes (1984).

### ANTI 18-20 kDa MONOSPECIFIC ANTIBODY PREPARATION

Pathogen free 2.5 kg New Zealand White (NZW) female rabbits were used to raise monospecific antibody against the electroeluted 18-20 kDa protein. Rabbits received four booster doses of 125 µg purified and concentrated protein mixed with 1 ml of Freund's incomplete adjuvant, via intramuscular and subcutaneous injection at different sites, with ten days intervals. Rabbits were bled before immunization and two weeks after the last injection. Collected sera were tested against the purified 18-20 kDa protein by immunoblotting (Towbin *et al.*, 1979).

### ELECTRON MICROSCOPIC STUDIES

Excysted *C. parvum* sporozoites were aliquoted in 150 µl volume and mixed with an 850 µl aliquot of glutaraldehyde formaldehyde fixative solution throughout processing. The samples were proceeded for electron microscopic examination following the method of Roth *et al.*, 1981 and Armbruster *et al.*, 1982. Briefly, samples were centrifuged, washed three times and left in buffer overnight at 4°C. The samples were dehydrated in a graded series of alcohol, then sectioned, picked up on nickel grids and floated on 1 % bovine serum albumin (BSA) in phosphate buffer saline (PBS) pH 7.4 for 15 min. They were left to react with a drop of monospecific anti 18-20 kDa antibody, or control rabbit serum, diluted 1:100 with 1 % BSA in PBS for 30 min

at room temperature, then with 0.5 % BSA for 10 min and further incubation for 30 min with 10 nm goat anti-rabbit IgG-gold colloidal particles (E.Y labs Inc. San Mateo, CA.94401). Samples were washed and then stained with 5 % uranyl acetate. They were examined in a Philips model 410 electron microscope at an accelerating potential of 80 kv.

### AFFINITY BLOTTING

Twenty five µg of the electroeluted 18-20 kDa protein was analyzed by SDS-PAGE and transferred to the nitrocellulose paper in Tris-glycine-methanol buffer (Towbin *et al.*, 1979). Electrophoresis was performed for 30 min at constant current of 400 mA, and the blot was then blocked with 5 % nonfat milk in TBS (tris-buffered saline; 0.15 M NaCl, 0.05 M tris-HCl, pH 7.6) for one hour at room temperature. After washing in TBS, the blot was incubated with concanavalin A (Pharmacia; 0.005 % w/v in TBS containing 0.0005M Ca Cl<sub>2</sub>, 0.5 % BSA) for two hours at room temperature, washed again in TBS, and incubated 90 min in 0.005 % (w/v) horse-radish peroxidase (Sigma Type VI). Color was developed by addition of 0.05 % diaminobenzidines tetrahydrochlorid (Sigma) in TBS with H<sub>2</sub>O<sub>2</sub> (0.01 % w/v) for 2-3 min. The stained blot was washed in water and dried (Scott & Dodd, 1990). Alternatively, some blots were stained with biotinylated jacalin (Vector Lab, Inc. Co) according to the supplier's instructions (Rohringer & Holden, 1985).

## RESULTS

### ULTRASTRUCTURE

Using TEM, *Cryptosporidium* sporozoites demonstrated three layered pellicle, together with an underlying double unit membrane. The conoid and crystalloid bodies were observed at the anterior and posterior ends respectively. Meanwhile, sporozoites characteristically demonstrated three sets of secretory organelles. At their leading end two rhoptries and many narrow rhoptries ducts were demonstrated. Rhoptries were found with two distinct regions; a basal bulbous part and narrow apical duct. Small fusiform or flask-shaped micronemes were found in a large number. The third demonstrated vesicles were the dense granules which were scattered in few numbers in the sporozoites. All these vesicles appeared as electron dense bodies in the nonexcysted forms (Fig. 1A & B).

### IMMUNOLocalIZATION

*C. parvum* oocysts containing non excysted, non released sporozoites did not show any internal or external labelling when they were incubated with monospecific

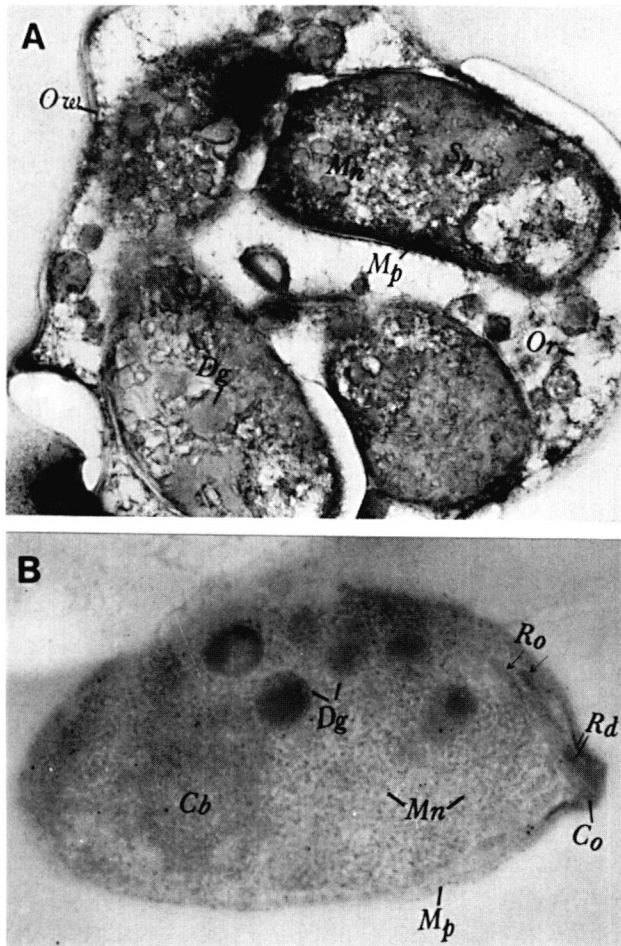


Fig. 1. – (A) Transmission electron micrograph of intact sporulated *C. parvum* oocyst, demonstrating four sporozoites (Sp) with electron dense micronemes (Mn), dense granules (Dg), and membrane pellicle (Mp); Or, Oocyst residuum; Ow, oocyst wall.  $\times 17,000$ .

(B) Transmission immunoelectron micrograph of nonexcysted sporozoite demonstrating the conoid (Co); two club shaped rhoptries (arrow heads), and rhoptries ducts (Rd). Micronemes (Mn), dense granules (Dg), crystalloid body (Cb) and membrane pellicle (Mp). All these organelles appear as electro-dense structures.  $\times 24,000$ .

18-20 kDa antibody using immunoelectron microscopy. On the other hand, transverse micrographs in the excysted, but unreleased sporozoites demonstrated minimal labelling at their secretory organelles with no labelling at their trilaminar pellicles. Meanwhile their secretory vesicles were found less electron-lucent than those of the excysted and completely released sporozoites (Fig. 2A, B, C). Excysted and released *C. parvum* sporozoites appeared to have a characteristic labelling pattern. Gold particles were concentrated principally at the apical end of the excysted sporozoites as well as at the trilaminar pellicle. Diffuse labelling was also detected as anterior extrusion of these sporozoites. Many electron-lucent vacuoles with variable sizes and with membrane whorls inside were observed among the labelled sporozoites (Fig. 3A). Some excysted sporozoites showed labelling over their entire bodies (circum-sporozoite) with varying intensity; this was demonstrated in scanning electron microscopy (Fig. 3B).

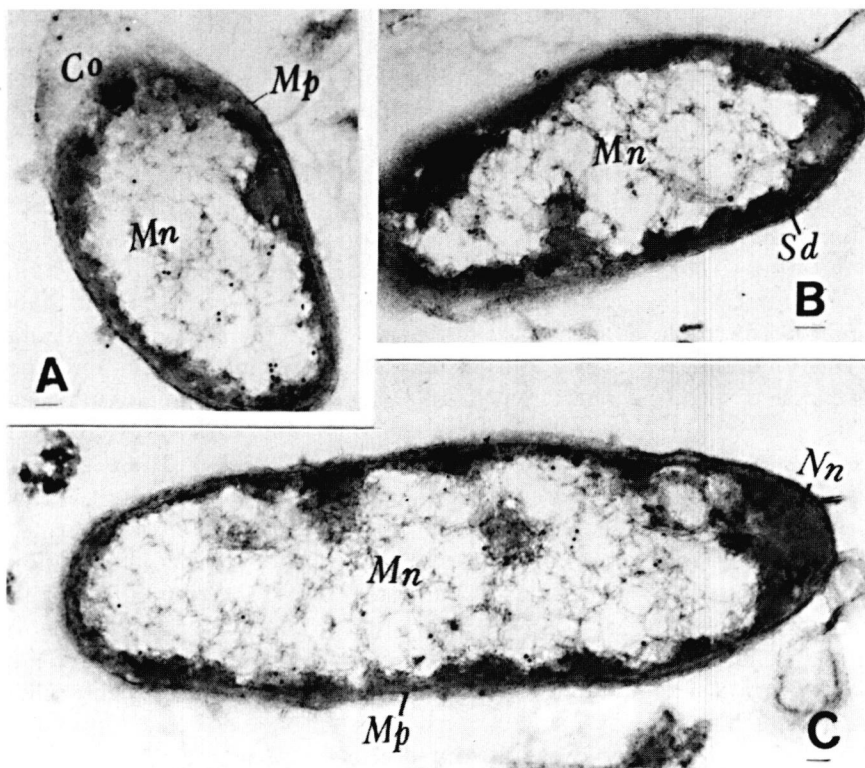


Fig. 2. – (A, B & C) represent different transverse immuno-electron micrographs of intact excysted non-released sporozoite reacted with anti-18-20 kDa monospecific antibody, showing minimal scattered gold particles. These sections demonstrate the fine ultra-structures of the *Cryptosporidium* sporozoite (Sp) with less electron-dense micronemes (Mn), dense granules (Dg). Nn, nucleus; Sd, subpellicular ducts; Co, conoid, and membrane pellicle (Mp).  $\times 24,000$ .

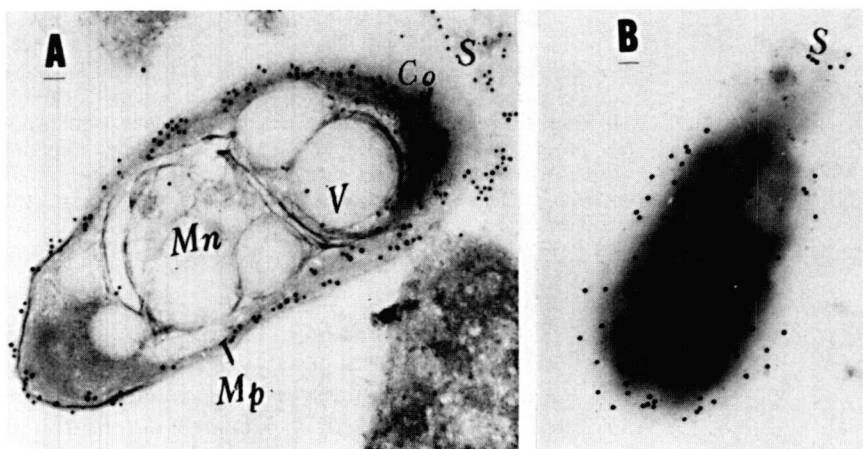


Fig. 3 – (A) Immunoelectron micrograph of excysted and completely released *C. parvum* sporozoite, reacted with monospecific anti-18-20 kDa antibody, demonstrating gold particles clustered on the conoid (Co) concentrated and distributed over the membrane pellicle (Mp) and forming a trail in front of the released sporozoites (S). Note electron-lucent vacuole (V) and micronemes (Mn), with many whorls.  $\times 24,000$ .

(B) Scanning immunoelectron micrograph of *C. parvum* excysted and released sporozoite, reacted with monospecific anti-18-20 kDa antibody, showing gold particles coating the entire sporozoite surface (circumsporozoite), and in front of the sporozoite (S).  $\times 11,000$ .

#### AFFINITY BLOTTING

Affinity staining of the 18-20 kDa protein revealed the presence of an O-linked oligosaccharide moiety. A positive staining was detected when this protein was treated with biotinylated jacalin, but not with concanavalin A suggesting the absence of an N-linked oligosaccharide moiety (data not shown).

#### DISCUSSION

Previously, an 18-20 kDa protein was detected in stool eluates from infected human and calves with *C. parvum*, but not in non infected control samples (El-Shewy *et al.*, 1994). In this report the origin and nature of this protein was elucidated. The invasive sporozoites of the apicomplexan parasites possess secretory organelles located at the apex and pellicle (Blackman & Bannister, 2001). Among these organelles, *C. parvum* sporozoites reported to have only one rhoptry (Tetley *et al.*, 1998). However, herein we demonstrated two rhoptries with many narrow delicate ducts during TEM examination of the *C. parvum* sporozoites.

Our findings with respect to the immunolocalization of the 18-20 kDa protein included an anterior end protein extrusion, as detected on some excysted sporozoites, while others were coated with this protein. This labelling pattern could suggest a secretory nature of the 18-20 kDa protein. It may be produced internally by the secretory organelles and then translocated over the surface or extruded via pellicle structures and rhoptries ducts. A similar finding has been reported in *Plasmodium* sporozoites, where an anterior trail of circumsporozoite protein was detected and believed to be synthesized internally, stored and released outside by appropriate host signals (Stewart & Vandenberg, 1991).

The detection of completely electron-lucent secretory organelles only in excysted and released sporozoites after being electron-dense in the non excysted form may support the secretory role of these organelles. A similar phenomenon has been observed in *Isoospora*, *Eimeria*, and in *Plasmodium* merozoites (Bannister *et al.*, 1979).

SDS-PAGE analysis of sporulated *C. parvum* sporozoites revealed more than 50 proteins of which at least 40 appeared to be of sporozoites origin (Tilly *et al.*, 1991; Reperant *et al.*, 1992). However, it was difficult to evaluate the functional significance of these proteins in the development of the sporozoites. With TEM, the detected pattern was significantly related to the state of functional activity of the sporozoites, as the non excysted sporozoites did not show any labeling. However excysted, but not released sporozoites demonstrated minimal labelling, finally, the typical gold labelling was observed when sporozoites were completely released after excystation.

Despite the effect of excystation on the release of the 18-20 kDa protein we do not believe that it plays a role in the excystation process. No labelling was detected in excysted not released sporozoites. More likely the detected 18-20 kDa protein has the same functions as those secreted in the apicomplexan parasites. In this respect, similarities in structures and functions of the apical proteins within apicomplexan genera have been documented (Grellier *et al.*, 1994; Sam-Yellowe *et al.*, 1988; Hehl *et al.*, 2000). These proteins appear to be generally responsible for the selective attachment to the host cells, formation of the parasitophorous vacuole, and the sporozoites motility (Blackman & Bannister, 2001). The distribution of 18-20 kDa protein over the whole surface and in front of the excysted sporozoites, represent a feature probably shared by several apicomplexan parasites. They glide over surfaces on a carpet of protein secreted from their apical ends and distributed over their bodies (Nus-

senzweig & Menard, 2000; Sultan *et al.*, 2001). Accordingly we anticipated that the detected protein might act as a lubricant or substrate for continuous gliding motility of the *C. parvum* sporozoites.

The glycosylated nature detected by the affinity staining denotes the potential immunogenicity of this protein. In other studies, *C. parvum* has been reported to possess glycoprotein located on the surface of its sporozoites and having molecular weights range from 15 to 900 kDa (Tilly *et al.*, 1991; Barnes *et al.*, 1998). In a previous trial for further characterization of this 18-20 kDa protein, an enterotoxic activity was remarkably observed on using Ussing chamber. The enterotoxic effect of this protein was time and dose dependent, heat labile and chloride dependent (El-Shewy *et al.*, 1999).

The data presented in this study could throw lights on a *Cryptosporidium* glycosylated exoantigen with an enterotoxic activity that could be potential target for vaccine development. Molecular cloning and further characterization of this antigen protein is being undertaken in our laboratory. Our long aim is to develop a protective element for neutralization and/or invasion inhibition of the *Cryptosporidium* parasite.

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