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Diagnosing Human Anisakiasis: Recombinant Ani s 1 and Ani s 7 Allergens versus the UniCAP 100 Fluorescence Enzyme Immunoassay▽

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Commercially available serological methods for serodiagnosis of human anisakiasis either are poorly specific or do not include some of the most relevant *Anisakis* allergens. The use of selected recombinant allergens may improve serodiagnosis. To compare the diagnostic and clinical values of enzyme-linked immunosorbent assay (ELISA) methods based on Ani s 1 and Ani s 7 recombinant allergens and of the UniCAP 100 fluorescence enzyme immunoassay (CAP FEIA) system, we tested sera from 495 allergic and 25 non-food-related allergic patients. The decay in specific IgE antibodies in serum was also investigated in 15 positive patients over a period of 6 to 38 months. Considering sera that tested positive by either Ani s 1 or Ani s 7 ELISA, the CAP FEIA classified 25% of sera as falsely positive, mainly in the group of patients with the lowest levels of anti-*Anisakis* IgE antibodies, and 1.28% of positive sera as falsely negative. Considering allergens individually, the overall sensitivities of Ani s 7 ELISA and Ani s 1 ELISA were 94% and 61%, respectively. The results also showed that anti-*Anisakis* IgE antibodies can be detected in serum for longer with Ani s 1 ELISA than with Ani s 7 ELISA and CAP FEIA ($P < 0.01$). Our findings suggest that ELISA methods with Ani s 7 and Ani s 1 allergens as targets of IgE antibodies are currently the best option for serodiagnosis of human anisakiasis, combining specificity and sensitivity. The different persistence of anti-Ani s 1 and anti-Ani s 7 antibodies in serum may help clinicians to distinguish between recent and old *Anisakis* infections.

Anisakiasis (also called anisakiosis) is an important emerging human nematodosis caused by several species of the genus *Anisakis* (30, 35). Although the ability of these parasites to infect humans has been known for more than 40 years (28, 36), their relevance as etiologic agents of systemic allergic reactions (i.e., urticaria, angioedema, and anaphylaxis), including gastroallergic anisakiasis, was not recognized until the end of the last century (5, 10). Unlike nematodes that complete their biological cycle in humans, *Anisakis* organisms do not develop beyond the L4 stage, and it is believed that most of the larvae are either expelled in the first 24 h (2) or die within 3 weeks of infecting the host (19). However, the IgE immune response, induced against some allergens released by the *Anisakis* larvae while they are still alive, can be detected in sera from infected patients over long periods (11), which may be prolonged when patients suffer one or more reinfections.

Throughout the past 15 years, several methods of detecting *Anisakis*-induced specific IgE antibodies in serum have been developed (4), among which only the UniCAP 100 fluorescence enzyme immunoassay (CAP FEIA) method is commercially available. In a previous study (23), we demonstrated the excellent sensitivity but low specificity of CAP FEIA and suggested that the latter is probably due to the use of a complex antigenic mixture of cross-reactive antigens as a target in the immunoassay. In contrast to CAP FEIA, a capture ELISA with *O*-deglycosylated *Anisakis* antigens (22), based on the use of the monoclonal antibody (MAb) UA3, which captures two high-molecular-mass *Anisakis* allergens, of 139 and 154 kDa (17, 21), displayed high sensitivity and specificity with positive sera from Spanish patients and negative sera from *Anisakis*-free populations. However, the assay was found to be somewhat limited, mainly due to the difficulty in obtaining large amounts of parasites and antigen uniformity between batches. Interestingly, the major *Anisakis* allergen recognized by MAb UA3 (Ani s 7) was recently characterized (31), and an internal portion of its sequence (t-Ani s 7) was cloned and expressed in *Escherichia coli*. Previous results obtained with this allergen as a target in indirect ELISA showed it to be highly specific (3), with a similar sensitivity to that obtained with UA3-capture ELISA (31). However, the t-Ani s 7 ELISA has not yet been tested under field conditions. In addition to Ani s 7, the *Anisakis* allergen Ani s 1 also appears to be of interest for serodiagnosis of human anisakiasis, as it was also reported to be a major allergen, recognized by about 85% of patients with *Anisakis*-induced IgE antibodies, when used as a target in ELISA in either the native (26) or recombinant (16) form.

The recent availability of recombinant forms of Ani s 7 and Ani s 1 allergens (rAni s 7 and rAni s 1, respectively) raises the possibility of developing new commercial tests for serodiagnosis of human anisakiasis, which may improve the specificity of the CAP FEIA method. To explore this possibility, in the present study we compare the diagnostic values of ELISA

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TABLE 1. General characteristics of the patients evaluated in the study

Clinical manifestation	No. (%) of patients
Urticaria	322 (65.05)
Angioedema	123 (24.85)
Anaphylaxis	4 (0.81)
Eosinophilia	35 (7.07)
Pruritus	24 (4.85)
Gastroenteritis, gastrointestinal lesions, dyspepsia, or intestinal pseudoclusions	56 (11.39)
Other allergies (respiratory, hymenoptera)	25 (5.05)

methods based on Ani s 1 and Ani s 7 recombinant allergens and of CAP FEIA and discuss the clinical significance of our findings.

MATERIALS AND METHODS

Patients and sera. The 495 serum samples used in this study were obtained from patients attending the Allergy Service of the Hospital del Aire (Madrid, Spain) between September 1995 and December 2001. The patients included 173 males (35%) and 322 females (65%), with a mean age of 44.3 years (range, 5 to 81 years). All patients lived in Madrid (Spain), a region where there is a high prevalence of *Anisakis* infections (30). To avoid bias due to patient selection, we investigated all available serum samples from patients with clinical symptoms who were suspected of having food-related allergies and who were included in one or more of the following groups: (i) patients presenting allergic symptoms related to consumption of fish in the 48 h prior to appearance of the symptoms; (ii) patients with allergic symptoms not related to recent consumption of fish; (iii) patients with erosive/obstructive gastrointestinal lesions; and (iv) patients with urticaria, pruritus, and/or eosinophilia of unknown etiology. In addition, 25 patients with non-food-related allergy were also included as controls. The overall clinical manifestations of the patients enrolled in this study are shown in Table 1. Four hundred ninety-three of 495 patients were included in the study, and the 25 control subjects reported consuming raw fish (mainly "boquerones," i.e., anchovies in vinegar) several times a year.

Following anamnestic evaluation of the selected patients in the above-mentioned period, serum samples were obtained from each patient, divided into aliquots, and stored at −80°C until *in vitro* analysis for the presence of specific anti-*Anisakis* IgE antibodies (see below).

When several serum samples from the same patient were available, only the first available sample was considered for sensitivity comparisons between the Ani s 1 and Ani s 7 ELISA methods (see below).

CAP FEIA determinations. Determination of anti-*Anisakis* IgE antibody levels with the CAP FEIA (Phadia AB, Uppsala, Sweden) was carried out following the manufacturer's instructions. Briefly, this system consists of ImmunoCAP flexible hydrophilic CNBr-activated cellulose derivative to which the target allergen preparation is attached. Specific IgE antibodies are first detected by incubation with serum samples and then revealed using an anti-IgE MAb labeled with β-galactosidase, which generates fluorescence by splitting the fluorogenic substrate 4-methylumbelliferyl-β-D-galactoside into 4-methylumbelliferone. If performed with undiluted reagent, the CAP FEIA quantifies specific serum IgE antibodies in the range of 0.35 to 100 kU/liter. However, for comparative purposes, the above range is often converted into 6 "scores," according to the internal calibrator system, as follows: class 0, negative; class 1, 0.35 to 0.7 kU/liter; class 2, 0.7 to 3.5 kU/liter; class 3, 3.5 to 17.5 kU/liter; class 4, 17.5 to 50 kU/liter; class 5, 50 to 100 kU/liter; and class 6, >100 kU/liter. IgE values of ≥0.35 kU/liter in serum are considered positive by the manufacturer.

Isolation of *Anisakis simplex* Ani s 1 and Ani s 7 recombinant allergens. The internal ^{435}Met-^{713}Arg fragment of the rAni s 7 (t-Ani s 7) allergen was cloned and expressed in *Escherichia coli* as previously described (31). The Ani s 1 recombinant allergen (rAni s 1), lacking the peptide signal, was obtained as follows. First, whole mRNAs were obtained from L3 larvae of *Anisakis simplex* by use of a Fast Track mRNA isolation kit (Invitrogen, Carlsbad, CA), following the instructions provided with the kit; the corresponding cDNAs were then synthesized by use of a Marathon cDNA amplification kit (Clontech, Palo Alto, CA). Once the corresponding cDNAs were obtained, two primers (forward, 5′-GAG AGAGA**GGATCC**GCCGATAGAACGGAATGTCAGC-3′; and reverse, 5′-G AGAGAGA**AAGCTT**TTATTTACAACAATTTGCAAATAATTTGC-3′) were designed from the nucleotide sequence of Ani s 1 (GenBank accession no. AB100095), previously described by Shimakura et al. (33). In such primers, two restriction sites (marked in bold and underlined), for BamHI and HindIII, respectively, were introduced for further cloning into the pRSET A plasmid polylinker (Invitrogen). A sequence of 516 bp, encoding the complete Ani s 1 sequence without the signal peptide (see Fig. 1C), was amplified by PCR with these primers and the *Anisakis simplex* cDNAs as a template. The PCR product was purified with a GFX PCR DNA and gel band purification kit (Amersham Bioscience, Buckinghamshire, United Kingdom) and cloned into the pRSET A plasmid with the same BamHI and HindIII restriction enzymes. The recombinant plasmid was sequenced in an Applied Biosystems 377 DNA sequencer (PE Biosystems, Foster City, CA) (see Fig. 1C) and then used to transform competent *E. coli* BL21(DE3)pLys cells (Novagen, Madison, WI) according to the supplier's instructions. Finally, the cells were grown at 37°C until an absorbance of 0.6 was reached; expression of the recombinant peptide was induced by addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and the cells were grown for another 4 h.

Isolation and purification of Ani s 1 recombinant polypeptide from inclusion bodies. Pelleted cells corresponding to 250 ml of culture were obtained by centrifugation at 10,000 × *g* at 4°C for 15 min, and the recombinant polypeptide, obtained in the form of inclusion bodies, was purified with the B-Per reagent (Pierce, Rockford, IL) according to the supplier's instructions. The protein was solubilized in 0.1 M sodium phosphate buffer, pH 8.0, containing 8 M urea and centrifuged at 10,000 × *g* for 15 min, and the supernatant was used for further purification on a Ni-nitrilotriacetic acid (Ni-NTA) affinity column (Qiagen, Izasa S.A., Madrid, Spain), following the manufacturer's instructions. The eluted protein was dialyzed and refolded by the oxidative refolding procedure previously described by Ibarrola et al. (16), without further purification. To monitor the efficacy of the affinity column purification step, aliquots of the retained and nonretained proteins were isolated in a 5 to 20% SDS-PAGE linear gradient gel (reducing conditions) and then analyzed by Coomassie blue staining and by Western blotting (WB) with an anti-His-tag monoclonal antibody (Sigma-Aldrich, Madrid, Spain; dilution, 1:20,000).

The presence of rAni s 1 oligomers in the refolded protein sample was investigated by (i) SDS-PAGE and WB, as indicated above, but with samples electrophoresed under nonreducing conditions; and (ii) size-exclusion chromatography with a fast-performance liquid chromatograph (FPLC) equipped with a Superose 12 HR column (Pharmacia, Uppsala, Sweden), with the protein concentration monitored at 280 nm. Finally, the immune recognition of recombinant Ani s 1 monomers and oligomers was investigated by WB (23), with a positive-control human serum diluted 1:4 and 1:25.

ELISA determinations. Specific anti-*Anisakis* IgE antibodies were detected by indirect ELISA, with t-Ani s 7 or rAni s 1 as the target. Wells in columns 1, 4, 7, and 10 of the 96-well microtiter plates (Greiner Bio-One, Frickenhausen, Germany) were filled with 100 μl of phosphate-buffered saline (PBS) containing rAni s 1 at a concentration of 5 μg/ml, and wells in columns 2, 5, 8, and 11 were filled with 100 μl of 0.1 M Tris buffer, pH 10.5, containing 0.6 μg/ml of t-Ani s 7. The wells in the remaining columns (controls) were filled with PBS alone. After incubation of the plates at 4°C overnight and blocking of nonreactive sites, 100 μl of undiluted serum was added to each well and the specific IgE detected as previously described (23). Optical densities (ODs) at 492 nm were calculated by subtracting the OD value produced by the same serum in the absence of antigen.

Cutoff calculations. The cutoff value for Ani s 1 ELISA was calculated as the mean OD plus four times the standard deviation for 200 human serum samples from healthy blood donors (aged 18 to 65 years). The samples were provided by the Galician Blood Transfusion Centre (Santiago de Compostela, Spain). The sera were selected at random from among blood donors who reported that they did not consume raw fish, and the sera were then confirmed to be negative (class 0) by CAP FEIA. The calculated cutoff value for Ani s 1 ELISA was an OD of 0.09. The cutoff value for t-Ani s 7 (OD = 0.05) was established previously (31).

Gold standard. In comparing the sensitivities and specificities of the Ani s 1 ELISA, Ani s 7 ELISA, and CAP FEIA methods, we considered a serum to be truly positive (gold standard) when it tested positive by Ani s 1 ELISA, Ani s 7 ELISA, or both methods. This criterion was based on the following: (i) Ani s 7 and, probably, Ani s 1 are excretory-secretory allergens that are released and recognized by the host immune system only in the course of an *Anisakis* infection (3, 6); (ii) both allergens are highly specific, as deduced from their primary amino acid sequences, which have no significant homologies with any recognized human allergen; (iii) these methods have proven high sensitivities, as they are reported to recognize about 85% and 100% of well-characterized positive sera, respectively (8, 16, 23, 31); and (iv) it is expected that the combined use of the Ani s 1 and Ani s 7 allergens in ELISA will increase the individual sensitivity of each

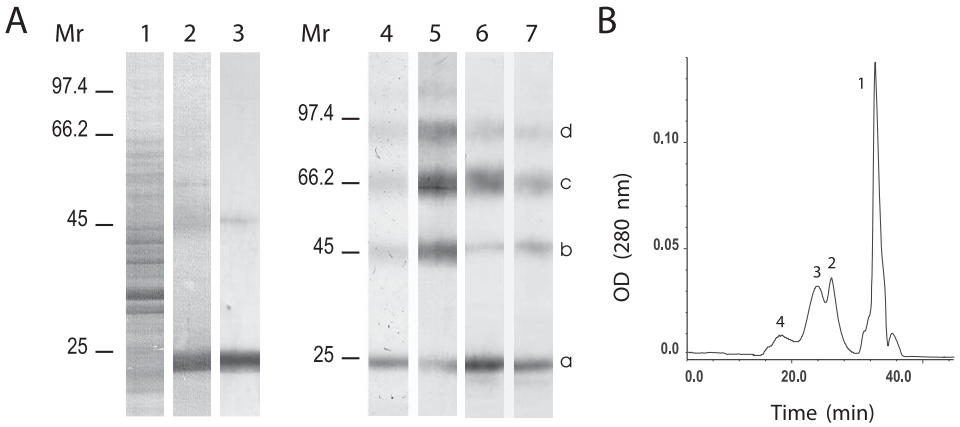

FIG. 1. (A) SDS-PAGE and WB analysis of Ani s 1 purified protein after oxidative refolding under reducing (lanes 1 to 3) and nonreducing (lanes 4 to 7) conditions. Lane 1, unretained fraction, Coomassie staining; lane 2, retained fraction, Coomassie staining; lane 3, retained fraction revealed by WB with a monoclonal anti-His antibody by a standard procedure; lane 4, retained fraction, Coomassie staining; lane 5, retained fraction revealed by WB with an anti-His antibody as described above; lanes 6 and 7, retained fraction revealed by WB with a positive human serum at 1:4 and 1:25 dilutions, respectively. (B) Chromatogram obtained after size-exclusion chromatography, using a Superose 12 HR column (Pharmacia), of the retained protein fraction of Ani s 1 after purification by Ni-NTA affinity chromatography. Peak 1 corresponds to the monomer fraction. (C) Complete amino acid sequence of Ani s 1 encoded by the pRSET A vector and expressed in BL21(DE3)pLys cells. The specific *Anisakis* expressed sequence is shown in boldface type. Note that the Ani s 1 sequence differs by one amino acid (Arg to Gln; boxed) from that described by Shimakura et al. (19).

individual assay because Ani s 1 and Ani s 7 have no sequence homologies, thus increasing the number of epitopes available to bind to IgE antibodies in serum.

Statistical analysis. Row-matched (paired) comparisons of the results obtained with CAP FEIA, Ani s 1 ELISA, and Ani s 7 ELISA after follow-up of 15 patients for a period of 6 to 38 months (see Table 3) were made with the Tukey-Kramer multiple-comparison test (included in GraphPad InStat, version 3.05, statistical software; GraphPad Software Inc.). Differences were considered significant at P values of ≤ 0.05. Predictive values for each serological method were calculated with Epidat 3.1 software (Consellería de Sanidade, Dirección Xeral de Saúde Pública, Xunta de Galicia, Spain).

RESULTS

Isolation of Ani s 1 recombinant allergen. After expression of the Ani s 1 allergen in *E. coli* and its purification by affinity chromatography on a Ni-NTA column, the homogeneity of the recombinant protein was analyzed by Coomassie staining and WB of samples isolated by SDS-PAGE. Under reducing conditions, the immunopurified antigen was revealed as a single major band that migrated in accordance with the theoretical mass of the protein (22,986 Da) (Fig. 1A, lane 2, and C). The same major band was also recognized by WB with an anti-His monoclonal antibody (Fig. 1A, lane 3). However, when the same sample was analyzed under nonreducing conditions, in addition to the major band corresponding to monomeric molecules (23 kDa; band a), three additional bands that appeared to correspond to dimers (46 kDa; band b), trimers (69 kDa; band c), and tetramers (92 kDa; band d) were also observed after Coomassie staining (Fig. 1A, lane 4). All of these bands were recognized by the anti-His MAb (lane 5) and, more importantly, were also recognized by the positive human serum used as a control (Fig. 1A, lanes 6 and 7). Maximal signals were obtained with monomers (a) and trimers (c) as targets of WB for both dilutions of the human serum (Fig. 1A, lanes 6 and 7). The presence of monomers (majority form; peak 1), dimers (peak 2), trimers (peak 3), and oligomers (peak 4) in the sample was also demonstrated by size-exclusion chromatography (Fig. 1B).

Recognition of sera from allergic patients by CAP FEIA and ELISAs with *Anisakis* recombinant allergens. A total of 495 sera were available for the present study. Most of them corresponded to patients who presented allergic symptoms compatible with food-related allergies, although sera from some patients with non-food-related allergy, eosinophilia, pruritus, or gastrointestinal manifestations were also included in the study. Urticaria and/or angioedema was the most frequent allergic manifestation observed (Table 1).

Among the 495 sera investigated, the CAP FEIA classified 235 sera as negative and 260 sera as positive (Table 2 and Fig. 2). The positive sera belonged to the following categories: class 1 (38 sera), class 2 (67 sera), class 3 (50 sera), class 4 (42 sera), class 5 (40 sera), and class 6 (23 sera). However, compared

TABLE 2. Diagnostic performances of CAP FEIA, Ani s 1 ELISA, and Ani s 7 ELISA[a]

Method	No. of sera[b]			
	TP	FP	TN	FN
CAP FEIA	195	65	232	3
Ani s 1 ELISA	121	0	297	77
Ani s 7 ELISA	186	0	297	12
Ani s 1/Ani s 7 ELISA	198	0	297	0

[a] Determined with 495 sera (198 truly positive sera plus 297 truly negative sera) and with respect to the proposed gold standard (combined results obtained with Ani s 1 and Ani s 7 ELISAs [see Materials and Methods]).

[b] TP, true positive; FP, false positive; TN, true negative; FN, false negative.

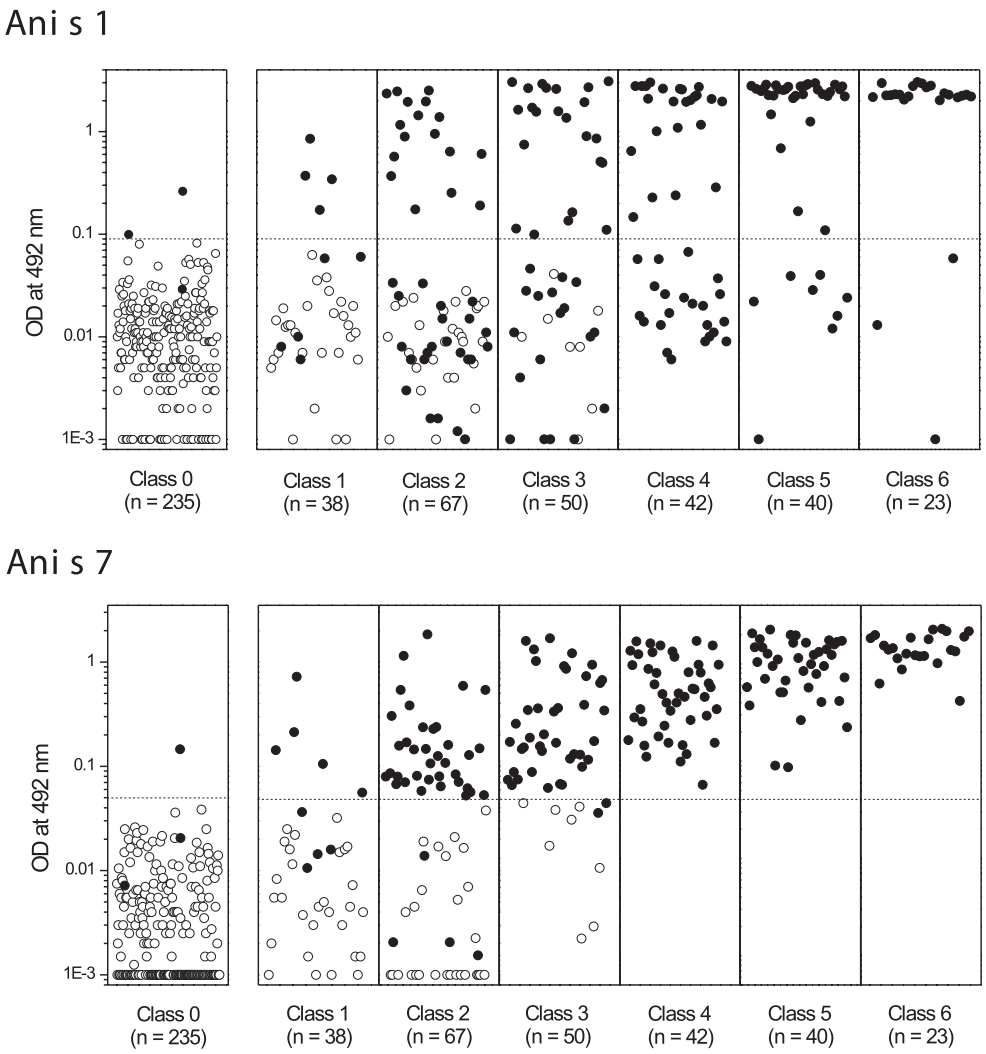

FIG. 2. Optical density values obtained for the 495 sera investigated in the Ani s 1 ELISA and the Ani s 7 ELISA. Each circle corresponds to an individual serum sample. The sera were grouped into CAP FEIA classes as described in Materials and Methods.

with our gold standard, 25% of positive sera (from 65 individuals) and 1.27% of negative sera (from 3 individuals) were classified as falsely positive and falsely negative, respectively (Table 2). The data in Fig. 2 and 3B also show that most false-positive results corresponded to classes 1 (76.3%; 29 sera) and 2 (41.8%; 28 sera), while only 16% (8 sera) of the false-positive sera belonged to class 3. No false-positive results were obtained for sera belonging to classes 4 to 6 or for sera from subjects with non-food-related allergy (negative controls). However, among the 260 sera classified as positive by CAP FEIA, 31 sera (11.9%) also tested positive (classes 1 to 3) against other allergens, such as *Ascaris*, *Toxocara*, and/or some kind of fish (i.e., cod, salmon, hake, tuna, and/or megrim [whiff]) (data not shown).

Individually, the Ani s 1 ELISA correctly classified 61.1% of truly positive sera (121 of 198 sera recognized by our gold standard), or 66.7% (98 of 147 sera) if considering only highly positive serum samples belonging to CAP FEIA classes 3 to 6 (Table 2 and Fig. 2A). Comparatively, the Ani s 7 ELISA (Table 2 and Fig. 2B) was more sensitive, as it correctly classified 93.9% of truly positive sera (186 of 198 sera), or 98.64% of truly positive sera (145/147 sera) if testing sera belonging to classes 3 to 6. The Ani s 7 ELISA, like CAP FEIA, correctly classified all sera with high levels of anti-*Anisakis* IgE antibodies, i.e., those belonging to classes 4 to 6 (Fig. 2B). When discordant values between the Ani s 1 and Ani s 7 ELISA methods were analyzed, differences for sera belonging to all CAP FEIA classes were observed, although maximal discrepancies were observed with sera included in classes 2 to 5 (Fig. 3A). As observed with CAP FEIA, all sera corresponding to subjects with non-food-related allergy (negative controls) were correctly classified as negative by the Ani s 1 and Ani s 7 methods.

Decay in anti-*Anisakis* IgE antibody levels in serum. The decay in antibody levels in serum is a key parameter for consideration in selecting antigens for developing serological assays, as it determines the time during which the assay recognizes the patient serum as positive. To investigate whether Ani s 1 ELISA, Ani s 7 ELISA, and CAP FEIA recognize anti-*Anisakis* IgE antibodies in serum for different lengths of time,

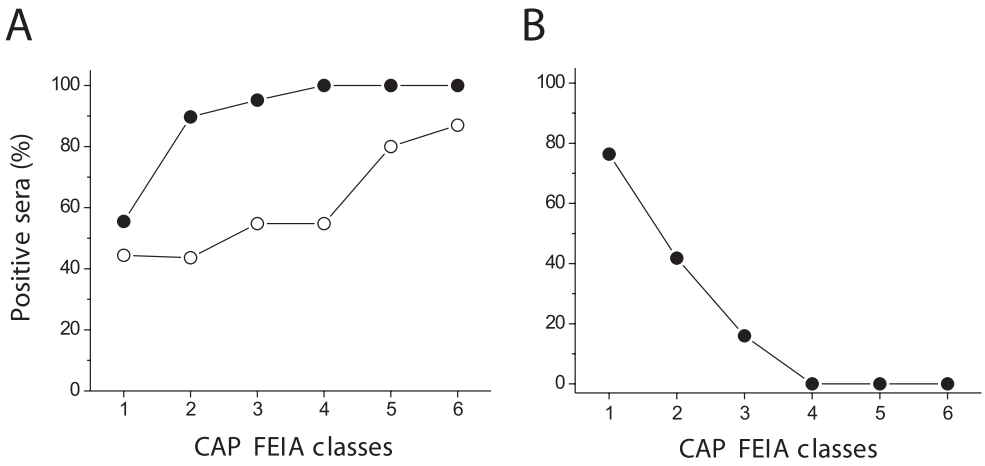

FIG. 3. (A) Percentages of positive serum samples, as determined by Ani s 1 ELISA (open circles) and Ani s 7 ELISA (closed circles), in CAP FEIA classes 1 to 6, according to the proposed gold standard (see Materials and Methods). The numbers 1 to 6 refer to CAP FEIA classes 1 to 6. (B) Percentage of false-positive sera for each CAP FEIA class, assuming the same gold standard.

we investigated the decay in IgE levels by each method over a period of 6 to 38 months for 15 selected patients who were classified positive by the three methods. To carry out the comparisons, we considered only the IgE values obtained at the start (when the first sample was obtained) and at the end of the observation period for each selected patient. The decreases in levels of specific IgE antibodies for each patient at the end of the observed period are shown in Table 3. Paired data analysis by use of the Tukey-Kramer multiple-comparison test revealed that the Ani s 1 ELISA was able to detect anti-*Anisakis* IgE antibodies in serum for a longer time (mean decay of 35.12%; $P < 0.01$) than the Ani s 7 ELISA (mean decrease, 64.56%) or the CAP FEIA (mean decrease, 60.41%). In more than 75% of patients (11 of 15 patients), the mean decrease in IgE antibodies measured by Ani s 1 ELISA was only 22.2% for a mean period of 15.6 months.

TABLE 3. Decreases in anti-*Anisakis* IgE antibody levels in CAP FEIA, Ani s 1 ELISA, and Ani s 7 ELISA observed for sera from 15 positive patients at the end of the follow-up period[a]

Patient no.	% Decrease in IgE antibody			Elapsed time (months)
	CAP FEIA	Ani s 1 ELISA	Ani s 7 ELISA	
1	52.52	52.52	82.62	38
2	73.05	28.52	73.00	38
3	41.60	88.04	82.36	21
4	77.33	28.77	69.14	16
5	65.76	14.00	54.16	6
6	35.75	12.84	69.36	13
7	90.53	32.76	74.00	35
8	72.78	12.33	45.09	10
9	65.47	41.12	58.00	9
10	94.32	78.67	70.50	30
11	58.28	29.56	78.44	14
12	53.44	17.20	17.76	7
13	70.49	38.37	81.72	10
14	71.15	0.00	20.10	14
15	46.01	52.23	30.03	9
Mean ± SE	60.41 ± 5.77	35.12 ± 6.35	64.56 ± 4.34	18.00 ± 2.97

[a] The percent variations in IgE values were calculated using the first and last available samples from each investigated patient. Significant differences ($P < 0.01$) between Ani s 1 and Ani s 7 ELISAs and between CAP FEIA and Ani s 1 ELISA were observed.

DISCUSSION

This is the first study to test the usefulness of recombinant *Anisakis* allergens and CAP FEIA for serodiagnosis of human anisakiasis. According to the proposed gold standard, the results obtained with CAP FEIA revealed a high sensitivity (S = 98.42%; 95% confidence interval [CI95] = 96.53 to 100%) and a highly negative predictive value (NPV = 98.72%; CI95 = 97.08 to 100%). However, this method showed poor specificity (Sp = 78.11%; CI = 73.24 to 82.99%) and a low positive predictive value (PPV = 75%; CI95 = 69.54 to 80.46%), which is in accordance with previous observations made with this method (18, 23, 29). This lack of specificity has led some investigators to consider CAP FEIA-positive results in the range of 0.35 to 0.75 kU/liter (20, 27), and even values of up to 1.5 kU/liter, as not clinically relevant (12).

Since the clinical symptoms associated with almost all sera used in this study were similar and since the sera were obtained from patients with risk factors for *Anisakis* infections, it was not possible to establish a clinical pattern for the 65 CAP FEIA-positive sera that tested negative by our gold standard method. Indeed, on the basis of previous reports (9, 27), it could be argued that in addition to *Anisakis* infections, CAP FEIA detects IgE antibodies induced by free *Anisakis* allergens that are present in fish contaminated with this parasite and categorizes the patients as positive. However, this hypothesis appears unlikely, considering the negative results obtained in provocation test studies carried out on sensitized patients, using frozen (1, 2, 14), freeze-dried (32), or excretory-secretory (6) antigens, as well as in experiments conducted in a murine model of anaphylaxis (7). In consequence, it appears more likely that the large number of positive results obtained with CAP FEIA is the result of the detection of IgE antibodies induced by other allergens that share common epitopes with *Anisakis* allergens, as previously reported (15, 18, 29). Differentiation of *Anisakis* infections from those induced by cross-

reacting allergens is of great clinical relevance because the former can easily be prevented by avoiding eating raw or undercooked fish, even with very low levels of parasites (2, 34).

In the comparison between Ani s 1 and Ani s 7 recombinant allergens, the Ani s 7 ELISA showed the highest overall sensitivity (S = 93.94%; CI95 = 90.36 to 97.52%), with only 12/198 positive sera not being recognized by this method (Table 2), and its sensitivity increased to 98.64% if considering sera corresponding to CAP FEIA classes 3 to 6. These data highlight the reported immunodominance of this allergen (23) and suggest it to be of great clinical relevance. In contrast to Ani s 7 ELISA, the Ani s 1 ELISA detected only 61% of positive sera (S = 61.1%; CI95 = 54.07 to 68.15%), and this increased to only 66.7% when positive sera corresponding to classes 3 to 6 were tested. The low sensitivity of Ani s 1 ELISA was surprising *a priori*, because previous studies with either native (26) or recombinant (16) forms of this allergen reported detection of more than 85% of selected patients with clinically confirmed anisakiasis. It could be argued that the discrepancy may be due to the different methods used in this study for the expression and purification of the Ani s 1 allergen. In this regard, Ibarrola et al. (16) indicated that the monomeric form of rAni s 1 is preferable to oligomeric and polymeric aggregates for IgE binding. The data shown in Fig. 1 confirmed the presence of dimers, trimers, and tetramers in the Ani s 1 preparation. However, because of the excellent OD signals obtained with the Ani s 1 ELISA for most positive sera, and since all oligomers, and especially trimers, were readily recognized by positive-control serum (Fig. 1A), exclusion of Ani s 1 oligomers in Ani s 1 preparations does not appear necessary. Furthermore, the fact that two of the sera classified as negative (class 0) by the CAP FEIA were classified as positive by Ani s 1 ELISA also indicates that the Ani s 1 ELISA provides good detection of IgE. In consequence, it appears that the low sensitivity obtained for the Ani s 1 ELISA in the present study relative to that obtained in previous studies was probably due to differences in the characteristics of the patients investigated and to a bias introduced in some studies by the exclusion of patients with low levels of anti-*Anisakis* IgE antibodies (i.e., sera corresponding to classes 1 and 2). Recent studies carried out in our laboratory have shown 82% sensitivity for Ani s 1 ELISA in a transverse study with sera from nonsymptomatic subjects and with the same Ani s 1 preparation (unpublished results), which appears to confirm the above hypothesis.

Another interesting finding of this study concerns the different lengths of time that the Ani s 1 ELISA, Ani s 7 ELISA, and CAP FEIA methods are able to detect anti-*Anisakis* IgE antibodies in sera from positive patients, which appear to depend on the target antigen used. In particular, the IgE response to Ani s 1 was significantly more prolonged than the response to either Ani s 7 or the allergens included in the CAP FEIA method. This suggests that the Ani s 1 and Ani s 7 ELISAs have different capabilities with regard to detecting old (Ani s 1) and recent (Ani s 7) *Anisakis* infections and may partly explain why some sera with low levels of IgE antibodies against Ani s 1 were not recognized in the Ani s 7 ELISA. Individual variations in the decay of the anti-*Anisakis* IgE antibodies probably reflected the different ability of each patient to respond to *Anisakis* allergens but may also have been related to the number and frequency of previous *Anisakis* infections suffered by each patient.

Previous studies on the lifetime of IgE memory cells showed that some human parasites, such as filariae, induce production of specific IgE antibodies, which decrease over time in the absence of reinfection but remain detectable in serum for several years (25). A long permanency of circulating IgE antibodies is typical of *Anisakis* infections, as observed in previous studies (13) and in the follow-up of the patients in the present study (Table 3). However, unlike other nematode infections, such as filariasis and trichinellosis, in which the parasite antigens are released regularly for long periods and stored in lymphoid tissues, the Ani s 1 and Ani s 7 allergens are excretory-secretory antigens, produced only by the *Anisakis* larvae during a limited period (3). This suggests that *Anisakis* allergens are able to induce long-lived IgE-secreting plasma cells that survive and secrete IgE antibodies for long periods, as previously reported for inhaled allergens (24).

In summary, the results of the present study show that *Anisakis* infections induce long-lasting secretion of IgE antibody and suggest that laboratory determination of specific IgE antibodies against recombinant *Anisakis* Ani s 1 and Ani s 7 allergens is currently the best choice for serodiagnosis of human anisakiasis in terms of specificity and sensitivity. In addition, the different times of persistence of anti-Ani s 1 and anti-Ani s 7 IgE antibodies in serum may help clinicians to distinguish whether or not current allergic symptoms presented by a patient are related to previous infection by this nematode. Although requiring confirmation, further data obtained in our laboratory appear to indicate that sera classified as positive by the Ani s 1 ELISA and negative by the Ani s 7 ELISA frequently correspond to old *Anisakis* infections.

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