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Research article

CHARACTERISTIC BAND PATTERN IN WESTERN BLOTS FOR SPECIFIC DETECTION OF ANTI-*TRICHINELLA SPIRALIS* ANTIBODIES IN DIFFERENT HOST SPECIES

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Western blot (Wb) is considered to be the gold standard test for *Trichinella* infection serology, since this method allows specific *Trichinella* antigens to be distinguished from cross-reactive antigens. This is not the case with widely used antibody assay techniques - indirect immunofluorescence and ELISA - which are sensitive, but subject to cross-reactions that make the interpretation of weakly positive results difficult. Application of *Trichinella spiralis* muscle larvae excretory-secretory (ES) antigens for the specific antibody detection in ELISA resulted in improved specificity compared to that of crude worm extract that was previously in use, but since production of ES has not yet been standardized, differences among laboratories occur. For this reason, the Wb profile of serum samples from different *T. spiralis* infected host species: human, horse, swine and dog, was investigated in the Serbian National Reference Laboratory for Trichinellosis (NRLT). The common feature of the obtained Wb profiles was the appearance of a triad of bands with molecular masses (Mw) of 45, 49, and 53 kDa. The very same triad was recognized by a monoclonal antibody (mAb) 7C2C5 specific for an immunodominant epitope unique to the muscle larvae stage of all species in the genus *Trichinella*. Inhibition studies confirmed that mAb and anti-*Trichinella* antibodies from sera competed for the same parasite epitope. Based on the obtained results, the NRLT introduced the recognition of the above mention triad as the basis for specific anti-*Trichinella* antibodies detection in the sera of infected hosts.

Key words: band triad, anti-*Trichinella* antibodies, trichinellosis, Western blot

INTRODUCTION

Nematodes of the genus *Trichinella* are zoonotic parasites with a cosmopolitan distribution. Infection in domestic and wild animals does not result in disease while in humans it induces trichinellosis. The severity of trichinellosis in humans can range from subclinical to fatal, and the estimated worldwide number of cases is over 2,500 people annually [1]. Infection initiates a strong humoral immune response both in humans and animals that can be recognized by specific antibody detection in the sera or body fluids

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[2-4].

In Serbia, 2430 cases of trichinellosis (current data of NRLT) occurred as of the beginning of this century (2001-2012). The number of infected people varied over the years, with a significant reduction of human infections being observed in between 2006 and 2010 (692 cases), as compared to the period 2001–2005 (1565 cases). The prevalence of *Trichinella* infection in pigs (the main source of infected meat in human infections in Serbia) was 0.11% in the period 2001-2005, with a two-fold decrease between 2006 and 2008 (0.05%), and reaching the level of only 0.02% in 2010 (5). The only reported *Trichinella* species isolated from domestic swine was *Trichinella spiralis* (*T. spiralis*) [5-8], while *T. spiralis* and *Trichinella britovi* were found in wildlife [5,9,10].

As reported by Cuperlovic et al. [7,11], trichinellosis is the most serious foodborne parasitic disease in Serbia. Transmission occurs in both the sylvatic and domestic cycle. While domestic pigs and synanthropic rats represent the most important hosts in maintaining the transmission cycle, other hosts such as dogs and horses can also be involved [12].

Although serological methods for the detection of *Trichinella* infection are not considered suitable for meat inspection, they are important for the surveillance of the infection and epidemiological investigations in animal populations, particularly in the areas where prevalence is high [13]. Serological methods are also useful for the presumptive or retrospective diagnosis and surveillance of human infection [14-16]. However, conventional serodiagnostic methods, such as the immunofluorescence antibody assay (IFA) and the enzyme-linked immunosorbent assay (ELISA), can yield results that vary due to antigen quality and may differ in the level of sensitivity. To overcome problems with contradictory or equivocal results, many laboratories use Western blot (Wb) as a confirmatory test. The purpose of this work was to define a Western blot profile characteristic for infection with *T. spiralis* in humans and other host species important in the epidemiology of *Trichinella* infection in Serbia.

MATERIALS AND METHODS

Parasites and antigens

T. spiralis infective muscle larvae (L1, ISS 161) were recovered from infected Wistar rats by artificial digestion [17]. ES products were obtained by cultivation of muscle larvae (5.000 *T. spiralis* L1 larvae/ml in DMEM media supplemented with 10 mM Hepes, 2mM L-glutamine, 1mM Na-pyruvate and peni/strep 50 U/ml) for 18-20 h at 37ºC and with 10% CO_2. Culture fluid was harvested, filtered through a 0.2 μm filter, concentrated and stored at -20ºC until use.

7C2C5 monoclonal antibody

Hybridoma cell line 7C2C5, generously supplied by HR Gamble, was maintained, cloned at NRLT and used for 7C2C5 monoclonal antibody (mAb) production. This mAb recognizes a unique immunodominant epitope characteristic for the muscle larvae of *Trichinella* genus and present in a triad of proteins with Mw of 45, 49 and 53 kDa

[18]. It was regularly used (in 1: 2000) as positive control in Wb analyses.

Serum samples

Human sera - Sera collected from patients with well-established clinical disease were obtained from a bank of *T. spiralis* positive human sera maintained at INEP. All sera were positive by IFA with different anti-*Trichinella* antibody titers. A pool of serum samples from 50 human blood donors was used as the negative control.
Horse sera - Reference serum samples (kindly supplied by E. Pozio, International *Trichinella* Reference Centre, Rome, Italy) included four samples from *Trichinella*-free horses (parasitologically and serologically negative), one sample from a *Trichinella*-free horse (parasitologically negative and serologically false positive), two samples from experimentally infected horses (sera collected 4 and 17 weeks post infection, p.i.) and one sample from a *Trichinella*-positive horse imported to Italy from Eastern Europe (parasitologically positive and serologically negative).
Swine sera - Serum samples were collected from experimentally inoculated pigs at different stages of infection (9 pigs, 500 *T. spiralis* L1/pig, 12 weeks infection duration). A pool of 50 sera collected from *Trichinella*-free pigs was used as a negative control. Additional sera were collected from 17 pigs that were parasitologically confirmed to be infected with *T.spiralis*, from a group of 726 examined pigs. These naturally infected pigs originated from small private farms in the vicinity of the town of Pozarevac (administrative centre of the *T. spiralis* endemic District of Braničevo, Serbia).
Dog sera - Serum samples were collected from three experimentally infected dogs (3000 L1/dog, 12 weeks infection duration) and from ten *Trichinella*-free dogs (all kindly supplied by M Djordjevic, ITHM, Belgrade, Serbia). Additional dog sera were collected from 30 stray dogs that originated from the field (*Trichinella* endemic district of Braničevo, Serbia, kindly supplied by M Živojinović, Veterinary Specialist Institute, Požarevac, Serbia). In 11 dogs, the presence of specific antibodies was detected by ELISA.

Polyacrylamide gel electrophoresis and Western blot

ES products were resolved by SDS-polyacrylamide gradient (5-20%) gel electrophoresis under reducing conditions and then subjected to Western blot on a PVDF membrane. Following transfer, membranes were blocked with TBS (50 mM Tris-HCl, 150 mM NaCl) pH 7.6 / 1% BSA and thereafter incubated for 48 hr with serum samples from humans and animal species (diluted 1:50). The 7C2C5 mAb was used as the positive control, while a pool of *Trichinella* free human or animal sera was applied as the negative control. Inhibition of specific 7C2C5 binding was performed by pre-incubation of blots with undiluted sera from an experimentally infected horse (generously supplied by F. van Knapen). After this and all subsequent steps, membranes were washed with TBS. Secondary antibodies (INEP, Belgrade, Serbia), including anti-human IgG, anti-horse IgG, anti-mouse IgG and anti-swine IgG were all labelled with HRP and used in 1:1000 dilution, while protein A-HRP was in dilution of 1:3000 and used to detect reactive IgG in dog serum samples. Blots were incubated with secondary reagents for 2 hours. Bands

were visualized with 0.05% solution of diaminobensidine (DAB, 0.01% H2O2 in 0.2 M Tris-HCl pH 7.6).

RESULTS

Interaction of monoclonal antibody 7C2C5 with ES products in Wb resulted in the appearance of a specific band triad at 45, 49 and 53 kDa. Since this MoAb recognizes a *Trichinella*-specific epitope, the occurrence of this triad may indicate the presence of anti-*Trichinella* antibodies in the examined sera. The reactivity of human serum samples with *T. spiralis* ES antigens showed, among other bands, the presence of the above triad (Mw 45, 49, 53 kDa) (Fig.1). These sera were considered positive for anti-*T. spiralis* IgG. The specific triad was absent in *Trichinella*-negative sera.

Western blots with horse serum samples indicated the presence of the anti-*Trichinella* IgG triad only in sera from an artificially infected horse (Fig.2 Lines 3 and 8). Sera from *Trichinella* free horses (previously confirmed to be serologically negative in both ELISA with ES or tyvelose-BSA antigen, and by IFA, in Rome and Belgrade respectively) did not exhibit the band triad (Fig.2 Lines 4, 5, 6 and 7). Serum sample obtained from a *Trichinella* free horse that was false positive in ELISA and IFA did not produce the characteristic band triad in Wb (Fig.2 Line 2). A false negative serum sample taken from a naturally infected horse with no specific antibodies detected by ELISA and IFA also appeared negative in Wb (Fig.2 Line 9).

Sera from experimentally infected pigs did not produce the characteristic band triad at Day 0. The characteristic band pattern was present at day 21 p.i., and persisted until day 85 p.i (end of the experiment) (Fig. 3). Results were consistent with those previously obtained by ELISA with ES antigen (data not shown).

Sera from naturally infected pigs gave positive reactions in Wb (Fig. 4). Sera from these pigs, parasitologically confirmed positive for *T. spiralis*, were also positive for anti-*Trichinella* IgG in ELISA using ES antigen.

Trichinella-specific bands also appeared when sera from artificially and naturally infected dogs were analyzed in Wb (Fig. 5). In only one of the 11 dog sera did the results obtained by ELISA (using ES antigens) differ from the Wb i.e. ELISA was negative while Wb was positive.

The 45, 49 and 53 kDa band triad was not seen in Wb analyses with *Trichinella* negative control sera of human and animal origin, indicating no false positive result.

Reactivity of undiluted sera from *Trichinella* infected humans [20] and from each animal strain (ten randomly selected ELISA positive sera from pigs or dogs, and one ELISA positive sera from an experimentally infected horse) completely inhibited binding of mAb 7C2C5 to ES antigens as shown in Fig. 6, with a representative serum sample used for inhibition. In this way the specificity of anti- *Trichinella* antibodies for 45, 49 and 53 kDa antigen triad was confirmed.

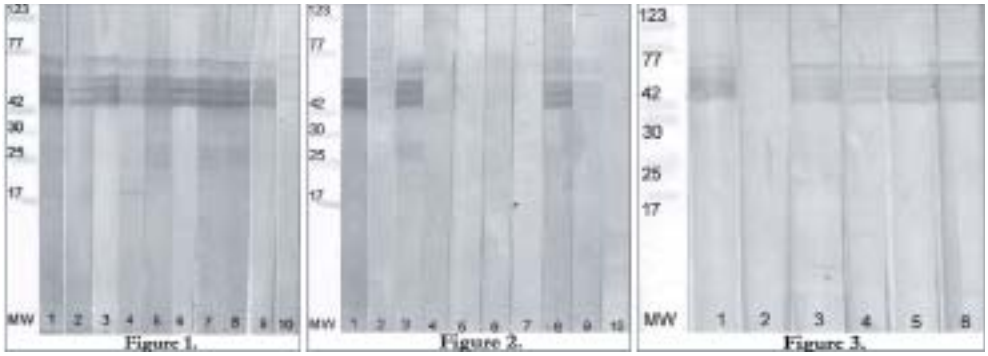

Figure 1. Western blot analyses of serum samples from a *T.spiralis* infected human. A representative Wb profile with *T.spiralis* characteristic band triad pattern in human sera is shown. Line 1. 7C2C5 mAb (MW 45, 49 and 53 kDa), Lines 2-9. Serum samples from infected people with different anti *Trichinella* antibody titers: Line 2. titer 1:80, Line 3. titer 1:160, Line 4. titer 1:320, Line 5 and 6. titer 1:640, Line 7 and 8. titer 1:1280, Line 9. titer 1:2560, Line 10. Negative human serum sample (antibody titer <1:40). **Figure 2.** Western blot analyses with reference horse serum samples (from the International *Trichinella* Referent Center, Rome, Italy). Line 1. 7C2C5 mAb (MW 45, 49, 53 kDa), Line 2. Serum sample from a *T.spiralis* parasitologically negative horse, false positive in IFA and ELISA, Line 3. Serum from a *T.spiralis* artificially infected horse (4 weeks p.i.), Lines 4-7. Sera from parasitologically negative horses, Line 8. Serum from a *T.spiralis* artificially infected horse (17 weeks p.i.), Line 9. Serum from a parasitologically positive horse, false negative according to serology, Line 10. Serum from an artificially infected horse (day 0 p.i.), used as a negative control. **Figure 3.** Western blot analyses of serum samples from pigs experimentally infected with *T.spiralis*. A representative Wb profile showing a *T.spiralis* characteristic band triad pattern is presented. Line 1. 7C2C5 mAb (MW 45,49,53 kDa), Line 2. Pig serum sample, Day 0 of infection, Line 3. Pig serum sample, day 21 p.i., Line 4. Pig serum sample, day 42 of infection, Line 5. Pig serum sample, day 64 of infection, Line 6. Pig serum sample, day 85 of infection.

DISCUSSION

This study revealed that sera from either human or animal species infected with *T. spiralis* reacted with *T. spiralis* ES antigens in Wb and generated the appearance of a characteristic band triad at 45, 49 and 53 kDa. These proteins bear an immunodominant epitope shared by all species of the genus *Trichinella*, which is recognized by the 7C2C5 mAb. The fact that 7C2C5 mAb and antibodies in the sera obtained from *T. spiralis* infected hosts compete for binding to the same epitope, indicates the specificity of polyclonal anti-*Trichinella* antibodies, and confirms the diagnostic significance of the band triad finding.

All human sera that were considered positive for *T. spiralis* infection by IFA also bound to the 45, 49, 53 kDa bands in Wb. However, previous investigations revealed that a number of sera from pregnant women or malignancies [19], as well as from patients with autoantibodies [20,21] and other parasitoses [22-26] gave false positive results in ELISA. When examined in Wb, sera from patients with *Trichinella*-irrelevant infections or other diseases showed presence of antibodies reactive with *T. spiralis* ES components of different Mw, some of which belong to the *Trichinella*-characteristic triad. However, this cross-reactivity never resulted in the appearance of a *Trichinella*-specific band triad

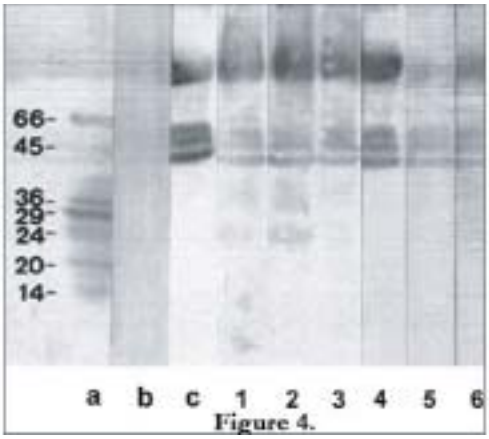

Figure 4.

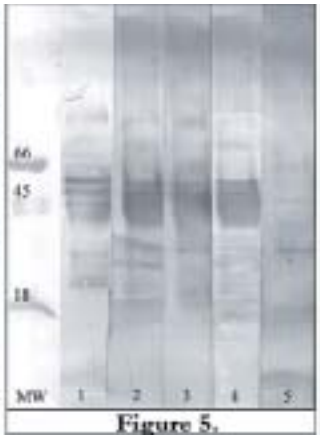

Figure 5.

Figure 6.

Figure 4. Western blot analyses of serum samples from pigs naturally infected with *T.spiralis*. Picture shows representative Wb profile with a *T.spiralis* characteristic band triad pattern in sera of 6 out of 17 infected animals: Line a) MW standards, Line b) Negative pig control serum sample, Line c)7C2C5 mAb (MW 45, 49 and 53 kDa), Lines 1-6- serum samples from naturally infected pigs. **Figure 5.** Western blot analyses of serum samples from dogs experimentally or naturally infected with *T.spiralis*. Picture shows representative Wb profile with a *T.spiralis* characteristic band triad pattern with: Line 1) 7C2C5 monoclonal antibody (Gamble & Graham, 1984); Line 2) serum of experimentally infected dog; Line 3) and Line 4) serum samples from *T.spiralis* naturally infected dogs, Line 5) Negative control dog sera pool. **Figure 6.** Inhibition of 7C2C5 mAb binding to ES components (representative example). Line 1) 7C2C5; Line 2) Membrane pre-incubated with undiluted sera with polyclonal anti-*Trichinella* antibodies and subsequently incubated with 7C2C5.

at 45, 49 and 53 kDa. Cross-reactivity occurs because of the existence of evolutionary highly conserved epitopes which are common for different pathogens and can also be recognized by some autoantibodies [21]. Sera from healthy blood donors, that tested negative in ELISA for the detection of anti-*Trichinella* antibodies, were used as negative controls. No false positive results were obtained by Wb analyses in any of these sera.

In our experience, the IFA has proved to be the most reliable method for early detection of anti-*Trichinella* antibodies in human infections [2,27]. Ivanoska and colleagues [27] demonstrated that the specific antibody could be detected by IFA in 85% of patient sera collected 20 – 30 days after exposure to *Trichinella* infection and reaching 100% by 30-60, while there was a significant delay of antibody detection by ELISA or CIA (ELISA 47,5% and CIA 41,7 % or 77,5% and 87% for the above-mentioned time intervals, respectively). Other authors [2] have reported that out of the 689 examined patients, antibodies were detected in 210 by IFA but only in 43 by ELISA, indicating a higher sensitivity of IFA compared to ELISA. Based on the results obtained in this study, Wb may also be a valuable tool in the laboratory diagnosis of human infection, especially in cases of doubtful results obtained by other antibody detection methods.

Surveillance for *Trichinella* infection in animals frequently employs serological methods. Widely used serological tests such as ELISA and Wb, as well as less commonly used tests, often give ambiguous results (e.g. in some cases antibodies are detected by only one of several tests) [28-30]. This has been an especially frustrating problem in sero-epidemiological studies in horses. In studies conducted with experimentally infected

horses, results on detection of anti-*Trichinella* IgG varied among authors and among the methods used [29,31-33]. Our previous results indicated that specific antibodies could be detected in experimentally infected horses until 32 weeks p.i. by IFA and Wb, but not by ELISA [12,30]. In the present study, sera from experimentally-infected horses, examined by Wb, reacted with components of muscle larvae ES antigens and led to the appearance of a 45, 49 and 53 kDa band triad. This finding confirmed positive results obtained by IFA.

Regardless of the problems with the sensitivity of ELISA testing of *Trichinella* infection in horses, this method is still most commonly used. Although Hill et al. [34] have proved that the persistence of the parasite in horse musculature is similar to that observed in swine and can extend well beyond one year, serological responses to muscle larvae ES antigens in most horses decreased below the cut-off value by 6 month p.i., when measured using a commercially available ELISA. Results obtained in our laboratory showed that an in-house ELISA based on ES antigens failed to detect anti-*Trichinella* IgG in sera of experimentally infected horses after 5 months p.i. On the other hand, IFA on paraffin embedded sections of muscle larvae and Wb on ES antigens showed greater sensitivity, since these tests allow detection of anti-*Trichinella* IgG antibodies for at least 8 months [30].

The results of anti-*Trichinella* IgG detection in two additional species - swine and dogs – suggested that Wb was a suitable method for accurate detection of infection in both experimental and naturally infected animals. Wb provided a definitive result even when values obtained by ELISA were close to cut-off values. We can conclude that Wb may be used as a confirmatory test for distinguishing between true positive and false positive sera, yielding unequivocal results.

Scientists from different laboratories are trying to establish a reliable system for detection of *Trichinella* infection using Wb i.e. different bands have been reported as *Trichinella*-specific by different authors. For example, Yera et al. [35] claimed *T. spiralis* antigenic components with Mw of 43-44 and 64 kDa as discriminative for *Trichinella* infection. In a study performed by Gomes-Morales and colleagues [36] another three-band pattern, ranging in size from 53–72 kDa (for human sera) and 48–72 kDa (for pig sera) was chosen for it discriminative characteristics. Our laboratory defined a band triad with Mw of 45, 49 and 53 kDa as specific for the detection of anti-*Trichinella* antibodies. Each laboratory endeavors to define characteristic antigenic components for the serodiagnosis of *Trichinella* infection according to the known properties of TSL-1 group of antigens, and using their own reference materials. Our experience relies on the usage of the 7C2C5 monoclonal antibody specific for the immunodominant epitope characteristic for the genus *Trichinella* which is present on the above-mentioned band triad, as well as on the application of a large number of reference sera from different species in Wb analyses. The absence of false positive and false negative results reinforced our belief in the significance of the 45, 49 and 53 kDa bands for the detection of *Trichinella* infection.

In summary, Wb allows the identification of specific antibodies that recognize components of *T. spiralis* ES antigens and can be a valuable tool for the serological detection of infection in animals and humans. Based on our previous experience and

the results presented here, all examined host species demonstrate positive reactions by the appearance of a characteristic (triad) banding pattern in the Wb. In accordance to the results presented here, the Serbian NRLT introduced the recognition of the triad of 45, 49 and 53 kDa bands, as the basis for specific anti-*Trichinella* antibody detection in the sera of infected hosts (as a confirmatory or gold standard test).

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OBRAZAC TRAKA U WESTERN BLOT ANALIZI KARAKTERISTIČAN ZA DETEKCIJU SPECIFIČNIH ANTI-*TRICHINELLA SPIRALIS* ANTITELA KOD RAZLIČITIH VRSTA DOMAĆINA

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Western blot (Wb) metoda se smatra zlatnim standardom u serološkoj dijagnostici trihineloze obzirom da ova metoda omogućava razlikovanje specifičnih *Trichinella* antigena od onih koji daju ukrštenu reakciju. Ovo nije slučaj sa drugim, u praksi češće korišćenim tehnikama baziranim na detekciji antitela – indirektna imunofluorescencija i ELISA, koje su senzitivne ali podložne unakrsnoj reaktivnosti što otežava interpretaciju slabo pozitivnih rezultata. Primena ekskretorno-sekretornog (ES) antigena larvi u mišićima, *Trichinella spiralis* u detekciji specifičnih antitela u ELISA testu, rezultirala je povećanom specifičnošću testa u odnosu na prethodni koji je bio baziran na primeni totalnog solubilnog ekstrakta parazita. Ipak, obzirom da proizvodnja ES antigena još

uvek nije standardizovana, postoje izvesne razlike između rezultata dobijenih u različitim laboratorijama. Iz ovog razloga, u Nacionalnoj Referentnoj Laboratoriji Srbije za Trihinelozu (NRLT) ispitivali smo Wb profil uzoraka seruma različitih vrsta domaćina inficiranih sa *T. spiralis*, i to ljudi, konja, svinja i pasa. Zajedničko svojstvo dobijenih Wb profila bila je pojava tripleta traka koje odgovaraju proteinima sa molekulskim masama (Mw) od 45, 49, 53 kDa. Isti triplet prepoznaju i monoklonska antitela (mAt) 7C2C5 specifična za imunodominantni epitop karakterističan za stadijum mišićnih larvi svih vrsta parazita iz genusa *Trichinella*. Eksperimentalne studije inhibicije su pokazale da postoji kompeticija između mAt i anti-*Trichinella* antitela iz seruma za isti epitop parazita. Na osnovu dobijenih rezultata, NRLT uvodi prepoznavanje pomenutog tripleta traka kao osnovu za detekciju specifičnih anti-*Trichinella* antitela u serumima inficiranih domaćina.