Evaluation of Specific Antibody Responses in Early Stage Human Trichinellosis

Erken Dönem İnsan Trişinellozunda Özgün Antikor Yanıtlarının Değerlendirilmesi

Songül BAYRAM DELİBAŞ,[a]
Selma USLUCA,[b]
Serap ARCAK,[a]
Soykan ÖZKOÇ,[a]
Çiler AKISÜ[a]

[a]Department of Parasitology,
Dokuz Eylül University
Faculty of Medicine, İzmir
[b]Parasitology Laboratory,
Refik Saydam National Institute of Health,
Ankara

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Yazışma Adresi/*Correspondence:*
Songül BAYRAM DELİBAŞ
Dokuz Eylül University
Faculty of Medicine,
Department of Parasitology,
İzmir,
TÜRKİYE/TURKEY
songul.bdelibas@deu.edu.tr

ABSTRACT Objective: Trichinellosis is a zoonotic disease in humans caused by Trichinella sp. Since the signs and symptoms due to Trichinellosis are not pathognomonic, the detection of serum antibodies is useful for the early diagnosis. The present study was undertaken to evaluate the specific antibody responses in confirmed cases of a trichinellosis outbreak, caused by T. britovi in Turkey. **Material and Methods:** Serum samples from 171 trichinellosis patients, 127 healthy individuals and 32 patients with other parasitosis were examined for IgG, IgA and IgM antibodies by an enzyme linked immunosorbent assay (ELISA) using excretory/secretory (ES) antigen. **Results:** IgG responses were positive in all patients, whereas IgA and IgM responses were positive in 50.9% and 59.6%, respectively. The specificity of antibody assays was 97.5% for IgA and IgM and 99.4% for IgG antibodies. According to the receiver-operator characteristic (ROC) curve analysis, area under the ROC curve (AUC) values of IgG, IgA and IgM ELISA were 99.7%, 74.2% and 78.6%, respectively. **Conclusion:** The results of this study suggest that IgG ELISA using ES antigen is the choice for diagnosis and in house IgM ELISA is more sensitive than IgA ELISA in diagnosing trichinellosis about 3-6 weeks after consumption of suspected meat. To the best of our knowledge, this is the first report presenting specific IgA and IgM antibody responses in such a large group of serum samples obtained in a T. britovi outbreak.

Key Words: Trichinella; early diagnosis; enzyme-linked immunosorbent assay

ÖZET Amaç: Trişenolloz, insanda Trichinella sp.'nin etken olduğu zoonotik bir hastalıktır. Trişinelloza ait bulgu ve semptomlar patognomonik olmadığından, hastalığın erken tanısında serum antikorlarının saptanması önemlidir. Bu çalışma, Türkiye' de T. britovi'nin etken olduğu bir salgında kesin tanı almış hastaların özgün antikor yanıtlarının değerlendirilmesi amacıyla yapılmıştır. **Gereç ve Yöntemler:** Çalışmada 171 trişinelloz hastası, 127 sağlıklı insan ve 32 diğer parazitozlu hastadan alınan serum örneklerinde salgısal (excretory/secretory-ES) antijenin kullanıldığı enzim aracılı immünosorbent deney (ELISA) yöntemiyle IgG, IgA ve IgM antikorları araştırılmıştır. **Bulgular:** Çalışmada kesin tanı almış trişinelloz hastalarının tamamında IgG cevabı gözlenirken, IgA cevabı %50,9'unda ve IgM cevabı %59,6'sında gözlenmiştir. Antikor testlerinin özgüllüğü IgA ve IgM için %97,5, IgG için %99,4 bulunmuştur. İşlem karakteristik eğrisi (ROC curve) analizi yapıldığında ROC eğrisinin altında kalan alan (EAA) değerleri IgG, IgA ve IgM antikorları için sırasıyla %99,7, %74,2 ve %78,6 saptanmıştır. **Sonuç:** Bu bulgular, şüpheli etin tüketilmesinden 3-6 hafta sonra trişinelloz tanısı için ES antijeninin kullanıldığı IgG ELISA'nın tercih edilebilecek bir test olduğunu desteklerken, hastalığın erken dönemde tanısında "in house" IgM ELISA testinin IgA ELISA testinden daha duyarlı olduğunu göstermiştir. Bildiğimiz kadarıyla bu çalışma, *T. britovi*'nin neden olduğu bir salgında, en fazla serum örneğinde özgül IgA ve IgM antikor cevaplarının ortaya konulduğu bir çalışmadır.

Anahtar Kelimeler: Trişinella; erken tanı; immunoenzimatik yöntem

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Trichinellosis is induced by nematode worms of the genus *Trichinella* and is acquired through the consumption of raw or undercooked meat or meat products that harbor *Trichinella* larvae. Although pork

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is the most common source of infection, meat from a variety of other animals has also been implicated, including wild boars, horses, bears and foxes.[1,2]

Since the symptoms and signs due to *Trichinella sp.* infection are not pathognomonic, the serological diagnosis is very important, in order to start the appropriate antihelmintic treatment as soon as possible, which is useful mostly at the early stages of the infection, i.e. when larvae and adults are in the gut or when newborn larvae migrate from the gut vessels to striated muscles.[3] Based on this, class specific antibody responses have been investigated in different human outbreaks, to determine the antibody class useful for the diagnosis.[4-6]

In the present study, our aim was to examine IgG, IgM, and IgA antibody responses and to demonstrate the diagnostic potential of IgM and IgA antibodies by an indirect ELISA with sera from patients involved in an early stage *T. britovi* outbreak in Turkey.

MATERIAL AND METHODS

DESCRIPTION OF THE OUTBREAK

In January-February 2004, a trichinellosis outbreak, caused by *T. britovi*, occurred in Izmir, Turkey. Six hundred nineteen patients were infected by eating raw minced meatballs, one of the most favorable traditional foods made from both beef and pork.[7,8]

SERUM SAMPLES

Serum samples were collected from 171 cases (73 females and 98 males; mean age 34 years) of confirmed human trichinellosis, involved in the outbreak and fulfilled the criteria of The European Center for Disease Control for the diagnosis of acute trichinellosis, approximately 3-6 weeks after the consumption of suspected meat product.[1] Control serum samples were collected from 159 individuals, 127 healthy volunteers and 32 patients with various other diseases (3 systemic lupus erythematosus, 2 rheumatoid arthritis, 5 toxoplasmosis, 3 leishmaniasis, 10 toxocariasis, 2 fascioliasis and 7 cystic echinococcosis). All participants gave informed consent for the study, which was approved by the local Clinical and Laboratory Researches Ethical Committee.

ANTIGEN

ES antigen was prepared from *Trichinella spiralis* muscle larvae recovered after HCl-pepsin digestion of infected rat muscles, according to the method of Gamble.[9] Briefly, *T. spiralis* muscle larvae were washed three times in phosphate buffered saline (pH: 7.2) with penicillin and streptomycin. The larvae were then washed four times by allowing them to settle in RPMI medium. Five thousand larvae per ml were then incubated in RPMI medium supplemented with penicillin and streptomycin with 10% CO_2 in a 75 cm^2 culture flask at 37°C for 18h. After centrifugation, the supernatant was collected and was filtered through filters (0.22 μm) and stored at -80°C until used.

ELISA ASSAYS

Detection of IgG, IgM and IgA antibodies were performed by in-house indirect enzyme-linked immunoassay (ELISA). Flat-bottomed, 96-well microtiter plates of high binding (Nunc, Germany) were used. Each well was sensitized overnight with 100 μl of 5mg/ml *T. spiralis* ES antigen diluted in phosphate buffered saline (PBS). After incubation, plates were washed three times with PBS containing 0.05% Tween-20 (PBS-T). The plates were then blocked with 1% bovine serum albumin (BSA) for 1 hour and were washed with PBS-T.

One hundred ml of the test or control sera diluted 1:100 in PBS-T were then placed in wells in duplicate. After 1 hour incubation at 37°C and washing steps, each well was filled with 100ml of anti-human IgG, IgM and IgA conjugated with alkaline phosphatase (Sigma-Aldrich,USA), diluted with PBS-T at the optimal concentration (1:10000). The plates were again incubated and washed. One hundred ml of the substrate indicator reagent (pNPP in Diethanolamine substrate buffer, Thermo Scientific, USA) was added and was incubated for 20 min at room temperature. The readings were taken at 405 nm. by ELISA plate reader. The serum dilutions that resulted in an absorbance higher than the mean absorbance of wells containing negative control samples plus three standard deviations were considered positive.

STATISTICAL ANALYSIS

Data were statistically analyzed using SPSS version 11 to obtain descriptive and analytical statistics. Results of ELISA tests were compared with the Mc Nemar's test. The diagnostic performances of tests were evaluated using Receiver Operating Characteristic (ROC) curve analysis. ROC curves were compared with Med Calc 12.2.1.0. statistics programme.

RESULTS

The absorbances of each immunoglobulin ELISA test for both study and control groups were shown in box plot and scatter plot graphics (Figure 1). The median absorbance values for IgG, IgM and IgA ELISA tests in the patient group were 700 (min.410-max.1900), 440 (min.120-max.1600) and 350 (min.150-max.1400), respectively. In the control group, the median absorbance values for IgG, IgM and IgA ELISA tests were 180 (min.123-max.460), 186 (min.109-max.580) and 197 (min.90-max.940), respectively.

All trichinellosis patients had anti-*Trichinella* IgG antibodies detected by ELISA while IgM antibodies were detected in 59.6% of trichinellosis patients and IgA antibodies were detected in 50.9% (Table 1). Mc Nemar's test revealed that IgA and IgM ELISA test results were significantly different from the expected results ($p<0.001$) unlike IgG ELISA test results ($p=1.000$). When IgA and IgM ELISA results were compared with the Mc Nemar's test regarding their accordance with expected results, IgM ELISA test was found more consistent than IgA ELISA test ($p=0.044$). The ELISA results showed cross-reactivity in all immunoglobulin ELISA assays. IgM and IgA antibodies were detected in 4 control sera (one toxoplasmosis, one cystic echinococcosis and two healthy individuals) while IgG antibody was found only in one (a helathy individual). The only serum cross-reactive with IgG antibody belonged to a toxocariasis patient. The specificity of antibody ELISA assays was 97.5% for IgM and IgA antibodies and 99.4% for IgG. Positive predictive values were 96.2% and 95.6%, and negative predictive values were 69.1% and 64.8% for IgM and IgA ELISA tests, respectively.

According to the ROC curve analysis, area under the ROC curve (AUC) value of IgG, IgA and IgM ELISA were 99.7% ($p<0.001$), 74.2% ($p<0.001$)

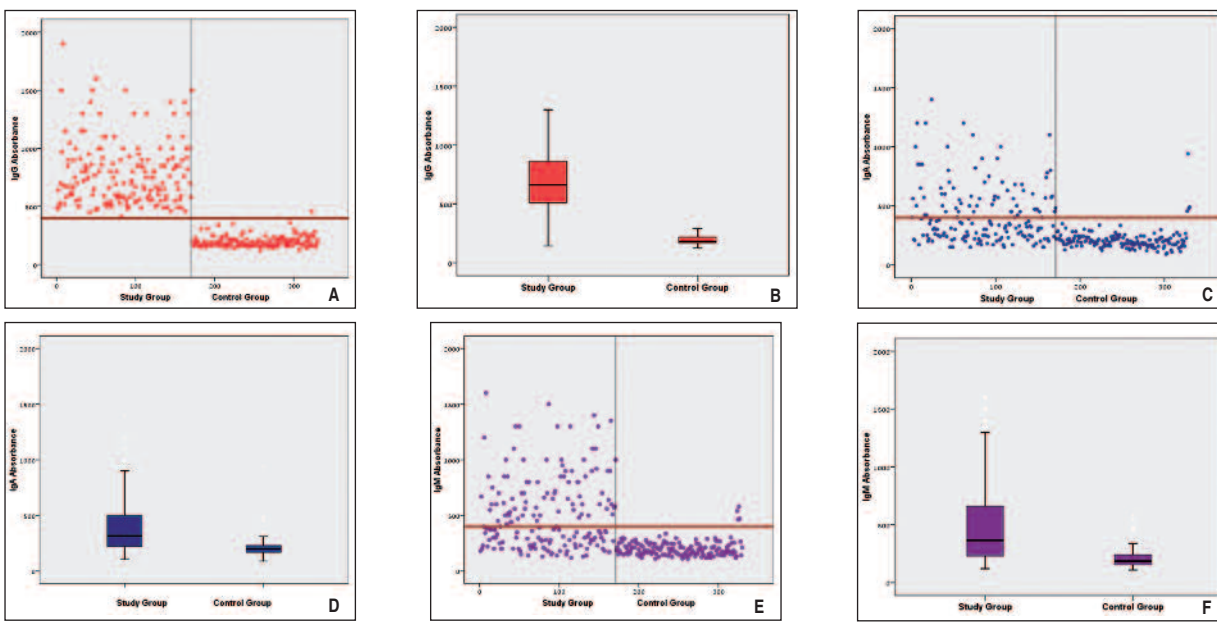

FIGURE 1: Box plots and scatter plots of IgG, IgM and IgA antibody responses of the study and the control groups. Continuous horizontal lines represent the cut-off value of the tests.

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TABLE 1: IgG, IgA and IgM antibody ELISA results of the study and the control groups.

ELISA result	Study Group (n=171)	Control Group (n=159)
IgG positive	171 (100.0%)	1 (0.6%)
IgG negative	0 (0.0%)	158 (99.4%)
IgA positive	87 (50.9%)	4 (2.5%)
IgA negative	84 (49.1%)	155 (97.5%)
IgM positive	102 (59.6%)	4 (2.5%)
IgM negative	69 (40.4%)	155 (97.5%)

ELISA: Enzyme-linked immunosorbent assay.

and 78.6% ($p<0.001$), respectively (Figure 2). When pairwise comparison of ROC curves were done for the diagnostic performances, the differences were all statistically significant (for IgG vs IgM and IgG vs IgA $p< 0.001$ and IgM vs IgA $p= 0.043$).

DISCUSSION

Early diagnosis of human trichinellosis is important since early treatment could prevent severe pathology. For this purpose, so far many serological studies have been done using different antigens and different sera obtained in outbreaks with different species.[4,10-12]

To the best of our knowledge, this is the first report presenting specific IgA and IgM antibody responses in such a large group of serum samples obtained in a *Trichinella britovi* outbreak.

The humoral immun response involves the production of anti-Trichinella antibodies of the IgE, IgG, IgM and IgA classes. IgG antibody is the most commonly used antibody in diagnosis. Several studies have shown high sensitivity for IgG antibodies in acute trichinellosis, while others have not.[11-17] In the present study, as in our previous study, all trichinellosis patients had anti-Trichinella IgG antibodies by ELISA using the ES antigen.[7,18] The main reasons for this high sensitivity may be the relatively long time between the consumption of suspected meat product and collection of sera, number of ingested larvae, individual immune response and genetic factors.[2,19,20]

Serological tests measuring other classes of antibodies (IgA, IgM) also resulted with different sensitivity rates.[4,6,10,11] van Knapen *et al.* found 86% IgM sensitivity and 62% IgA sensitivity using first month sera. In1990, 110 serum samples collected in the second month and 69 sera in the third month of *T. britovi* outbreak were included in a study by Bruschi *et al.* to examine the presence of IgG, IgM and IgE antibodies using ELISA; IgM positivity in the second and third months was 78.2% and 82.6%, respectively.[15]

In another study, Mendez-Loredo B *et al.* suggested the IgA-ELISA assay was the most sensitive (81.3% and 94.1% in 3 and 5 weeks, respectively) and IgA antibodies against newborn larvae (NBL) antigens might be useful for early diagnosis and epidemiological studies in human trichinellosis. However, in contrast to the high IgA response, the sensitivity for IgM was low (18.8% and %47.1 in 3 and 5 weeks, respectively) in the same study.[4] In our study, the sensitivity of the IgM and IgA ELISA test was 59.6% and 50.9%, respectively. This difference may be due to the different antigens used to assess antibody response and the stage of infection.[2]

The specificities of serological tests depend on the type of antigen and cross-reactions.[21,22] In order to reduce the risk of cross-reactions, ES antigen, instead of crude antigen, has been used for a long time in most laboratories.[23] Cross-reactive antibodies have been found in patients infected with other parasites and autoimmune diseases as well as

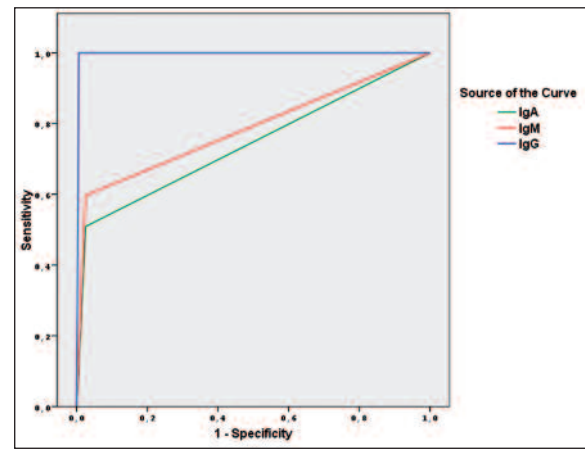

FIGURE 2: ROC curve analysis of IgG, IgM and IgA ELISA tests.
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healthy people.[12,23,24] Morakote *et al.* reported that with IgG-ELISA and IgM-ELISA, false positivity rates in the group with other parasitic infections were 20.93% and 9.30%, respectively.[18] Gomez Morales *et al.* had 1.9% false positivity in healthy people and 14.3% false positivity in a group of patients with health disturbances other than trichinellosis.[23] In this study, specificities of anti-Trichinella IgG, IgM and IgA ELISA tests were high; only 5 patients with other parasitoses and 3 healthy people had cross reactions in different antibody assays.

When IgM and IgA antibodies are compared, our results suggest that in-house IgM and IgA ELISA tests have similar specificity and positive predictive values. Although the AUC values of ROC curve analysis in IgM and IgA ELISA tests were similar, IgM ELISA had a significantly higher sensitivity for early stage (3-6 weeks) trichinellosis. However, the low negative predictive values of IgM and IgA ELISA results indicated that those antibody tests were not suitable for diagnosis of trichinellosis especially in isolated cases and IgG ELISA using the ES antigen was the choice.

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