TRICHINELLA BRITОВI HUMAN INFECTION IN SPAIN: ANTIBODY RESPONSE TO SURFACE, EXCRETORY/SECRETORY AND SOMATIC ANTIGENS

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Summary:

A third outbreak of *Trichinella britovi* with 140 people involved, occurred in Granada Spain (December 1998). The source of infection was sausage made from uninspected wild boar meat. Fifty-two patients agreed to participate in this study. An elevated eosinophil level (> 5 %) was detected in 59.6 % of patients, and persisted in most of these cases for two months. A moderate IgG response was observed. At the onset of symptoms, Western blot (WVB) test detected more positive cases than Enzyme linked immunosorbent assay (ELISA) and indirect immunofluorescence (IIF). Six months from infection, ELISA revealed fewer positive cases than the other two tests. It would appear that the response to somatic antigens starts earlier than those to cuticular and excretory/secretory (ES) antigens and that the response to ES antigens is the first to decrease.

KEY WORDS : *Trichinella britovi*, outbreak, Spain, antibody response, ELISA, IIF, WVB.

Résumé : ÉPIDÉMIE DE TRICHINELLOSE À *TRICHINELLA BRITОВI* EN ESPAGNE: RÉPONSE EN ANTICORPS AUX ANTIGÈNES CUTICULAIRES, AUX ANTIGÈNES D'EXCRÉTION/SÉCRÉTION ET AUX ANTIGÈNES SOMATIQUES

Une troisième épidémie de *Trichinella britovi* impliquant 140 personnes a eu lieu à Grenade en Espagne en décembre 1998. La source de l'infection a été du saucisson fait à partir de viande de sanglier non contrôlée. Cinquante deux patients ont accepté de participer à cette étude. Un taux élevé d'éosinophiles (> 5 %) a été détecté chez 59,6 % des patients, et a persisté pendant deux mois dans presque tous ces cas. Une réponse modérée en IgG a été observée. Au début des symptômes, le Western blot (WVB) a permis de détecter plus de cas positifs que l'"Enzyme linked immunosorbent assay" (ELISA) et l'immuno fluorescence indirecte (IFI). Cependant, six mois après l'infection, l'ELISA a révélé moins de cas positifs que les deux autres tests. Il semble que la réponse sérologique vis-à-vis des antigènes somatiques commence plus tôt et que la réponse aux antigènes ES est la première à décroître.

MOTS CLÉS : *Trichinella britovi*, épidémie, Espagne, réponse en anticorps, ELISA, IFI, WVB.

INTRODUCTION

Trichinellosis is a disease caused by *Trichinella* spp., which affects humans and many other mammals. Humans are infected by ingestion of raw or undercooked meat contaminated with *Trichinella*. Pork and game animals meat were considered to be the main sources of trichinellosis infections in humans. However, in 1975 a trichinellosis outbreak affecting 89 persons was found to be caused by ingestion of horse meat (Mantovani *et al.*, 1980). Since then, other trichinellosis outbreaks resulting from contaminated horse meat have been described (Ancelle *et al.*, 1998), although none have occurred in Spain. Spain is at risk from silvatic trichinellosis and is also recognized as an endemic area of domestic trichinel-

losis. In Spain, trichinellosis is an obligatory notifiable disease that, despite the legal rules established to control it, remains a major public health threat because the parasite is enzootic in domestic pigs and wild animals (Pozio, 1998). In Spain, from January 1980 to May 1990, 1,261 cases of trichinellosis were reported, and 362 new cases occurred between January 1991 and May 1999 (Anonymous, 1996-1999). Most human *Trichinella* infections to date have been attributed to *Trichinella spiralis*. However, now that it is possible to type the parasite genetically and a more precise characterization of the various species is possible, others species such as *T. pseudospiralis*, *T. nativa* and *T. britovi* have been associated with such infections (Pozio *et al.*, 1988; Carneri *et al.*, 1989; Andrews *et al.*, 1993; Gari-Toussaint *et al.*, 1994). Until recently in Spain, only *T. spiralis* infections had been reported in epidemics, but in 1995, the two first outbreaks of trichinellosis caused by *T. britovi* were reported (Rodríguez *et al.*, 1995). Serology is a helpful tool for the diagnosis of human trichinellosis as clinical signs of this helminth infection are non-specific (Nunez *et al.*, 2000; Costantino *et al.*, 2001; Andiva *et al.*, 2002).

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In this paper, we present epidemiological and serological data from a third *T. britovi* outbreak that occurred in the province of Zaragoza, Spain, in 1998.

MATERIALS AND METHODS

DESCRIPTION OF THE OUTBREAK

In December 1998, the Epidemiological Surveillance Section of the Health Service of Zaragoza (Spain) reported an outbreak of human trichinellosis that occurred in Zaragoza in the Aragon region of Spain, involving 140 people who had eaten sausages made from uninspected wild boar meat (Anonymous, 1999). The parasite was isolated from the sausages and characterized as *T. britovi* at the Health Institute Carlos III, Madrid. According to the classification system for *Trichinella* infections (Kassur & Januszkiewicz, 1968), patients involved in this outbreak exhibited moderate forms. There were no severe complications and no patient death.

From the 140 people involved in this outbreak, 52 consulted at the Hospital Clínico Universitario of Zaragoza and were serologically studied. They were interviewed following an epidemiological study protocol used at this Hospital. All people reported eating the suspect products and had some signs and/or symptoms related to trichinellosis. Over the following days they were cited for further examinations and laboratory tests. The clinical incubation period was 15–20 days.

PATIENTS AND SAMPLES OF SERUM

Fifty-two people, 21 females and 31 males aging from 14 to 59 years, were studied. Antibodies to cuticular, excretion/secretion (ES) and somatic antigens were investigated in all patients. Serum samples were collected in December 1998, at the onset of symptoms, and again monthly until June 1999. Samples were divided into five groups: 1, 2, 3 and 4 correspond to sera taken in December 1998, January, February and March 1999 respectively, and five to sera taken from April to June 1999. From the 52 patients, samples from only 11 patients were available at all the above mentioned times post-infection allowing serologic evolution. Patients gave no history of suffering from other parasitic disease.

ANTIGENS

T. spiralis, MSUS/ES/59/LASO (ISS112) and *T. britovi* M CAN/ES/76/ISS113/ L1 larvae, maintained in Swiss mice and recovered by acid-pepsin digestion (Blair, 1983), were used as antigenic sources. Crude extracts

were prepared by homogenization of L1 larvae in phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulfonylfluoride (PMSF) and 5 mM EDTA. The homogenate was then sonicated, centrifuged at 12,000 g for 30 min and the protein content of supernatant quantified (Bradford, 1976). All steps were carried out at 4°C. Extracts were used for Western blot (WB) analysis. Whole *T. spiralis* larvae were employed in indirect immunofluorescence test (IIF). *Trichinella* species were maintained with the minimum possible suffering to the animals involved and in accordance with the bioethical rules of the European Union and the Kingdom of Spain.

SEROLOGICAL TESTS

IgG response to *T. britovi* and *T. spiralis* cuticular antigens was measured by the IIF procedure using whole L1 larvae (Sadun *et al.*, 1962). An IIF IgG titre $\geq 1:80$ was considered positive (Faubert *et al.*, 1981), but in patients in which serologic evolution was studied, titres of 1:20 and 1:40 were also considered positive. Detection of antibody to somatic antigens was carried out by WB using crude extract from *T. spiralis* and *T. britovi* L1; 600 µg of protein/gel from both extracts were separated in a 10 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), following the method previously described (Towin *et al.*, 1979).

After electrophoretic blotting, nitrocellulose sheets were blocked in 5 % skim milk in PBS for 1 h and reacted with a 1:100 dilution of human sera in PBS/1 % skim milk/0.3 % Tween-20 for 1 h. After washing, a 1:3,000 dilution of goat anti-human IgG horseradish peroxidase conjugate (Biorad, Hercules, CA 94547, USA) in PBS/0.3 % Tween-20 was added for 1 h and, after a new washing cycle, the reaction was developed with hydrogen peroxide and 4-chloro-1-naphthol. A pre-stained molecular weight marker was included. Two negative and two positive sera from patients with confirmed trichinellosis and high and low IIF titres were used as controls in the test. Serum samples were considered positive when their recognition pattern was the same or very similar to the control sera, and negative, when no bands were detected.

The IgG response to ES antigens was carried out with "Melotest triquinosi", a commercial ELISA-sandwich kit (Melotec, Barcelona, España), following the indicated protocol. Three replicates of each serum diluted at 1:32, two high and low positive control sera, and two negative control sera were used. Serum samples with an R value > 1 were considered positive, R being the ratio between the mean serum sample OD and the cut-off value (mean value of negative control sera plus 0.4). In all serological tests, 20 negative sera were used as control.

EOSINOPHILIA AND MUSCULAR ENZYME

At 30 and 60 days after infection, eosinophil levels (normal value < 5 %) were measured and a muscular enzymatic profile (creatinine phosphokinase [CPK], normal values < 195 U/L), was determined.

STATISTICAL ANALYSIS

A classical two proportions comparison test (Rios, 1972) was applied to the results.

RESULTS

The classical signs and frequent symptoms observed in the 52 patients, were myalgia (98 %), periorbital edema (40 %), fever (80.2 %) and diarrhea (35.4 %). Eosinophil levels of > 5 % of the total leukocytes were found in 59.6 % of patients, with a maximum value of 30 % detected in one patient. Elevated levels of CPK were detected in 34.5 % of patients, with only one showing a value above 1,000 U/L. The incubation period ranged from 15 to 20 days.

Taking into account all the periods studied, results were as follows: of the 52 patients, 29 (55.8 %) were IgG-ELISA positive (R values ranging from 1.10 to 8.71). Samples from 32 patients (61.5 %) were positive using the IgG-IIF test, with titres ranging from 1:80 to 1:1,280. In the negative control group, sera were negative with both tests. Thirty-nine patients (75.0 %) were positive using WB, with crude antigens from *T. britovi* and *T. spiralis*. The percentages of positivity obtained with the three tests were not significantly different at the 95.0 % confidence level. The western blot response was directed to poly-

Fig. 1. – Western blot results with *Trichinella spiralis* and *Trichinella britovi* antigens in two patients.

peptides with molecular mass ranging from 31.7 kDa to 205 kDa. Immunodominant antigens with molecular mass superior to 42.5 kDa were observed with both extracts. The negative sera used as control showed no bands. A representative recognition pattern from two positive patients is shown in Figure 1.

Figure 2 represents the positivity results of sera taken from the 52 patients at the different time post-infec-

Fig. 2. – Anti-*Trichinella* IgG response in fifty-two patients at different times post-infection.

tion. At the onset of symptoms, the number of positive results was low, with WB analysis detecting the highest number. As expected, positivity increased as the infection progressed, thus by January, February and March, the number of positive cases increased notably. In the April-June period, a low number of positive patients were detected with ELISA.

The diagnosis sensitivity of the associated techniques is on Table I. The highest sensitivity was reached when ELISA and WB were considered.

Tests	Positive patients	Positivity rate (%)
IIF + ELISA + WB	41	78.8
IIF + ELISA	34	35.4
IIF + WB	40	76.9
ELISA + WB	41	78.8

IIF = Indirect Immunofluorescence.

ELISA = Enzyme Linked Immunosorbent Assay.

WB = Western Blot.

Table I. – Diagnosis sensitivity of the associated techniques in the 52 patients.

The results from 11 patients on which the three tests were performed at all the determined time post-infection are in Table II. At the onset of symptoms, the response to cuticular and ES antigens, measured by IIF and ELISA, is practically null, with only one patient, (number 32), showing a titre and R value indicative of infection. However, in response to somatic antigens, five patients showed a WB recognition pattern similar to the control positive serum. The highest immune response was detected in January 1999, when the highest IIF-titres and ELISA-R values were observed.

In February 1999, the response to ES antigens began to decrease, a trend which continued until the end of the study, when three patients become negative. The

response to cuticular antigens was also found to decrease and, by April-June 1999 period, seven patients showed titres at the limit of positivity. As far as the response to somatic antigens is concerned, only one patient was found to be negative by April-June 1999 period, in all other patients, the recognition pattern was similar to those obtained in previous periods although bands were fainter. With respect to the positivity rate at the different time post-infection, IIF showed significantly differences ($p \leq 0.1$) between the first period and all others. With ELISA, we only found that the first period was significantly different ($p \leq 0.1$) with respect the second, third and fourth period.

DISCUSSION

The outbreak reported here is the third human *T. britovi* trichinellosis outbreak recorded in Spain and, according to the classification of *Trichinella* infections (Kassur & Januszkiewicz, 1968), is considered to be moderate, in that there was no severe manifestations, and no death. On the other hand, IIF-IgG titres supported this view, they were low, maximum of 1:1,280 in three patients; and only seven patients showed R values ≥ 5 by ELISA (data not shown). The severity of the clinical course of trichinellosis depends on factors such as the number of living larvae ingested and the *Trichinella* species. There are important differences among species in the number of new born larvae produced by females. *T. britovi* have a lower prolificity than *T. spiralis* (Pozio *et al.*, 1992). The number of days between ingestion of contaminated meat and the apparition of clinical signs is similar to those periods reported in other *T. britovi* outbreaks (Rodríguez *et al.*, 1995; Pozio *et al.*, 1993).

In a recent outbreak caused by infected wild boar meat, in southern Spain, where the clinical and epi-

Patient	December (onset of infection)			January			February			March			April-June		
	IIF	ELISA	WB	IIF	ELISA	WB	IIF	ELISA	WB	IIF	ELISA	WB	IIF	ELISA	WB
3	–	–	+	80	–	+	40	–	+	–	–	+	–	–	+
9	–	–	–	40	–	+	–	–	+	–	–	–	–	–	–
10	–	–	–	640	3.35	+	640	2.52	+	320	1.75	+	80	–	+
16	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
22	–	–	+	320	7.63	+	320	6.22	+	320	5.46	+	80	2.53	+
23	–	–	–	1,280	8.71	+	640	7.93	+	640	7.41	+	80	1.55	+
30	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
31	–	–	+	160	1.81	+	80	1.88	+	80	1.85	+	80	1.98	+
32	320	1.66	+	640	7.20	+	160	3.45	+	80	2.36	+	40	–	+
33	–	–	–	160	6.61	+	320	5.28	+	320	4.03	+	80	–	+
40	–	–	+	160	3.30	+	160	3.0	+	160	2.71	+	80	1.82	+
Total positive	1	1	5	9	7	9	8	7	9	7	7	8	7	4	8

Table II. – Anti-*Trichinella* IgG response in eleven patients at different times post-infection.

demological features were consistent with the hypothesis that the etiological agent was *T. spiralis* (Rodríguez-Osorio *et al.*, 1999) the incubation period was shorter. Although this difference may be due to the number of ingested larvae, it is worth noting that *T. britovi* infection is characterized by a lower infectivity. It is also worth noting that when the incubation period is brief, the course of infection is more severe (Pawlowski, 1983). A great number of asymptomatic patients (81 out of 140) and a lower number of patients with periorbital edema (21 out of 52) was found in the present outbreak, probably as a result of this lower infectivity.

Eosinophils levels, higher than 5 % of the total leucocytes population, was observed in 59.6 % of the 52 patients, and in most of these cases persisted for two months. Similar results were obtained in patients from *T. britovi* outbreaks reported in Spain and Italy (Rodríguez *et al.*, 1995; Pozio *et al.*, 1993), but differ from other reported outbreaks (Gari-Toussaint *et al.*, 1994).

Significant CPK values were obtained in 34.5 % of patients. This result does not agree with that obtained in an earlier *T. britovi* outbreak, initially diagnosed as *T. nelsoni* (Ferraccioli *et al.*, 1988, 1989), in which, two months after initial infection, 62 % of patients showed high enzymatic levels. However, in the 1995 outbreak mentioned above, 90 % of patients had an elevated CPK value (Rodríguez *et al.*, 1995).

Trichinella possesses surface, secreted (some stage specific) and somatic antigens that induce both a humoral and cellular immune response. In this study, three tests, IIF, ELISA and WB were used to detect the anti-IgG humoral response to these antigens. The positivity rate obtained in the 52 patients with all three tests was low, which may be explained by a low *T. britovi* infectivity.

An acceptable diagnosis sensitivity was reached when IIF/ELISA/WB, IIF/WB and ELISA/WB were associated. The lowest percentage of positive patients was obtained with the association of IIF/ELISA (Table I). WB analysis revealed a more intense response with *T. britovi* than with *T. spiralis* antigen. This may be because antigens from *T. britovi*, even those with the same molecular weight, have more specific epitopes. Slight qualitative differences in the antigenic profiles have been demonstrated (Fig. 1). With some sera we observed an additional antigen having a molecular mass of approximately 69 kDa with *T. britovi* crude extract, but not *T. spiralis* extract. An antigen with similar molecular weight was found by others (Pozio *et al.*, 1993). Such antigen could correspond to the *T. britovi* 71.3 kDa protein identified as the 70 kDa heat shock protein (Vayssier *et al.*, 1999).

One of the main distinctions between *T. spiralis* and *T. britovi* infections is a longer duration of parasite-spe-

cific IgG in patients infected with *T. spiralis* than in patients infected with *T. britovi* (Pozio *et al.*, 1993). In a recent outbreak in southern Spain (Rodríguez-Osorio *et al.*, 1999), probably caused by *T. spiralis*, 75 % of patients were identified as anti-IgG positive two years after infection using IIF test (Rodríguez-Osorio and others, unpublished data). This long term persistence of IgG in patients, even up to 39 years after infection (Froscher *et al.*, 1988), probably reflects a progressive destruction of *T. spiralis* larvae in muscle tissue, and a long antigenic stimulation of the immune system (Ivanoska *et al.*, 1989). The results in Figure 2 show a shorter duration of the immune response. After two months from the onset of symptoms, IIF and ELISA tests begin to show a decrease in anti-*Trichinella* IgG, and by six months, patients were either negative or showed values at the limit of positivity, WB detected IgG positive patients throughout the study, although six months after the infection, the recognition pattern showed faint bands. These results support those obtained by other authors (Bruschi *et al.*, 1990; Pozio *et al.*, 1993), and suggest a rapid elimination of *T. britovi* larvae from human tissues.

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