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Original Article

Immunodetection of specific IgE (immunoblotting) in a prevalence study of *Anisakis simplex* sensitisation in Spain

Background: The possibility of a high rate of sensitisation to *Anisakis simplex*, together with that of geographic bias and confounding factors in population-based diagnostic and prevalence data prompted the Spanish Allergology Society (SEAIC) to undertake a multicentre trial based on immunodetection analysis of the serum samples. **Material and Methods:** Twenty-one centres located in different geographic areas participated in the study. All the patients referred to the Allergology services because of urticaria, angioedema or anaphylaxis were included in the study (as cases), independently of the suspected aetiology. Sera from normal subjects with no history of urticaria, angioedema or anaphylaxis were used as controls. The serum samples were processed by immunoblotting tests, the authors being blind as to the clinical outcomes, skin test results or CAP values. The data were transmitted back to the centres of origin and to the study coordinator. **Results:** The most striking result was the very high rate of positivities among the sera from the cases as compared to those of the controls, suggesting the relevance of *Anisakis simplex* as an aetiological agent, and the significant geographic variation in the number of positive immunoblots among the samples. This geographic influence is underscored by the fact that there were no positive results among the case or control samples from Vigo, while the rate of positive samples exceeded 50% in two centres in Madrid. The positivity rates among the control subjects were always less than 10%. **Conclusion:** This study of different geographic areas leads to the conclusion that sensitisation is not due to fish intake per se but to active infestation as a consequence of the habit of eating raw fish. Thus, risk factor suppression might lead to the eradication of this disease condition.

Key words: *Anisakis simplex*. Immunoblotting. Immunodetection.

Inmunodetección de IgE específica (immunoblotting) en un estudio de prevalencia de sensibilización a *Anisakis simplex* en España

Introducción: La posibilidad de que existiera una alta prevalencia de sensibilización frente a *Anisakis simplex*, asociada a la existencia de posibles sesgos geográficos y de factores de confusión, llevó a la SEAIC a proponer un estudio

multicéntrico en el que se incluyera el análisis por inmunodetección de las muestras. **Material y Métodos:** En total han participado en este proyecto 21 centros de distintas áreas geográficas. Se seleccionaron como casos a los pacientes remitidos a los servicios de Alergología por episodios de urticaria, angioedema o anafilaxia, cualquiera que fuese la sospecha etiológica. Se incluyeron como controles sueros de personas que nunca hubieran padecido urticaria, angioedema o anafilaxia. Los sueros fueron analizados mediante *immunoblotting*, sin conocimiento alguno de los resultados obtenidos por los investigadores clínicos ni los datos de pruebas cutáneas o valores de CAP de las muestras, remitiéndose posteriormente los resultados a los centros de origen y a la coordinadora del estudio. **Resultados:** Como resultado cabe destacar que existe una abrumadora mayoría de sueros positivos entre los casos en comparación con los controles, lo que sugiere la importancia de *Anisakis* como agente etiológico, y que existen enormes variaciones en la cifra de sueros con blot positivo que dependen de la zona geográfica de procedencia. Esta influencia geográfica hace que no se detecten ningún caso ni ningún control positivos en las muestras recibidas de Vigo, mientras que dos centros de Madrid superan el 50%. La aparición de individuos positivos entre los controles no supera en ningún caso el 10%. **Conclusión:** El estudio de diferentes zonas geográficas permite concluir que la sensibilización no ocurre por la ingesta de pescado sino por la parasitación activa debida al hábito de consumo de pescado crudo. Esto implica que esta patología puede ser erradicada si se eliminan factores de riesgo.

Palabras clave: *Anisakis simplex*. *Immunoblotting*. Inmunodetección.

INTRODUCTION

The existence of clinical disease caused by *Anisakis simplex* sensitisation has been known only relatively recently¹. Already many years ago, the existence of a chronic or acute disease termed "anisakiasis" or "anisakidosis" had been reported; this complaint was induced by active parasitic infestation by this nematode. Yet, the possibility that individuals might exist that were strongly sensitised, through an IgE-mediated mechanism, to allergens from the parasite had been suspected in only a few patients².

The initial study of the first patients reported in Spain was carried out with the immunodetection (immunoblot) technique. The usefulness of the method in the diagnosis of this sensitisation was soon evident. Its ability not only to discriminate between positive and negative sera but also to provide information as to the antigens recognised by each serum has not been improved to date by any other technique^{3,4}. This method also was able to reveal the existence of clinically unrelated false positive results both in the skin prick tests and in the CAP, in most cases due to the recognition of a ca. 40-kDa band that is periodate-sensitive. However, in our experience in studying hundreds of sera that were prick test- and CAP-negative, we did not find any positive results in the immunodetection⁵. This demonstrates that both the prick test and the CAP System have a very high sensitivity, turning them into very useful methods for large studies. On the other hand, their low specificity renders it advisable to use them as primary sampling methods, with later confirmation of the results by immunodetection.

The possibility of a high prevalence of sensitisation to this parasite, of possible geographic bias and of confounding factors in the prevalence diagnosis or estimation induced the Spanish Society of Allergology and Clinical Immunology (SEAIC) to propose a multicentre study including the immunodetection analysis of the samples. In order to prevent variations attributable to the method, all samples were to be analysed at the "Carlos III" Hospital. Overall, 21 centres from different geographic areas in Spain participated in this project.

METHODS

Cases and controls

According to the investigators' instruction manual, the various participating centres selected as cases "all patients referred to the Allergology services because of episodes of urticaria, angioedema or anaphylaxis, regardless of the suspected aetiology". Sera from individuals who had never had urticaria, angioedema or anaphylaxis were included as controls.

The various sera were sent by courier service to the "Carlos III" Hospital, where they were processed without any knowledge of the results of the clinical investigations, of the skin tests or of the CAP results of the samples; the results were then transmitted back to the centres of origin of the samples and to the Study Coordinator.

For the present report, only those centres have been included for which all the "per protocol" samples were available or, if this condition was not met, those for which all those samples were available that would have been positive by skin prick test or by CAP analysis. This procedure, which aimed at preventing bias, reduced the total number of centres included to 16.

Preparation of the *Anisakis simplex* protein extract

Anisakis simplex larvae were obtained by direct extraction from infested fish, mainly hake (*Merluccius merluccius*) and blue whiting (*Micromesistius poutassou*), in which the larvae were found free in the peritoneal cavity, on the intraperitoneal organs or inside them (mainly in the liver), forming large numbers of tangles adherent to the peritoneum or penetrating the muscles in the shape of flat spirals, and in some cases even perforating the fishes' skin in an attempt to attain freedom. The raw extract was prepared by homogenisation of the larvae in phosphate-buffered saline (PBS), followed by centrifugation (4,500 g) for 10 minutes; the supernatant was collected and frozen at -20°C.

Protein electrophoresis in sodium dodecylsulfate-polyacrylamide gel (SDS-PAGE)

This was carried out in a discontinuous system according to the procedure described by Laemmli⁶, using 16% polyacrylamide minigels with 4% concentrating gels. The protein extract samples were dissolved in application buffer (125 mM Tris, pH 6.8; 4% SDS; 20% glycerol, and 0.042% bromophenol blue)⁷. Electrophoresis was carried out at a constant 150 V in a Mighty Small System electrophoresis device (Hoefer Scientific Instruments, San Francisco, California, USA), the protein bands were detected by staining the gel with Coomassie brilliant blue (BioRad). The excess stain was eliminated by repeated washing with a decolourant solution (10% acetic acid; 7.5% methanol).

Transfer of the proteins to nitrocellulose membranes

This was essentially done according to the procedure described by Petit et al.⁸, with some modifications⁹. After the development of the SDS-PAGE had been completed, the gels were placed between two nitrocellulose membranes (Supported Nitrocellulose Membrane 0.45 µm, BioRad) and immersed in the transfer buffer (50 mM NaCl; 2 mM Tris HCl, pH 7.4) in a transfer apparatus (Hoefer Scientific Instruments) under stirring. The proteins migrated by

diffusion from the polyacrylamide gel to the nitrocellulose membrane for 18 hours and not by electrotransfer; this allowed us to prepare two nitrocellulose copies from each gel.

Immunodetection of specific IgE (immunoblotting)

The nitrocellulose membranes were incubated for 30 minutes in a 3% Nonidet P-40 solution (Amresco) in PBS so as to achieve renaturation of the transferred proteins and thus prevent the loss of recognition by IgE that might have resulted from the conformational changes experienced by the epitopes in the course of the electrophoresis, transfer and incubation processes with various different buffers¹⁰. The nitrocellulose membranes were then incubated overnight with human sera diluted 1:5 in a diluent solution (10 mM Tris-saline, pH 7.4; 5% foetal calf serum; 1% Tween-20). After three washings with a solution of 0.5 M NaCl, 2 mM Tris-HCl (pH 7.4) and 0.1% Tween-20, the membranes were incubated for three hours with a monoclonal IgG anti-IgE antibody (Ingenasa, Madrid, Spain) diluted 1:1000 in the diluent solution. After washing, the membranes were again incubated for one hour with an alkaline phosphatase-conjugated mouse anti-IgG antibody (BioSource International, Camarillo, California, USA) diluted 1:2000. Finally, and after several washings in washing solution and a last one in normal saline, a commercial mixture of BCIP-NBT (Amresco) was added as substrate.

RESULTS

All the results presented have been achieved using the same detection method and the same reagents. The samples have been classed into only two groups: (a) sera with high positive levels and recognition of several bands, which are compatible with *Anisakis simplex* sensitisation, and (b) the remainder of the sera, which evidenced absent or only weak band recognition. Figure 1 once again demonstrates how an intense blot is associated to high CAP results and how the reverse is the case when the blot is negative, thus confirming the results of previous studies. It can also be observed that intermediate CAP values (0 – 20 kU/l) are very often associated to recognition of a single band in the blot, suggesting the existence of cross reactivity based on that protein.

Figures 2 – 8 illustrate the main results of the present study, grouping the data according to geographic location. The following data should be stressed because of

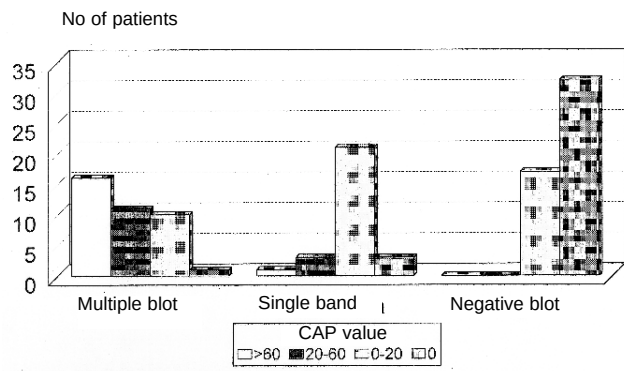


Fig. 1. CAP values according to the type of blot.

their interest:

1. There is an overwhelming majority of positive sera among the cases as compared to the controls, suggesting the importance of *Anisakis simplex* as an aetiological agent.
 2. There are very considerable variations in the numbers of sera with positive blots, which are dependent on the geographic area of origin.
- As the various participating centres sent us, according to their own criteria, either all the samples or only the positive ones, the relative frequency of positive re-

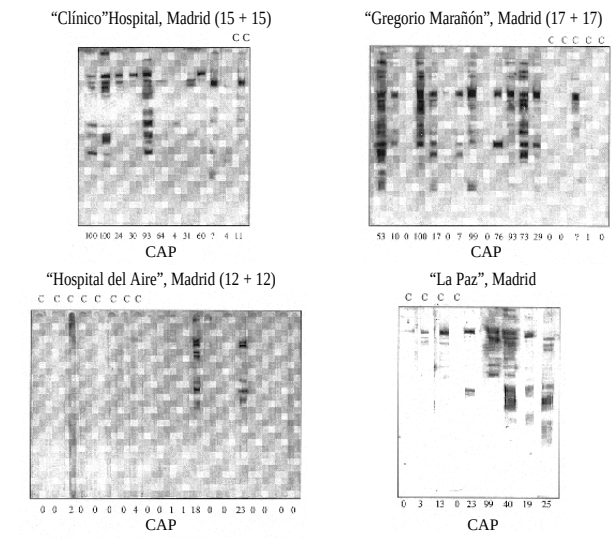


Fig. 2. Results of specific *Anisakis simplex* IgE immunodetection. When known, the CAP values are inserted below each of samples. The control samples. The control samples have been marked with a "C" on top of the lane. These results are not the final ones, as some of the participating centres only sent sera from positive patients while others sent all the collected samples irrespective of the results.

sults has been ordered and expressed as percentages in Figure 9. This clearly reflects the geographic influence, which leads to no positive cases or controls having been detected in the samples from Vigo, while two centres in Madrid clearly exceed 50%. The detection of positive individuals among the controls is never in excess of 10%.

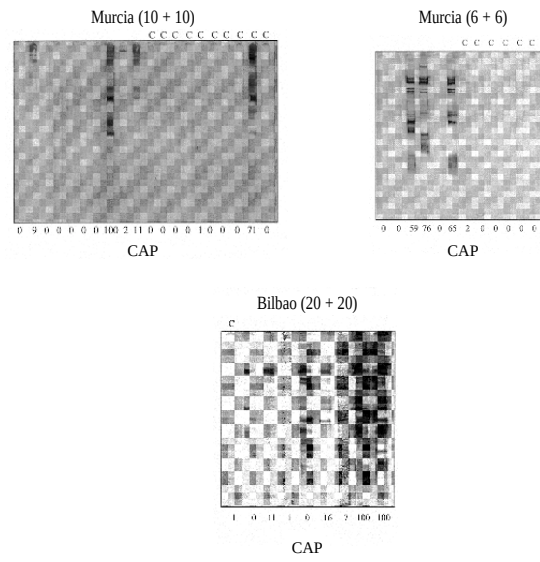


Fig. 3. Results of specific *Anisakis simplex* IgE immunodetection.

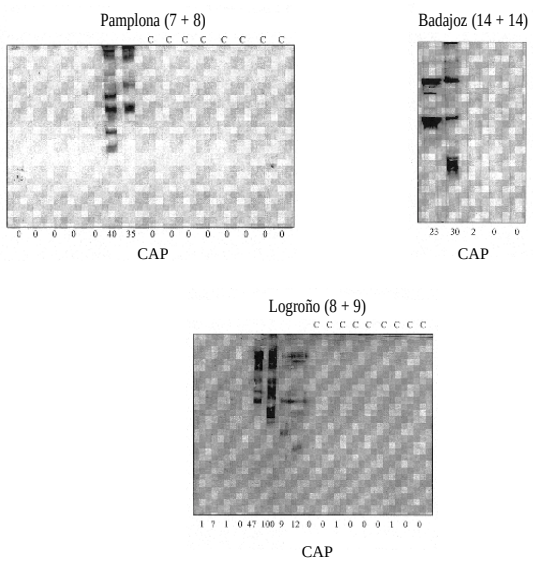


Fig. 4. Results of specific *Anisakis simplex* IgE immunodetection

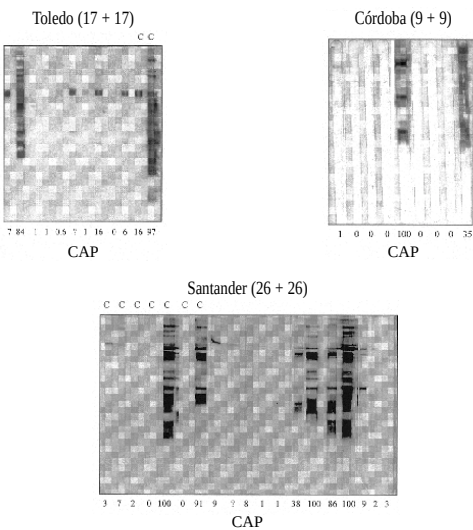


Fig. 5. Results of specific *Anisakis simplex* IgE immunodetection.

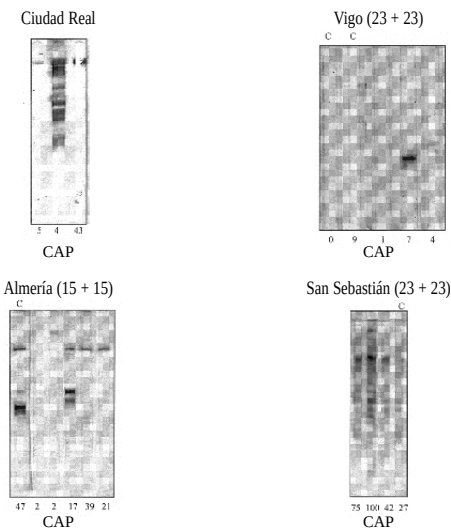


Fig. 6. Results of specific *Anisakis simplex* IgE immunodetection.

DISCUSSION

The main objective of this multicentre study was a double one: on the one hand, it aimed at assessing the frequency with which *Anisakis simplex* is involved in the causation of urticaria, angioedema and/or anaphylaxis; this particular aim would be addressed by the study of sequential patients with these conditions. On the other hand, the study of a paired control population would provide know-

ledge of the prevalence of sensitisation among the general population. The comparative analysis of the various geographic zones within Spain would furthermore disclose whether differences between the various zones exist, and whether such differences might be attributable to a given concrete factor. The immunodetection study of the sera was not formally required in order to fulfil all these aims, as the use of the skin prick tests or of the CAP assessment, or both together, might have sufficed. However, as

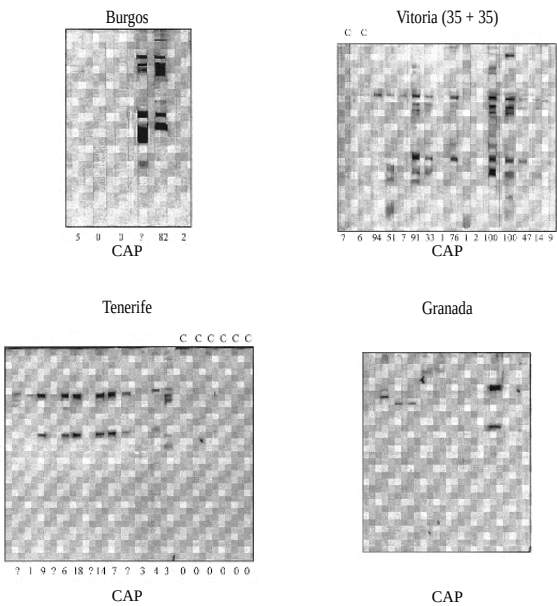


Fig. 7. Results of specific *Anisakis simplex* IgE immunodetection.

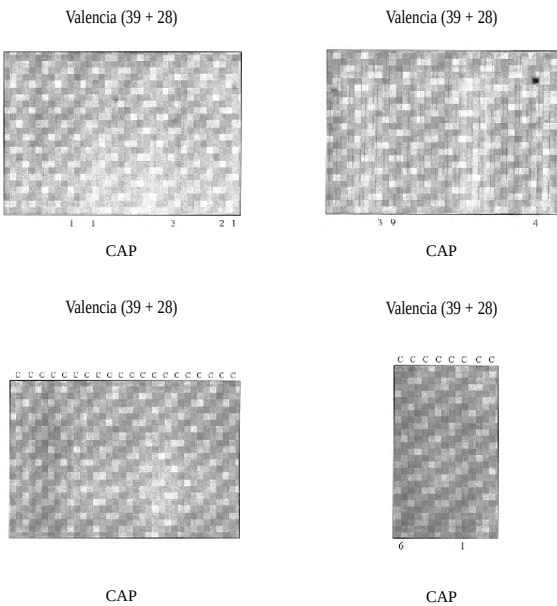


Fig. 8. Results of specific *Anisakis simplex* IgE immunodetection.

immunodetection is a more restrictive method than either the skin prick tests or the CAP, it did provide supplementary information to the former two rendering possible the elimination of eventual confounding factors.

This multicentre study has made it possible to ascertain the very considerable geographic variation regarding this disease condition and its high prevalence in some regions. At the same time, this sensitisation has been demonstrated to be low or unknown in other areas, in agreement with the experience of the various groups (Fig. 9). The reasons explaining these findings may perhaps be derived from the data collected in the protocol of the epidemiologic study, but they may be as simple as the existence of varying gastronomic habits among the various regions. If this were the case, and according to the results observed in the present study, a change in the alimentary habits might suffice for eliminating the problem. The non-existence of sensitisation in certain areas definitively rules out the assumption that sensitisation to the parasite may occur through the simple ingestion of infested fish, and lends support to the hypothesis that the synthesis of specific IgE results from one or more active parasitic infestation episodes. In our opinion, this particular finding is fundamental

towards possible prevention, as the actual findings demonstrate that the ingestion of fish and the absence of parasitic infestation are fully compatible.

On the other hand, the frequency of sensitisation observed among the control population, even though considerably lower than that seen among the urticaria/angioedema cases, would appear to be a cause for concern, particularly in certain areas. If the samples have been collected with no bias, the apparently healthy but at risk population might exceed 5% in some regions. Subclinical or forgotten anisakidosis episodes, or sensitisations that were in progress at the time of sampling might both be responsible for these findings among the control individuals, but there is no doubt that exposure to high amounts of the allergen might trigger clinically overt sensitisation episodes in these patients considering the very high levels of specific IgE antibodies they evidence. Only the follow-up of these individuals over time may reveal whether, sometime in the future, they shall have to cease to be considered as controls and enter into the category of truly sensitised patients. In this context, it should be remembered that individuals who develop episodes of acute anisakidosis already evidence high titres of specific antibodies that cannot

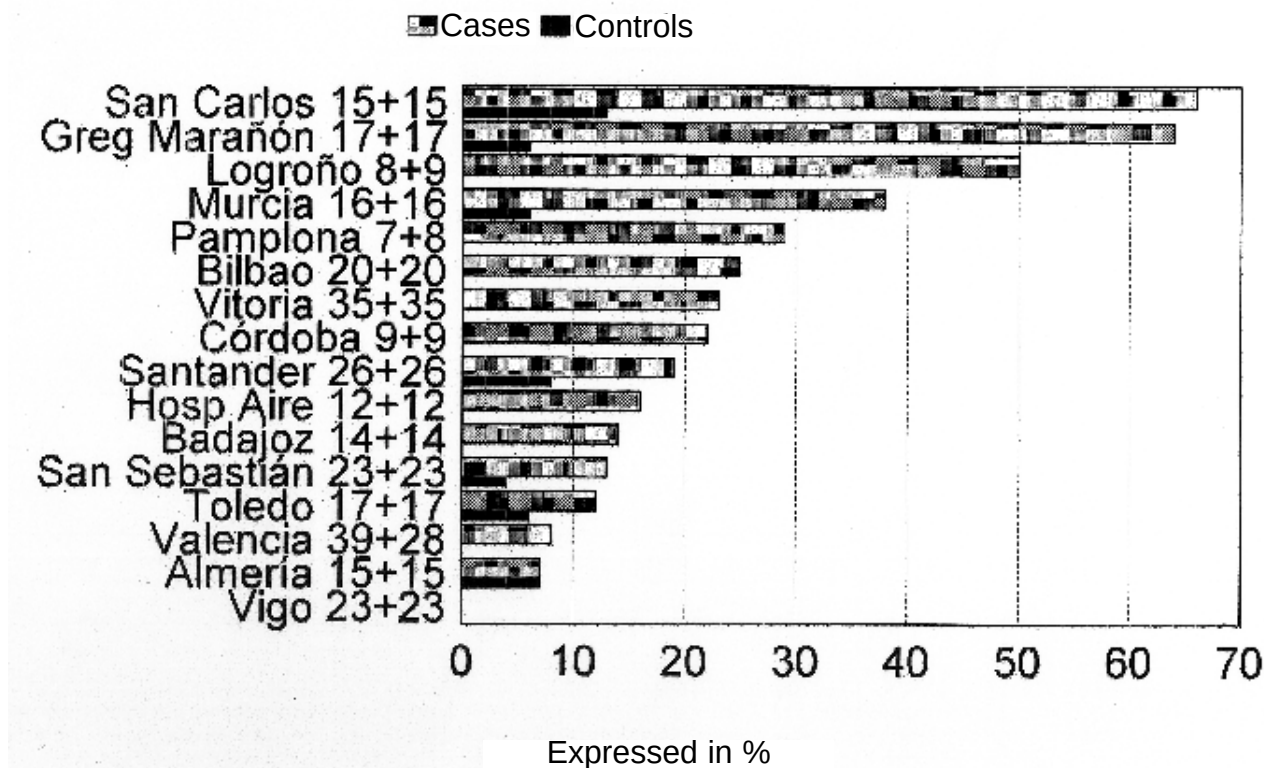


Fig. 9. Percentage representation of the blot-positive individuals. For each centre, the number of cases and controls included in the study is specified in parentheses, ordered by decreasing percentage of positive cases.

have been synthesised in the few hours elapsed between the ingestion of the fish vector and the development of symptoms. If this were the case, this multicentre study would have allowed us to at least in part ascertain the natural history of this sensitisation/parasitosis.

It is our considered opinion that this multicentre study has afforded us an overall view of the degree of involvement of the parasite in urticaria/angioedema episodes, and at the same time it has provided a study of sensitisation among the apparently healthy population. The comparative study of the various geographic areas allows us to rule out the possibility that sensitisation to parasite allergens may occur through the simple ingestion of infested fish, and suggests a requirement for active parasitic infestation. This observation is particularly interesting, as it opens the way to effective prevention measures for a sensitisation that affects a considerable number of individuals among the apparently healthy population.

PARTICIPATING CENTRES

Xeral Cíes" Hospital, Vigo; "Marqués de Valdecilla" Hospital, Santander; "Santiago Apóstol" Hospital, Vitoria; "Basurto" Hospital, Bilbao; "Aránzazu and Amara" Hospital, San Sebastián; "Virgen del Camino" Hospital, Pamplona; "General Yagüe" Hospital, Burgos; Hospital Complex of Ciudad Real; "Gregorio Marañón" Hospital, Madrid; "La Paz" Hospital, Madrid; "Hospital del Aire", Madrid; "San Carlos" University Hospital, Madrid; "Virgen del Valle" Hospital, Toledo; "Infanta Cristina" Hospital, Badajoz; "Nuestra Señora de la Candelaria" Hospital, Tenerife; "To-

rrécárdenas" Hospital, Almería; "Reina Sofía" Hospital, Córdoba; Clinical University Hospital, Granada; "Virgen de la Arrixaca" University Hospital, Murcia; "La Fe" University Hospital, Valencia; "San Millán" Hospital, Logroño.

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