Parasitol Res (2007) 100:1205–1211
DOI 10.1007/s00436-006-0390-x

ORIGINAL PAPER

Seroprevalence of anti-*Kudoa* sp. (Myxosporea: Multivalvulida) antibodies in a Spanish population

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Received: 3 November 2006 / Accepted: 9 November 2006 / Published online: 20 December 2006

Abstract A majority of *Kudoa* species infect the somatic muscle of fish establishing cysts. Because there is no effective method to detect infected fish without destroying them, these parasitised fish reach the consumer. The elevated humoral responses detected previously by us in BALB/c mice immunised with *Kudoa* sp. pseudocyst extracts and the high IgG1 and IgE levels induced by the oral administration of *Kudoa* pseudocysts to BALB/c mice showed the possible immunopathological effects in man from the ingestion of *Kudoa*-infected fish. In this work, we investigated the seroprevalence of anti-*Kudoa* sp. antibodies in a Spanish healthy population and the possible association between the manifestation of allergic reactions after fish consumption and the humoral responses to *Kudoa* sp. antigens. Specific anti-*Kudoa* sp. antibody levels in sera of patients diagnosed with several digestive pathologies were also determined, studying their possible association with the alteration of analytic parameters in these patients.

Introduction

A majority of *Kudoa* species infect the somatic muscle of marine and estuarine fish, establishing cysts which contain many spores. As the parasite grows, it produces proteolytic enzymes (Patashnik et al. 1982; Tsuyuki et al. 1982), thus destroying the filaments of the muscle fiber (Stehr and Whitaker 1986). It is during this stage, when the parasite contains both developing and mature spores and the infected fibers appear white. Whilst the parasite is within a muscle fiber, it remains undetected by the immune system of the host. However, as the parasite grows, it breaks the sarcolemma and is duly recognised by the host (Moran et al. 1999). This recognition results in the rapid development of a fibroblast layer around the parasite (Stehr and Whitaker 1986; Morado and Sparks 1986), and the cyst, more properly, pseudocyst, quickly acquires a black appearance. However, because the process of resorption is slower than the development of the pseudocysts, the net effect is an accumulation of black pseudocysts as the infection progresses (Kabata and Whitaker 1986). In contrast, in unusual host species such as salmonids, no cellular responses are seen, and pseudocysts can completely fill muscle fibers and rupture them, releasing spores and other parasitic materials.

In recent years, the farming of salmonids has become an important industry in several countries. Until 1990, salmonid infections by genus *Kudoa* were unusual. However, since that time, the increase in the number of cases has been spectacular (Whitaker and Kent 1991; Barja and Toranzo 1993; Holliman 1994). Because there are currently no effective methods to detect parasitised fish without destroying them, inevitable infected fish reach the consumer. Despite the black or white appearance of pseudocysts in fish meat, they frequently go unnoticed, and the myoliquefaction process may not be sufficiently advanced for the infection to be detected.

In Spain, *Kudoa*-infected fish have lately been detected in both fresh and frozen imported Chilean hake (*Merluccius gayi gayi*; Guichenot 1848) destined for human consumption. We have previously shown (Martínez de Velasco et al.

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2002) that BALB/c mice immunised with 'white' and 'black' *Kudoa* sp. pseudocyst soluble extracts or orally administered with 'white' *Kudoa* sp. pseudocysts developed high levels of IgG1 and IgE antibodies, suggesting that some of the components of the parasite may be allergenic. These components could potentially be responsible for type-I hypersensitivity reactions after their ingestion and might thus pose a risk to human health.

To continue the research of the possible immunopathological effects in man after the ingestion of *Kudoa*-infected fish, we investigated the seroprevalence of anti-*Kudoa* sp. antibodies in a healthy Spanish population and the possible association between the manifestation of allergic reactions after fish consumption and the humoral response to *Kudoa* sp. antigens. Specific anti-*Kudoa* sp. antibody levels in sera of patients diagnosed with several digestive pathologies were also determined, studying their possible association with the alteration of analytic parameters in these patients.

Materials and methods

Asymptomatic adults

Two-hundred and fifty-one sera from asymptomatic adults of several health services around Spain were randomly selected: four samples from the Laboratory Service of the Cabueñes Hospital (Asturias), 36 samples from the Hematology Service of the Virgen de la Arrixaca Hospital (Murcia) and 211 samples from the Analytic Department of the Mutua Castilla assurance (Seville). There was no evidence of any clinical signs or symptoms of disease in the patients included in this group.

Patients diagnosed with digestive pathologies

Fifty-nine patients with Crohn's disease (CD) were selected for this study. They were recruited at the Gastroenterology Service of the La Paz Hospital. This is a University Hospital that covers part of the metropolitan area of Madrid (ca. 650,000 patients). All CD patients were diagnosed according to the clinical, radiological, endoscopical and histopathological criteria of Lennard-Jones (1989). Disease activity was assessed according to the index of Harvey and Bradshaw (1980). Sera from patients with different diseases were also selected: 55 from patients with acute appendicitis, 37 from patients with gastrointestinal cancer, 14 from patients with ulcerous colitis and 82 from patients with digestive hemorrhage attended in the Digestive Hemorrhage Service of the same hospital. Other collected samples were from patients with cholecystitis (one sample), diverticulitis (one sample), abdominal pain (two samples), intestinal obstruction (three samples), peritonitis (two samples) and hydatid cyst (one sample). For all patients, the following analytic parameters were taken: leukocytes, eosinophils, haemoglobin, prothrombin time (PT), partial thromboplastin time (PTT), fibrinogen, platelets and erythrocyte sedimentation rate. The localisation, evolution and treatment with or without immunosuppressive therapy of the disease were registered for each patient.

Patients with allergic reactions after the fish consumption

Thirty-five sera were collected from patients treated at the Allergology Service of the Hospital del Aire from Madrid, who had presented allergic reactions after the fish ingestion.

Kudoa sp. pseudocysts

'White' *Kudoa* sp. pseudocysts were manually obtained from the skeletal musculature of Chilean hake (*M. gayi gayi*) from local fish markets and destined for human consumption. Pseudocysts were carefully separated from any associated fish tissue and then homogenised in a hand-operated glass tissue grinder in PBS at 4°C.

Soluble extracts

To release the spore contents, glass beads of 425–600 μm in diameter (Sigma, St. Louis, MO, USA) were added (0.1 g 100 μl^{-1} sample) to 1.5 ml vials containing the homogenate of 'white' *Kudoa* sp. pseudocysts and then shaken in a FastPrep™ shaker (FP 120, BIO 101, Savant) by three shakings of 5.5 m/s, 30 s each, with a 5-min rest of the vials in an ice-water bath between each shaking. The homogenate was extracted in PBS at 4°C overnight and then centrifuged at 8,500×*g* for 30 min at 4°C (Biofuge 17RS: Heraeus Sephatech, GmbH, Osterode, Germany). The supernatant was dialysed overnight at 4°C in PBS. The protein content of the extract was estimated by the Bradford (1976) method using BSA as standard, and the extract was frozen at −20°C until used.

Specific antibody levels

Specific antibody levels were measured by ELISA. The 96-well microtitre plates (Nunc-Immuno Plate Polysorp™, Brand Products, Denmark) were coated overnight at 4°C, with 10 μg protein/ml per well of the 'white' *Kudoa* sp. pseudocyst extract diluted in a 0.1-M carbonated buffer, pH 9.6. Several wells were kept uncoated as a control for nonspecific reactions. After washing the plates three times with 0.05% PBS-Tween 20 (PBS-Tween), blocking was carried out by adding 200 μl per well of 0.1% BSA (Sigma, St Louis, MO, USA) in PBS, incubating for 1 h at 37°C.

After washing, 100 μl of serum samples was diluted 1/100 (1/2 for the IgE measures) in PBS-Tween, 0.1% BSA, added to duplicate wells and incubated at 37°C for 2 h. Three sera were included as negative controls. The criterion for the inclusion of the control sera is lately explained. Once the plates were washed, 100 μl per well of affinity-isolated, peroxidase-conjugated, goat anti-human immunoglobulins (Ig*s*) (γ, μ, α and light chains), IgG (γ), IgM (μ) and IgA (α) (BioSource International, Camarillo, CA, USA) was added and incubated for 1 h at 37°C. For the IgE determination, 100 μl per well of a mouse IgG1 specific to human IgE (ε) (BioSource International, Camarillo, CA, USA) was added in PBS-Tween, 0.1% BSA, and incubated for 1 h at 37°C, followed by 100 μl per well of a goat anti-mouse IgG1 (γ1) horseradish peroxidase conjugate added in PBS-Tween, 0.1% BSA, and incubated for 1 h at 37°C. After washing, 100 μl per well of substrate (*O*-phenylene-diamine; Sigma, St Louis, MO, USA) was added at 0.04% in a phosphate–citrate buffer (pH 5.0) with 0.04% hydrogen peroxide. The reaction was stopped with 3 N sulphuric acid, and the plates were read at 490 μm.

Evaluation of the results

We used the diagnostic index (DI) as the mean of the ratios resulted of dividing the optical density (OD) of the test sera once their corresponding non-specific reaction with BSA used in the post-coating was subtracted by the mean OD of the negative controls minus their corresponding non-specific reaction with BSA. For the asymptomatic group, the three sera of each plate with the lowest OD levels were selected as negative controls. Sera were randomly included in each plate. The 18 sera with the lowest Ig*s*, IgG, IgM, IgA and IgE DIs were selected of the asymptomatic adults group. These 18 sera were randomly divided in three groups, mixing the sera of each group and thus resulting in three sera that were used as negative controls for the determination of the antibody levels of the sera from the patients diagnosed with digestive pathologies or allergic reactions after fish consumption. In this group, IgE determinations could only be assessed in 37 of the 290 samples due to the big volume of serum required for the test and the scarce quantity of each sample.

Criteria of positiveness

Values higher than the mean of the DIs of the group of asymptomatic adults obtained for each immunoglobulin plus twice their respective standard error were considered as positive. The same values were applied as criterion of positiveness to the results of the sera of the patients diagnosed of digestive pathologies or with allergic reactions after fish consumption.

Statistical analysis

Data were analysed with SPSS© (version 11.0) software. To study the dependence or independence of the variables, contingency tables were performed and, depending on the number of the samples analysed, the Fisher's test to determine the chi-square values (bilateral exact significance) or the Pearson's chi-square test was applied. When the data had a normal distribution, the independent sample *t* test was used to compare the mean of two or more groups into the same dependent variable. The significant differences among the means of two or more groups were confirmed by the one-factor ANOVA. Multiple comparisons were performed with the Bonferroni method. *P* values <0.05 were considered to be statistically significant. Correlation studies using the Pearson's correlation coefficient (PCC) were performed to compare immunoglobulin levels. According to Colton (1974), a 0–0.25 PCC implies no correlation. A 0.25–0.5 value points to a low or moderate correlation. Finally, 0.5–0.75 is considered to be a moderate to good correlation, and >0.75 implies good to excellent correlation.

Results

Asymptomatic adults

We examined a total of 251 sera although IgE levels could only be determined in 223 of them. DIs of the Igs response >4.48 were considered as Igs positive, DIs of the IgG response >8.84 as IgG positive, DIs of the IgM response >5.22 as IgM positive, DIs of the IgA response >10.15 as IgA positive and DIs of the IgE response >12.31 as IgE positive (Fig. 1a). The antibody prevalences in this group were 5.2, 4.8, 5.6%, 4.4 and 7.6% for the Ig*s*, IgG, IgM, IgA and IgE responses, respectively. The IgE prevalence refers to the 223 samples analysed.

Correlation studies between the DIs of the Ig*s*, IgG, IgM, IgA and IgE responses of the asymptomatic group were performed. Good significant correlations were found between the Igs DIs and the IgG DIs (PCC=0.732) and moderate between the Igs DIs and the IgM DIs (PCC=0.620).

Patients diagnosed with digestive pathologies or allergic reactions after fish consumption

Considering the 292 samples of patients diagnosed with digestive pathologies or with allergic reactions after fish consumption as one group, the antibody prevalences were 9.6, 7.2, 1.7, 7.9 and 9.3% for the Ig*s*, IgG, IgM, IgA and IgE responses, respectively. Only 75 samples were tested for IgE in this group. The same values applied as criteria of

positiveness for the asymptomatic group were used (Fig. 1b). In the IgM response, Pearson's chi-square test indicated that there is a connection between an IgM-positive DI and the presence or absence of disease. The Fisher's exact test was $p=0.018$ (bilateral exact significance). Considering all the samples from the asymptomatic adults and from the patients diagnosed with digestive pathologies or with allergic reactions after fish consumption, good significant correlations were found between the Igs DIs and the IgG DIs (PCC=0.715) and moderate between the Igs DIs and the IgA DIs (PCC=0.513).

Patients diagnosed with digestive pathologies

Two-hundred and fifty-seven sera were examined (Fig. 1c). The antibody prevalences were 10.1, 7, 0.8, 8.2 and 8.9% for the Ig*s*, IgG, IgM, IgA and IgE responses, respectively. The IgE prevalence refers to the 67 samples that could be analysed in this group.

When the DIs of the asymptomatic group were compared with the DIs of the patients diagnosed with digestive pathologies (not including patients with allergic reactions after fish consumption) for each response, the independent sample *t* test indicated statistically significant differences in the IgM response ($p=0.000$). The Igs response was near to signification ($p=0.058$). However, the one-factor ANOVA did not confirm this significance.

Considering the sera from the asymptomatic adults and from the patients diagnosed with digestive pathologies, good significant correlations were found between the Igs DIs and the IgG DIs (PCC=0.722) and moderate between the Igs DIs and the IgA DIs (PCC=0.605) and between the IgG DIs and the IgA DIs (PCC=0.529).

When the positive and negative DIs of the patients diagnosed with digestive pathologies were compared in each response with the analytic parameters of these patients, the independent sample *t* test indicated statistically significant differences for the leukocytes ($p=0.010$) and for the PTT ($p=0.022$) in the IgG response, and for the hemoglobin ($p=0.024$) and for the PT ($p=0.004$) in the IgA response.

Results by pathologies

Patients with allergic reactions after fish consumption

Thirty-five patients were examined (Fig. 2a). The antibody prevalences were 5.7, 8.6, 8.6, 5.7 and 12.5% for the Igs, IgG, IgM, IgA and IgE responses, respectively. The IgE prevalence refers only to the eight samples whose IgE levels could be determined. When the correlations between the DIs obtained in the Igs, IgG, IgM, IgA and IgE responses of the patients with allergic reactions after fish consumption were studied, excellent significant correlations were observed

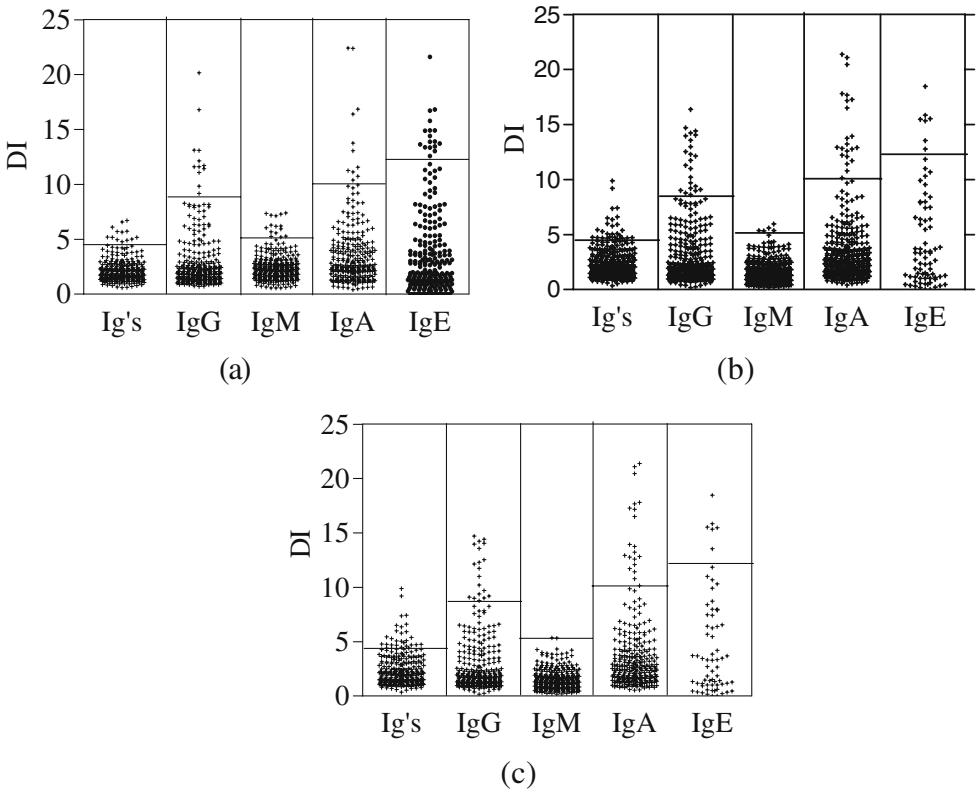

Fig. 1 Diagnostic indexes (DIs) obtained in the Igs, IgG, IgM, IgA and IgE responses in the group of asymptomatic adults (**a**), in the patients diagnosed with digestive pathologies or allergic reactions after fish consumption (**b**) and digestive pathologies (**c**). The *horizontal strike* represents the limit between the positive DIs (*upper part*) and the negative DIs (*lower part*)

between the Igs DIs and the IgG DIs (CCP=0.879) and good between the Igs DIs and IgM DIs (CCP=0.686).

Patients diagnosed with acute appendicitis

Fifty-five patients were tested (Fig. 2b). The antibody prevalences were 7.3, 1.8, 0, 3.6 and 0% for the Igs, IgG, IgM, IgA and IgE responses, respectively. The IgE prevalence refers to the nine samples analysed for the IgE levels. Moderate significant correlations were observed in this group between the Igs DIs and the IgG DIs (PCC= 0.609), between the Igs DIs and the IgM DIs (PCC=0.596) and between the Igs DIs and the IgA DIs (PCC=0.593).

Patients diagnosed with gastrointestinal cancer

Thirty-seven patients were studied (Fig. 2c). The antibody prevalences were 10.8, 5.4, 0, 5.4 and 0% for the Igs, IgG, IgM, IgA and IgE responses, respectively. Seven samples were tested for IgE levels. In this group, good significant correlations were observed between the Igs DIs and the IgG DIs (PCC=0.731) and between the Igs DIs and the IgA DIs (PCC=0.694) and moderate between the IgG DIs and the IgA DIs (PCC=0.563).

Patients diagnosed with ulcerous colitis

For the 14 patients investigated (Fig. 2d), the antibody prevalences were 14.3, 7.1, 0, 7.1 and 0% for the Igs, IgG, IgM, IgA and IgE responses, respectively. The IgE

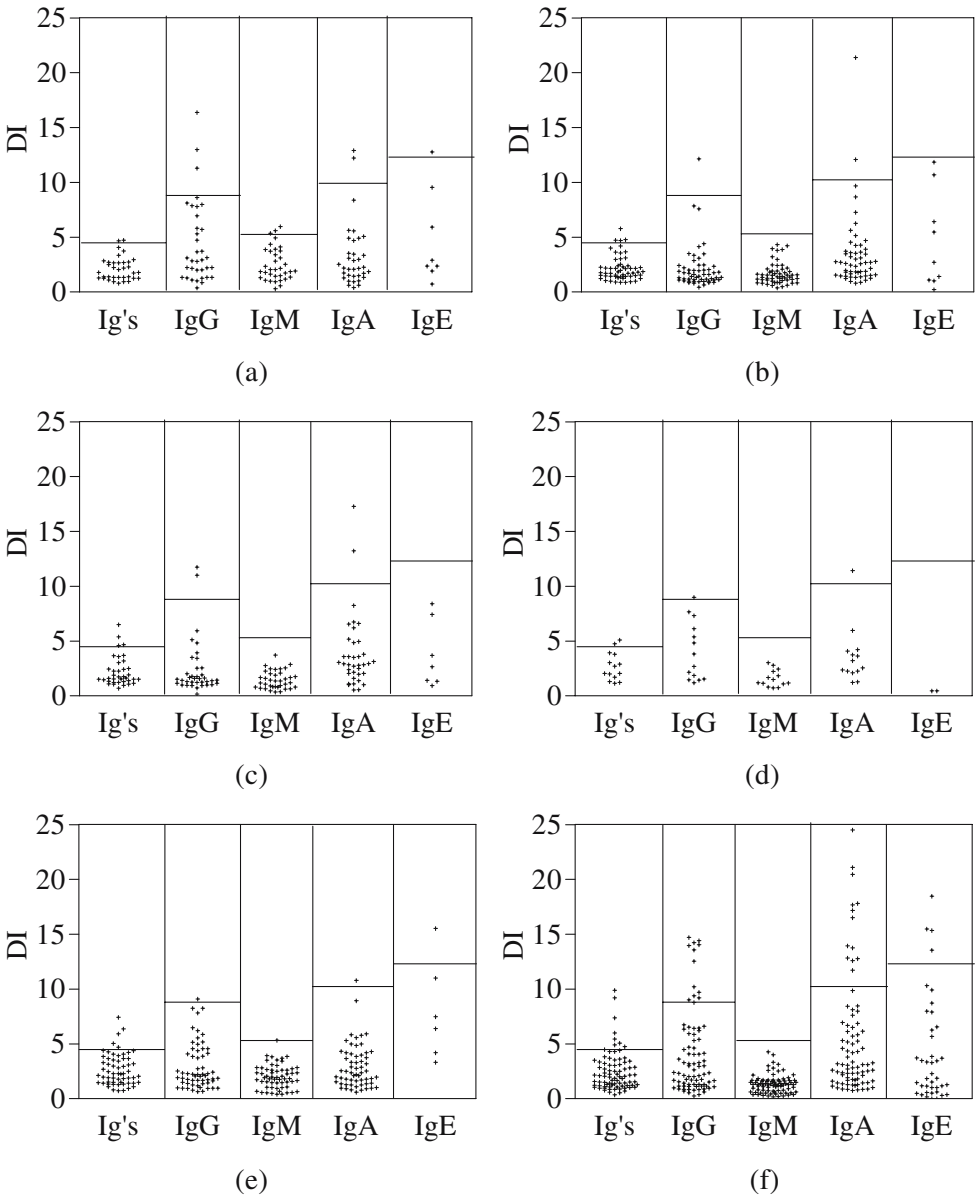

Fig. 2 Diagnostic indexes (DIs) obtained in the Igs, IgG, IgM, IgA and IgE responses in the group of patients with allergic reactions after fish consumption (**a**), acute appendicitis (**b**), gastrointestinal cancer (**c**), ulcerous colitis (**d**), Crohn's disease (**e**) and digestive haemorrhage (**f**). The *horizontal strike* represents the limit between the positive DIs (*upper part*) and the negative DIs (*lower part*)

prevalence refers to the two samples tested for this immunoglobulin. When the correlations between the DIs obtained in the Igs, IgG, IgM, IgA and IgE responses of the patients diagnosed with ulcerous colitis were studied, excellent significant correlations were observed between the Igs DIs and the IgG DIs (PCC=0.948), very good between the Igs DIs and the IgA DIs (PCC=0.752) and good between the IgG DIs and the IgA DIs (PCC=0. 685).

Patients diagnosed with Crohn's disease

Fifty-nine patients were studied (Fig. 2e). The antibody prevalences were 8.5% for the Igs response, 1.7% for the Igs, IgG, IgM and IgA responses and 16.6% for the IgE response (six samples tested). Good significant correlations were observed between the Igs DIs and the IgG DIs (PCC= 0.680) and moderate between the Igs DIs and the IgM DIs (PCC=0.510). Besides, a very good correlation between the disease activity index and the IgE DIs was observed (PCC= 0.775).

Patients diagnosed with digestive hemorrhage

Fifty-five patients were tested (Fig. 2f). The antibody prevalences were 11, 14.6, 0, 15.8 and 10% for the Igs, IgG, IgM, IgA and IgE responses, respectively. The IgE prevalence refers to the nine samples analysed for IgE levels. When the correlations between the DIs obtained in the Igs, IgG, IgM, IgA and IgE responses of the sera from the patients diagnosed with digestive hemorrhage were determined, very good significant correlations were observed between the Igs DIs and the IgG DIs (PCC=0.780), good between the Igs DIs and the IgA DIs (PCC=0.694) and moderate between the IgG and the IgA DIs (PCC= 0.526) and between the IgM DIs and the IgE DIs (PCC= 0.559).

Other digestive pathologies

Patients with cholecystitis (one sample), diverticulitis (one sample), abdominal pain (two samples), intestinal obstruction (three samples), peritonitis (two samples) and hydatid cyst (one sample) were considered as a single group. The antibody prevalences were 20% (intestinal obstruction and peritonitis), 10% (hydatid cyst), 10% (peritonitis), 20% (peritonitis and hydatid cyst) and 33.3% (hydatid cyst) for the Igs, IgG, IgM, IgA and IgE responses, respectively. The IgE prevalence refers to the three samples analysed for IgE levels. Excellent significant correlations were observed between the Igs and the IgA DIs (PCC=0.889) and between the IgG and the IgE DIs (PCC=0.994), very good between the IgG DIs and the IgA DIs (PCC=0.774) and between the IgM DIs and the IgA DIs (PCC=0.752), good between the Igs DIs and the IgG DIs (PCC=0.689) and moderate between the Igs DIs and the IgM DIs (PCC=0.558).

When the relation between a positive or negative DI for each response and the presence of allergic symptoms after fish consumption or diverse digestive pathologies or the absence of disease was studied, the Pearson's chi-square test indicated a relation between the two variables in the IgG (p=0.028), the IgM (p=0.043) and the IgA (p=0.005) responses. The one-factor ANOVA confirmed these significant differences with p values of 0.001, 0.000 and 0.001 for the IgG, the IgM and the IgA responses, respectively.

When the positive and negative DIs from the sera with the same digestive pathology were compared against the clinical parameters of the patients of this group, the independent sample t test showed statistically significant differences for the cephaline time (p=0.049) in the IgG response of the patients diagnosed with digestive bleeding.

Discussion

According to the data from the Spanish Nutrition and Feeding Study of 1991, published in 1995, fish represents a considerable percentage of the proteic and energetic support in the Spanish daily diet, being this percentage in increase. Thus, from 63 g person^{-1} day^{-1} consumed in 1964, 72 g was reached in 1991. In fact, the results on the presence of anti-*Kudoa* antibodies in a random healthy population showed a high prevalence.

The sera from people from Asturias or Murcia were not strictly from healthy people, because they went to the hospitals of Cabueñes or Virgen de la Arrixaca, respectively, and conducted blood tests by medical prescription. However, the number of samples obtained from these locations represents 15.9% of the total analysed samples, and only five of them were positive against one of the assayed immunoglobulins. On the other hand, the sera from Seville (211 samples) were obtained from medical exams of healthy workers from several companies. According to the Spanish Nutrition and Feeding Study (1995), Asturias and Seville are two of the provinces where fish consumption is greater than in the rest of the Spanish territory, with 81.5 and 75.4 g person^{-1} day^{-1}, respectively, whilst Murcia is one of the lowest, with 55.5 g person^{-1} day^{-1}. However, although these data are important, it is likely that the high glycosylation of *Kudoa* sp. antigens was the one really responsible of the high number of positive samples. If the antibodies produced by phylogenetically distant immunogens can react because of their sugared residues (Yamaga et al. 1978; Bayne et al. 1987), it is then possible that the great prevalence detected was due to cross-reactions with other mixosporidia or, inclusively, with antibodies against other parasites or microorganisms also in contact with the human

being, which include the same carbohydrates in their antigenic determinants. The work of Muñoz et al. (1999) showed the presence of manose and/or glucose in the valves of most of the examined mixosporidia, such as *Kudoa* sp. Several parasites of mammals, such as *Entamoeba histolytica*, *Acantahmoeba* sp., *Trichomonas vaginalis*, *Pneumocystis carini* or *Leishmania* sp., also have residues of manose which have been associated to virulence or infectivity in several cases Slifkin and Doyle (1990). In contrast, the criterion employed to consider a DI value as positive seems to be strictly enough to eliminate most of the cross-reactions.

Whilst in the Igs, IgG, IgA and IgE responses the percentage of positive samples was higher in the group of patients with digestive pathologies or allergic reactions after fish ingestion than in the group of presumable healthy population, in the IgM response, the opposite situation occurred. In fact, although there were no many differences between the DIs from both groups of population, the mean DIs from the patient group were slightly superior in all the responses except in the IgM. Against this immunoglobulin, higher differences between the mean DIs of the negative from both groups and between the mean DIs of the positive from the same above-mentioned groups were appreciated. The lowest mean DI was obtained, in both cases, in the group of patients. Curiously, the chi-square tests indicated that a relation between an IgM-positive DI and the presence or absence of disease existed (Fisher's exact statistic=0.018, bilateral exact significance). The same phenomenon was repeated when the control group was compared to the other from patients diagnosed with digestive pathologies, without the inclusion of the patients with allergic reactions after fish ingestion. In fact, when the DIs from the healthy population group were compared to the DIs from the digestive patient group in each response, the independent sample *t* test indicated statistically significant differences in the IgM response (p=0.000). Besides, the high correlation rates obtained among the Igs, IgG, IgM and IgA DIs confirmed the coherence of the reported results. Moreover, a very good significant correlation between the activity index of CD and the IgE DIs was observed.

Acknowledgements This work was supported in part by research grants PR238/00-9444 (DANONE, S.A.) and PR27/05-13950 (BSCH). We also are grateful to Luis García Palacios and Dr. Gutiérrez Ramos for collecting the serum samples. The experiments comply with the current laws of Spain.

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