AEM Accepts, published online ahead of print on 4 September 2009
Appl. Environ. Microbiol. doi:10.1128/AEM.01555-09

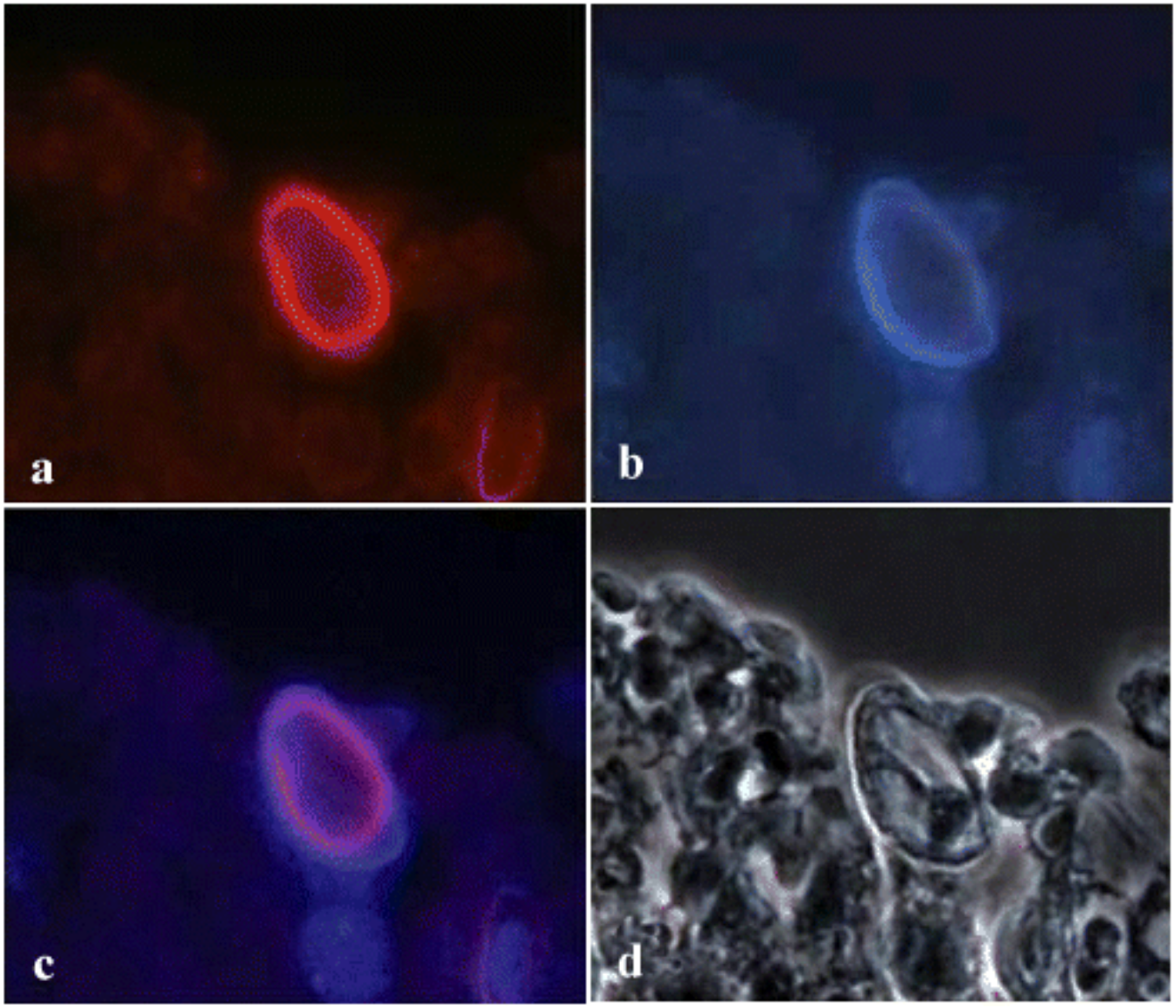
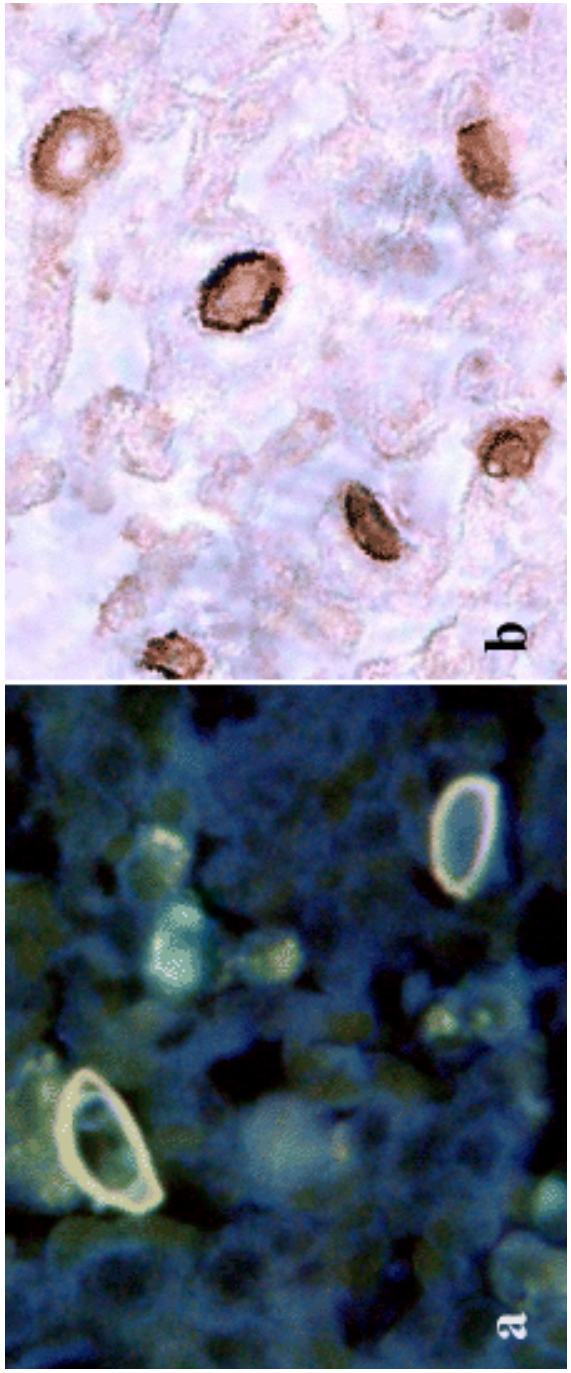


Figure legends

Figure 1. Localization of cellulose in *Acanthamoeba* cyst wall after D-CBD staining with two different conjugates of frozen section of cornea from patient with keratitis. Note distinct binding to the inner cyst wall and orange autofluorescence of the outer cyst wall after staining with D-CBD-Alexa Fluor® 350 blue conjugate. Some blue autofluorescence of the connective tissue background is seen (a). Identification of *Acanthamoeba* cyst in corneal tissue stained with biotinylated D-CBD (b).

Figure 2. Detection of *Acanthamoeba* cyst in paraffin section of experimentally infected mouse tissue with fluorescent D-CBD conjugate: Red staining of the cyst wall with D-CBD coupled to Alexa Fluor® 568 (a). Autofluorescence of *Acanthamoeba* cyst wall when viewed under UV excitation light (b). Alexa Fluor® 568 staining superimposed on the blue autofluorescence shows cellulase binding at the inner aspect of the autofluorescent cyst wall (c). The same area in phase contrast illumination (d).



Labelled *Trichoderma reesei* cellulase as marker for *Acanthamoeba* cyst wall cellulose in infected tissues

Monika Derda,[1*] Jadwiga Winiecka-Krusnell,[2] Markus B. Linder,[3] Ewert Linder,[2,4]

[1] Department of Biology and Medical Parasitology, University of Medical Sciences, 61-701 Poznan, Poland, mderda@ump.edu.pl

[2] Swedish Institute for Infectious Disease Control, 171 82 Solna, Sweden, jadwiga.krusnell@smi.se

[3] VTT Biotechnology, VTT Technical Research Centre of Finland, FIN 02044-VTT, Espoo, Finland, Markus.Linder@vtt.fi

[4] Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, 171 82 Solna, Sweden, ewert.linder@smi.se

Corresponding author:
Monika Derda
Department of Biology and Medical Parasitology,
Poznan University of Medical Sciences,
10 Fredry Sreet, Poznan 61-701, Poland,
e-mail: mderda@ump.edu.pl

Abstract

Some protozoa are able to encyst as a protective response to a harmful environment. The cyst wall usually contains chitin as its main structural constituent. *Acanthamoeba* is exception since its cyst wall contains cellulose. Specific cytochemical differentiation between cellulose and chitin by microscopy has not been possible, due to the similarity of their constituent β-1,4-linked hexose backbones. Thus, various fluorescent brightening agents and lectins bind to both cellulose and chitin. The identification of *Acanthamoeba* spp. which is primarily based on morphological and biochemical features, is labor-intensive and requires cloning and axenization. We described a novel immunocytochemical method for the identification of *Acanthamoeba* spp. based on selective binding of *Trichoderma reesei* cellulase to protozoan cyst wall cellulose. A recombinant cellulose-binding protein consisting of two cellulose-binding domains (CBDs) from *Trichoderma reesei* cellulases was coupled to the fluorescent dyes Alexa Fluor® 350 and 568 or labelled with the biotin using EZ-Link Sulfo-NHS-Biotin. No staining reaction was observed with the chitin-containing preparations of Fungi. Thus, the recombinant CBD can be used as a marker to distinguish between cellulose and chitin. This allows for rapid demonstration of *Acanthamoeba* cysts wall cellulose in paraffin or frozen sections of infected tissues.

Introduction

Laboratory diagnostics of infections with *Acanthamoeba* spp. is based on identification of the parasite in infected tissue. Although various techniques including immunocytological and molecular methods have been described, recovery of viable parasites by cultivation on agar is still the basic procedure (16). This method is usually associated with histopathological examination of the specimen to prove tissue invasion by the parasite.

Recognition of parasites in tissue sections is often difficult and depends on expertise of the pathologist. In addition to traditional histological staining methods, immunohistology using parasite-specific antibodies, lectin conjugates and calcofluor white have been used for the visualization of parasites in tissue sections (3).

Some protozoan parasites have the ability to protect themselves by forming a cyst wall, which is resistant to environmental stress such as desiccation, lack of nutrients, and variations in temperature and pH. In most pathogenic protozoa studied, chitin is the carbohydrate polymer conveying the required structural toughness to the cyst wall. *Acanthamoeba* spp. are exceptions, as their cysts are made up of cellulose. Recently, cellulose has also been identified as a cyst wall component in a closely related amoeba *Balamuthia mandrillaris* (15). Cellulose consists of β-D-glucosyl units linked by β -1,4-glucosidic bonds. Chitin is very similar but contains N-acetylglucosamine as the monomer. Both polymers form very similar crystalline macroscopic structures. Specific cytochemical differentiation between cellulose and chitin by microscopy has not been possible due to the similarity of the constituent β-1,4-linked hexose backbones. This is especially true for various fluorescent brightening agents, such as calcofluor white, used as a cytochemical marker in microscopic diagnostics of protozoan and fungal infections. A two-domain structural organization is often observed in cellulose-degrading enzymes. Most

Trichoderma reesei cellulases consist of a catalytic domain and a cellulose-binding domain (CBD) joined by a linker. The catalytic domain contains the active site with the amino acid residues responsible for the hydrolytic mechanism. The role of the CBD is binding to the solid cellulose. The ability of CBDs to attach to cellulose can be utilized in various applications. Individual types of CBDs can vary significantly in their properties, such as affinity, preference for crystalline or amorphous cellulose, and cross-reactivity with other similar carbohydrates (7, 8, 9, 10).

We have previously described a novel immunocytochemical method for the identification of *Acanthamoeba* spp. based on selective binding of *Trichoderma reesei* cellulase to protozoan cyst wall cellulose (12). In that study we used a recombinant dimeric CBD fusion protein in indirect immunofluorescence to specifically stain the cellulose and visualize its localization in the cyst wall. In preliminary studies, this method was also shown to be useful for the detection of parasites in tissue sections (11).

The aim of the present study was to simplify the detection method by preparing D-CBD as fluorescent and biotinylated conjugates to be used for direct and rapid detection of cellulose in *Acanthamoeba* by both fluorescence and ordinary light microscopy.

Materials and methods

Cellulase

D-CBD obtained as a recombinant fusion protein (8) was coupled to the fluorescent dyes using Alexa Fluor® 350 (blue)- and Alexa Fluor® 568 (red) – Protein Labeling Kit (Molecular Probes, Eugene, Oregon, USA), or labelled with the biotin using EZ-Link Sulfo-NHS-Biotin (Pierce, Rockford, Illinois, USA). All coupling procedures were performed according to the methods provided by manufacturers.

Samples of *Acanthamoeba* spp.

Corneal samples from human *Acanthamoeba* keratitis and tissues from mice infected by intranasal inoculation with trophozoites of *Acanthamoeba* spp. (13) were fixed in formalin and embedded in paraffin or in freezing medium (Tissue-tek, Miles, Naperville, Ill.), and sectioned (5 µm). Prior to the staining, paraffin sections were deparaffinized and rehydrated in double changes of xylene and ethanol (99.9%, 95.5%, and 70%), 5 min. each, while frozen sections were fixed in the cold acetone for 20 min. The reference preparation of *Acanthamoeba* cysts was obtained from axenic culture with PYG medium (2). Cysts and empty cyst walls were collected from prolonged cultures, washed three times with sterile Page's saline and immunostained in suspension or after air drying and fixation in cold acetone for 20 min on microscope slides.

Samples of Fungi

Samples from following species were used for investigations:

Division: Zygomycota, Class: Zygomycetes (*Absidia corymbifera)* (6); Phylum: Ascomycota, Class: Pezizomycetes (*Aspergillus fumigatus, Aspergillus niger, Penicillium notatum, Phylum: Ascomycota, Euascomycetes (Scedosporium apiospermum*) (5); Phylum: Ascomycota, Class: Saccharomycetes (*Candida albicans, Candida dubliniensis, Candida famata, Candida inconspicua, Candida kefyr, Candida rugosa, Candida sake, Candida utilis, Candida zeylanoides, Geotrichum candidum, Geotrichum capitatum, Kluyveromyces fragilis, Pichia membranaefaciens, Pichia* spp.); Phylum: Basidiomycota, Class: Agaricomycotina (*Cryptococcus neoformans*) and Phylum: Basidiomycota, Class: Urediniomycetes (*Rhodotorula* spp., *Sporobolomyces roseus*) (1, 4). Fungi were cultivated on Sabouraud agar (14). Than hyphea and spores of Fungi were collected from cultures, washed three times with sterile Page's saline and immunostained in suspension, after air drying and fixation in cold acetone for 20 min on

microscope slides or as a frozen sections prepared in freezing medium (Tissue-tek, Miles, Naperville, Ill.).

D-CBD–AlexaFluor conjugates

For staining with D-CBD coupled to the fluorescent dyes, slides were immersed with PBS and incubated with D-CBD for 30 min at room temperature, washed three times with PBS and mounted with Vectashield non-fading medium (Vector Laboratories Inc., Burlingame, CA, USA). For microscopy a Leica DMRB fluorescence microscope (Leica Mikroskopie und Systeme GmbH) equipped with dicroic mirror filter combinations for UV excitation light (A) and green TRITC excitation light (K3). For photography, a Nikon-Kodak (Eastman Kodak Inc.) digital camera was used.

D-CBD-biotin conjugates.

In order to reduce non-specific reaction due to the presence of endogenous biotin in both tissues and *Acanthamoeba* cysts slides with tissue sections and control cysts preparation were incubated with streptABComplex/HRP (Dako, Denmark), for 1 hour at 37°C and washed three times in PBS. Endogenous peroxidase was blocked by incubation with 5% H_2O_2 in PBS for 30 min at room temperature. After blocking slides were rinsed with PBS and incubated with biotinylated D-CBD for 30 min. at room temperature. Subsequent washings in 3 changes of PBS were followed by the incubation with Strept ABComplex/HRP (Dako, Denmark), for 30 min. at room temperature. After washing with PBS, 3,3 diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, Missouri, USA) was added for 15 min. Slides were rinsed with water, dried, mounted permanently with coverslips and examined in the light microscope.

Results and discussion

One of the problems we encountered in the previous study was exposing the cellulose located in the inner wall of the *Acanthamoeba* cyst. Using paraffin or frozen sections however, allow CBD to access the target molecules more easily. Figures show that conjugated D-CBD reagents can be used for the demonstration of *Acanthamoeba* cysts in infected cornea of eye (Fig. 1 a,b) and in infected mouse tissues (Fig. 2 a,c). D-CBD prepared as a biotinylated protein can be easily used in the light microscopy (Fig. 1 b). Due to the high affinity of the binding, avidin-biotin technology is widely used in histochemical methods. However, the presence of endogenous biotin in various cells and tissues and its potential interference with the test can cause false positive results (17). With the blocking step included in the test we could significantly reduce the background staining.

Although, the additional incubations increased the time needed to accomplish the test, blocking resulted in satisfactory resolution of the staining. Using the fluorescent conjugates, detection of *Acanthamoeba* cysts is a single step procedure, which allows for a rapid and specific demonstration of parasites. Alexa Fluor® 568 conjugate appears to be superior to Alexa Fluor® 350 in the present application, especially when used in tissue sections. Bright autofluorescence of *Acanthamoeba* ectocyst in the UV excitation light interferes with the blue staining of cellulose when Alexa Fluor® 350 is used (Fig. 1 a and Fig. 2 a,c).

The red fluorochrome in Alexa Fluor® 568 conjugate however, offers a possibility to distinguish between the autofluorescence of the outer wall and specific reaction in the inner wall and allow to demonstrate the precise localization of cellulose in the cyst of *Acanthamoeba* spp. (Fig. 2 b,c).

A positive staining reaction was seen only with *Acanthamoeba* cysts. No reactivity of D-CBD was seen on sections of Fungi. The negative result of reaction the D-CBD with Fungi which contain chitin in the cell wall confirms specificity of the D-CBD of *T. reesei* to cellulose.

158 The D-CBD reacts with cellulose, but it doesn't react with chitin. Earlier investigations showed
159 that the D-CBD doesn't react with chitin in wall of *Entamoeba dispar, Giardia isteninalis*,
160 *Pneumocystis carinii* cysts (11, 12).
161 The D-CBD conjugates for direct staining of *Acanthamoeba* cysts described here are alternative,
162 potentially useful diagnostic tools. Also, these conjugates may be useful markers for the
163 identification and classification of environmental amoebae.
164

165 **Acknowledgements**

166 M.D. received financial support from the Swedish Institute and M.L. acknowledges support from
167 the Academy of Finland. Corneal samples were obtained from Dr. Elisabet Holst, Department of
168 Infectious Diseases and Medical Microbiology, Lund University, Sweden and tissues from
169 infected mice were donated by Prof. Edward Hadas, Department of Biology and Medical
170 Parasitology, Poznan University of Medical Sciences, Poland. Fungal samples were a kind gift of
171 Dr. Victor Fernandez, Swedish Institute for Infectious Disease Control, Stockholm, Sweden.
172

173 **References**

174 1. **Adl, S. M., A. G Simpson, M. A. Farmer, R. A. Andersen, O. R. Anderson, J. R. Barta,**
175 **S. S. Bowser, G. Brugerolle, R. A. Fensome, S. Fredericq, T. Y. James, S. Karpov, P.**
176 **Kugrens, J. Krug, C. E. Lane, L. A. Lewis, J. Lodge, D. H. Lynn, D. G. Mann, R. M.**
177 **McCourt, L. Mendoza, O. Moestrup, S. E. Mozley-Standridge, T. A. Nerad, C. A.**
178 **Shearer, A. V. Smirnov, F. W. Spiegel, and M. F. Taylor.** 2005. The new higher level
179 classification of eukaryotes with emphasis on the taxonomy of protists. J. Eukaryot.
180 Microbiol. **52**:99-451.

2. American Type Culture Collection. 1993. Catalogue of protists, p. 68. American Type Culture Collection, Manassas, Va.

3. **Bottone, E. J.** 1993. Free-living amebas of genera *Acanthamoeba* and *Naegleria*: an overview and basic micribiologic correlates. Mount. Sinai. J. Med. **60** (49):260-270.

4. **Eriksson, O. E. and K. Winka.** 1997. Supraordinal taxa of Ascomycota. Myconet http://www.fieldmuseum.org/myconet/

5. **Gilgado, F., J. Cano, J. Gené, D. A. Sutton, and J. Guarro.** 2008. Molecular and phenotypic data supporting distinct species statuses for *Scedosporium apiospermum* and *Pseudallescheria boydii* and the proposed new species *Scedosporium dehoogii*. J. Clinic. Microbiol. **46**:766-771.

6. **Hoffmann, K., S. Discher, and K. Voigt.** 2007. Revision of the genus *Absidia* (Mucorales, Zygomycetes) based on physiological, phylogenetic, and morphological characters, thermotolerant *Absidia* spp. form a coherent group, Mycocladiaceae fam. nov. Mycol. Res. **111**:1169-1183.

7. **Linder, M., I. Salovuori, L. Ruohonen, and T. T. Teeri.** 1996. Characterization of double cellulose-binding domain. J. Biol. Chem. **35**:21268-21272.

8. **Linder, M., K. Selber, T. Nakari-Setälä, M. Qiao, M. R. Kula, and M. Penttilä.** 2001 a. The hydrophobins HFBI and HFBII from *Trichoderma reesei* showing efficient interactions with nonionic surfactants in aqueous two-phase systems, Biomacromolecules. **2**:511-517.

9. **Linder, M., and T. T. Teeri.** 1996. The cellulose-binding domain of the major cellobiohydrolase of *Trichoderma reesei* exhibits true reversibility and a high exchange rate on crystalline cellulose. Proc. Natl. Acad. Sci. USA. **93**:12251–12255.

10. **Linder, M., and T. T. Teeri.** 1997. The roles and function of cellulose-binding domains. J. Biotechnol. **57**:15–28.

11. **Linder, M., J. Winiecka-Krusnell, and E. Linder.** 2001 b. Identification of *Acanthamoebae* in clinical and environmental samples using cellulose-binding domains of cellulase as immunocytochemical marker for cyst wall cellulose. Proceedings of IX-th Intern. Meeting on the Biology and Pathogenicity of Free-living Amoebae. Paris, July 2001:198-201.

12. **Linder, M., J. Winiecka-Krusnell, and E. Linder.** 2002. Use of recombinant cellulose binding domains of *Trichoderma reesei* cellulase as a selective immunocytochemical marker for cellulose in protozoa. Appl. Environm. Microbiol. **68** (5): 2503-2508.

13. **Mazur, T., E. Hadas, L. Gustowska, J. Winiecka-Krusnell, and E. Linder.** 1999. Secondary amoebic eye infections in mice due to *Acanthamoeba* spp. Parasitol. Res. **85**:776-778.

14. **Sandven, P., and J. Lassen**. 1999. Importance of selective media for recovery of yeasts from clinical specimens. J. Clinic. Microbiol. **37**:3731–3732.

15. **Siddiqui, R., N. A. Khan, and E. L. Jarroll**. 2009. The cyst wall carbohydrate composition of *Balamuthia mandrillaris*. Parasitol. Res. **104**:1439-1443.

16. **Visvesvara, S., H. Moura, and F. Schuster.** 2007. Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp., *Balamuthis mandrillaris*, *Naegleria fowleri* and *Sappinia diploidea*. FEMS Immunol. Med. Microbiol. **50**:1-26

17. **Wang, H., and J. Pevsner.** 1999. Detection of endogenous biotin in various tissues: novel functions in the hippocampus and implications for its use in avidin-biotin technology. Cell. Tissue. Res. **296**: 511-516.

Figure legends

Figure 1. Localization of cellulose in *Acanthamoeba* cyst wall after D-CBD staining with two different conjugates of frozen section of cornea from patient with keratitis. Note distinct binding to the inner cyst wall and orange autofluorescence of the outer cyst wall after staining with D-CBD-Alexa Fluor® 350 blue conjugate. Some blue autofluorescence of the connective tissue background is seen (a). Identification of *Acanthamoeba* cyst in corneal tissue stained with biotinylated D-CBD (b).

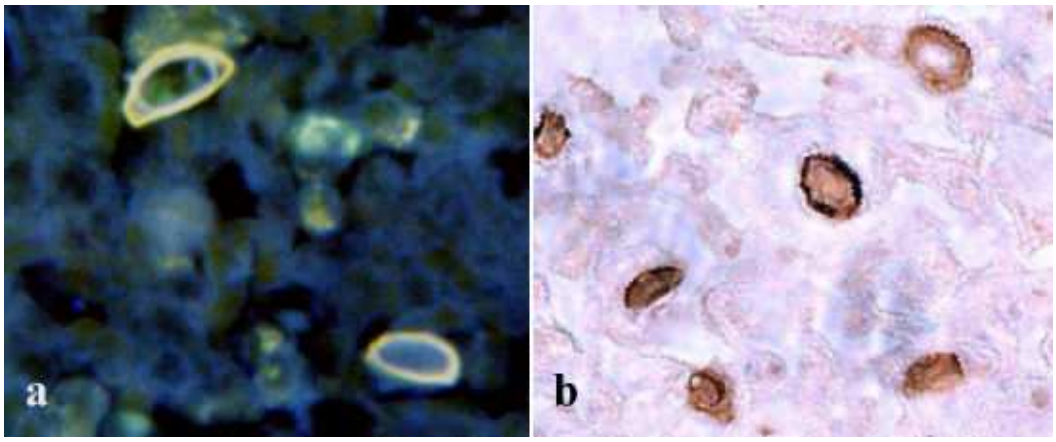
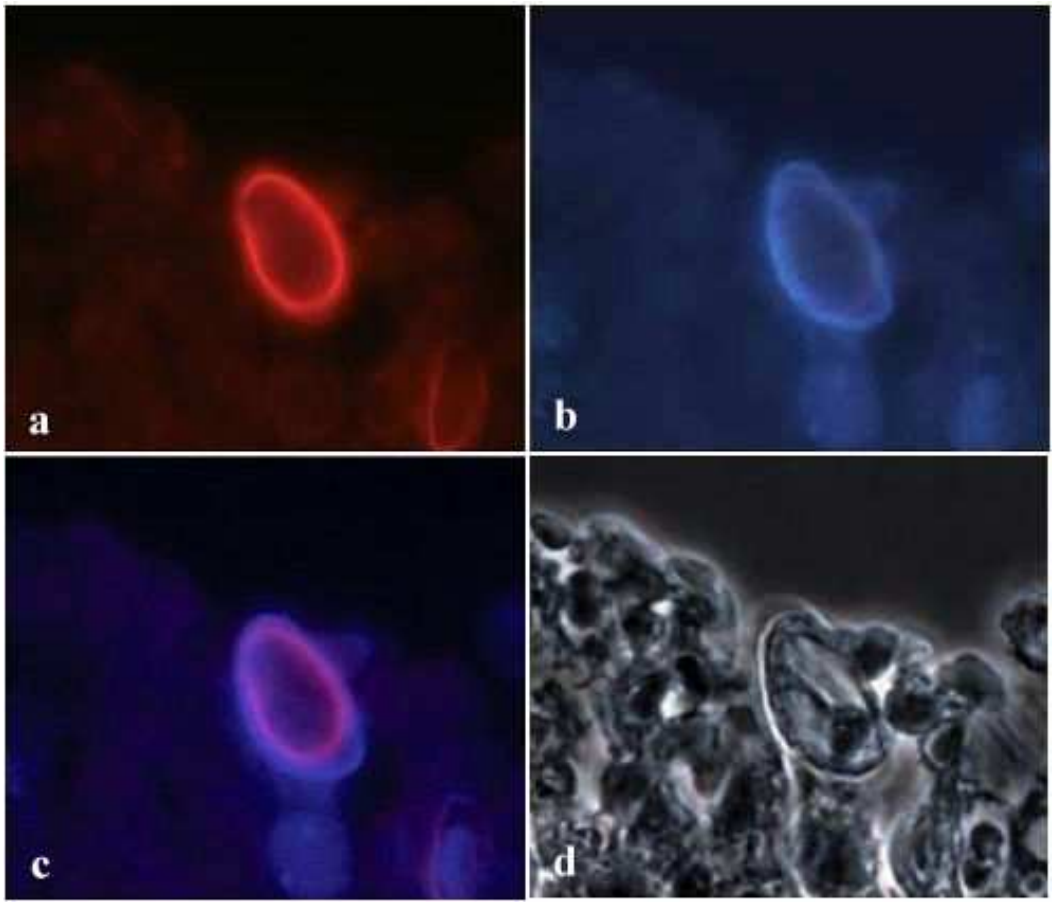


Figure 2. Detection of *Acanthamoeba* cyst in paraffin section of experimentally infected mouse tissue with fluorescent D-CBD conjugate: Red staining of the cyst wall with D-CBD coupled to Alexa Fluor® 568 (a). Autofluorescence of *Acanthamoeba* cyst wall when viewed under UV excitation light (b). Alexa Fluor® 568 staining superimposed on the blue autofluorescence shows cellulase binding at the inner aspect of the autofluorescent cyst wall (c). The same area in phase contrast illumination (d).



240