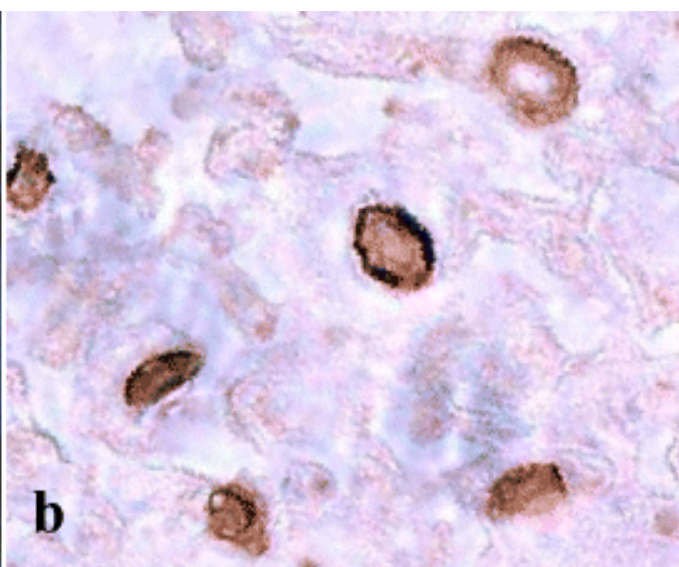
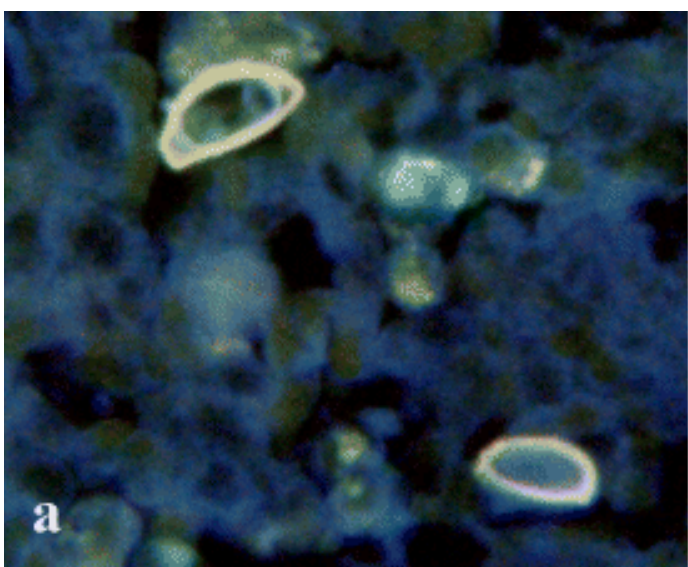


1 **Figure legends**

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

8

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



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

3

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23 **Abstract**

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

39 **Introduction**

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

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

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

62 *Trichoderma reesei* cellulases consist of a catalytic domain and a cellulose-binding domain
63 (CBD) joined by a linker. The catalytic domain contains the active site with the amino acid
64 residues responsible for the hydrolytic mechanism. The role of the CBD is binding to the solid
65 cellulose. The ability of CBDs to attach to cellulose can be utilized in various applications.
66 Individual types of CBDs can vary significantly in their properties, such as affinity, preference for
67 crystalline or amorphous cellulose, and cross-reactivity with other similar carbohydrates (7, 8, 9,
68 10).

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

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

78

79 **Materials and methods**

80 Cellulase

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

86 Samples of *Acanthamoeba* spp.

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

96

97 Samples of Fungi

98 Samples from following species were used for investigations:

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

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

112

113 D-CBD–AlexaFluor conjugates

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

121

122 D-CBD-biotin conjugates.

123 In order to reduce non-specific reaction due to the presence of endogenous biotin in both tissues
124 and *Acanthamoeba* cysts slides with tissue sections and control cysts preparation were incubated
125 with streptABCComplex/HRP (Dako, Denmark), for 1 hour at 37°C and washed three times in
126 PBS. Endogenous peroxidase was blocked by incubation with 5% H₂O₂ in PBS for 30 min at
127 room temperature. After blocking slides were rinsed with PBS and incubated with biotinylated D-
128 CBD for 30 min. at room temperature. Subsequent washings in 3 changes of PBS were followed
129 by the incubation with Strept ABCComplex/HRP (Dako, Denmark), for 30 min. at room
130 temperature. After washing with PBS, 3,3 diaminobenzidine tetrahydrochloride (Sigma-Aldrich,
131 St. Louis, Missouri, USA) was added for 15 min. Slides were rinsed with water, dried, mounted
132 permanently with coverslips and examined in the light microscope.

133

134 **Results and discussion**

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

145 Although, the additional incubations increased the time needed to accomplish the test, blocking
146 resulted in satisfactory resolution of the staining. Using the fluorescent conjugates, detection of
147 *Acanthamoeba* cysts is a single step procedure, which allows for a rapid and specific
148 demonstration of parasites. Alexa Fluor® 568 conjugate appears to be superior to Alexa Fluor®
149 350 in the present application, especially when used in tissue sections. Bright autofluorescence of
150 *Acanthamoeba* ectocyst in the UV excitation light interferes with the blue staining of cellulose
151 when Alexa Fluor® 350 is used (Fig. 1 a and Fig. 2 a,c).

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

155 A positive staining reaction was seen only with *Acanthamoeba* cysts. No reactivity of
156 D-CBD was seen on sections of Fungi. The negative result of reaction the D-CBD with Fungi
157 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

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171 Dr. Victor Fernandez, Swedish Institute for Infectious Disease Control, Stockholm, Sweden.

172

173 **References**

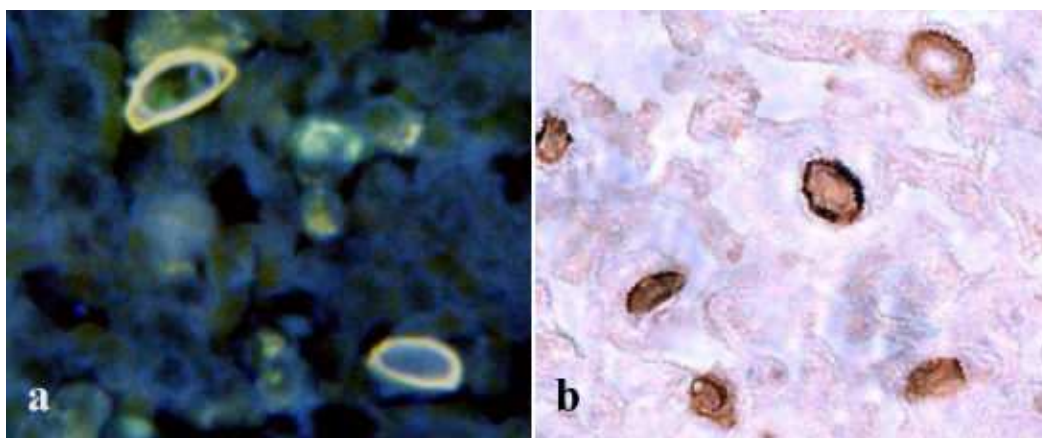
- 174 1. Adl, S. M., A. G Simpson, M. A. Farmer, R. A. Andersen, O. R. Anderson, J. R. Barta,
175 S. S. Bowser, G. Brugerolle, R. A. Fensome, S. Fredericq, T. Y. James, S. Karpov, P.
176 Kugrens, J. Krug, C. E. Lane, L. A. Lewis, J. Lodge, D. H. Lynn, D. G. Mann, R. M.
177 McCourt, L. Mendoza, O. Moestrup, 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.

- 181 2. American Type Culture Collection. 1993. Catalogue of protists, p. 68. American Type
182 Culture Collection, Manassas, Va.
- 183 3. **Bottone, E. J.** 1993. Free-living amebas of genera *Acanthamoeba* and *Naegleria*: an
184 overview and basic microbiologic correlates. Mount. Sinai. J. Med. **60** (49):260-270.
- 185 4. **Eriksson, O. E. and K. Winka.** 1997. Supraordinal taxa of Ascomycota. Myconet
186 <http://www.fieldmuseum.org/myconet/>
- 187 5. **Gilgado, F., J. Cano, J. Gené, D. A. Sutton, and J. Guarro.** 2008. Molecular and
188 phenotypic data supporting distinct species statuses for *Scedosporium apiospermum* and
189 *Pseudallescheria boydii* and the proposed new species *Scedosporium dehoogii*. J. Clinic.
190 Microbiol. **46**:766-771.
- 191 6. **Hoffmann, K., S. Discher, and K. Voigt.** 2007. Revision of the genus *Absidia* (Mucorales,
192 Zygomycetes) based on physiological, phylogenetic, and morphological characters,
193 thermotolerant *Absidia* spp. form a coherent group, Mycocladiaceae fam. nov. Mycol. Res.
194 **111**:1169-1183.
- 195 7. **Linder, M., I. Salovuori, L. Ruohonen, and T. T. Teeri.** 1996. Characterization of double
196 cellulose-binding domain. J. Biol. Chem. **35**:21268-21272.
- 197 8. **Linder, M., K. Selber, T. Nakari-Setälä, M. Qiao, M. R. Kula, and M. Penttilä.** 2001 a.
198 The hydrophobins HFBI and HFBI from *Trichoderma reesei* showing efficient interactions
199 with nonionic surfactants in aqueous two-phase systems, Biomacromolecules. **2**:511-517.
- 200 9. **Linder, M., and T. T. Teeri.** 1996. The cellulose-binding domain of the major
201 cellobiohydrolase of *Trichoderma reesei* exhibits true reversibility and a high exchange rate
202 on crystalline cellulose. Proc. Natl. Acad. Sci. USA. **93**:12251–12255.
- 203 10. **Linder, M., and T. T. Teeri.** 1997. The roles and function of cellulose-binding domains. J.
204 Biotechnol. **57**:15–28.

- 205 11. **Linder, M., J. Winiiecka-Krusnell, and E. Linder.** 2001 b. Identification of *Acanthamoebae*
206 in clinical and environmental samples using cellulose-binding domains of cellulase as
207 immunocytochemical marker for cyst wall cellulose. Proceedings of IX-th Intern. Meeting on
208 the Biology and Pathogenicity of Free-living Amoebae. Paris, July 2001:198-201.
- 209 12. **Linder, M., J. Winiiecka-Krusnell, and E. Linder.** 2002. Use of recombinant cellulose
210 binding domains of *Trichoderma reesei* cellulase as a selective immunocytochemical marker
211 for cellulose in protozoa. Appl. Environm. Microbiol. **68** (5): 2503-2508.
- 212 13. **Mazur, T., E. Hadas, L. Gustowska, J. Winiiecka-Krusnell, and E. Linder.** 1999.
213 Secondary amoebic eye infections in mice due to *Acanthamoeba* spp. Parasitol. Res. **85**:776-
214 778.
- 215 14. **Sandven, P., and J. Lassen.** 1999. Importance of selective media for recovery of yeasts from
216 clinical specimens. J. Clinic. Microbiol. **37**:3731-3732.
- 217 15. **Siddiqui, R., N. A. Khan, and E. L. Jarroll.** 2009. The cyst wall carbohydrate composition
218 of *Balamuthia mandrillaris*. Parasitol. Res. **104**:1439-1443.
- 219 16. **Visvesvara, S., H. Moura, and F. Schuster.** 2007. Pathogenic and opportunistic free-living
220 amoebae: *Acanthamoeba* spp., *Balamuthis mandrillaris*, *Naegleria fowleri* and *Sappinia*
221 *diploidea*. FEMS Immunol. Med. Microbiol. **50**:1-26
- 222 17. **Wang, H., and J. Pevsner.** 1999. Detection of endogenous biotin in various tissues: novel
223 functions in the hippocampus and implications for its use in avidin-biotin technology. Cell.
224 Tissue. Res. **296**: 511-516.

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230 background is seen (a). Identification of *Acanthamoeba* cyst in corneal tissue stained with
231 biotinylated D-CBD (b).



232

233

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