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Antigenic differences between *Trichinella spiralis* and *T. pseudospiralis* detected by monoclonal antibodies

I. Kehayov [1], Ch. Tankov [2], S. Komandarev [2], and S. Kyurkchiev [1]

[1] Department of Molecular Immunology, Institute of Immunobiology, Bulgarian Academy of Sciences, 73 Lenin Bld, 1113 Sofia, Bulgaria
[2] Central Laboratory of Helminthology, Bulgarian Academy Sciences, 12 Acad. Bonchev, 1113 Sofia, Bulgaria

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Abstract. Antigenic differences between *Trichinella spiralis* and *T. pseudospiralis* were established using two monoclonal antibodies (mAbs) that show different specificities to muscle larvae of the two variants. Enzyme-linked immunosorbent assay (ELISA) revealed that mAb 3G6 reacts positively against *T. spiralis*, *T. nelsoni*, *T. nativa* and *T. pseudospiralis*, whereas mAb 3E10 does not react with *T. pseudospiralis* under the same experimental conditions. These antigenic differences were confirmed after preabsorption of the antibodies with serial dilutions of extracts of *T. spiralis* or *T. pseudospiralis* muscle larvae. The indirect immunofluorescence technique showed that the antigen corresponding to mAb 3G6 is located in the stichosomes and the cuticle surface of both *T. spiralis* and *T. pseudospiralis*. In contrast, mAb 3E10 positively stained cryostat sections of *T. spiralis*, forming a dense reaction product on the surface of the whole larvae and the surrounding capsule. This antibody can be quite useful as a specific probe for distinguishing *T. spiralis* from *T. pseudospiralis* in taxonomic studies. Using an avidin-biotin system, we could prove that mAb 3G6 recognizes an excretory/secretory-type antigen.

In spite of the extensive investigations carried out for more than 20 years in search of morphological, biochemical and genetic variants of *Trichinella spiralis* Owen 1835, as yet there is no definitive opinion as to the existence of separate species in the genus *Trichinella* (Almond et al. 1986).

T. pseudospiralis, a variant previously described by Garkavi (1972), is particularly intriguing in that respect. Both larval and adult forms of this variant differ from the corresponding forms of *T. spiralis* (Boev et al. 1979). Differences in the morphological structure of the two variants have been observed by scanning electron microscopy by Hulinska and Shaikenov (1980). However, Lichtenfels et al. (1983) could not find any differences between the two variants, although they used the same technique. Certain differences between *T. spiralis* and *T. pseudospiralis* have been established in investigations of the life cycles of the worms (Kramar et al. 1981; Palmas et al. 1985). The differences are particularly well defined by the pathological alterations caused by the infectious larvae (Despommier 1975; Gustowka et al. 1980).

Almond et al. (1986) recently established definite biochemical differences between both the larval and the adult forms of the two variants using a sensitive comparative analysis of radiolabelled proteins of secretory and surface somatic origin. Some data from previous immunodiffusion analysis of the antigenic structures of *T. spiralis* and *T. pseudospiralis* tend to show definite differences between these variants (Boszon et al. 1976; Efremov and Ermolin 1980). Similar data obtained using two-dimensional electrophoresis analysis have been reported by Komandarev et al. (1986).

In the present paper, data are reported on the differences in the antigenic structures of *T. spiralis* and *T. pseudospiralis* as detected by specific monoclonal antibodies (mAbs). One of the antibodies used reacted against a secretory/excretory antigen (mAb 3G6), whereas the other recognized a somatic antigen (mAb 3E10).

Materials and methods

Experimental animals

BALB/c mice, Wistar rats and guinea pigs were supplied by the Experimental Animals Farm of the Bulgarian Academy of Sciences. *Trichinella spiralis* and *T. pseudospiralis* were isolated from animals that had been orally infected with 2000 muscle larvae each. Living larvae were recovered after digestion of muscle tissue with 1% pepsin-HCl for 2 h at 37° C. Isolated larvae were washed in phosphate-buffered saline (PBS, pH 7.4) at 37° C and resuspended in serum-free RPMI 1640 medium (Flow Labs, UK).

Offprint requests to: I. Kehayov

Soluble antigens

Extracts of *T. spiralis* or *T. pseudospiralis* were prepared after homogenization of larvae that had been frozen and thawed several times. The homogenate was centrifuged at 20000 rpm for 60 min at 4° C, and the clear supernatant was divided into aliquots and stored at −70° C for use in further experiments. The protein content of each extract was adjusted at 1 mg/ml according to the method of Lowry. Water-soluble extracts of *T. nelsoni, T. nativa, Ascaris suum, Echinococcus granulosus* (larvae), *Trichocephalus ovis* and *T. suis* were kindly donated by the Central Laboratory of Helminthology, Bulgarian Academy of Sciences (Sofia, Bulgaria).

Construction of hybridomas

BALB/c mice were infected orally with 200 live *T. spiralis* larvae and boosted with 200 µg larval saline extract. About 20 days after inoculation with *T. spiralis* larvae, BALB/c mice were injected i.p. with 20 µg crude *T. spiralis* extract per animal; 5–6 days later, the mice were bled and the sera obtained were tested by enzyme-linked immunoabsorbent assay (ELISA). All animals had developed a humoral immune response to *Trichinella* soluble antigens, and the titre of antibodies was between 160 and 320 in different animals. Splenocytes from highly reactive mice were fused with mouse myeloma cells according to the protocol adapted in the laboratory by Kyurkchiev et al. (1988), and supernatants from wells containing growing hybrid cells were screened by ELISA against saline extract of *T. spiralis* and/or guinea-pig muscle. Cells from wells that showed highly specific reactions were cloned and recloned to isolated, stable monoclonal hybridomas.

Enzyme-linked immunosorbent assay

The production of specific antibodies and the specificity of each supernatant were determined by a specific immunoassay. Wells of 96-well polyvinylchloride (PVC) microtitre plates were coated with either saline extract of *T. spiralis* larvae or control muscle extract with a protein content of 20 µg/ml. The plates were blocked with 10% inactivated calf serum for 1 h at room temperature, and purified antibody or supernatants were added and incubated for 2 h at room temperature. After extensive washing, wells were treated for 1 h at room temperature (RT) with anti-mouse Ig serum conjugated with horseradish peroxidase (Sevac, CSSR), after which the wells were washed again and the enzyme reaction was developed with *o*-phenylenediamine (Sigma Chemical, Co., USA). Optical density values were recorded by a MicroELISA reader (Dynatech Co., Switzerland).

The presence of a secretory/excretory antigen in tissue-culture medium of cultured muscle larvae was assayed by ELISA using a biotinylated mAb, which was purified by 43% ammonium precipitation of spent culture medium followed by fast protein liquid chromatography (FPLC) as described elsewhere (Stamenova et al. 1990). Purified preparations were dialysed overnight at 4° C against 0.1 M $NaHCO_3$ and then mixed with 60 µl biotin (1 mg/ml NHS-Biotin in dimethylsulfoxide; Sigma Chemical Co., USA). The mixture was incubated at room temperature for 4 h and then dialyzed overnight at 4° C against PBS. After blocking, larval extract-coated plates were treated with biotinylated antibody for 1 h at RT and washed with T-PBS, and avidin peroxidase diluted 1:5000 (Sigma Chemical Co., USA) was then added for 1 h at RT. The reaction was further developed and read as described above.

Indirect immunofluorescence technique

Paraffin-embedded sections of muscles from guinea pigs that had been infected with *T. spiralis* larvae were processed for immunofluorescence according to the method described by McLaren et al. (1987). In some experiments cryostat sections of tissues from infected animals were used. Sections were covered with the mAb, incubated for 60 min at RT, washed extensively in PBS (pH 7.4) and incubated for an additional 60 min with anti-mouse Ig serum conjugated with fluorescein isothiocyanate (FITC; Miles Laboratories, UK) diluted to 0.1 mg/ml. Stained sections were observed using an epifluorescent microscope Laborlux K (Leitz, Austria).

Results

After screening of 134 supernatants from wells containing growing hybrid cells, two hybridomas (designated as 3E10 and 3G6) were selected that secreted specific mAbs against saline extract of *Trichinella spiralis*. For a more detailed characterization, these antibodies were tested for cross-reactions against striated muscle samples from guinea pigs and rats and against various species of parasite worms: *Ascaris suum, A. lumbricoides, Echinococcus granulosus, Toxocara canis, Trichocephalus suis* and *T. ovis*. The results obtained using ELISA showed that mAbs 3E10 and 3G6 did not react with extracts from the tissues and parasites tested. Table 1 contains data about the reaction of the antibodies against crude saline extracts from four larval variants of the genus *Trichinella*: *T. spiralis, T. nelsoni, T. nativa* and *T. pseudospiralis*.

The most important finding in these experiments was that mAb 3E10 did not react with larval extract of *T. pseudospiralis* as determined by ELISA. This quite intriguing observation was confirmed when mAbs 3G6 and 3E10 were absorbed using varying concentrations of *T. spiralis* or *T. pseudospiralis* extracts. At protein concentrations of up to 5 mg/ml, crude saline extract from *T. pseudospiralis* did not inhibit the binding of mAb 3E10 to PVC plates coated with *T. spiralis* extract (Fig. 1). However, when the same antibody was absorbed with *T. spiralis* extract, a typical absorption curve was outlined that showed a well-defined dose-dependent correlation. Under these conditions, a sample containing 400 µg/ml total protein inhibited about 50% of the bind-

Table 1. Specificity of mAb 3G6 and mAb 3E10 against various species of nematodes as tested by ELISA

Nematode species	mAb 3G6	mAb 3E10
Trichinella spiralis	0.988	0.84
T. nelsoni	0.86	0.9
T. nativa	0.79	0.695
T. pseudospiralis	0.81	0.024
Toxocara canis	0.09	0.028
Ascaris lumbricoides	0.045	0.015
A. suum	0.08	0.03
Echinococcus granulosus	0.058	0.054
Trichocephalus ovis	0.045	0.038
T. suis	0.031	0.059
Negative control	0.03	0.035

Data represent the means of three independent experiments. mAb specificity is expressed as optical density at 492 nm

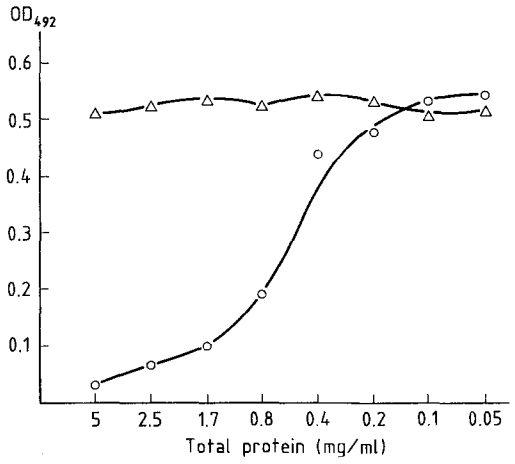

Fig. 1. Competitive enzyme-linked immunosorbent assay (ELISA). Titration curves for mAb 3E10 after absorption with serial dilutions of extracts of *Trichinella spiralis* (○—○) and *T. pseudospiralis* (△—△). Preabsorped mAb 3E10 was added to *T. spiralis* extract-coated PVC plates

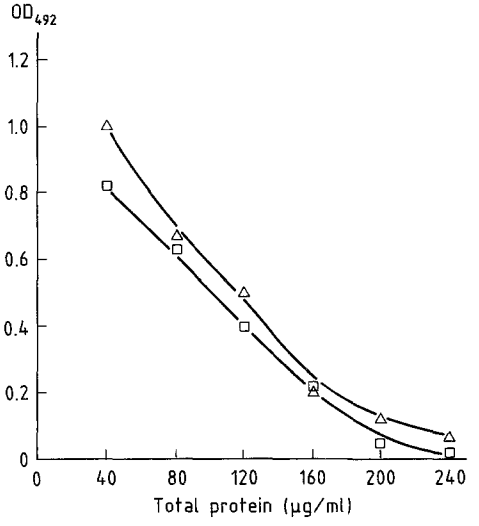

Fig. 2. Competitive enzyme-linked immunosorbent assay (ELISA). mAb 3G6 was preabsorbed with serial dilutions of extracts of *Trichinella spiralis* (△—△) and *T. pseudospiralis* (□—□) and then added to *T. spiralis* extract-coated PVC plates

ing of mAb 3E10. In contrast, the activity of mAb 3G6 was inhibited after absorption with extracts of both *T. spiralis* and *T. pseudospiralis*, as 120 µg/ml *T. spiralis* extract absorbed as much antibody as did 130 µg/ml *T. pseudospiralis* extract, which amounted to about 50% of the binding of mAb 3G6 (Fig. 2).

The differences in the antigenic composition of *T. spiralis* and *T. pseudospiralis* variants could be demonstrated in experiments on some of the biological features of antigens corresponding to mAbs 3E10 or 3G6, which primarily concerned the secretory/excretory status of the two antigens. For this purpose, larvae from the two variants were isolated from infected muscle tissue and incubated in RPMI 1640 medium containing 10% foetal calf serum. Samples from the tissue-culture medium were collected on days 1, 3, 5 and 7 and then tested for the presence of secretory *Trichinella* antigens. When Mab 3G6 was used for ELISA, its corresponding antigen was detected in samples from the 1st day of incubation, as the amount of antigen secreted seemed to increase until the 3rd day of culture. However, under the same conditions mAb 3E10 did not react with any of the samples from the culture medium of *T. spiralis* or *T. pseudospiralis* (Table 2).

Table 2. Secretion of the excretory/secretory antigen in culture supernatants of larvae monitored by specific mAbs

Nematodes	mAbs	Days of culture				
		1st	3rd	5th	7th	9th
Trichinella spiralis	3G6	0.232	0.69	0.51	0.48	0.35
	3E10	0.37	0.71	0.39	0.37	0.337
T. pseudospiralis	3G6	0.05	0.08	0.01	0.03	0.025
	3E10	0.01	0.05	0.023	0.08	0.02

Data are expressed as optical density at 492 nm

The data obtained by ELISA on the existence of antigenic differences between *T. spiralis* and *T. pseudospiralis* were confirmed by immunofluorescence studies on the cellular distribution of the antigens recognized by mAbs 3E10 and 3G6 in both paraffin-embedded and cryostat sections from muscles of guinea pigs that had been orally infected with *T. spiralis* or *T. pseudospiralis*. When mAb 3G6 was applied as the first anitbody, followed by FITC-labelled anti-mouse immunoglobulin serum, bright specific staining was seen on the stichosome of *T. spiralis* muscle larvae (Fig. 3a). Intensive fluorescence was observed on both longitudinal and cross-sections of the larvae (Fig. 3b). It should be pointed out that specific staining was located on the surface and that its intensity was greater in the portion corresponding to the stichosome. The larval capsule and the surrounding muscle fibres were not stained. An irrelevant mAb of the same immunoglobulin subtype was used on control sections (Fig. 3e). A quite similar pattern of staining was seen when paraffin-embedded sections from muscles of animals infected with *T. pseudospiralis* were treated with mAb 3G6. Again, the positive reaction product was located on the stichosome and the surface layer of the cuticle (Fig. 3c).

When mAb 3E10 was used as the first antibody in the indirect immunofluorescence test, a different pattern of staining was observed. Cryostat sections from muscles of guinea pigs infected with *T. spiralis* were treated with mAb 3E10, and homogeneously positive staining was seen on the surface of the whole larvae. In contrast to the reaction of mAb 3G6, in this case specific fluorescence was observed in the space between the stichosome and the larval cuticle. Quite weak fluorescence was seen on the larval capsule, which created the impression that in some cases the intracapsular space was stained (Fig. 3d). An important finding in these experiments was that mAb 3E10 did not stain *T. pseudospiralis* muscle

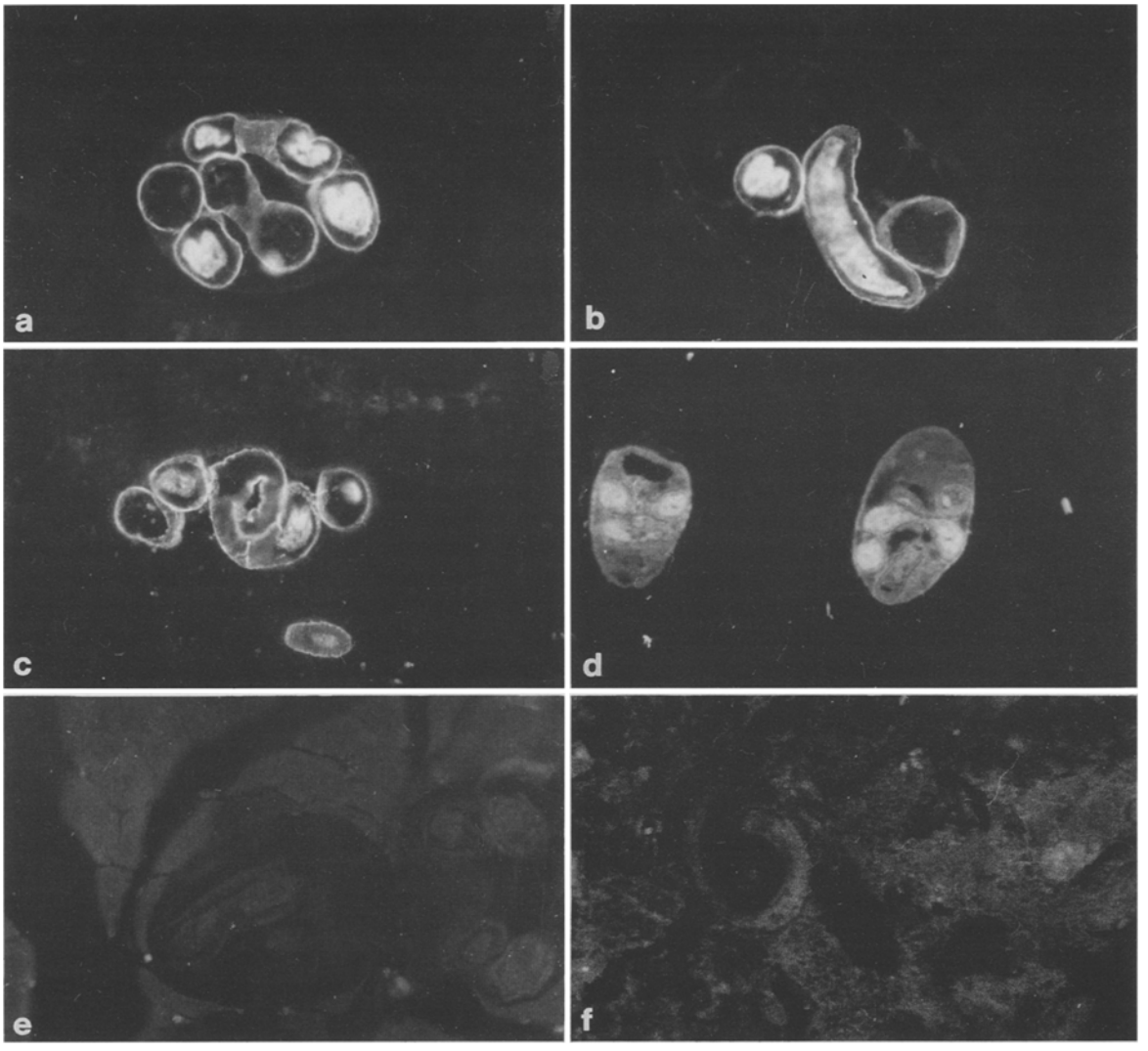

Fig. 3a–f. Indirect immunofluorescence on cryostat sections. **a** A muscle from a guinea pig infected with *Trichinella spiralis* that was stained with mAb 3G6. Bright immunofluorescence can be seen on the stichosome and the cuticle, which are clearly distinguished at the transverse section of the larvae. (× 500). **b** Longitudinal and transverse sections of *T. spiralis* muscle larvae that were treated with mAb 3G6 as the first antibody layer. An intensely positive reaction can be seen on the stichosome and the larval surface (× 500). **c** A guinea-pig muscle infected with *T. pseudospiralis*. mAb 3G6 applied as the first antibody layer positively stained an antigen associated with the stichocytes and the cuticle of muscle larvae (× 500). **d** A guinea-pig muscle infected with *T. spiralis* that was treated with mAb 3E10 as the first antibody. Positive immunofluorescence can be observed on the surface of the whole larvae and surrounding larval cuticle (× 500). **e** A muscle from a guinea pig infected with *T. spiralis* that was treated with an irrelevant mAb of the same immunoglobulin isotype as mAb 3G6. The section is completely negative, as no fluorescence can be seen (× 500). **f** A muscle from a guinea pig infected with *T. pseudospiralis*. Note that mAb 3E10 shows no reaction with any component of the muscle larvae of this parasite (× 500)

larvae (Fig. 3f). In several repeated experiments the finding was completely negative, as were those in sections treated with irrelevant mAb or normal mouse serum diluted 1:100.

Discussion

The present paper reports data on the characterization of two hybridoma lines that secrete specific mAbs against antigens of *Trichinella spiralis* muscle larvae. The immunization schedule used represented an attempt to imitate the natural infection of animals with *T. spiralis*, involving a single oral infection with 200 live larvae, followed 20 days later by a booster consisting of crude larval extract.

As mentioned earlier, after the fusion of splenocytes from immunized mice with myeloma cells, eight hybridomas were obtained that secreted antibodies that reacted against *Trichinella* extract but were completeley negative against striated muscle extract. For further experiments, two clones were selected that reacted in a specific manner with *T. spiralis* or *T. pseudospiralis*. The most important finding was that mAb 3E10 did not react with crude

saline extract of *T. pseudospiralis*; this observation was confirmed by direct ELISA tests (Table 1) and after absorption of mAb 3E10 with serial dilutions of extracts of the two variants of *Trichinella* (Fig. 1). The antigenic differences demonstrated between *T. spiralis* and *T. pseudospiralis* in these experiments are essential for the taxonomic definition of the two variants.

The cellular localization of 3G6 antigen, established in our experiments by indirect immunofluorescence, is quite similar to that of antigens described by Silberstein and Despommier (1984) and Gamble (1985). McLaren et al. (1987) recently reported that the mAbs previously obtained by Ortega-Pierres et al. (1984) recognized an antigen located in the stichosome, epicuticle and intestine of muscle larvae. These authors speculated that NIM-M1 and NIM-M2 antigens were of the excretory-secretory type. Quite similar features were revealed for 3G6 antigen in our experiments. Moreover, when biotinylated mAb 3G6 was used, its corresponding antigen could be detected in tissue-culture supernatant from *T. spiralis* and *T. pseudospiralis* muscle larvae cultured in vitro (Table 2). Analysis of the data available for 3G6 antigen leads to the assumption that this antigen is specific to *T. spiralis* species and that it is of the excretory-secretory type. The finding that 3G6 antigen can be detected in culture supernatants from *T. spiralis* and *T. pseudospiralis* grown in vitro as well as in sera from infected guinea pigs and rats (unpublished data) indicates the possibility of test systems being developed for early diagnosis of *Trichinella* infection in accidentally infected humans or animals.

Quite different is the characteristics of the antigen recognized by mAb 3E10. Even in the initial screening procedures, it was established that mAb 3E10 reacts positively with *T. spiralis*, *T. nelsoni* and *T. nativa* extracts but does not react with *T. pseudospiralis* extracts (Table 1). Attempts to determine the cellular localization of the 3E10 antigen on formalin-fixed, paraffin-embedded sections by indirect immunofluorescence were unsuccessful. However, when fresh cryostat sections were treated with mAb 3E10, the corresponding antigen was found to be located on the surface of the larvae. This finding is in agreement with the observation that 3E10 antigen cannot be detected in the culture media of *T. spiralis* and *T. pseudospiralis* larvae (Table 2). It should be mentioned that in these experiments we used the biotin-avidin system, which is reportedly several times more sensitive than systems in which a second antibody is applied.

Some differences in the organization and components of surface protein structures of *T. spiralis* and *T. pseudospiralis* have been established by Almond et al. (1986). These authors succeeded in distinguishing different surface proteins using radiolabelled markers. It would be quite acceptable that mAb 3E10 could recognize an antigen that is a surface component, not be secreted, and be specific only to muscle larvae. Provided that this is the case, after its purification and detailed characterization, this antigen could be used as the basis for development of a potent anti-*Trichinella* vaccine. Otherwise, mAb 3E10 can be used to distinguish *T. spiralis* from *T. pseudospiralis* variants in taxonomic studies.

In conclusion, in the present study data were obtained on the existence of antigenic differences between *T. spiralis* and *T. pseudospiralis* variants. These differences were established using specific mAbs that recognize antigens whose characteristics differ. A surface-somatic antigen identified by mAb 3E10 was found in *T. spiralis* but could be not detected in *T. pseudospiralis*.

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