Experimental Parasitology 197 (2019) 9–15

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Comparative analysis of excretory-secretory antigens of *Anisakis simplex*, *Pseudoterranova decipiens* and *Contracaecum osculatum* regarding their applicability for specific serodiagnosis of human anisakidosis based on IgG-ELISA

Maciej Kochanowski[a], Miguel González-Muñoz[b], María Ángeles Gómez-Morales[c], Bruno Gottstein[d], Joanna Dąbrowska[a], Mirosław Różycki[a], Tomasz Cencek[a], Norbert Müller[d,*], Ghalia Boubaker[d]

[a] Department of Parasitology and Invasive Diseases, National Veterinary Research Institute, Partyzantów Avenue 57, 24-100, Puławy, Poland
[b] University Hospital La Paz-FIBHULP, Paseo Castellana, 261, 28046, Madrid, Spain
[c] Department of Infectious Diseases, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161, Rome, Italy
[d] Institute of Parasitology, Vetsuisse Faculty, University of Bern, Länggass-Strasse 122, 3012, Bern, Switzerland

ARTICLE INFO

Keywords:
ELISA
Anisakidosis
Excretory-secretory antigens
Anisakis
Pseudoterranova
Contracaecum

ABSTRACT

Serodiagnosis of human anisakidosis is presently hampered by the current lack of standardised serological assays that allow sensitive and specific detection of Anisakidae-specific antibodies in human patients. In the present study, we comparatively evaluated the diagnostic value (by IgG-ELISA) of excretory-secretory antigens (ESAgs) of *Anisakis simplex*, *Pseudoterranova decipiens* and *Contracaecum osculatum*, representing the most frequently found genera responsible for human infection. In addition, we tested also a mix of the three ES preparations (Mix-ESAgs) as well as two recombinant allergens of *A. simplex*, rAni s 1 and rAni s 7. ES antigen from *C. osculatum* yielded the best diagnostic performance in IgG-ELISA-based serodiagnosis of the Spanish anisakidosis patients investigated in this study (relative serodiagnostic sensitivity 100%; specificity 89%) as compared to *A. simplex* ES-antigen (93% versus 57%) and *P. decipiens* (67% versus 93%) or a mix of the three ES antigens (100% versus 44%), respectively. Cross-reactions of *C. osculatum* ES antigen with serum-antibodies from patients suffering from other helminth infections were rare and were exclusively found with few sera from toxocariasis, ascariasis, and filariasis patients. The two recombinant allergens rAni s 1 and rAni s 7 did not prove sufficiently sensitive and specific in order to justify a further evaluation of these antigens regarding their suitability in IgG-ELISA-based serodiagnosis of human anisakidosis. In conclusion, the *C. osculatum*-ESAg-ELISA remains as key candidate to be further assessed for the serodiagnosis of symptomatic anisakidosis in different endemic regions.

1. Introduction

Human anisakidosis is a parasitic infection of the gastrointestinal tract caused by the third-stage larvae (L_3) of anisakid nematodes from predominantly three genera of the Anisakidae family: *Anisakis, Pseudoterranova*, and *Contracaecum* (Audicana and Kennedy, 2008).

The life cycle of anisakid nematodes involves marine fish, squid and crustaceans as intermediate and/or paratenic hosts and marine mammals as definitive hosts (Pozio, 2013). Humans are accidental hosts and become infected by consuming raw or undercooked fish parasitized by anisakid L_3 larvae, most commonly of *A. simplex, A. pegreffii* and *P. decipiens*; nevertheless, three sporadic cases due to *C. osculatum* have been recorded from Germany (Schaum and Müller, 1967), Japan (Nagasawa, 2012) and Australia (Shamsi and Butcher, 2011).

Human anisakidosis is highly endemic in Japan, which accounts for up to 90% of global cases (Hochberg and Hamer, 2010). In Europe, the burden of anisakidosis has been initially estimated to 500 cases per year (Arizono et al., 2012; D'Amico et al., 2014), however, this is likely to be an underestimate according to recent reports (Bao et al., 2017).

Pathogenic effects of Anisakidae worms in humans depend on their location within or outside the gastrointestinal tract, the anisakid genus (Arizono et al., 2012) and the immune status (Audicana and Kennedy, 2008; Nieuwenhuizen, 2016). Gastric anisakidosis is the most common subtype of the disease concerning 95% of cases, whereas intestinal

* Corresponding author. Institute of Parasitology, Vetsuisse Faculty, University of Bern, Länggass-Strasse 122, 3012, Bern, Switzerland.
E-mail address: norbert.mueller@vetsuisse.unibe.ch (N. Müller).

https://doi.org/10.1016/j.exppara.2018.12.004
Received 31 July 2018; Received in revised form 22 November 2018; Accepted 20 December 2018
Available online 22 December 2018

anisakidosis accounts for the majority of the remaining (Caramello et al., 2003). Anisakidosis cases of extra-gastrointestinal localization are extremely rare (Hochberg and Hamer, 2010; Ramanan et al., 2013).

Allergic reactions are common in humans upon exposure to *A. simplex* or *A. pegreffii* (Anadón et al., 2009; Mattiucci et al., 2013), however it remains yet unknown whether or not *Pseudoterranova* spp. and *Contracaecum* spp. can cause allergies in humans as well (Buchmann and Mehrdana, 2016).

After ingestion of infected fish, incubation periods can vary from hours (2–12 h) in gastric anisakidosis to days (2–5 days) in intestinal infection (Ishikura et al., 1993; Takabayashi et al., 2014). Acute and severe epigastric pain is the typical symptom of gastric anisakidosis, while clinical manifestation of intestinal anisakidosis is mostly unspecific, which makes an accurate diagnosis difficult (Miura et al., 2010; Shimamura et al., 2016; Tsukui et al., 2016).

Diagnosis of anisakidosis relies mainly on clinical features and a history of consuming seafood in the preceding hours or days (Zullo et al., 2010). Endoscopic visualization of L_3 larvae is the unique available approach to prove gastric and/or colon anisakidosis (Yoon et al., 2004). Computed tomography scan of the abdomen showing typical findings such as segmental edema of the intestinal wall and ascites (Shimamura et al., 2016) can be applied to detect intestinal infection. Nevertheless, intestinal anisakidosis remains the most commonly misdiagnosed subtype of the disease; indeed it is frequently confused with bowel obstruction, inflammatory bowel disease or appendicitis (Chikamori et al., 2004; Yasunaga et al., 2010). Molecular tools are only applied as confirmatory test or for genetic identification of the parasite once Anisakidae larvae are removed (Lim et al., 2015).

To date, serological testing is applied almost exclusively in diagnosis of allergic anisakidosis, thereby analyses of total and Anisakidae-specific IgE antibody levels are an integral part of the immunodiagnostic approach (Baeza et al., 2004; Shimamura et al., 2016). Several allergens have been characterised from *A. simplex* (Fæste et al., 2014), with Ani s 1 and Ani s 7 being major allergens found in the excretory-secretory (ES) products (Anadón et al., 2010). Nonetheless, confirmation of anisakidosis cases only based on testing of total serum IgE level and presence of Anisakidae-specific IgE antibodies is very challenging since respective immunoassays are often questioned for their diagnostic reliability. Specificity of parasite-specific IgE-tests is often low because numerous helminths, other than the anisakid nematodes stimulate IgE synthesis; furthermore IgE responses induced by environmental, food or helminth allergens share common immunological properties (Yman, 2000). In comparison to other immunoglobulin classes, in particular IgG, half-life of IgE antibodies is extremely short (2–3 days), which in turn significantly affects sensitivity and reproducibility of IgE-tests (Lawrence et al., 2017). In mice, a low anti-Ani s 7 IgE antibody response was induced subsequent to intraperitoneal challenge of mice with alive but not dead Anisakidae L_3 larvae, this reaction peaked on day thirty post infection and declined rapidly (Anadón et al., 2009).

Overall, current serodiagnosis of human parasitoses mostly relies on parasite-specific IgG detection assays where the antigen is one of the main factors that has the highest influence on both test sensitivity and specificity. To date, the necessity for a reliable Anisakidae-specific IgG detection assay is mainly based on the need of avoiding unnecessary surgery resulting from preoperative misdiagnosis of the aetiology of the gastrointestinal pain.

So far, to our knowledge, no study has been done on the comparative assessment of the IgG responses in anisakidosis patients against crude or ES antigens from different Anisakidae parasites.

2. Material and methods

2.1. Ethics statement

Rabbits purchased from the Center for Experimental Medicine (Katowice, Poland) were housed under standard conditions and experiments were conducted under the approval of the Local Ethical Commission for Animal Experimentation (licence no: 66/2012).

Concerning sera from Spanish patients, informed consent was obtained in all cases with approval of ethical committee (University Hospital La Paz-FIBHULP, Madrid, Spain; Carballeda-Sangiao et al., 2016).

The other human sera tested in this study were taken from a pre-existing biological collection at the Institute of Parasitology in Bern (University of Bern, Switzerland). These sera have been previously used in similar studies (serodiagnosis of human parasitoses) which have, respectively, been ethically approved by local committees (Pfister et al., 1999; Müller et al., 2007; Gottstein et al., 2014).

2.2. Anisakidae antigens

2.2.1. Anisakidae L_3 larvae collection and preparation of crude and ES antigens

Encapsulated or free L_3 larvae of *A. simplex* were collected from the abdominal cavity walls and from visceral organs of Atlantic mackerel (*Scomber scombrus*). Encapsulated *P. decipiens* and *C. osculatum* L_3 larvae were obtained from fillets of Atlantic cod (*Gadus morhua*) and from livers of Baltic cod (*Gadus morhua callarias*), respectively. Anisakidae larvae were purified by successive washes with sterile 0.01 M phosphate-buffered saline solution (PBS, pH 7.4). Anisakidae parasites were then genetically analysed for species identification by using PCR-restriction fragment length polymorphism (PCR-RFLP) (Zhu et al., 1998).

For *A. simplex* and *C. osculatum*, ES antigens were prepared by incubation of viable L_3 larvae in sterile PBS (~200 larvae, equivalent to ~0.6 g in 7 ml PBS). For *P. decipiens*, approximately 100 larvae (equivalent to ~2.5 g) were cultured under sterile conditions in 20 ml sterile PBS. After completion of the incubation period (12 h at 37 °C), larvae were removed. The media containing ES products were collected and centrifuged at 20′000 g for 15 min at 4 °C. Supernatants were subjected to ultrafiltration using polyether sulfone membrane of 3 K molecular weight cut-off at 4 °C (Thermo Fisher Scientific, Rockford, IL, U.S.A.).

Crude extracts of *A. simplex*, *C. osculatum* and *P. decipiens* were prepared as follows: ~0.6 g of larvae which have been previously collected from culture media were homogenized with 10 ml of sterile PBS in a small mortar. Afterwards, lysates were transferred to 15 ml Falcon tubes, sonicated in ice (10 μm amplitude for 30 s) and incubated for 1 h at 4 °C. Supernatants were collected after centrifuging at 20′000 g for 15 min at 4 °C.

To avoid proteolytic degradation, a protease inhibitor cocktail (Sigma, St. Louis, MO, U.S.A.) was added (100U/ml) to ES and crude extracts. Protein concentrations were determined using Bradford assay (BioRad, Hercules, CA, U.S.A.) and antigens were stored at −20 °C until use.

2.2.2. Cloning, expression and purification of recombinant Ani s1 and rAni s7

The truncated forms of the two *A. simplex* allergens: Ani s 1 and 7 (Ibarrola et al., 2008; Rodríguez et al., 2008) were recombinantly expressed according to our previously described protocol (Boubaker et al., 2014).

2.3. Sera

2.3.1. Rabbit sera

Rabbits were immunised by intramuscular injection of 2.0 mg of *A. simplex*-ESAg emulsified with Freund's complete adjuvant (Sigma, St Louis, MO, U.S.A.), followed by three booster injections at days 14, 28, and 56. Hyperimmune sera were obtained two weeks after the last injection. Preimmune serum was taken before immunisation and used as negative control.

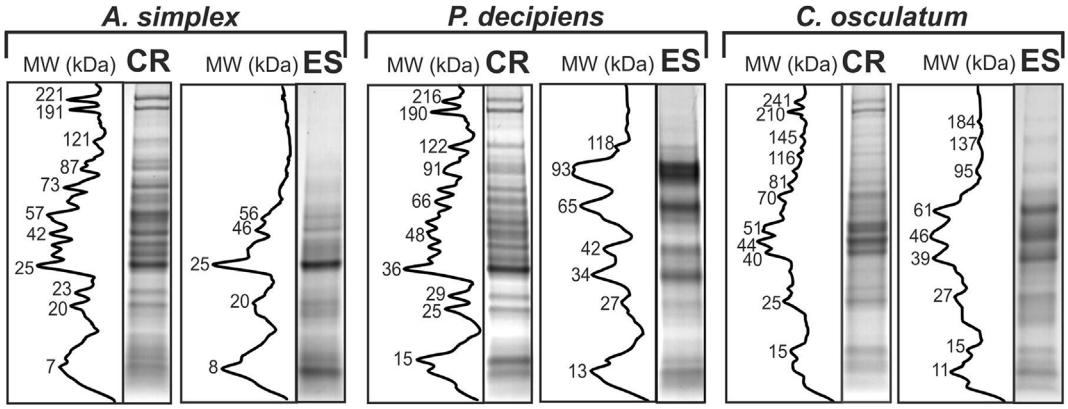

Fig. 1. Colloidal Coomassie-stained 4–15% SDS-PAGE analysis of crude (CR) and excretory-secretory (ES) antigens prepared from *A. simplex*, *P. decipiens* and *C. osculatum*. Densitometric analysis and molecular weights (MW) quantification presented in kilodaltons (kDa) on the SDS-PAGE gels performed by Bio-1D software.

2.3.2. Human sera

- *Healthy blood donors*: 18 serum samples of healthy blood donors were used to determine cut-off values.
- *Sera from patients exposed to anisakids:* 15 serum samples were obtained from Spanish patients with symptoms of anisakidosis and having Anisakidae-specific IgE antibodies. More detailed information on the patients is given in Supplementary Table S1.
- *Sera from patients with other parasitoses:* to assess the degree of cross-reactivity of different ELISAs regarding other parasitic diseases, a total of 61 serum samples were collected from patients with 13 different parasitoses including cystic echinococcosis; alveolar echinococcosis; cysticercosis; filariasis; strongyloidiasis; ascariasis; toxocariasis; trichinellosis; fasciolosis; schistosomiasis; amoebiasis; leishmaniasis and malaria.

2.4. SDS–PAGE and Western blot (WB)

Crude and ES antigens from *A. simplex*, *P. decipiens* and *C. osculatum* were analysed by 4–15% gradient SDS-PAGE (BioRad, Hercules, CA, U.S.A.) under reducing conditions (Laemmli, 1970) and gels were stained with colloidal Coomassie G-250 (PageBlue, Thermo Fisher Scientific, Rockford, IL, U.S.A.).

For WB analyses, ES antigens from the 3 species were first separated by 4–15% gradient SDS-PAGE and then transferred onto a 0.45 µm nitrocellulose membrane (Amersham Protran, GE Healthcare, Glattbrugg, Switzerland). Three nitrocellulose strips loaded with different ES were separately incubated with rabbit anti-*A. simplex*-ESAg hyperimmune serum diluted 1:500. Three additional strips were incubated with pre-immune rabbit serum. The anti-rabbit IgG antibody conjugated to alkaline phosphatase (Sigma, Baar, Switzerland) was used as secondary antibody.

SDS-PAGE and WB profiles were examined based on quantitative densitometric analysis using Bio-1D software (ver. 15.07, Vilber Lourmat, Marne-la-Vallée, France).

2.5. ELISA

Diagnostic performances of the three ES antigens and the two recombinant allergens were assessed separately by ELISA. In addition, we also evaluate the mixture (1:1:1 by protein content) of the three ES antigens (Mix-ESAgs).

ELISAs were basically performed as described earlier (Gottstein et al., 1983; Müller et al., 1989). Briefly, 96-well plates were coated for overnight at 4 °C with different antigens diluted into 0.05 M carbonate buffer, pH 9.6. The optimal concentration of each antigen was established by checker-board titration and was as follow: 9 µg/ml for *A. simplex* ES, 1.6 µg/ml for *P. decipiens* ES, 0.85 µg/ml for *C. osculatum* ES; for rAni s 1 and rAni s 7, a concentration of 1 µg/ml was used, respectively. Mix-ESAgs was prepared using the same concentration as in individual assays. The sera were diluted 1:100 and the anti-human-IgG-alkaline phosphatase conjugate (Sigma, Baar, Switzerland) was diluted 1:800. Absorbance values (A_{405nm}) were measured using a Magellan plate reader (Tecan, Männedorf, Switzerland).

2.6. Statistical analyses

The receiver operator characteristic (ROC) curve analyses were performed to determine optimal cut-off values for different ELISAs (Supplementary Table S2) using NCSS software (ver. 11.0.13, NCSS, LLC. Kaysville, Utah, U.S.A., ncss.com/software/ncss). For each ELISA, the cut-off value was adjusted in order to achieve an optimal sensitivity by testing 15 sera from anisakidosis patients without losing the maximal specificity (100%) by testing 18 blood donor sera.

For each ELISA, the distribution of $A_{405\ nm}$ values in the different groups (see section 2.3.2) of sera tested in the each ELISA was displayed on a dot plots graph.

3. Results

3.1. SDS–PAGE and WB analyses of ES and crude antigens from *A. simplex*, *P. decipiens* and *C. osculatum*

The SDS-PAGE profiles of ES and crude antigens from *A. simplex*, *P. decipiens* and *C. osculatum* are shown in Fig. 1. For both ES and crude antigens, protein bands were in the 7–241 kDa range. Multiple banding patterns of *A. simplex* and *P. decipiens* crude extracts were similar, whereas *C. osculatum* crude antigen was slightly different and characterised by smaller number of major bands located in the range of 40–51 kDa. Conversely, patterns of ES were clearly distinct between *A. simplex*, *P. decipiens* and *C. osculatum*.

In WB, the rabbit IgG polyclonal antibodies raised against *A. simplex* ES reacted in a distinctive manner with all three ES antigens (Fig. 2). While ES antigen from *A. simplex* and *P. decipiens* have similar reaction patterns; the pattern of *C. osculatum* ES antigen was distinct and basically characterised by two major bands corresponding to proteins with approximate sizes of 230 kDa and 15 kDa, respectively.

3.2. Diagnostic sensitivity of the IgG-ELISAs

Preliminary IgG-ELISA-based evaluations of crude antigens using selected samples from the above-described panel of anisakidosis and non-anisakidosis patients' sera (see section 2.3.2) revealed that these antigens did not fulfill the diagnostic criteria particularly as far as the

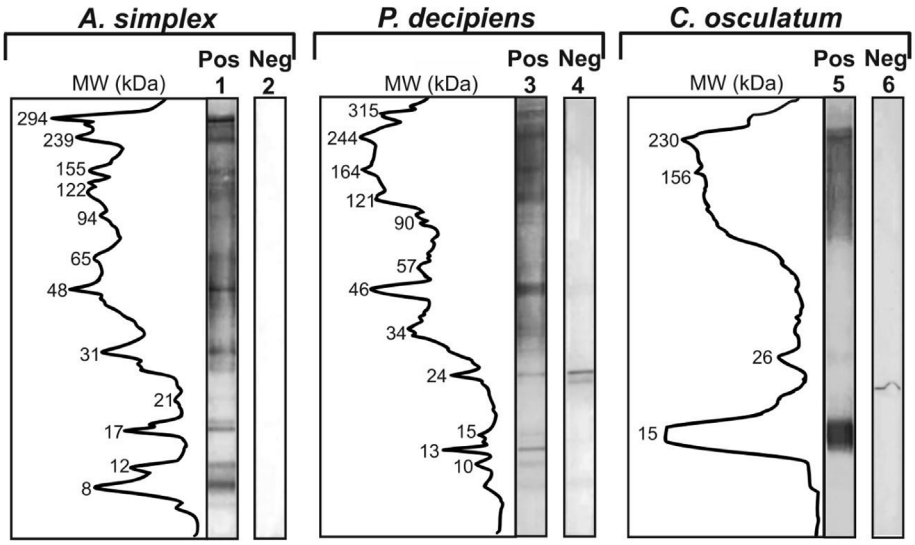

Fig. 2. Reactivity of anti-*A. simplex* ES rabbit IgG antibodies to ES antigens from *A. simplex*, *P. decipiens* and *C. osculatum* analysed by Western blot; with its densitometric analysis and molecular weights (MW) quantification presented in kilodaltons (kDa) (Bio-1D software).
Nitrocellulose strips contained ES of *A. simplex* (strip 1 and 2), ES of *P. decipiens* (strip 3 and 4) and ES of *C. osculatum* ES (strip 5 and 6). One strip from each ES antigen (strips 1, 3 and 5) was incubated with hyperimmune serum from rabbit immunised with *A. simplex* ESAg (Pos), whereas strips 2, 4 and 6 were incubated with rabbit preimmune serum (Neg).

specificity of the analyses are concerned (data not shown). Accordingly, we decided to concentrate our assessment of the diagnostic performance on those ELISAs that contained individual preparations, or a mix, of ES antigens and on recombinant antigens rAni s 1 and 7.

Respective ELISAs were named as follow: *A. simplex*-ESAg-ELISA, *P. decipiens*-ESAg-ELISA, *C. osculatum*-ESAg-ELISA, Mix-ESAgs-ELISA, rAni s 1-ELISA and rAni s 7-ELISA. For each ELISA, the diagnostic cut-off $A_{405\ nm}$ values were calculated based on ROC analysis and are listed in Supplementary Table S2.

In order to assess the diagnostic sensitivity of the six ELISAs listed above; we tested 15 sera from Spanish patients with clinically diagnosed allergic anisakidosis (Table 1). Detailed results demonstrating the immunoreactivity of serum IgG in each of the six tests is presented in Supplementary Table S3.

Both *C. osculatum*-ESAg-ELISA and Mix-ESAgs-ELISA reached a sensitivity of 100%, while *A. simplex*-ESAg-ELISA and *P. decipiens*-ESAg-ELISA displayed a sensitivity of 93% and 67%, respectively. Ten out of 15 cases of anisakidosis were positive in all three ELISAs based on ES antigen from *A. simplex*, *P. decipiens* and *C. osculatum*, respectively (Supplementary Table S3); $A_{405\ nm}$ values of 9 sera out of these 10 were higher in *C. osculatum*-ESAg-ELISA than in *A. simplex*-ESAg-ELISA or *P. decipiens*-ESAg-ELISA (Supplementary Table S3 and Fig. 3). The lowest sensitivities of 53% and 60% were obtained for the rAni s 1-ELISA and rAni s 7-ELISA, respectively (Table 1).

3.3. Specificity of the IgG-ELISAs

Specificity of the six IgG-ELISAs for serodiagnosis of human anisakidosis was investigated by testing 61 sera from patients with other parasitoses (caused by cestodes, nematodes, trematodes and protozoan parasites).

Among the ES antigens tested, Mix-ESAgs and *A. simplex* ESAg displayed the lowest specificities of 44% and 57%, respectively (Table 2). The *P. decipiens*-ESAg-ELISA provided the highest specificity of 93%, immediately followed by the *C. osculatum*-ESAg-ELISA exhibiting a 89% specificity.

As shown in Table 2, sera from individuals suffering from cystic echinococcosis, alveolar echinococcosis, strongyloidiasis and ascariasis, represented the major source of cross-reactions in the *A. simplex*-ESAg-ELISA. In contrast, no cross-reactivity with the tested cestode infections (cystic echinococcosis, alveolar echinococcosis and cysticercosis) was detected with ES antigen from *P. decipiens* or from *C. osculatum*, respectively.

For the *C. osculatum* ESAg-ELISA, a high degree of cross-reactivity was particularly observed with sera from toxocariasis patients (Table 2).

Specificities of the rAni s 1-ELISA and the rAni s 7-ELISA were 89% and 67%, respectively (Table 2). All sera that were cross-reacting with rAni s 1 exhibited only a borderline positivity (Supplementary Table S4 and Fig. 3). In contrast, 5 out of the 20 cross-reactions observed in rAni s 7-ELISA were characterised by high $A_{405\ nm}$ values ranging between approximately 3 to 4 times the cut-off $A_{405\ nm}$ value (Supplementary Table S4).

All $A_{405\ nm}$ values from the IgG-ELISAs are listed in Supplementary Table S3, Supplementary Table S4 and a dot plot graph illustrating the distribution of these values is shown in Fig. 3.

4. Discussion

Although human anisakidosis has been identified long time ago as a medical problem of global importance, the current diagnostic scheme of this disease still includes an urgent need for a standardised serological test, in particular an IgG-ELISA. Such an ELISA is essential in view of a targeted serodiagnosis to specifically detect anisakidosis in an individual patient but also as a screening test within an analytical spectrum suitable for diversified serological examinations of helminth infections in humans. Our present study therefore was aimed at the development of an Anisakidae-specific IgG-ELISA. For this purpose, we simultaneously assessed and compared the diagnostic performance of different ELISAs containing ES antigens from *A. simplex*, *P. decipiens* and *C. osculatum* which were chosen as representative species from the three genera of the Anisakidae family infectious for humans.

Pretesting of the ES antigens in Western blots using rabbit anti-*A. simplex*-ESAg hyperimmune serum revealed similarities in the

Table 1
Sensitivity testing of the six ELISAs with sera from patients suffering from anisakidosis.

Antigen used in the ELISA	*A. simplex*- ES	*P. decipiens*- ES	*C. osculatum*-ES	Mix- ESAgs	rAni s 1	rAni s 7
N° of positive sera (n = 15)	14	10	15	15	8	9
Sensitivity (in percent)	93.33	66.67	100	100	53.33	60

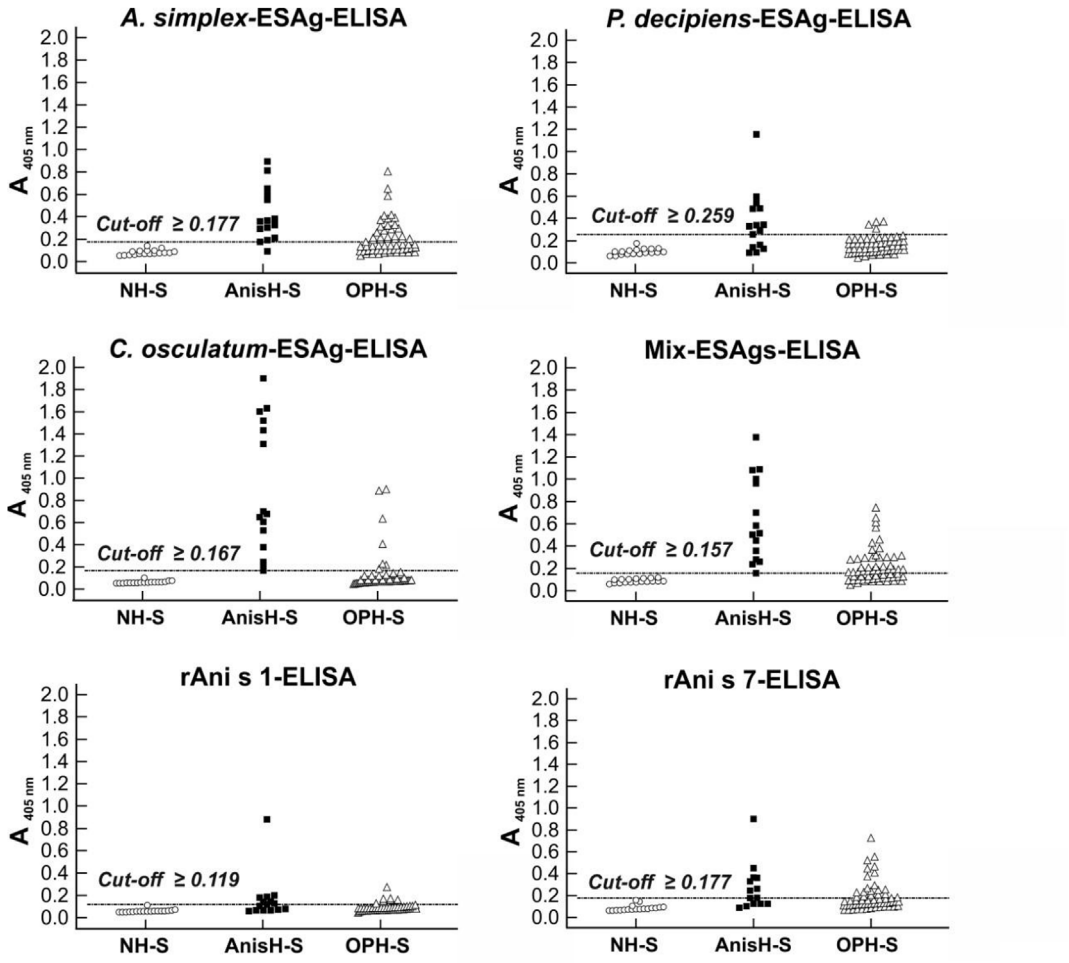

Fig. 3. Dot plot graph illustrating the distribution of $A_{405\ nm}$ values of all tested human sera in the six ELISAs. Sera were divided in 4 groups: healthy human sera (NH [HYPHEN]S), reference sera from anisakidosis patients (AnisH[HYPHEN]S), sera from patients with other parasitoses (OPH[HYPHEN]S

Table 2
Specificity testing of the six ELISAs with sera from patients suffering from parasitic infections other than anisakidosis.

Antigen used in the ELISA		*A. simplex*- ES	*P. decipiens*- ES	*C. osculatum*-ES	Mix- ESAgs	rAni s 1	rAni s 7
Disease	[a] N° Total	N° of positive sera	N° of positive sera	N° of positive sera	N° of positive sera	N° of positivesera	N° of positivesera
Type of infection/disease							
Cestodes							
Cystic echinococcosis	5	5	0	0	5	0	0
Alveolar echinococcosis	5	5	0	0	4	0	0
Cysticercosis	2	1	0	0	1	1	1
Nematodes							
Filariasis	4	2	1	1	4	3	1
Strongyloidiasis	5	4	2	0	5	0	3
Ascariasis	5	4	0	2	4	1	3
Toxocariasis	5	2	0	4	4	0	1
Trichinellosis	5	0	0	0	0	0	2
Trematodes							
Fasciolosis	5	0	0	0	2	0	0
Schistosomiasis	5	0	0	0	1	0	3
Protozoa							
Amoebiasis	5	2	1	0	3	1	2
Leishmaniasis	5	1	0	0	1	1	3
Malaria	5	0	0	0	0	0	1
N° of positive sera (n = 61)		26	4	7	34	7	20
Specificity (in percent)		**57.38**	**93.44**	**88.52**	**44.26**	**88.52**	**67.21**

[a] Total number of tested sera representing individual diseases.

immunoreactivity banding patterns for *A. simplex* and *P. decipiens* (see Fig. 2). *C. osculatum* ES antigen also cross-reacted with anti-*A. simplex*-ESAg antibodies, however, the respective banding pattern was clearly distinct. This observation is in line with molecular phylogeny inferred from mitochondrial DNA sequences where *Anisakis* spp. and *Pseudoterranova* spp. were clustered in the same clade, while *Contracaecum* spp. appeared in a separate clade (Nadler and Hudspeth, 2000; Nadler et al., 2005). Despite this apparent distinctness, however, ES antigen from *C. osculatum* surprisingly best met the criteria relevant for a reliable serodiagnosis of human anisakidosis. In this respect, we consider the following findings as most important: (i) as demonstrated in the Western blot incubated with anti-*A. simplex*-ESAg hyperimmune serum, *C. osculatum* ES antigen contain strong antigenic epitopes that seem to be shared between different Anisakidae species (see Fig. 2). (ii) Comparatively, the *C. osculatum* ES antigen exhibited the most balanced ratio between serodiagnostic sensitivity and specificity in our IgG-ELISA (see Tables 1 and 2). (iii) Cross-reactions of *C. osculatum* ES antigen with sera from patients suffering from other helminth infections were restricted to a few sera from toxocariasis, ascariasis, and filariasis patients (see Table 2). To a certain extent, false positive cases due to such antibody cross-reactions can be discriminated from real anisakidosis cases by including complementary serological testings, i.e. ELISAs for serodiagnosis of toxocariasis, ascariasis, and filariasis, respectively. Furthermore, the interpretation of the results from the *C. osculatum*-ESAg-ELISA may be facilitated by considering anamnestic information about the medical history of the patient associated with a possible consumption of fish/seafood. Finally, confirmation of allergic reactions possibly associated with production of Anisakidae-specific IgE antibodies to be determined by previously described methods (Anadón et al., 2010; Carballeda-Sangiao et al., 2016; Mattiucci et al., 2017) may provide complementary information in order to verify the results obtained from the *C. osculatum*-ESAg-ELISA. In summary, we expect that the combination of the entire serological and anamnestic data listed above will provide sufficient information allowing reliable diagnosis of anisakidosis in human patients.

Basically, the use of a mix of different antigen preparations can be a valuable strategy to improve the sensitivity of an ELISA-based serology as e.g. demonstrated for serodiagnosis of human alveolar echinococcosis (Gottstein et al., 1993). In our study however, this strategy turned out to be not recommendable mainly because the exclusive use of *C. osculatum* ES antigen as antigenic agent in ELISAs already allowed serodiagnosis of human anisakidosis with a maximal sensitivity (100%). Furthermore, the mix of all three ES antigen preparations exhibited a much lower specificity (44%) as compared to the ES products from *C. osculatum* alone (89%).

In our study, we also investigated previously described recombinant allergens Ani s 1 and Ani s 7 from *A. simplex* (Ibarrola et al., 2008; Rodríguez et al., 2008) regarding their suitability in IgG-ELISA-based serodiagnosis of human anisakidosis. We decided to test these allergens because they had demonstrated a relatively high sensitivity in IgE-ELISA-based diagnosis of cases of allergic anisakidosis (Moneo et al., 2000; Rodríguez et al., 2008; Anadón et al., 2010; Carballeda-Sangiao et al., 2016). However, in the present study, these antigens proved to be insufficiently sensitive to justify their serodiagnostic application on an IgG-ELISA basis.

In conclusion, our study showed that, based on serum IgG detection, firstly ES antigens from different Anisakidae species performed basically better than recombinant *A. simplex* allergens rAni s 1 and 7. Secondly, among the three ES antigens tested, *C. osculatum* yielded the best diagnostic performance regarding optimised diagnostic sensitivity and specificity. Nevertheless, since the present study included only a limited number of sera from human anisakidosis patients, and since this patients' cluster covered only a distinct geographical region (Spain), we consider our study as an explorative step towards the identification, evaluation and preliminary validation of antigens which should prospectively be further evaluated for the serodiagnosis of human anisakidosis, with a special focus on the *C. osculatum* ES antigen. From current literature it can be concluded that, for many geographic areas, symptomatic anisakidosis is frequently caused by *A. simplex* as e.g. reported in Japan (Umehara et al., 2007) and also in Spain (Repiso Ortega et al., 2003). Referring to the report from Spain (Repiso Ortega et al., 2003), we assume that our Spanish panel of *C. osculatum*-ESAg-ELISA-positive sera most likely originated from patients with an *A. simplex* infection. Thus, we assume that the *C. osculatum*-ESAg-ELISA allows a reliable diagnosis of *A. simplex* infections, but not necessarily of infections with the closely related *Pseudoterranova*, and *Contracaecum* species. Accordingly, extended serological examinations of cases with a diversified underlying aetiology determined on the Anisakidae species level will have to clarify if the diagnostic potential of the *C. osculatum*-ESAg-ELISA covers the entire spectrum of symptomatic anisakidosis in humans. Furthermore, future studies will have to address the question if recombinant analogues from predominant 230 kDa and 15 kDa C. *osculatum*-ES antigens (see Western blot in Fig. 2) will be suited for anisakidosis serology. In preliminary studies, particularly the 15 kDa antigen consistently exhibited a strong immunoreactivity with sera from patients with suspected anisakoidosis (data not shown). However, such an approach involving recombinant antigen production will be only realistic if the antigenicity of the protein is determined by peptide structures instead of sugar residues often representing covalently linked antigenic components of nematode ES proteins (Maizels et al., 1987; Dell et al., 1999). Furthermore, such an approach will also rely on a sophisticated mass spectrometrical determination of the amino acid sequence from the 15 kDa antigen to obtain the genetic information as a basis for recombinant production of this protein.

Conflicts of interest

The authors declare that there is no conflict of interest.

Acknowledgments

We would like to thank Bernadette Merkle, Ursula Kurath, and Xuan Lan Vu for their excellent technical assistance. Furthermore, we thank Joachim Müller for his valuable suggestions concerning our experimental approaches. This research was partially supported by a grant from the National Center for Research and Development under the Strategic Program Biostrateg (grant no. BIOSTRATEG2/296211/4/NCBR/2016). This paper is part of Maciej Kochanowski's Ph.D. dissertation (National Veterinary Research Institute, Puławy, Poland).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exppara.2018.12.004.

References

Anadón, A.M., Romarís, F., Escalante, M., Rodríguez, E., Gárate, T., Cuéllar, C., Ubeira, F.M., 2009. The *Anisakis simplex* Ani s 7 major allergen as an indicator of true *Anisakis* infections. Clin. Exp. Immunol. 156, 471–478.

Anadón, A.M., Rodríguez, E., Gárate, M.T., Cuéllar, C., Romarís, F., Chivato, T., Rodero, M., González-Díaz, H., Ubeira, F.M., 2010. Diagnosing human anisakiasis: recombinant Ani s 1 and Ani s 7 allergens versus the UniCAP 100 fluorescence enzyme immunoassay. Clin. Vaccine Immunol. 17, 496–502.

Arizono, N., Yamada, M., Tegoshi, T., Yoshikawa, M., 2012. *Anisakis simplex sensu stricto* and *Anisakis pegreffii*: biological characteristics and pathogenetic potential in human anisakiasis. Foodb. Pathog. Dis. 9, 517–521.

Audicana, M.T., Kennedy, M.W., 2008. *Anisakis simplex*: from obscure infectious worm to inducer of immune hypersensitivity. Clin. Microbiol. Rev. 21, 360–379.

Baeza, M.L., Rodríguez, A., Matheu, V., Rubio, M., Tornero, P., De Barrio, M., Herrero, T., Santaolalla, M., Zubeldia, J.M., 2004. Characterization of allergens secreted by *Anisakis simplex* parasite: clinical relevance in comparison with somatic allergens. Clin. Exp. Allergy 34, 296–302.

Bao, M., Pierce, G.J., Pascual, S., González-Muñoz, M., Mattiucci, S., Mladineo, I., Cipriani, P., Bušelić, I., Strachan, N.J.C., 2017. Assessing the risk of an emerging zoonosis of worldwide concern: anisakiasis. Sci. Rep. 7, 43699.

Boubaker, G., Gottstein, B., Hemphill, A., Babba, H., Spiliotis, M., 2014. *Echinococcus* P29 antigen: molecular characterization and implication on post-surgery follow-up of CE patients infected with different species of the *Echinococcus granulosus* complex. PLoS One 9, e98357.

Buchmann, K., Mehrdana, F., 2016. Effects of anisakid nematodes *Anisakis simplex* (*s.l.*), *Pseudoterranova decipiens* (*s.l.*) and *Contracaecum osculatum* (*s.l.*) on fish and consumer health. Food Wat. Parasitol. 4, 13–22.

Caramello, P., Vitali, A., Canta, F., Caldana, A., Santi, F., Caputo, A., Lipani, F., Balbiano, R., 2003. Intestinal localization of anisakiasis manifested as acute abdomen. Clin. Microbiol. Infect. 9, 734–737.

Carballeda-Sangiao, N., Rodríguez-Mahillo, A.I., Careche, M., Navas, A., Moneo, I., González-Muñoz, M., 2016. Changes over time in IgE sensitization to allergens of the fish parasite *Anisakis* spp. PLoS Neglected Trop. Dis. 10, e0004864.

Chikamori, F., Kuniyoshi, N., Takase, Y., 2004. Intussusception due to intestinal anisakiasis: a case report. Abdom. Imag. 29, 39–41.

D'Amico, P., Malandra, R., Costanzo, F., Castigliego, L., Guidi, A., Gianfaldoni, D., Armani, A., 2014. Evolution of the *Anisakis* risk management in the European and Italian context. Food Res. Int. 64, 348–362.

Dell, A., Haslam, S.M., Morris, H.R., Khoo, K.H., 1999. Immunogenic glycoconjugates implicated in parasitic nematode diseases. Biochim. Biophys. Acta 455, 353–362.

Fæste, C.K., Jonscher, K.R., Dooper, M.M.W.B., Egge-Jacobsen, W., Moen, A., Daschner, A., Egaas, E., Christians, U., 2014. Characterisation of potential novel allergens in the fish parasite *Anisakis simplex*. EuPA Open Proteom 4, 140–155.

Gottstein, B., Eckert, J., Fey, H., 1983. Serological differentiation between *Echinococcus granulosus* and *E. multilocularis* infections in man. Z. für Parasitenkd. 69, 347–356.

Gottstein, B., Jacquier, P., Bresson-Hadni, S., Eckert, J., 1993. Improved primary immunodiagnosis of alveolar echinococcosis in humans by an enzyme-linked immunosorbent assay using the Em2plus antigen. J. Clin. Microbiol. 31, 373–376.

Gottstein, B., Schneeberger, M., Boubaker, G., Merkle, B., Huber, C., Spiliotis, M., Müller, N., Garate, T., Doherr, M.G., 2014. Comparative assessment of ELISAs using recombinant saposin-like protein 2 and recombinant cathepsin L-1 from *Fasciola hepatica* for the serodiagnosis of human fasciolosis. PLoS Neglected Trop. Dis. 8, e2860.

Hochberg, N.S., Hamer, D.H., 2010. Anisakidosis: perils of the deep. Clin. Infect. Dis. 51, 806–812.

Ibarrola, I., Arilla, M.C., Herrero, M.D., Esteban, M.I., Martínez, A., Asturias, J.A., 2008. Expression of a recombinant protein immunochemically equivalent to the major *Anisakis simplex* allergen Ani s 1. J Investig. Allergol. Clin. Immunol. 18, 78.

Ishikura, H., Kikuchi, K., Nagasawa, K., Ooiwa, T., Takamiya, H., Sato, N., Sugane, K., 1993. Anisakidae and anisakidosis. Prog. Clin. Parasitol. 3, 43–102.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lawrence, M.G., Woodfolk, J.A., Schuyler, A.J., Stillman, L.C., Chapman, M.D., Platts-Mills, T.A.E., 2017. Half-life of IgE in serum and skin: consequences for anti-IgE therapy in patients with allergic disease. J. Allergy Clin. Immunol. 139, 422–428. e4. https://doi.org/10.1016/j.jaci.2016.04.056.

Lim, H., Jung, B.-K., Cho, J., Yooyen, T., Shin, E.-H., Chai, J.-Y., 2015. Molecular diagnosis of cause of anisakiasis in humans, South Korea. Emerg. Infect. Dis. 21, 342–344.

Maizels, R.M., Kennedy, M.W., Meghji, M., Robertson, B.D., Smith, H.V., 1987. Shared carbohydrate epitopes on distinct surface and secreted antigens of the parasitic nematode *Toxocara canis*. J. Immunol. 139, 207–214.

Mattiucci, S., Fazii, P., De Rosa, A., Paoletti, M., Megna, A.S., Glielmo, A., De Angelis, M., Costa, A., Meucci, C., Calvaruso, V., Sorrentini, I., Palma, G., Bruschi, F., Nascetti, G., 2013. Anisakiasis and gastroallergic reactions associated with *Anisakis pegreffii* infection. Italy. Emerging Infect. Dis. 19, 496–499.

Mattiucci, S., Colantoni, A., Crisafi, B., Mori-Ubaldini, F., Caponi, L., Fazii, P., Nascetti, G., Bruschi, F., 2017. IgE sensitization to *Anisakis pegreffii* in Italy: comparison of two methods for the diagnosis of allergic anisakiasis. Parasite Immunol. 39, e12440.

Miura, Tomofumi, Iwaya, A., Shimizu, T., Tsuchiya, J., Nakamura, J., Yamada, S., Miura, Tsutomu, Yanagi, M., Usuda, H., Emura, I., 2010. Intestinal anisakiasis can cause intussusception in adults: an extremely rare condition. World J. Gastroenterol. 16, 1804–1807.

Moneo, I., Caballero, M.L., Gómez, F., Ortega, E., Alonso, M.J., 2000. Isolation and characterization of a major allergen from the fish parasite *Anisakis simplex*. J. Allergy Clin. Immunol. 106, 177–182.

Müller, N., Gottstein, B., Vogel, M., Flury, K., Seebeck, T., 1989. Application of a recombinant *Echinococcus multilocularis* antigen in an enzyme-linked immunosorbent assay for immunodiagnosis of human alveolar echinococcosis. Mol. Biochem. Parasitol. 36, 151–159.

Müller, N., Frei, E., Nunez, S., Gottstein, B., 2007. Improved serodiagnosis of alveolar echinococcosis of humans using an in vitro-produced *Echinococcus multilocularis* antigen. Parasitology 134, 879–888.

Nadler, S.A., Hudspeth, D.S., 2000. Phylogeny of the Ascaridoidea (Nematoda: ascaridida) based on three genes and morphology: hypotheses of structural and sequence evolution. J. Parasitol. 86, 380–393.

Nadler, S.A., D'Amelio, S., Dailey, M.D., Paggi, L., Siu, S., Sakanari, J.A., 2005. Molecular phylogenetics and diagnosis of *Anisakis, Pseudoterranova*, and *Contracaecum* from northern Pacific marine mammals. J. Parasitol. 91, 1413–1429.

Nagasawa, K., 2012. The biology of *Contracaecum osculatum sensu lato* and *C. osculatum* A (Nematoda: Anisakidae) in Japanese waters: a review. Biosphere Sci 51, 61–69.

Nieuwenhuizen, N.E., 2016. *Anisakis* - immunology of a foodborne parasitosis. Parasite Immunol. 38, 548–557.

Pfister, M., Gottstein, B., Cerny, T., Cerny, A., 1999. Immunodiagnosis of echinococcosis in cancer patients. Clin. Microbiol. Infect. 5, 693–697.

Pozio, E., 2013. Integrating animal health surveillance and food safety: the example of *Anisakis*. Rev. Sci. Tech. 32, 487–496.

Ramanan, P., Blumberg, A.K., Mathison, B., Pritt, B.S., 2013. Parametrial anisakidosis. J. Clin. Microbiol. 51, 3430–3434.

Repiso Ortega, A., Alcántara Torres, M., González de Frutos, C., de Artaza Varasa, T., Rodríguez Merlo, R., Valle Muñoz, J., Martínez Potenciano, J.L., 2003. Gastrointestinal anisakiasis. Study of a series of 25 patients. Gastroenterol. Hepatol. 26, 341–346.

Rodríguez, E., Anadón, A.M., García-Bodas, E., Romarís, F., Iglesias, R., Gárate, T., Ubeira, F.M., 2008. Novel sequences and epitopes of diagnostic value derived from the *Anisakis simplex* Ani s 7 major allergen. Allergy 63, 219–225.

Schaum, E., Müller, W., 1967. Die heterocheilidiasis. Dtsch. Med. Wochenschr. 92, 2230–2233.

Shamsi, S., Butcher, A.R., 2011. First report of human anisakidosis in Australia. Med. J. Aust. 194, 199–200.

Shimamura, Y., Muwanwella, N., Chandran, S., Kandel, G., Marcon, N., 2016. Common symptoms from an uncommon infection: gastrointestinal anisakiasis. Chin. J. Gastroenterol. Hepatol. 5176502.

Takabayashi, T., Mochizuki, T., Otani, N., Nishiyama, K., Ishimatsu, S., 2014. Anisakiasis presenting to the ED: clinical manifestations, time course, hematologic tests, computed tomographic findings, and treatment. Am. J. Emerg. Med. 32, 1485–1489.

Tsukui, M., Morimoto, N., Kurata, H., Sunada, F., 2016. Asymptomatic anisakiasis of the colon incidentally diagnosed and treated during colonoscopy by retroflexion in the ascending colon. J. Rural Med. 11, 73–75.

Umehara, A., Kawakami, Y., Araki, J., Uchida, A., 2007. Molecular identification of the etiological agent of the human anisakiasis in Japan. Parasitol. Int. 56, 211–215.

Yasunaga, H., Horiguchi, H., Kuwabara, K., Hashimoto, H., Matsuda, S., 2010. Clinical features of bowel anisakiasis in Japan. Am. J. Trop. Med. Hyg. 83, 104–105.

Yman, L., 2000. Specific IgE in the diagnosis of parasite-induced allergy. Allergy 55, 14–17.

Yoon, S.W., Yu, J.S., Park, M.S., Shim, J.Y., Kim, H.J., Kim, K.W., 2004. CT findings of surgically verified acute invasive small bowel anisakiasis resulting in small bowel obstruction. Yonsei Med. J. 45, 739–742.

Zhu, X., Gasser, R.B., Podolska, M., Chilton, N.B., 1998. Characterisation of anisakid nematodes with zoonotic potential by nuclear ribosomal DNA sequences. Int. J. Parasitol. 28, 1911–1921.

Zullo, A., Hassan, C., Scaccianoce, G., Lorenzetti, R., Campo, S.M., Morini, S., 2010. Gastric anisakiasis: do not forget the clinical history!. J. Gastrointestin. Liver Dis. 19 359–359.