SEROLOGICAL DIFFERENTIATION OF MICROSPORIDIA WITH SPECIAL REFERENCE TO *TRACHIPLEISTOPHORA HOMINIS*

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Summary :

Myositis is a common clinical syndrome in advanced stages of AIDS. *Trachipleistophora hominis* (phylum Microsporida) has been detected in several cases of painful, immobilising myositis in AIDS patients. Enzyme linked immunosorbent assays (ELISAs) and Western blotting of protein profiles separated by SDS PAGE were used to determine whether this species could be detected and differentiated by serology. Sixteen microsporidia, including several species known to infect man and species infecting fish, crustaceans and a mosquito, were used as antigen. Each species had a unique profile of SDS PAGE-separated proteins. In Western blots, mouse antiserum, raised to *T. hominis* and selected for its high ELISA specificity, bound to antigens ranging from less than 25 kDa to greater than 250 kDa with major bands at 39-44 kDa and 98-150 kDa on *T. hominis* protein profiles. The serum also recognised some high molecular weight antigens in the profiles of *Vavraia culicis*, *Heterosporis anguillarum*, and three species of *Pleistophora* but none in the remaining genera examined. It was concluded that ELISA and Western blotting could be used to detect and differentiate *T. hominis* in muscle biopsy tissue from patients with myositis. However, sera from *T. hominis*-infected patients in the terminal stages of AIDS would not be useful for detection of infections because of a sharp decline in antibody level.

KEY WORDS : *Trachipleistophora hominis*, microsporidia, human myositis, fish, crustaceans, mosquitoes, ELISA, SDS PAGE, Western blot.

Résumé : DIFFÉRENCIATION SÉROLOGIQUE DES MICROSPORIDIES ET NOTAMMENT DE *TRACHIPLEISTOPHORA HOMINIS*

La myosite est un syndrome fréquent des stades avancés du SIDA. *Trachipleistophora hominis* (phylum Microsporida) a été détecté dans plusieurs cas de myosite douloureuse, immobilisant les patients atteints du SIDA. L'ELISA et le Western blot des protéines séparées par SDS PAGE ont été utilisés pour déterminer si cette espèce peut être détectée et différenciée par sérologie. Outre *T. hominis*, quinze autres espèces trouvées respectivement chez l'homme, des poissons, des crustacés ou des moustiques ont été utilisées comme matériel antigénique. Le profil des protéines séparées par le SDS PAGE était propre à chaque espèce. En Western blot, un sérum de souris dirigé contre *T. hominis* et choisi pour sa haute spécificité en ELISA s'est lié à des antigènes compris entre < 25 kDa et > 250 kDa avec des bandes principales à 39-44 kDa et 98-150 kDa dans les profils de protéines obtenus à partir de *T. hominis*. Ce même sérum a également reconnu des antigènes de haut poids moléculaire de *Vavraia culicis*, d'*Heterosporis anguillarum* et de trois espèces de *Pleistophora*, mais aucun dans les autres genres testés. En conclusion, l'ELISA et le Western blot peuvent être utilisés pour détecter et différencier *T. hominis* dans les biopsies musculaires des patients atteints de la myosite. Toutefois, le sérum des patients contaminés par cette microsporidie aux stades terminaux du SIDA serait inutilisable pour sa détection en raison de la chute du taux des anticorps sériques.

MOTS CLÉS : *Trachipleistophora hominis*, microsporidies, myosite humaine, poisson, crustacé, moustiques, ELISA, SDS PAGE, Western blot.

INTRODUCTION

The microsporidian parasite *Trachipleistophora hominis* Hollister, Canning, Weidner, Field, Kench & Marriott, 1996 was found infecting the skeletal muscle and cornea and was present in nasal discharge of an AIDS patient (Field *et al.*, 1996). The patient had become immobilised by severe muscular pain and wasting but his condition improved after treatment with a therapeutic combination which included albendazole (400 mg twice daily), a drug with known anti-microsporidial activity. The patient died five

months after admission to hospital of progressive HIV disease without recurrence of myositis. The microsporidia were isolated *in vitro* and described as a new genus and species (Hollister *et al.*, 1996).

In numerous genera of microsporidia, sporogony culminates in a large (more than eight) and variable number of spores packaged in an envelope of presumed parasite origin (polysporous in sporophorous vesicles). Of these only *Trachipleistophora* from man and *Glugea*, *Heterosporis* and *Pleistophora* from fish are known from vertebrates. *Pleistophora*-like microsporidia also infect edible crustaceans – shrimps and crabs. *Glugea* and *Heterosporis* infections are strictly localised in their hosts by being retained within hypertrophied host cells modified to form xenomas. *Pleistophora* and *Trachipleistophora* infections are diffuse. Species of the genera *Loma* and *Spraguea* are also parasites of fish. In *Loma*, sporogony gives rise to four or more spores retained within a parasitophorous vacuole bounded by

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a membrane of presumed host origin (Lom & Pekkarinen, 1999). In *Spraguea* there are two sporogonic sequences which give rise to two types of spores, both lying free in host cell cytoplasm (no enveloping membrane).

In two cases of microsporidian myositis in AIDS patients, the parasites were identified as *Pleistophora* spp. (Chupp *et al.*, 1993; Grau *et al.*, 1996) but are more likely to belong to *Trachipleistophora* than *Pleistophora* (Canning, 2001) and another *T. hominis* infection has been diagnosed recently (A. Curry, personal communication). A second species *Trachipleistophora anthropophthera* has caused a multisystem disseminating infection, especially infecting the brain, in two more AIDS patients (Yachnis *et al.*, 1996; Vavra *et al.*, 1998). The occurrence of several of these deep tissue infections, which cannot be transmitted directly between humans, at least not from muscle or brain, has raised the question of their origin, and ingestion of infected fish or crustaceans seemed a likely possibility. However, in a recent study of generic relationships revealed by a molecular phylogeny, *Vavraia culicis* a parasite of anopheline and culicine mosquitoes was found to be closest to *T. hominis*, raising the possibility of microsporidian myositis being a vector-borne disease (Cheney *et al.*, 2000). All the *Trachipleistophora* infections so far detected have been at an advanced stage, only investigated because of the pain and weakness of muscles suffered by the patients. Buskila & Gladman (1990) reported that 35 % of AIDS patients

suffer some degree of myositis. In this paper we report on an approach to serological detection of *T. hominis* and differentiation of polysporous microsporidia by enzyme linked immunosorbent assay (ELISA) and Western blotting. These tests might be developed further to detect early infections of *T. hominis* in man and thus evaluate its role as a cause of myositis in AIDS patients.

MATERIALS AND METHODS

ANTIGEN

Microsporidia, spores of which were tested for cross reactivity with *T. hominis* in ELISAs, Western blots or both, are listed with their hosts in Table I. Those of human origin were propagated *in vitro* in the authors' laboratory after previous isolation from human tissue. *Vavraia culicis* was propagated in large lepidopteran larvae and purified spores were provided by courtesy of Dr J.J. Becnel. The other microsporidia, of fish, crustacean or insect origin, were obtained from their natural hosts, either as purified spores or infected host tissues. Spores were purified from culture supernatants or by mechanical disruption of host tissue, then lysis of host cells in water for one hour. The lysate was spun at 500 g for 10 min, the pellet was resuspended in Minimal Essential Medium (MEM) and spun on a 50 % Percoll gradient in PBS at 900 g for 30 min. After washing three

Microsporidia	Hosts	ELISA ¹ titre	Western blot ²
<i>Trachipleistophora hominis</i>	<i>Homo sapiens</i> (man) ³	12,800	+
<i>Vavraia culicis</i>	<i>Aedes albopictus</i> (mosquito) ⁴	800/1,600	+
<i>Heterosporis anguillarum</i>	<i>Anguilla japonica</i> (eel)	200	+
<i>Pleistophora hippoglossoides</i>	<i>Hippoglossoides platessoides</i> (dab)	400	ND
<i>Pleistophora mirandellae</i>	<i>Rutilus rutilus</i> (roach)	800	+/-
<i>Pleistophora typicalis</i>	<i>Myoxocephalus scorpius</i> (bull rout)	400	+
<i>Pleistophora</i> sp. (TB)	<i>Taurulus bubalis</i> (sea scorpion)	200	+
<i>Pleistophora</i> sp. (LS)	<i>Litopenaeus setiferus</i> (shrimp)	800	-
<i>Pleistophora</i> sp. (FA)	<i>Farfantepenaeus aztecus</i> (shrimp)	400	ND
<i>Spraguea lophii</i>	<i>Lophius americanus</i> (angler fish)	200	-
<i>Glugea anomala</i>	<i>Gasterosteus aculeatus</i> (stickleback)	ND	-
<i>Loma acerinae</i>	<i>Gymnocephalus cernuus</i> (ruff)	ND	-
<i>Encephalitozoon cuniculi</i>	<i>Canis familiaris</i> (dog) ⁵	800	-
<i>Encephalitozoon bellem</i>	<i>Homo sapiens</i> (man) ⁶	400	ND
<i>Vairimorpha mesnili</i>	<i>Pieris brassicae</i> (moth)	200	ND
<i>Vittaforma corneae</i>	<i>Homo sapiens</i> (man) ⁷	400	ND

¹ELISA titres are those obtained with mouse serum 483 (titre = 1:12,800).

²Western blotting was carried out with serum 443 (titre = 1:3,200). Results are given as positive (+), negative (-) or borderline (+/-). ND = not done.

³Propagated in RK13 cells after isolation from human muscle (Hollister *et al.*, 1996).

⁴Propagated in larvae of *Helicoverpa zea* (Lepidoptera).

⁵Propagated in MDCK cells after isolation from dog (Stewart *et al.*, 1979).

⁶Wainwright isolate, propagated in MDCK cells from human nasal polyp (Hollister *et al.*, 1993).

⁷Propagated in MDCK cells from human corneal biopsy (Shaddock *et al.*, 1990; Silveira & Canning, 1995).

Table I. - Microsporidia and their hosts used in ELISA and Western blotting.

times the spore pellet was resuspended in MEM and stored at 4°C.

ANTISERA

Balb C euthymic mice were inoculated intraperitoneally (ip) or subcutaneously (sc) with 5×10^6 or 10^7 whole or disrupted spores of *T. hominis* with or without the adjuvant monophosphoryl lipid A plus trehalose dicorynomycolate emulsion (MPL+TDM, Sigma). Two to four inoculations were performed according to responses of individual mice. Titres were determined by ELISA using the dilution which gave an optical density reading equivalent to twice that of the control (normal serum) at the starting dilution. A single sample of human serum from an AIDS patient with *T. hominis* infection was also tested, with normal human serum as control.

ELISA

Spores of each species were added at 2×10^5 spores per well (4×10^5 spores of *Encephalitozoon cuniculi*, which has smaller spores) in carbonate/bicarbonate buffer overnight at 4°C. Non-adherent spores were removed with the buffer and the remainder fixed to the plate with a 1:1 mixture of ethanol and methanol. The blocking agent was 1 % horse serum (HS) in PBS. Normal mouse serum (control) and mouse antiserum to *T. hominis* were serially diluted in 1 % HS in PBS from 1/200 to 1/100,000 and incubated with antigen for one hour at room temperature (RT). For visualization Vectastain® ABC-AP kits for mouse IgG (Vector Laboratories) and, in one experiment the kit for mouse IgM, were used according to the manufacturer's instructions and the optical densities were read 10 min and 30 min after addition of substrate in a Dynatek MR 60 Microplate reader. Controls were *E. coli* (strain JM109) as non-microsporidian antigen and coating buffer only in place of serum. Vectastain® ABC-AP kits for human IgG and, in one experiment, the kits for human IgM, IgA, IgE and kappa and lambda were used for visualising reactions of the human serum.

SDS-PAGE

10^8 spores of each species (2×10^8 spores of *E. cuniculi*) were suspended in 200 µl of modified Laemmli reducing buffer (50 mM TRIS-HCl pH 6.8, 100 mM dithiothreitol, 10 % glycerol). Spores were disrupted in a bead beater (Biospec Products), 10 % SDS was added and the sample was boiled for 5 min, spun at 1,200 g for 10 min and the supernatant containing the soluble spore proteins was collected. A 10 µl aliquot was used for determination of protein concentration (Protein Assay, BioRad). Bromophenol blue (0.1 %) was added to the remainder for SDS-PAGE in a BioRad

mini-gel apparatus, using 4 % polyacrylamide stacking gel and 10 % reducing polyacrylamide separating gel. Samples were added as 10 µl aliquots containing 0.5 µg protein to the slots, as well as 5 µl of a prestained 10-250 kDa molecular weight protein standard (Amersham, Rainbow Catalogue). Gels were run in at 120 V for 5 min and at 70 V for a further $3^{1/2}$ hours. Gels were silver stained (BioRad).

WESTERN BLOTTING

Unstained proteins separated by SDS-PAGE were transferred electrophoretically to Blot PVDF paper, pore size 0.2 µm (BioRad) in a Mini Trans Blot system (BioRad), for 1 h at 100 V at 5°C in running buffer (48 mM TRIS pH 9.2, 39 mM glycine, 20 % methanol). The paper with transferred proteins was dipped into methanol, incubated for 1 h in 5 % non fat dry milk powder in PBS, washed in PBS and incubated overnight with mouse or human antiserum. Reactive proteins were detected with Vectastain® ABC-AF kit (Vector Laboratories).

RESULTS

TITRES OF MOUSE SERA

Using different combinations of inoculation route (ip or sc), whole or disrupted spores of *T. hominis*, with or without MPL + TDM, it was determined that whole spores inoculated i.p. stimulated a good antibody response, if given with adjuvant. The titres and details of inoculation are given in Table II. Serum from mouse 485 was virtually non-reactive and was discarded.

ELISA

Antiserum 483 with a titre of 1:12,800, was used to test cross reactivity with the full range of microsporidia available. The titres are given in Table I and show that the serum was strongly reactive on *T. hominis* and had some cross reactivity with several of the other species tested. It was at least eight times more reactive for IgG with *T. hominis* antigen (1:12,800) than with *V. culicis* (1:800-1,600). Reactivity was less on *Pleistophora mirandellae*, *Pleistophora* sp. (LS) from *Litopenaeus setiferus* and *E. cuniculi* (1:800), *Pleistophora hippoglossoides*, *Pleistophora typicalis*, *Pleistophora* sp. (FA) from *Farfantepenaeus aztecus*, *Encephalitozoon hellem* and *Vitiforma corneae* (1:400) and was non-reactive on *Heterosporis anguillarum*, *Pleistophora* sp. (TB) from *Taurulus bubalis*, *Spraguea lophii* and *Vairimorpha mesnili*. When tested for IgM on *T. hominis*, the titre of serum 483 was 1:800. Serum from the AIDS patient who had been infected with *T. hominis* gave a titre of

1:800 for IgG in the homologous test but was negative for IgM, IgA, IgE, kappa and lambda light chains. As there had been some cross reactivity between serum 483 and most of the microsporidia tested, all the other sera that had been raised in mice against *T. hominis* except serum 485 (Table II) were retested using *T. hominis* and *V. culicis* as antigen, to assess cross reactivity with the latter. In this test, serum 483 was eight times more specific for *T. hominis* (1:12,800 versus 1:1,600) but serum 443, with a lower titre against *T. hominis*, showed the highest specificity i.e. 16× more reactive on *T. hominis* than on *V. culicis* (1:3,200 versus 1:200). The highest titre serum (486) had almost the lowest specificity for *T. hominis* being only twice as reactive on *T. hominis* as on *V. culicis* (1:25,800 versus 12,800). Serum 443 was selected for Western blotting because of its high specificity and because it revealed a greater number of bands than did other sera in preliminary Western blots (data not shown).

SDS-PAGE AND WESTERN BLOTS

The protein profiles of 11 microsporidia separated by SDS PAGE are shown in Figure 1. The bands revealed in *T. hominis* are a group of four or five between 100 and 70 kDa, one at 62 kDa, a group of three between 54 and 50 kDa, a major band at 44 kDa, others between 40 and 34 kDa, another major band at 32 kDa and several below 30 kDa. A clear profile was not obtained for *Pleistophora* sp. (TB) from *T. bubalis*. All profiles were unique.

When identical protein profiles were blotted with mouse serum 443 (Fig. 2), major immunogenic antigens of *T. hominis* were displayed between 150 and 98 kDa and between 44 and 39 kDa and there were others above 250, at 80, 66, 38, 36, 33 and below 25 kDa. Cross reactive antigens were also demonstrated in *V. culicis*, *P. typicalis*, *Pleistophora* sp. (TB) from *T. bubalis* and *H. anguillarum* but there was no binding to the low molecular weight bands of these spe-

Mouse	Number of spores per inoculation	Number of inoculations	Condition of spores	Solution	Route	Titre
443	5×10^6	4	whole	PBS	i.p.	3,200
445	5×10^6	4	whole	MPL+TDM	i.p.	3,200
447	5×10^6	4	disrupted	MPL+TDM	s.c.	6,400
483	1×10^7	2	whole	MPL+TDM	i.p.	12,800
484	1×10^7	3	whole	MPL+TDM	i.p.	12,800
485	5×10^6	3	whole	MPL+TDM	i.p.	200
486	5×10^6	3	whole	MPL+TDM	i.p.	25,600

Table II. – Methods for raising antisera in mice against *T. hominis* and titres of the sera.

Fig. 1. – Silver-stained SDS-PAGE separated protein profiles of *T. hominis* in comparison with profiles of eleven microsporidia of fish, crustacean, insect and mammalian origin. Lanes are: a. *Trachipleistophorus hominis*, b. *Vavraia culicis*, c. *Pleistophora typicalis*, d. *Pleistophora* sp. (TB) from *Taurulus bubalis*, e. *Heterosporis anguillarum*, f. *Pleistophora mirandellae*, g. *Pleistophora* sp. (LS) from *Litopenaeus setiferus*, h. *Glugea anomala*, i. *Loma aceriniae*, j. *Spraguea lophii*, k. *Encephalitozoon cuniculi*.

Fig. 2. – Western blot of SDS PAGE-separated protein profiles of *T. hominis* and eleven other microsporidia with mouse antiserum 443 raised against *T. hominis*. Lanes a-k are as in Figure 1.

cies. In *V. culicis* bands were visible above 250 kDa, at 170-130 kDa and at 88 kDa. Major antigens of 140-100 kDa, 90 kDa and 65 kDa were identical in *P. typicalis* and *Pleistophora* sp. (TB) from *T. bubalis*. Reactive antigens from *H. anguillarum* were in the region of 250-170 kDa and between 130 and 65 kDa. A single band at about 135 kDa was given by *P. mirandellae*. No antigens in common with *T. hominis* were revealed by antiserum 443 in *Pleistophora* sp. (LS) from *L. setiferus*, *Glugea anomala*, *Loma acerinae*, *S. lophii* or *E. cuniculi*. Serum from the AIDS patient was non-reactive on *T. hominis* antigens even at 1:10 dilution.

DISCUSSION

In the present study all the microsporidia investigated were found to have unique protein profiles. Some epitopes of immunogenic antigens were common to *Trachipleistophora*, *Vavraia*, *Heterosporis* and *Pleistophora* but none of these were shared by the remaining genera, *Glugea*, *Loma*, *Spraguea* and *Encephalitozoon*, as revealed in Western blots. There was also no reactivity with *Pleistophora* sp. (LS) from the shrimp *L. setiferus*, suggesting that this species does not belong to the genus *Pleistophora* and should be assigned to another genus after morphological re-examination.

Comparison of the ELISA and Western blot results obtained with mouse anti-*T. hominis* sera revealed some anomalies. *H. anguillarum*, *P. typicalis* and *Pleistophora* sp. (TB) gave strong profiles in Western blots but were negative or of very low titre by ELISA. The close relationship between *P. typicalis* and *Pleisto-*

phora sp. (TB) indicated by phylogenies based on 16S rDNA (Cheney *et al.*, 2000; Nilsen, 2000) was confirmed by their very similar profiles in Western blots (Fig. 2). Unfortunately the SDS PAGE profile of *Pleistophora* sp. (TB) was not sufficiently clear to support this. *P. mirandellae* gave an ELISA titre of 1:800, greater than that of *P. typicalis* (1:400) yet there was barely a reaction in the Western blot, even though the SDS PAGE profile, a duplicate of that which was blotted, revealed a good range of proteins. Furthermore *Pleistophora* sp. (LS) and the *Encephalitozoon* spp. were moderately positive by ELISA but totally negative in the Western blot. Some of the low titres in the ELISA could have occurred because spores were dislodged from the plastic walls of the ELISA plates during processing. We conclude that the ELISA with serum 483 simply indicated that there was strong homologous reactivity and very little heterologous reactivity, without accurate differentiation of the other species. The Western blot results are considered to be more reliable because the amount of protein applied to the gels was adjusted to 0.5 µg for each species, making each profile comparable and because they support the phylogenetic relationships indicated by sequences of the 16S rDNA gene (Cheney *et al.*, 2000; Nilsen, 2000) and of the RPB1 gene (Cheney *et al.*, in press). The 16S rDNA phylogenies showed that the species which were strongly cross reactive in the present Western blot results are more closely related to one another than any of these are to *Pleistophora* sp. (LS) or to the genera *Glugea*, *Loma* and *Spraguea*. These relationships were upheld in a phylogeny based on sequences of the A-G region of the largest subunit of RNA polymerase (RPB1) (Cheney *et al.*, in press).

Mouse serum 443 used for Western blotting was the only serum that had been obtained after i.p. inoculation of spores in PBS. In other cases spores had been administered in adjuvant. Intraperitoneal inoculation has been demonstrated as a route of infection in athymic mice (Hollister *et al.*, 1996). It is likely that in mouse 443 there had been an active, though transient, infection and the serum would have contained antibodies specific for internal spore antigens as well as antigens of the spore coat. This is supported by the greater number of bands revealed in Western blots by serum 443 than by the other sera. Also, as mouse serum 443 was used for Western blotting of proteins from the range of microsporidian species, while serum 483 was used in the ELISA tests against these species, the results are not strictly comparable. In spite of the differences, it is clear that *T. hominis* can be differentiated from closely related microsporidia by its unique SDS-PAGE profile and that highly specific antisera raised to *T. hominis* could be used to screen muscle biopsies from AIDS patients with myositis to detect early infections and determine whether or not *T. hominis* is involved in the pathology. Such sera would also differentiate *T. hominis* from the other microsporidia which have caused myositis in AIDS patients i.e. *Pleistophora* sp. (Ledford *et al.*, 1985) and *Brachiola vesicularum* (Cali *et al.*, 1998).

Serum from one patient, with severe myositis due to *T. hominis*, who was in the terminal stages of AIDS, was mildly reactive on *T. hominis* antigen by ELISA but non-reactive on Western blots. In a previous study of an AIDS patient with *E. hellem*, from whom serum samples had been kept over a period of four years, there had been a marked drop in antibody level in the terminal stages of AIDS, as revealed by Western blotting of culture-derived spore antigens (Hollister *et al.*, 1993): most of the characteristic proteins of this species shown by the serum samples taken earlier in the patient's illness were no longer recognised by the last sample. In the description of *E. hellem* which was differentiated from *E. cuniculi* by SDS-PAGE and Western blotting (Didier *et al.*, 1991), sera from three patients with keratoconjunctivitis due to *E. hellem* were tested on SDS-PAGE protein profiles of *in vitro* culture-derived spores of *E. hellem*. Serum from one patient who was at a slightly earlier stage of AIDS reacted strongly against the *E. hellem* protein profiles, whereas the reactions of the sera of the other two patients, who were in the terminal stages of AIDS, were much weaker. Although these sera were derived from separate individuals, whose immune responses would not have been identical, the results are in accord with the decline in antibody level, which was tracked by Hollister *et al.* (1993) in one patient, as AIDS progressed. These results suggest that late stage sera might not be useful in diagnosis of microsporidial infections

such as *T. hominis* or *T. anthropophthera*, as these infections are only likely to be problematic (and detected) in the terminal stages of AIDS. However, should *T. hominis* be detected in AIDS patients in future, retrospective examination of sera obtained at an earlier stage of AIDS might reveal high levels of specific antibodies. Such sera would be valuable in detection of *T. hominis* antigen in biopsies or as a control in serological surveys of patients with myositis, using the patient's sera against culture derived *T. hominis* antigen.

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