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A NOVEL DOT BLOT TEST FOR *TRICHINELLA SPIRALIS* ANTIGEN DETECTION

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The Trichinella spiralis excretory-secretory (ES) antigen complex contains components characteristic for the whole genus. Besides their strong immunomodulatory effect, they are key targets for the host immune system. These antigens are currently used for research purposes and also in serological diagnostics. Immunodiagnostic tools, based on ES antigen application, provide an indirect confirmation of parasitism, ensure specific antibody detection and accurate diagnosis in infected humans and are recommended for surveillance programs in animals. If recognized in antibody fluids of the host, the ES antigen is direct evidence of infection. In our study, polyclonal anti-ES antibodies were produced, characterized and analyzed for application in a dot blot test. Applied as capture antibodies, they were successfully combined with monoclonal antibody 7C2C5 which recognizes a Trichinella genus specific epitope. The lowest detectable antigen concentration, i.e. dot blot test sensitivity was 156 ng/ml. This dot blot test may be considered specific and sufficiently sensitive. Validated in an experimental model system, it is expected to have a possible application in practice in Serbia with the ongoing reemergence of trichinellosis.

Key words: dot blot, ES antigen, polyclonal antibodies, Trichinella spiralis

INTRODUCTION

The excretory-secretory (ES) antigen of *Trichinella spiralis* first stage, infective muscle larvae (L1), has a number of biological and immunological functions. It is considered likely that such secreted products are involved in the survival and development of parasites within the host cell (Despommier, 1998). Also, they are strong stimulators of the host immune system (Sofronic-Milosavljevic *et al*., 1997). Recently, it has been shown that one of the ES antigen components is a homologue of the host macrophage migration inhibitory factor (MIF). In that way it could interfere with the activity of human monocytes-macrophages, and their migration into infected tissue (Wu *et al*., 2003). By being a strong stimulator of the adaptive immune response, the ES antigen is very useful in serological diagnosis of trichinellosis. The applicability of ES antigens in serodiagnosis enables the development of specific, sensitive and rapid tests for

detection of *T. spiralis* ES antibodies in the circulation of infected people and many animal species. Conventional serodiagnostic methods are the immunofluorescence antibody test (IFAT), western blot analyses (WBA), complement fixation test (CFA), hemagglutination test (HAT) and enzyme-linked immunosorbent assay (ELISA) (*Nöckler et al*., 2000). IFAT, HAT and CFT are based on the use of cuticular and/or somatic antigens from *Trichinella* larvae and some cross-reactions may occur (Saathoff *et al*., 1978). In WBA and ELISA both crude extracts and ES antigen are in use. ELISA is such a sensitive test that it can detect infection levels as low as 0.01 larva/1 g of tissue (Gamble, 1996a). Also, results from experimental studies indicate that tissue fluids (meat juice) from infected animals may be suitable for serological examinations using ELISA for antibody detection (Gamble and Patrascu, 1996; Kapel *et al*., 1998). Meat juice can also be used for anti *T. spiralis* antibody detection by the lateral flow card test in infected pigs (Patrascu *et al*., 2000). The ELISA method is recommended for herd surveillance programs and is useful for detecting ongoing transmission of *Trichinella* at the farm level (Gamble, 1996b). However, this test may fail to detect infected pigs during both the early and very late stages of infection (Nöckler *et al*., 1995). Most immunoassays to diagnose trichinellosis are based on the demonstration of antibodies against muscle larval antigens. The detection of antigens would be of value to support the diagnosis of an active infection, and would probably allow its diagnosis in an earlier phase. The few reports considering the detection of circulating *T. spiralis* antigens in infected humans and animals showed a heterogeneous kinetic pattern as well as different sensitivities and patterns of applicability of the tests examined (Ivanoska *et al*., 1986, De La Rosa *et al*., 1996).

This work includes the characterization of polyclonal antibodies against *T. spiralis* ES antigen, obtained by immunization of a rabbit, with the aim of developing a dot blot test for detection of *T. spiralis* ES antigen as a potential new tool for serodiagnosis of trichinellosis. The dot blot test developed could be used for ES antigen detection in body fluids (serum and meat juice) of infected animals while *T. spiralis* larvae are viable, which includes early and very late stages of infection.

MATERIALS AND METHODS

Parasites and antigens

T. spiralis L1 larvae were recovered from infected Wistar rats by digesting the carcasses in prewarmed gastric juice (1% pepsin in 1% HCl, pH 1.6 -1.8). After 4 h stirring at 37°C, the larvae were allowed to sediment. The pepsin-HCl solution was removed by aspiration and L1 larvae were washed with saline. ES products were obtained by cultivation (5.000 *T. spiralis* L1 larvae/ml in DMEM media supplemented with 10 mM Hepes, 2mM L-glutamine, 1mM Na-pyruvate and pen/strep 50 U/ml) for 18-20 h at 37°C and with 10 % CO_2. The culture fluid was harvested, filtered through a 0.2 μm filter, concentrated and stored at -20°C until use.

Immunization protocol

For the purpose of polyclonal antibody production, a rabbit was immunized with *T. spiralis* L1 larvae ES antigen by the following protocol: each dose contained 100 μg of *T. spiralis* ES antigen. The first dose was incomplete Freund's adjuvant (CFA, Torlak, Serbia), while all the others were in incomplete Freund's adjuvant. They were administered intramuscularly in the region of the back left thigh in a total volume of 0,5 ml of mixture with adjuvant. The second and the third dose were applied at 3 week intervals. The first sample for testing was taken 7 days after the last injection and after that samples were taken at three week intervals. *T. spiralis* antibodies were detected in the blood of immunized animals by double immunodiffusion in agar gel (DID).

Rabbit IgG isolation and purification

When the greatest titer (1:32) of anti ES antibodies was obtained in DID, the animal was bled and antiserum was separated by centrifugation at 3000 rpm and stored at -20°C until use. Precipitation with ammonium sulphate was applied for the isolation of rabbit IgG. In the first step antibodies were partially purified by precipitation with 50%, and in the second step with 18% ammonium sulphate. The treatment was repeated 2 times. After that, the sediment was dissolved in PBS (half the volume of the starting serum) and dialyzed. The protein concentration was determined by spectrophotometry, and the immunoreactivity of the partially purified antibodies was investigated by ELISA and dot blot assay. Rabbit IgG was stored at -20°C until required.

ELISA for T.spiralis antibody detection

ELISA was performed on flat bottomed polystyrene microtiter plates coated with *T. spiralis* ES antigen, according to the manufacturer's instructions (INEP, Zemun).

Immunoreactivity of the polyclonal anti ES antibodies in the dot blot test

T. spiralis ES antigen was bound to a nitrocellulose (NC) membrane (0.45 nm) in a series of double dilutions (500 μg/ml – 31.25 μg/ml). The membranes were cut into 8x8 mm pieces and incubated with the polyclonal antibodies at 100, 50 and 25 μg/ml for 48 h at 4°C. All the incubations were performed in glass tubes (volume 4 ml). The secondary antibody was HRPO-coupled sheep anti-rabbit IgG, applied at the dilution of 1:1000, and incubated for 2 h. After each incubation the membranes were washed with TBS (50 mM Tris-HCl, 150 mM NaCl) at pH 7.6. Dot blots were developed with a 0,05% solution of diaminobenzidine (DAB, 0.01% H_2O_2 in 0.2 M Tris-HCl pH 7.6).

Reactivity of mAb 7C2C5 with the polyclonal antibody-ES antigen complex, in the dot blot test system.

Polyclonal antibodies (rabbit IgG with the fraction of specific antibodies against *T. spiralis* ES antigen) were bound to NC membranes in the concentration

of 100 μg/ml, and the membranes were incubated with *T. spiralis* ES antigen (500 μg/ml). Ascites fluid, containing monoclonal antibody 7C2C5, was tested in 5 different dilutions: 1:100, 1:200, 1:500, 1:1000 and 1:2000 in TBS buffer which alone also served as the negative control.

Conditions for the detection of T. spiralis ES antigens in animal body fluids by the dot blot test

Polyclonal antibodies (100 μg/ml) were bound to NC membranes (8x8 mm) and incubated with *T. spiralis* ES antigen (series of dilutions) for 48 h. After that the membranes were incubated with the chosen dilution of monoclonal antibody (mAb) 7C2C5 (Gamble and Graham, 1984) for 48 h, followed by incubation with secondary HRPO-coupled goat anti-mouse antibodies (diluted 1:1000) (previously determined as optimal in WB, Ilić, 2002), for 2 h. After each incubation step, the membranes were washed with TBS as described above. Dot blots were developed with a 0,05% solution of diaminobenzidine (DAB, 0.01% H_2O_2 in 0.2 M Tris-HCl pH 7.6).

Analysis of serum samples from horses artificially infected with T. spiralis by the novel dot blot test

In order to validate the dot blot test, serum samples from experimentally infected horses as well as from *T. spiralis* free horses were analyzed in 1:50 dulitions by dot blot assay (under the conditions described above).

Sera from experimentally infected horses. Three adult DMB horses (designated as Horse I, II and III) were infected with a low dose of *T.spiralis* infective muscle larvae (L1) (1100 larvae / per horse). Muscle larvae were recovered in the tongue muscles of all three animals 32 weeks after exposure to infection (pi), by the standard pepsin-digestion method (Gamble *et al*. 2000), and reported as larvae per gram of muscle sample (lpg). (Horse I – 0,97 lpg, Horse II 0 - 0.11 lpg, and Horse III – 0,81 lpg) (Murrell *et al*., 2004). Serum samples were collected on day 0 (before infection), after 8 weeks (when the *T.spiralis* L1 muscle infection was expected to be established) and after 32 weeks pi (end of experiment). They were analyzed for *T.spiralis* antigen by the novel dot blot test.

Sera from *T. spiralis* free horses – Sera were collected from horses slaughtered at the *"Damjanovic"* slaughterhouse, Mladenovac, and originated from animals shown to be *Trichinella* free by the artificial digestion method.

RESULTS

The immunoreactivity of the rabbit IgG polyclonal antibody fraction was investigated by ELISA. According to the manufacturer's instructions, samples with OD index values over 3 are considered positive for *T.spiralis* specific antibody presence. The polyclonal antibody reactivity was found to be 6.2 (OD index), which was very satisfactory.

Another quality control test for IgG polyclonal antibody reactivity with ES antigen was performed on NC membranes. ES antigen was bound to the NC

membrane at five different concentrations, each of them followed by incubation with three different concentrations of IgG polyclonal antibodies. The reaction was visible at all dilutions of antigen and antibody, but was absent with the negative control (TBS) (Figure 1.). The intensity of the reaction of the polyclonal antibodies with ES antigen was dose dependent. The most intensive staining was obtained at the 100 μg/ml antibody concentration, but the reactivity was not dependent on the antigen concentration. Consequently, the first component of the dot blot system for *T. spiralis* ES antigen detection in body fluids was defined and implemented by binding 100 μg/ml of IgG polyclonal antibodies to the NC membranes.

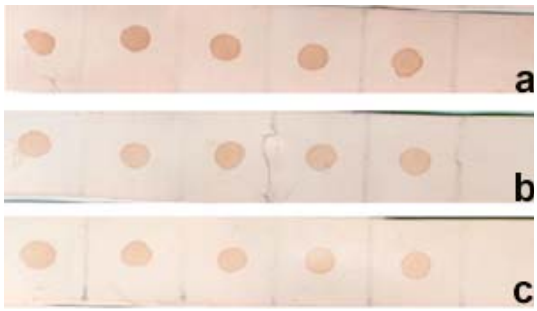

Figure 1. Immunoreactivity of the polyclonal antibodies. Binding of ES antigen to the membrane at concentrations of: 500, 250, 125, 62,5; 31.25 μg/ml. Incubation with polyclonal antibodies in concentrations of: a) 100 μg/ml, b) 50 μg/ml, c) 25 μg/ml

The second important dot blot test component - mAb 7C2C5 which provides test specificity, was analyzed for its reactivity with the polyclonal antibody-ES antigen complex in the dot blot test system. The results presented in Figure 2. indicated the ascites dilution of 1:500 for mAb 7C2C5 as the one that gave the optimal staining and intensity of reaction for all 5 dilutions of ascites tested.

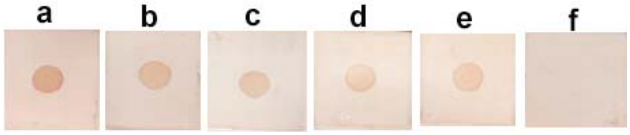

Figure 2. Reactivity of mAb 7C2C5 with the polyclonal antibody-ES antigen complex, in the dot blot test system. Dilutions of mAb 7C2C5: a) 1:100, b) 1:200, c) 1:500, d) 1:1000, e)1:2000, f) incubation with buffer (TBS) as the negative control

The results of the dot blot test sensitivity determination are presented in Figure 3. For this purpose, membranes with fixed polyclonal antibodies were incubated with *T. spiralis* ES antigen in a series of double dilutions, until the visible reaction disappeared, covering concentrations from 500 μg/ml to 78 ng/ml. The

lowest concentration of *T. spiralis* ES antigen that could be detected by the described dot blot test was 156 ng/ml. This way, the sensitivity of the test was defined and the basis for a potential serodiagnostic test was established.

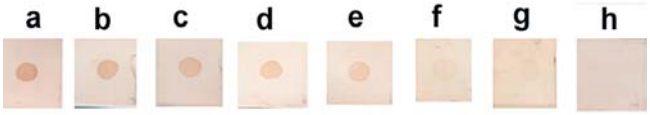

Figure 3. Determination of the sensitivity of the dot blot test for *T.spiralis* antigen detection. *T. spiralis* ES antigen was applied in the following concentrations: a) 500 μg/ml, b) 250 μg/ml, c) 125 μg/ml, d) 62,5 μg/ml, e) 31,25 μg/ml, f) 10 μg/ml, g) 5 μg/ml, h) 2.5 μg/ml.

Analysis of serum samples from three experimentally infected horses by the new dot blot test, indicated that *T.spiralis* ES antigens were not present either in the sera of horses at day 0 of experiment or in the serum samples of *T. spiralis* free horses (Figure 4). However, ES antigens were detected at weeks 8 and 32 pi.

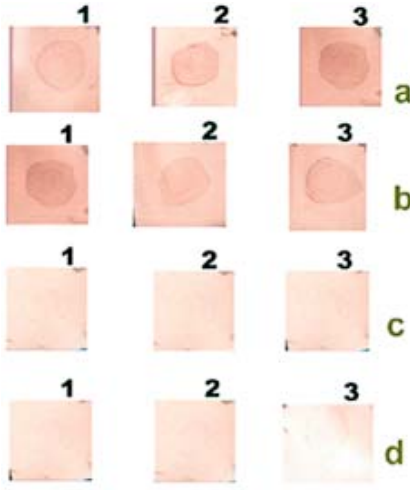

Figure 4. Detection of *T.spiralis* ES antigens in serum samples from artificially infected horses by the novel dot blot test: a) Week 8 pi – 1. Horse I, 2. Horse II, 3. Horse III; b) Week 32 pi – 1. Horse I, 2. Horse II, 3. Horse III; c) Day 0 – 1. Horse I, 2. Horse II, 3. Horse III; d) Serum samples from 3 *T. spiralis* free horses

DISCUSSION

The diagnosis of trichinellosis relies either on direct parasitological finding of L1 larvae present in the muscle of infected humans or animals or on a combination of numerous factors. Where human trichinellosis is concerned, those factors include: a history of exposure, clinical signs and symptoms, laboratory tests and muscle biopsy. If specific antibodies are detected by ELISA and other recognized specific and sensitive tests, the finding is accepted as final proof of diagnosis of human trichinellosis. Detection of *Trichinella* infection in animals is influenced by the purpose: meat safety or a surveillance program. The ICT Recommendations on Control in Food Animals strongly indicate direct parasitological methods: trichinelloscopy and artificial digestion for meat inspection programs. The ELISA, based on ES antigen use for specific antibody detection, is the only serological method recommended by OIE for surveillance and ante-mortem diagnosis of trichinellosis in animals. The suitability of a serological detection method depends on the specific properties of the test system and the characteristics of host immunity if the test detects specific antibodies. In that case, the test antigen is considered to be an important factor for the specificity of the result. The use of *T. spiralis* somatic antigens may cause false positive results (Gamble *et al*., 1983). Although ES antigens of the parasite are more specific, their use in the detection of anti ES antibodies could give false negative results, in the early or very late stage of infection. There are also host-specific factors that may influence the evaluation of the test results. Thus, differences in the individual response, the presence of maternal antibodies and immunodeficiency syndromes compromise interpretation of the test results (Nöckler *et al*., 1995). The reliability of such a test is affected by the time of seroconversion and the presence of antibodies in the host circulation.

In the absence of antibodies, the finding of *Trichinella* antigens in body fluids by immunodiagnostic tools could be of diagnostic value. *T. spiralis* specific antigens are present in the body fluids of infected animals during the whole infection, from the very early to the very late stage, while muscle larvae are viable. From this point of view, a test based on two combined anti-ES antibodies (polyclonal for capture and mAb as specific) for the detection of ES antigen in body fluids, could be of value in everyday practice for diagnostic or surveillance needs in those countries with re-emerging trichinellosis.

In the dot blot test presented here, monoclonal antibody 7C2C5 was applied. It recognizes the immunodominant epitope on proteins with molecular weights 45, 49 and 53 kDa, that belong to TSL-1 group of antigens (found in the stihocyte cells and on the surface of the parasite cuticle, actively secreted by first stage L1 larvae) (Gamble and Graham, 1984). Recent data show that this mAb provides the most specific reaction when compared with many other mAbs, and that the use of mAb 7C2C5 in ELISA gives an extremely sensitive result of 1 ng ES antigen/ml (Li and Co, 2001). Although dot blot test sensitivity is limited compared to ELISA or IRMA (Ivanoska *et al*., 1986), the test could provide valuable results concerning antigen presence. The sensitivity of the dot blot test is satisfactory as it

is a test on paper, and compares favourably with the results of other investigators for this kind of test (Dzbenski *et al*., 1994).

The applicability of the described dot blot test was investigated in a model system using horses artificially infected with *T. spiralis*. ES antigens were detected in serum samples from all the animals, even though they were infected with very low doses of *T. spiralis* larvae. The results obtained for ES antigen presence demonstrated *T.spiralis* establishment during the early phase of infection (8 weeks pi) and correlated well with the L1 larvae parasitological finding at the end of the experiment. No false positive reactions occured either with initial sera from the experimentally infected or samples from uninfected animals. Thus, cross-reactivity with components that are not specific for *T. spiralis* in the examined test system was excluded. This point should be further verified and evaluated in practice with more samples. It should always be kept in mind that, although detection of antigen or antibody in sera or body fluids confirms the diagnosis of trichinellosis, their absence does not exclude infection with *Trichinella spp*.

Further work will be directed to wards improvement of test performance (by converting it to be fast), if it appears valuable in surveillance studies.

This type of diagnostic dot blot test, based on the application of polyclonal anti-ES antibodies and specific mAb, is not commercially available, and standardization of such a test would be a favourable contribution to the serodiagnosis of trichinellosis.

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NOVI DOT BLOT TEST ZA DETEKCIJU ANTIGENA *TRICHINELLA SPIRALIS*

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SADRŽAJ

Kompleks ekskretorno-sekretornih (ES) antigena *T. spiralis* sadrži u sebi komponente karakteristične za čitav genus. Pored snažnog imunoregulatornog dejstva, oni su moćni detektori specifičnog imunskog odgovora domaćina. Ovi antigeni se danas u svetu koriste kako u istraživačke svrhe, tako i u serološkoj dijagnostici. Imunodijagnostička sredstva, bazirana na primeni ES antigena, omogućavaju indirektno dokazivanje prisustva parazita, kroz detekciju specifičnih antitela čime se potvrdjuje dijagnoza infekcije kod ljudi, a preporučuju se i za praćenje prevalencije ove zoonoze kod životinja. Ako se detektuje u telesnim tečnostima domaćina, ES antigen predstavlja direktnu potvrdu da je infekcija prisutna. U našem radu, prikazana je priprema poliklonskih ES antitela, njihova karakterizacija i primenljivost u dot blot testu. Upotrebljena kao antitela – hvatači, ona su uspešno kombinovana sa 7C2C5 monoklonskim antitelima koja prepoznaju specifični epitop karakterističan za genus *Trichinella.* Najniža koncentracija ES antigena koja se na ovaj način može detektovati tj. osetljivost dot blot testa je 156 ng/ml. Ovakav dot blot test se može smatrati specifičnim i senzitivnim. Izvršena je validacija u eksperimentalnom model sistemu, a planira se i njegovo ispitivanje u praksi u cilju utvrdjivanja njegove primenljivosti za otkrivanje trihineloze koja se u Srbiji još uvek širi.