# Immunoproteomic analysis of *Trichinella spiralis* larval crude antigens recognized by sera from patients with trichinellosis after treatment with albendazole

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**Abstract.** Antibody responses and antigen recognition were monitored during and after treatment with albendazole (ABZ) in nine patients selected from a trichinellosis outbreak that occurred in north-west Poland in 2007. Seven out of the nine patients yielded positive serum IgG response during treatment. One month after treatment, the IgG response decreased in most patients. Serum levels of ABZ and main metabolites greatly varied among patients without correlation with the IgG response. Two-dimensional electrophoresis and western blot with serum from each patient showed highly immunoreactive spots located between 3–10 pI and 45–97 kDa in all patients. Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI–TOF) and MALDI–TOF/time-of-flight mass spectrometry (TOF MS) analysis identified actine, enolase, p49 protein, *Caenorhabditis elegans*-targeted antigen, and serine protease as the most reactive proteins. A minor spot located at 6 pI and 26 kDa identified as annexin I failed recognition in most patients showing decline in IgG response and clinical improvement after treatment. This protein could constitute a sensitive marker for the effectiveness of ABZ against trichinellosis.

## INTRODUCTION

At present, specific chemotherapy of intestinal as well as muscular infection by *Trichinella* species relies on the use of albendazole (ABZ) [methyl [(5-propylsulfanyl-3H- benzoimidazol-2-yl) carbamate], a benzimidazole–carbamate compound of broad antihelminthic spectrum (Gottstein *et al.*, 2009).

The effectiveness of ABZ-based chemotherapy is influenced by several key factors, such as oral bioavailability, which mainly depends on the solubility, dosage of therapy, the host biotransformation, and enantioselectivity patterns (López *et al.*, 1997; López-García *et al.*, 1998; Virkel *et al.*, 2002; Solana *et al.*, 2009) as well as time of onset of treatment after infection (Pozio *et al.*, 2003).

On the other hand, serum antibody responses during trichinellosis have been widely investigated and it was found that a specific IgG response measure represents the most important laboratory contribution for diagnosis (Pinelli *et al.*, 2007). Several components of the excretory/secretory (ES) products of the *Trichinella* L-1 larvae

(TSL-1) such as 43–45 and 64 kDa glyco-proteins, and some other gylcoproteins have been found to be relevant for the serodiagnosis of trichinellosis by ELISA or western blot assays (Yera *et al.*, 2003; Bruschi *et al.*, 2005; Mitreva & Jasmer, 2006; Cui *et al.*, 2015). In addition, by using capture and inhibition ELISA assays with monoclonal antibodies, it has been demonstrated that in *Trichinella*-infected patients the immune response to TSL-1 antigens is mostly directed against glycans with tyvelose-containing epitopes (Escalante *et al.*, 2004). Further-more, glycan microarray profiling of *Trichinella* infection sera has been assessed as useful technology for the specific sero-diagnosis of trichinellosis (Aranzamendi *et al.*, 2011).

Therefore, the aim of the present work was to study the biotransformation patterns of ABZ in humans as well as serological follow-up with the identification of prominent antigenic changes using proteomics as a new strategy for future optimization of treatment protocols and proper assessment of the effectiveness of these treatments.

MATERIALS AND METHODS

**Patients**
Nine patients (five male and four female) were selected from an outbreak of trichinellosis that occurred in north-west Poland (West Pomerania) in June 2007. All epidemiological and clinical data have been published elsewhere (Golab *et al.*, 2007a, 2007b).

Blood samples were taken during the treatment period (serum sample I) and serological tests were carried out by an in-house IgG ELISA test developed and performed at the Department of Medical Parasitology at the National Institute of Hygiene in Warsaw (Golab *et al.*, 2007a, 2007b).

Specific treatment was carried out with ABZ (Zentel®, *GlaxoSmithKline*) at a dose ranging between 800 and 1 600 mg/day taken with food for 5–10 days according to the severity of clinical symptoms.

The nine patients included in this study were selected on the basis of their attendance for medical follow up 1 month after being diagnosed and treated (serum sample II) according to the calendar indicated in Tables 1 and 2.

**High-performance liquid chromatography (HPLC) assay**
A modular liquid chromatograph equipped with a Jasco PU-1580 pump, an automatic sampler (Gilson 231 XL) fitted to a 100 µl sampler loop (Rheodyne), a variable wavelength detector (UV-1575, Jasco), and Borwin HPLC software (*JMBS*

Table 1. Anti-*Trichinella* IgG serum levels as measured by indirect ELISA using excretory-secretory antigen

| Patient Nº | Aged | Sex | Serum sample I | | Serum sample II | |
| | | | Date | ELISA OD values (cut-off) | Date | ELISA OD values (cut-off) |
| --- | --- | --- | --- | --- | --- | --- |
| B.H./1 | 43 | F | 25.06.07 | 1.313 (0.646) | 23.07.07 | 1.154 (0.593) |
| B.L./2 | 44 | M | 25.06.07 | 0.666 (0.646) | 23.07.07 | 0.738 (0.593) |
| D.E./3 | 26 | F | 25.06.07 | 1.246 (0,646) | 23.07.07 | 1.123 (0.593) |
| H.D./9 | 56 | F | 20.06.07 | 0.820 (0.642) | 19.07.07 | 0.854 (0.593) |
| J.P./4 | 24 | M | 18.06.07 | 0.791 (0.646) | 18.07.07 | 0.554 (0.593) |
| K.G./8 | 47 | F | 22.06.07 | 0.717 (0.646) | 27.07.07 | 0.648 (0.586) |
| K.J./7 | 50 | M | 22.06.07 | 1.224 (0.646) | 27.07.07 | 1.129 (0.586) |
| L.P./5 | 16 | M | 20.06.07 | 0.552 (0.646) | 05.07.07 | 0.426 (0.696) |
| W.M./6 | 37 | M | 20.06.07 | 1.210 (0.646) | 20.07.07 | 1.447 (0.483) |

M: male, F: female

Table 2. Treatment schedule and measurement of serum concentration of ABZ and its main metabolites by HPLC in trichinellosis patients

| No. case | Age | Sex | First day treatment | Daily doses in tablets/ period | Uptake serum sample I date | Drug concentration ($\mu$g/ml) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | ABZ | ABZSO | ABZSO$_2$ | % Enantiomer (+) |
| B.H./1 | 43 | F | 15.06.07 | 2x1 t/5 days | 25.06.07 | <0.05 | <0.05 | ND | ND |
| B.L./2 | 44 | M | 15.06.07 | 2x1 t/10 days | 25.06.07 | 2.4 | 144.2 | 0.56 | 50 |
| D.E./3 | 26 | F | 15.06.07 | 2x1t/5 days | 25.06.07 | <0.05 | <0.05 | ND | ND |
| H.D./9 | 56 | F | 19.06.07 | 2x2 t/5 days | 20.06.07 | <0.05 | 0.13 | ND | ND |
| J.P./4 | 56 | M | 18.06.07 | 2x2 t/ 7 days | 18.06.07 | <0.05 | <0.05 | ND | ND |
| K.G./8 | 47 | F | 17.06.07 | 2x2 t/ 5 days | 22.06.07 | <0.05 | 0.44 | <0.05 | 50 |
| K.J./7 | 50 | M | 15.06.07 | 2x1 t/ 7 days | 22.06.07 | 0.2 | 0.87 | <0.25 | 66 |
| L.P./5 | 16 | M | 20.06.07 | 2x2 t/7 days | 20.06.07 | ND | ND | ND | ND |
| W.M./6 | 37 | M | 20.06.07 | 2x2 t/ 7 days | 20.06.07 | ND | ND | ND | ND |

M: male; F: female; ND: not determined; t: tablet

*Developpements* S.A. of Grenoble, France) were used.

In order to precipitate proteins of serum samples, 2 mL methanol was added to aliquots (0.4 mL) of plasma samples. After vortex-mixing for 1 min, samples were centrifuged at 3 000 x g for 10 min and filtered through a PVDF Durapore® 0.45 mm filter (Millipore, Merck KGaA, Darmstadt, Germany). In order to quantify ABZ concentration, 100 $\mu$l aliquots of the filtered fractions were injected into the HPLC system. ABZ assay was performed according to the HPLC methods described in USP 24 (2000). To this end, a 5 $\mu$m C$_{18}$ (Gemini® Phenomenex, Torrance, CA 90501-1430, USA) 250 × 4.6 mm column and a mobile phase containing 5.5 g of Na$_2$HPO$_4$ dissolved in 400 mL of water and mixed with 600 mL of methanol at a flow rate of 1 mL/min were used. Samples were assayed at 291 nm. Under these conditions, the retention time was 15 min. Validation data of this method have been previously reported (García-Rodríguez *et al.*, 2001).

ABZSO and ABZSO$_2$ are the two main metabolites of ABZ and both of them were assayed based on the method previously reported (García-Rodríguez *et al.*, 2001). A Gemini® ODS2 column (250 × 4.6 mm, 5 $\mu$m) and a mobile phase containing 800 mL of a water solution with 188 $\mu$l of phosphoric acid 85% and 200 mL of acetonitrile at a flow rate of 1.5 mL/min were used. Samples were measured at 290 nm. Under these conditions, the retention times were 8.5 (ABZSO) and 13 (ABZSO$_2$) min. Validation of this method has been previously reported (García *et al.*, 1999).

In order to proceed to the quantitative assay of (+) ABZSO and (–) ABZSO, the samples were first assayed by the non-chiral procedure described before and liquid samples were collected at the retention time of ABZSO. These samples were concentrated to dryness by using vacuum at 70ºC in a Savant Speedvac® concentrator. Samples were then dissolved and filtered through a PVDF Durapore® 0.45 $\mu$m filter (Millipore). A chiral-AGP column (100 × 4 mm, 5 $\mu$m) (Agilent Technologies Spain SL, Madrid) and a mobile phase containing sodium phosphate buffer (8 mM, pH 7.0) with 1.25 mL of 2-propanol at a flow rate of 0.9 mL/min were used. Samples were measured at 290 nm. Under these conditions, the retention times of (+) ABZSO and (–) ABZ SO were 2.0 and 3.1 min, respectively.

**Two-dimensional electrophoresis and western blotting**

Crude larval extracts (CLE) from muscle larvae from the reference isolate (ISS-48 MFEL/SP/62/GM1) of *Trichinella spiralis* were prepared as previously described (Dea-Ayuela *et al.*, 2007). Eight hundred micrograms of CLE protein were diluted in

340 µL of rehydration buffer (7 M urea, 2 M thiourea, 2% Chaps, 0.75% IPG buffer 3–10, bromophenol blue), adsorbed onto 18 cm immobilized pH 3–10 gradient (IPG) strips (Amersham Biosciences, Piscataway, NJ 08855-1327, USA) at 20ºC for 12 h and 50 V, and then focused on an IPGphor IEF unit (Amersham Biosciences) using this setting: 2 h at 150 V; 1 h at 500 V; 1 h at 1 000 V; 2 h at 2 000 V and 6 h at 8 000 V. Then the strips were incubated in 10 mL equilibrating buffer (50 mM Tris-ClH pH 8.8, 6 M Urea, 30% glycerol, 2% SDS) containing 10 mg/mL DTT for 15 min. Following this, a further equilibration step was performed with equilibrating buffer containing 25 mg/mL iodoacetamide for 25 min. For separation in the second dimension, the IPG strips were placed onto 12.5% SDS–PAGE gels and run at 15 mA/gel. After separation, proteins were visualized by colloidal Coomassie blue staining.

Proteins from 2DE gels were transferred to a nitrocellulose membrane (Amersham Biosciences) on a Trans-blot semidry Transfer Unit (Amersham Biosciences). The membranes were rinsed with TBS–Tween buffer (20 mM Tris, 500 mM NaCl, pH 7.4) and incubated with blocking buffer (5% w/v skim milk buffer) overnight. The blotted membranes were incubated with either pooled or individual serum samples at 1:100 dilution in 1% w/v milk blocking buffer for 2 h at room temperature. After washing three times in TBS–Tween (TBS-T) for 15 min, the membranes were incubated with goat anti-human IgG antibody conjugated with horseradish peroxidase (*Caltag Laboratories* Burlingame, CA 94010, USA) diluted 1:1 000 in blocking buffer for 2 h at room temperature. Specific immunoreactive spots were colour-revealed by using a highly sensitive soluble 3, 3'-diaminobenzidine (DAB) (Sigma-Aldrich Co., USA) as a substrate.

## MALDI–TOF and MALDI–TOF/TOF mass spectrometry analysis and database searching

The spots of interest were manually excised from silver-stained 2-DE gels after being distained as described by Gharahdaghi *et al.* (1999). The gel pieces were incubated with 12.5 ng/µL sequencing-grade trypsin (Roche Molecular Biochemicals) in 25 mM AmBic overnight at 37°C. After digestion, the supernatants were separated. Peptides were extracted from the gel pieces first into 50% ACN, 1% trifluoroacetic acid, and then into 100% ACN. Then, 1 µL of each sample and 0.4 µL of 3 mg/mL α-cyano-4-hydroxycinnamic acid matrix (Sigma) in 50% ACN, 0.01% trifluoroacetic acid were spotted onto a Matrix-assisted laser desorption ionization (MALDI) target. Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) analyses were performed on a Voyager-DE STR mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA). The following parameters were used: cystein as s-carbamidomethyl derivative and methionine in oxidized form. Spectra were acquired over the m/z range of 700–4 500 Da. Tryptic, monoisotopic peptide mass lists were generated and exploited for database searching. The peptide mass was searched against the Swiss-Prot/TrEMBL non-redundant protein database (www.expasy.ch/sprot) and using the Mascot (www.matrix science.com) software program. MS/MS sequencing analyses were carried out using the MALDI–tandem time-of-flight mass spectrometer 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA). Mass spectrometry was performed at the University Complutense of Madrid Proteome Facility.

## RESULTS

### Monitoring serum IgG responses in trichinellosis patients during and after treatment

The results are summarized in Table 1. Positive IgG response was recorded in eight patients across the observation period (samples I and II taken 1 month apart after diagnosis and treatment) ranging from 1.313 to 0.738 (cutoff 0.642–0.593). One patient (numbered L.P. /5) remained negative. In most patients, the optical density (OD) values for IgG response experienced a slight decrease from sample I to sample II, excepting the

patient numbered W.M. /6 for whom a clear increase was observed (from 1.210 to 1.447 OD with 0.646 to 0.483 cutoff, respectively) and sample I from patient number B.L. /2 turned equivocal but had positive seroconversion in sample II .

**Measuring ABZ and ABZ metabolite serum levels in treated trichinellosis patients**
Drug concentration was measured only in serum sample I. The results are summarized in Table 2. Overall, great variation in drug concentration levels was observed among patients. ABZ was undetected (below 0.05 µg/mL) in the sera from most patients and only traces were present in two of them (numbered B.L. /2 and K.J. /7). ABZSO was detected in four patients with concentration levels ranging from 144 µg/mL (sample number B.L. /2) to 0.13 µg/mL (sample number H.D. /9). The inactive metabolite $ABZSO_2$ was detected in only one sample (number B.L. / 2). Enantioselectivity could be assessed only in sample number K.J. /7, where a slight predominance (66%) of the (+) enantiomeric form was observed.

For patients L.P. /5 and W.M. /6, drug concentration levels were not tested as serum samples were taken just at the start of the treatment.

**Immunoproteomic analysis of *Trichinella* antigens recognised by sera from patients with trichinellosis, during and after treatment**
A relatively high number of protein spots appeared well resolved following staining of the 2D gel with colloidal Coomassie blue. They were regularly distributed across 3–10 pI pH and from below 20.1 up to 66 kDa molecular weight (MW), with the higher concentration being recorded at 4–10 pI and 45–66 kDa (Figure 1A). A minor group was resolved around 97 kDa and 5 pI.

For immunoproteomic analysis, two sets of assays were performed. First, in order to establish a reference immunoproteomic map for trichinellosis patients, all positive samples from patients undergoing treatment (serum samples I) were pooled and probed in western blotting with CLE. The most antigenic protein spots were concentrated from 3 to 10 pI and above 45 up to 97 kDa (Figure 1B).

Thereafter, and for a proper follow-up of each patient, the individual positive or negative samples during and after the treatment period (samples I and II, respectively) were probed in western blotting with CLE. Nine spots from the highly immunoreactive area plus one spot located around 26 kDa and 6 pI that exhibited a differential recognition pattern by individual serum samples were selected for MS analysis.

Five spots were identified either by MS or MS/MS as antigen targeted in *C. elegans*, an actine from *Wuchereria bancrofti*, the enolase, and P49 antigen from *Trichinella*. A serine proteinase (spot number 7) and annexin I-like (spot number 10), respectively, were identified by de novo sequencing. The remaining four spots were not identified (Table 3). Additional spots matched with those already identified in the ES antigen as isoforms of serine proteases (Robinson & Connolly, 2005) (see encircled spots in Figure 1B).

The representative patterns for the individual sera testing are summarized in Figure 2. The IgG-negative sera did not show any antigen recognition (Figure 2A, 2D) and therefore served as negative control. For the IgG-positive sera, no major differences were apparent within the higher immunoreactive area of the gel during and after treatment with ABZ but a differential pattern was shown regarding annexin I. The sera samples from patients showing either a sustained or decreased antibody response failed to recognize annexin I after treatment (Figure 2B, 2E) whereas, for samples showing an increased IgG response after treatment with ABZ (e.g. number W.M./6), its recognition was maintained (Figure 2C, 2F).

DISCUSSION

Clinical diagnosis of trichinellosis still remains difficult due to the lack of pathognomonic signs or symptoms, so it relies on serodiagnosis and muscle biopsy.
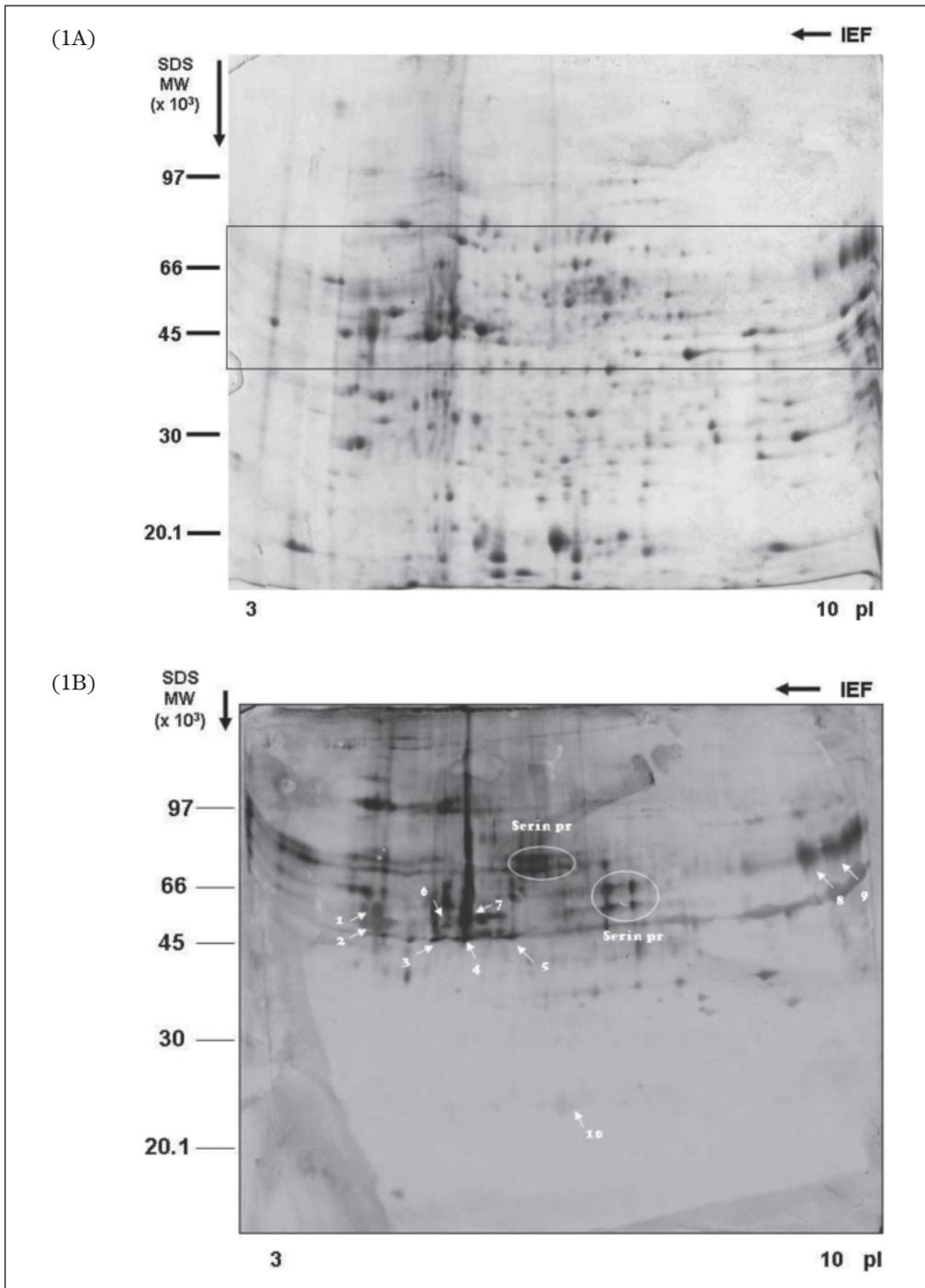
Figure 1. Two-dimensional electrophoresis (2DE) and western blot of proteins from crude larval extract (CLE) with pooled human serum samples. Proteins were separated in 3–10 pI and 12.5% SDS–PAGE gels and then transferred to nitrocellulose paper. Figure 1A shows the 2DE separated spots following staining with colloidal Coomassie blue. The highly immunoreactive spots corresponding to the TSL-1 antigens are highlighted. Figure 1B shows the western blot with pooled sera revealed by the 3, 3'-diaminobenzidine (DBA) substrate. Spots 1–10 were selected for MALTI–TOF mass spectrometry and those encircled correspond to serine proteases previously identified in excretory/secretory products (Robinson & Connolly, 2005) (see Table 3).

Table 3. Identification of relevant immunoreactive proteins in non-redundant sequence databases using data from MALDI TOF and MALDI TOF/TOF mass spectrometry analysis and *de novo* sequencing

| Protein spots | Accesion number | Name | Analysis way | Mowse score | de novo sequence plus homology searching in Blast | MW (x10³) | pI |
|---|---|---|---|---|---|---|---|
| 1 | gi\|404638 | Antigen targeted by protectives antibodies (*C. elegans*) | MS-MS/MS/COMB | 342 | GIGTDEDAIIEIIVTR | 53 | 4.0 |
| 2 | | No identified | MS-MS/MS | – | | 49 | 4.0 |
| 3 | Q9NJ12 | Homology with Actin from *Wuchereria bancrofti* | MS-MS/MS | 107 | | 47 | 4.8 |
| 4 | Q967U0 | Enolase *Trichinella* | MS | 146 | | 47 | 5.1 |
| 5 | Q27076 | P49 antigen *Trichinella* | | 73 | | 47 | 5.8 |
| 6 | | No identified | MS-MS/MS | – | | 53 | 4.1 |
| 7 | AY028974.1 | Serine proteinase *Trichinella* | MS-MS/MS de-novo | | LNEPHR | 53 | 5.1 |
| 8 | | No identified | MS-MS/MS | – | | 65 | 9.5 |
| 9 | | No identified | MS-MS/MS | – | | 65 | 9.8 |
| 10 | gi\|32450112 | Homology with annexin | MS-MS/MS de novo | | GIGTDEDAIIEIIVTR | 23 | 6.5 |

MW: molecular weight

In our study, serological analysis revealed the presence of anti-*Trichinella* IgG response in eight patients whereas, in one, despite showing clinical manifestations, the response remained negative. One month after first diagnosis and treatment, the IgG levels tended to decrease in most patients except one, who exhibited a clear increase. This differential behaviour in antibody responses could be attributed to a different evolution of the course probably due to a varied effectiveness of treatments. Therefore, attempts have been made to monitor drug levels in the blood of treated patients. A proper pharmacokinetics analysis and monitoring were impeded by the lack of the required samples, as only two samples (I and II) taken 1 month apart from each patient were available and some in sample II were excluded from the assay because they fell outside of the treatment period. Therefore, the information provided by the drug analysis is of limited value. It is accepted that the effectiveness of specific drugs against trichinellosis is largely dependent on the time at which they are administered according to the course of the infection (Dupouy-Camet, 2002). This is a critical point, as the
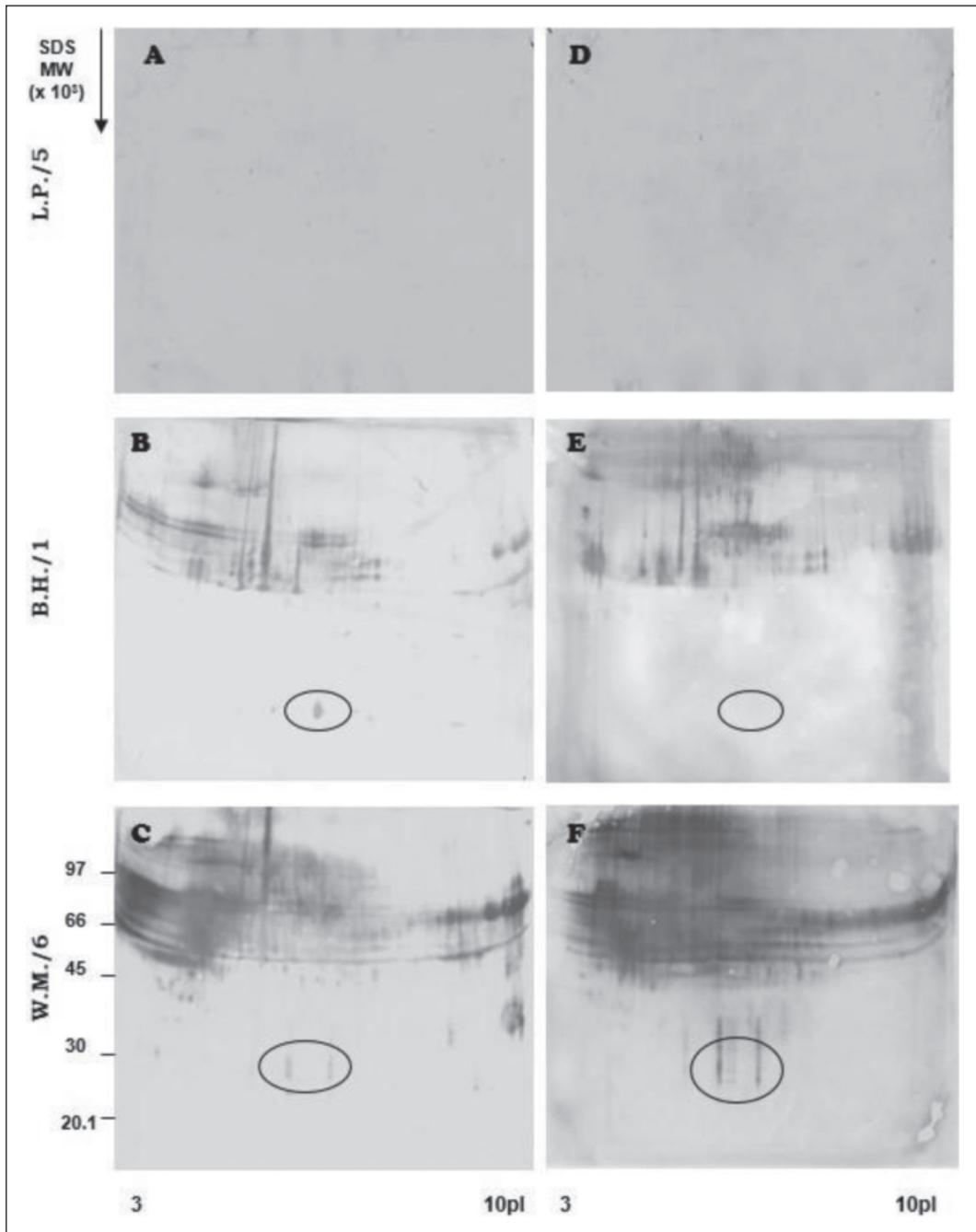
Figure 2. Comparative analysis of antigen recognition profiles in 2D western blots of CLE with representative individual serum samples during (A, B, C) and after treatment (D, E, F) with ABZ as revealed by 3, 3'-diaminobenzidine (DBA) substrate . Encircled are the differentially recognized spots identified as annexin I.

incubation period varies from patient to patient, so treatment cannot be applied equally. In our study, the case outcome period lasted from 2 to 18 July 2007. For all patients, treatment started between 15 and 20 July and the duration varied from 5 to 10 days (Golab *et al.*, 2007a). In addition, individual physiological factors together with the type of food ingested (fatty food helps absorption) may substantially affect the bioavailability and biotransformation of ABZ. All these features may explain the great variation in ABZ and its metabolite concentration among patients taken from the analysis of one single sample. Furthermore, no correlation can be established between the kinetics of serum antibody responses and drug levels.

Consequently, the monitoring of the pharmacokinetics of drugs used for the specific treatment of trichinellosis is still a required task for validating treatment protocols as, so far, treatment schedules rely on medical criteria without a normalized therapeutic guide (Ambrosioni *et al.*, 2006).

By contrast, the kinetics of IgG response seem to correlate with the clinical evolution of the disease, as those patients showing a decline in IgG levels experienced clear clinical improvement whereas the patient with an increasing tendency for IgG response manifested persistent myalgia (data not shown).

Proteomic analysis of antigens (immuno-proteomics) may provide further clues for the evolution of trichinellosis following specific treatment. Our study focused on the highly immunoreactive area of the 2D gel including both acid and basic proteins in the range of about 40–66 kDa. These proteins have previously been reported as TSL-1 antigens with relevant immunological as well as physiological roles (Bolás-Fernández & Del Corral-Bezara, 2006; Yépez-Mulia *et al.*, 2007). Other relevant proteins from other *Trichinella* life cycle stages have been tagged by Mitreva *et al.* (2005) and by other workers as summarized by Mitreva & Jasmer (2006). Following MS analysis and database searching, we were able to identify some highly antigenic relevant proteins such as actine, enolase, the p49 proteins, and particularly an apparently highly glycosylated serine protease together with other ES antigens identified by Robinson & Connolly (2005). Besides, some other very highly immunoreacting proteins located in the basic area of the gel remain to be identified. Some of them may match with those recently identified by Wang *et al.* (2013, 2014) on ES products and by Liu *et al.* (2014) on the surface of the larvae using a similar technological approach.

No major differences were seen in the recognition patterns of these proteins by sera from patients during and after treatment. By contrast, a clear differential recognition pattern by a protein spot located outside the TSL-1 region was shown. This spot failed to be recognized by sera from patients showing a decreased IgG response after ABZ treatment with no change in those showing maintenance or even increased response patterns. MS analysis allowed this spot to be identified as an annexin I-like protein.

Annexins are a superfamily of calcium binding proteins identified in major eukaryotic phyla but they are absent from yeast and prokaryotes (Moss & Morgan, 2004). Functionally, annexin I in vertebrates has been associated with glucocorticoid-mediated anti-inflammatory responses (Buckingham *et al.*, 2006) as well as with cell signalling, cell differentiation, and apoptosis, and its altered function was related to some organic disorders such as cancer (Hsiang *et al.*, 2006; Yan & He, 2008). Recently, the parasite annexins have been reviewed and, according to their predicted additional secondary structure elements that may confer unique functional properties, they are considered as new potential targets for drug and vaccine development (Hofmann *et al.*, 2010).

In *Trichinella* infections, it is assumed that, soon after muscle invasion, the developing larvae secrete proteins that induce de-differentiation of muscle cells into a new unique entity called a nurse cell (Despommier, 1998; Wu *et al.*, 2008; Guiliano *et al.*, 2009). Annexin I could be one of these early secreted proteins playing a role in the formation and maintenance of the nurse cell. The killing of larvae at an early developmental stage by effective treatment

would stop the production and secretion of this and other related proteins, consequently avoiding their ability to trigger the immune response. Should this be the case, annexin I could constitute a sensitive marker of the early effect of drugs in developing muscle larvae. Further studies including cloning and expression of this protein are now in progress and they will be of great help for future functional studies.

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