

Human antibody recognition of Anisakidae and *Trichinella* spp. in Greenland

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ABSTRACT

High levels of total IgE are observed among children in Greenland. To evaluate the extent to which Anisakidae and *Trichinella* spp. contribute to the high total IgE level, an ELISA and a western blot were developed for the detection of IgG antibodies to Anisakidae, based on excretory/secretory antigens from Anisakidae larvae. Western blots with Anisakidae and *Trichinella* antigens discriminated between Anisakidae and *Trichinella* infections, enabling cross-reactivity between the two parasite infections to be eliminated. Serum samples from 1012 children in Greenland were analysed for specific antibodies to Anisakidae and *Trichinella*. Eleven children were IgG-positive for *Trichinella* and nine were IgG-positive for Anisakidae, indicating a relatively low prevalence of both infections among children in Greenland. Faecal samples from 320 children were also examined for other intestinal parasites. *Enterobius vermicularis* was found in one sample and *Blastocystis hominis* in 32 samples, but no other intestinal parasites were identified. In total, 304 children had elevated total IgE levels. There was a significant association between *Trichinella* seropositivity and high levels of total IgE, but not between Anisakidae seropositivity and total IgE. The data indicate that parasitic infections alone do not explain the high level of total IgE observed among children in Greenland.

Keywords Anisakidae, ELISA, Greenland, IgE, immunoblot, *Trichinella* spp.

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INTRODUCTION

Nematodes of the Anisakidae family are found worldwide in marine fish, fish-eating birds and mammals [1–4]. Infected sea mammals excrete Anisakidae eggs; the larvae infect fish after hatching and are then transmitted to other fish or mammals by predation. Anisakidae live in the abdominal cavity of fish, but may migrate into the muscles when the fish die [5]. *Anisakis simplex* and *Pseudoterranova decipiens* infect humans primarily following consumption of raw seafood or fish harbouring third-stage larvae. Following human

consumption of infected seafood, the larvae penetrate the human stomach wall, causing an inflammatory reaction, with infiltration of eosinophils and elevated levels of total IgE in serum [6,7]. Exposure to antigens from the dead larvae of *A. simplex* and *P. decipiens* has been associated with allergic reactions in humans, but it is not clear whether sensitisation requires pre-exposure to live larvae, or whether it can be induced after exposure to dead larvae, e.g., after cooking [8].

Fish are a major food source in Greenland, and are often eaten without adequate cooking. Anisakidae infections have been recorded in Atlantic cod (74%), Greenland cod (73%) and Greenland halibut (3–76%) [9] from waters surrounding Greenland, and infection with Anisakidae is therefore expected to be present in humans;

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however, no data exist concerning the prevalence of Anisakidae infections among humans in Greenland.

Parasitic infections among the inhabitants of Greenland are thought to be common, since elevated levels of serum IgE antibodies are found in children from Greenland [10–12]. Such findings cannot be explained exclusively by atopy or allergy, and could instead reflect frequent helminth infections [13]. The possible aetiological agents include a number of foodborne worms in addition to Anisakidae, e.g., tapeworms from fish (*Diphyllobothrium* spp.) or roundworms from game meat (*Trichinella nativa*) [14].

The aims of the present study were to investigate the extent to which children in Greenland showed serological evidence of exposure to the foodborne helminths Anisakidae and *Trichinella* spp., and to determine whether such exposure could explain the high levels of total IgE in their serum. As no commercial test is available for detection of Anisakidae antibodies, a serological assay was developed and tested for reactivity to *Trichinella* spp. antigens.

MATERIALS AND METHODS

Subjects

The study material consisted of serum and stool samples collected during 2001 from children in Greenland, after informed consent was obtained from their parents. A cross-sectional study on allergic diseases in children had been performed previously [12] in the towns of Ilulissat, Aasiaat, Sisimiut and Maniitsoq, located on the west coast of Greenland. The inhabitants of these towns comprise 32% of the total population of Greenland. All children (aged 8–14 years) in these towns were eligible for participation, and there were no exclusion criteria. Of 1536 children, 1012 (74%) individuals agreed to participate and a venous blood sample was taken. After separation by centrifugation, the serum was immediately frozen at -20°C until analysis. All samples were initially analysed for total IgE; if additional serum remained (1012 samples), these were then tested for IgG antibodies against *Trichinella* spp. and Anisakidae. All 1012 children were asked to give a stool sample for microscopy and 320 complied.

Seropositive and seronegative controls

Trichinella-exposed subjects. Serum samples were obtained from five individuals exposed to *Trichinella*, of whom four had been diagnosed with trichinellosis. All samples originated from two game meat-related outbreaks in west Greenland during 2001 and 2002, respectively [15]. One male and three females (aged 11, 30, 40 and 46 years, respectively) were highly seropositive for IgG antibodies against *Trichinella* spp.

Subjects unexposed to Anisakidae and Trichinella. Serum samples from 200 adult Danes, submitted to Statens Serum Institut (SSI; Copenhagen, Denmark), who were presumed never to have been exposed to live Anisakidae or *Trichinella* spp., constituted a panel of negative controls.

Laboratory analyses

Anisakidae excretory and secretory (E/S) antigens. Twenty herrings caught in Danish waters were dissected, with 18 yielding third-stage Anisakidae larvae from the abdominal cavity. The larvae were washed three times in phosphate-buffered saline (PBS) and once in RPMI-1640 medium (SSI) supplemented with penicillin and streptomycin, and were then incubated in 100 mL of RPMI-1640 medium for 19 h in the dark at room temperature. Following filtration through a $0.22\text{-}\mu\text{m}$ filter (Millipore, Billerica, MA, USA), the filtrate was frozen at -18°C for a minimum of 24 h. The E/S products from the larvae contained in the medium were concentrated using Amicon Biomax PBGC cells (Millipore) under pressure (60 psi). The protein concentration was then measured using the Bradford technique [16] and adjusted to a concentration of 1 mg/mL. The resulting fluid was designated Anisakidae E/S antigen.

Trichinella antigens. Two different antigens specific for *Trichinella* were used: a synthetic tyvelose antigen [17], and *Trichinella* E/S antigens manufactured as described by Kapel and Gamble [18]. Both antigens were adjusted to a concentration of 1 mg/mL.

IgG ELISAs. Microtitre plates (NUNC, Roskilde, Denmark) were coated with either Anisakidae E/S antigen 1:1000, tyvelose antigen 1:800, or *Trichinella* E/S antigens 1:4000 diluted in carbonate buffer, and were then left overnight at 4°C . The plates were then washed three times in PBS containing Tween-20 1% v/v. Serum samples were diluted 1:200 according to Møller *et al.* [19], and 100 μL was added per well. The ELISA plates were gently agitated for 1 h, and then washed three times. A rabbit anti-human IgG alkaline phosphatase-labelled conjugate (cat. no. D0336; Dako, Glostrup, Denmark) was diluted 1:1000, and 100 μL was added per well, after which the plates were gently agitated for a further 1 h. The plates were then washed three times and 100 μL of alkaline phosphatase substrate (Sigma-Aldrich, Brøndby, Denmark) was added to each well. The reaction was stopped after 30 min by adding 100 μL of 1 M NaOH and the colour intensity was read at 405 nm.

Cut-off values. ELISA cut-off values for Anisakidae E/S, *Trichinella* E/S and tyvelose antigens were defined as the 98% percentiles of the OD distribution of the test results of the 200 presumed seronegative Danish control sera. This resulted in cut-off values of OD 0.19 for Anisakidae E/S, OD 0.26 for *Trichinella* E/S, and OD 0.24 for tyvelose antigen. As a control for the validity of the Anisakidae ELISA cut-off value, western blot (WB) analyses of five of the control sera with the highest ELISA OD Anisakidae E/S values were performed. Of the five sera with the highest ELISA OD values, only one showed weak smear bands at c. 35, 55 and 60 kDa in the WB; the remaining four sera were negative. None of the controls was positive in the *Trichinella* E/S, the tyvelose ELISA or the corresponding WB.

Western blots. WBs were performed on all specimens with *Trichinella* and Anisakidae ELISA results above the cut-off values. E/S antigens were used for both *Trichinella* and Anisakidae WBs. The E/S antigen (1 mg/mL) was diluted 1:2, separated electrophoretically on a stacking 5% w/v gel and an SDS-PAGE 10% w/v gel, and then transferred to nitrocellulose membranes. Serum samples were diluted 1:100 and a secondary antibody rabbit anti-human IgG labelled with alkaline phosphatase (cat. no. D0336; Dako) was diluted 1:1000 and used as a conjugate. The immunoblots were visualised using nitroblue-tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich) as substrate.

Total IgE. Total serum IgE was analysed by Pharmacia (Copenhagen, Denmark), using the UniCAP total IgE assay. The lowest detectable value was 9 kU/L and there was no upper detection limit. Normal values were defined by Pharmacia as levels <100 kU/L.

Faecal samples. The 320 stool samples were collected in formalin 10% v/v in PBS and were then stored frozen (−18°C) until analysis at SSI. The samples were concentrated using an in-house formol–ether concentration technique [20], and were then examined by microscopy for helminth eggs and, after staining with iodine, for protozoan cysts at a magnification of up to ×400. A direct smear before concentration was stained with the modified Ziehl–Neelsen stain and examined for *Cryptosporidia* cysts.

Statistical analyses

The association between parasitic status and serum IgE concentration was analysed using the Wilcoxon rank sum test (Mann–Whitney *U*-test) and SAS for Windows v.8.02.

RESULTS

Study population and serological analyses

The demographical characteristics of the 1012 participating children, in total and according to *Trichinella* and Anisakidae seropositivity, are summarised in Table 1. With the *Trichinella* ELISA, 20 samples were above the cut-off value (OD 0.26) using E/S antigens, while 19 samples were above the cut-off value (OD 0.24) using tyvelose. Only six samples were above the cut-off values in both tests. WB analysis identified *Trichinella*-associated bands in the sera of 11 children with OD values above the cut-off value (five boys and six girls; mean age 11 years, range 9–12 years; Table 1).

Using the Anisakidae ELISA, 244 samples were above the cut-off value of OD 0.19, but no samples below an OD value of 0.267 were positive according to the confirmatory WB. Using an OD value of ≥0.267 as the cut-off

Table 1. Demographical and serological characteristics of 1012 children from Greenland who participated in the study

Variables	Participants (<i>n</i> = 1012), <i>n</i> (%)	<i>Trichinella</i> - seropositive (<i>n</i> = 11), <i>n</i> positive (%)	Anisakidae- seropositive (<i>n</i> = 9), <i>n</i> positive (%)
Age (years)			
8	18 (1.8)	0	3 (16.7)
9	196 (19.4)	3 (1.5)	1 (0.5)
10	197 (19.5)	0	3 (1.5)
11	202 (20.0)	4 (2.0)	0
12	219 (21.6)	4 (1.8)	2 (0.9)
13	179 (17.7)	0	0
14	1 (0.1)	0	0
Gender			
Boys	504 (49.8)	5 (0.99)	2 (0.40)
Girls	508 (50.2)	6 (1.18)	7 (1.40)
Place of residence			
Ilulissat	251 (24.8)	0	0
Aasiaat	219 (21.6)	7 (3.2)	0
Sisimiut	359 (35.5)	3 (0.9)	3 (0.9)
Maniitsoq	183 (18.1)	1 (0.6)	6 (3.3)
Place of birth			
Greenland	980 (96.8)	11 (1.09)	9 (0.89)
Elsewhere	32 (3.2)	0	0

value, 31 sera were positive, of which nine (two boys and seven girls; mean age 11 years, range 9–13 years) yielded Anisakidae-associated bands in the WB (Table 1).

Of the five individuals exposed to *Trichinella*, one was seropositive for both antigens, while four were seropositive for one, but not the other (three seropositive for *Trichinella* and one for Anisakidae). No children were positive for both Anisakidae and *Trichinella*, but of 54 samples that yielded non-specific bands in the Anisakidae WB, and were therefore considered to be Anisakidae-negative, five were positive in the *Trichinella* WB.

A 40-kDa band and a double band of *c.* 60 kDa were considered to be diagnostic for Anisakidae. The samples were considered negative if there were smear bands or no reactions against the three antigens. The serum from one Danish individual in the control group showed a weak reaction with Anisakidae antigens, with smear bands of *c.* 35, 50 and 60 kDa, but showed no reaction with the *Trichinella* antigens.

Analysis for intestinal parasites

All 320 faecal samples were negative for helminth eggs and protozoan cysts, with the exception of one (0.3%) child who was infected with *Enterobius vermicularis*, and 32 (10%) children who were infected with *Blastocystis hominis*. Stool examination was undertaken using the formol–ether

concentration technique, which is not the method of choice for diagnosis of infection with *E. vermicularis*. Therefore, the results show only that *E. vermicularis* is present in Greenland.

Total IgE and Anisakidae/*Trichinella* seropositivity

The median total IgE value of all 1012 children was 44.5 kU/mL. From the material analysed for Anisakidae and *Trichinella* seropositivity (1012 samples), 304 children (30%) had total IgE levels >100 kU/mL. There was a significant association between seropositivity for *Trichinella* and increased total IgE (median total IgE concentration of 187 kU/mL for seropositive children, compared with 43.9 kU/mL for seronegative children; $p < 0.001$). In contrast, there was no statistically significant association between increased total IgE and Anisakidae seropositivity. The median total IgE concentration for children seropositive for Anisakidae was 80.9 kU/mL, compared with 44.3 kU/mL for seronegative children ($p = 0.56$).

DISCUSSION

During the course of the present study, a new ELISA was developed for detection of IgG antibodies against Anisakidae. As no sera were available from humans with confirmed Anisakidae infections, the cut-off value for a positive result was defined according to the frequency distribution of the OD values for a population with a low risk of Anisakidae infection. The assay is simple and particularly suitable for screening purposes, as the E/S antigens from L3 larvae of Anisakidae, on which the test is based, are inexpensive and simple to produce.

A particular problem in serological tests for nematodes is cross-reactivity. Such cross-reactivity has been described previously, both among *A. simplex* and other nematodes [21,22], and among the zoonotic helminthes *Trichinella spiralis*, *Fasciola gigantica* and *Echinococcus granulosus* [23]. Although such cross-reactions may be caused by the use of crude antigens (parasite homogenates) which have less specificity than the E/S antigens used in the present study, crude antigens can be useful in combination with a subsequent confirmatory test such as a WB. The present study demonstrated that a WB test could distinguish between Anisakidae and *Trichinella* seropositivity.

Thus, of nine sera with Anisakidae ELISA OD values above the cut-off value and Anisakidae-associated bands in the WB, none showed *Trichinella*-associated bands by WB. However, five serum samples with Anisakidae ELISA OD values above the cut-off value, but with non-specific bands in the Anisakidae WB, showed *Trichinella*-associated bands in the WB. The latter five samples may represent cross-reactivity in the ELISA test, and demonstrate the importance of using the WB as a confirmatory test.

For the purpose of this study, and based on the WB results, the Anisakidae tests appeared to be reliable. A cut-off value was chosen, based on the 98% percentile reactivity level for sera from 200 Danish individuals who were presumed never to have been exposed to live Anisakidae. Sera from the five individuals with the highest OD values were tested by WB and, except for one weak non-specific reaction, were negative by WB. The Anisakidae samples positive by WB each showed bands at c. 60 kDa (two bands) and 40 kDa. A response to the antigens of c. 60 kDa and 40 kDa in size was observed with the sera from all nine children that reacted in the WB, and also in the two positive samples from the trichinellosis outbreaks. Based on the WB results, the Anisakidae test appears to be useful for diagnostic purposes.

Only nine (0.9%) of 1012 children had Anisakidae-associated antibodies. The relatively high percentage of the youngest children (age 8 years) found to be seropositive for Anisakidae may be a statistical artefact related to the low number of children in that age group. However, it could also reflect true exposure and, if so, might indicate that the specific IgG response is short-lived and that the seroprevalence declines in older age groups.

In Greenland, fish is one of the staple ingredients of the local diet [24], and is often eaten raw or dried, which poses a risk of infection [6,25]. As no previous study of the prevalence of Anisakidae in humans in the Arctic has been conducted, it is not known whether the observed human seroprevalence in Greenland is higher or lower than expected. However, high prevalence levels have been found in fish. The population living in the four study towns represented 32% of the total population of Greenland in 2001, and as there is no indication that consumption of fish in these towns differs markedly from that in other parts of the country, the present study can be taken to

indicate that the overall prevalence of anisakiasis is low among children of school age in Greenland. The duration of the Anisakidae-specific IgG response is not known, nor is it known whether it is elicited only when the larvae invade the stomach wall or whether intestinal exposure can stimulate an IgE response without resulting in an IgG response that is measurable in serological assays.

The apparent seropositivity to *Trichinella* (1.1%) is not surprising, as trichinellosis is well-known in Arctic communities, and outbreaks have been reported recurrently in Greenland and other Arctic countries (e.g., Alaska and Canada) [26–30]. In Greenland, such outbreaks were most frequent in the early 20th century, although two minor outbreaks involving six individuals have been recorded in recent years [16]. It is not known whether the apparent decrease in incidence is associated with a change in dietary habits, with less traditional food being eaten, or is caused by under-reporting.

There is no population-based information concerning seroprevalence among children in Greenland; thus, it is unknown whether the prevalence of 1.1% observed in this study among school-age children is as expected. However, a study using a commercial ELISA kit coated with E/S antigen to examine sera from children and young adults (aged 1–23 years) from Bali in Indonesia found an overall seroprevalence of 19.5% [31]. In contrast, a study from Chile, in which 12 882 individuals were tested for trichinellosis using a precipitin test and an indirect haemagglutination test, found that 1.5% were infected, with an increasing prevalence with age [32]. Although living conditions in Greenland differ markedly from those in these other countries, it appears that the seroprevalence among children in Greenland is relatively low, given that *Trichinella* is found frequently in meat from polar bears and walrus [33–35]. However, the seroprevalence may be higher in other areas of Greenland in which polar bear and walrus are eaten on a more regular basis. In addition, the seroprevalence may be lower among school-age children than among older individuals who may have been exposed to *Trichinella* for a longer period.

Anisakidae infection induces a strong cellular immune response, with elevated levels of eosinophils. IgE levels against Anisakidae and total IgE levels increase rapidly following infection,

and total IgE appears to peak *c.* 30 days after the infection, and then gradually decreases [36]. Children in Greenland generally have high levels of total IgE compared with children in European countries, which is a finding that cannot be explained solely by allergy [13]. Instead, it has been hypothesised that parasitic infections might be involved [37]. Microscopy of faecal samples for parasites other than Anisakidae and *Trichinella* spp. revealed no intestinal protozoans or eggs from intestinal helminths, e.g., *Ascaris* or *Diphyllobothrium* spp.; however, since *Diphyllobothrium* spp. have been found in Greenland fish, further studies to detect this parasite are required.

The detection of one child infected with the pinworm *E. vermicularis* indicates that *Enterobius* is found in Greenland, but examination of faecal samples is not the optimum method for detection of this parasite, and thus the present study does not provide information concerning the prevalence of *Enterobius*. The clinical importance of the yeast *B. hominis* is unclear [38].

It should be noted that the present study may not have detected some parasitic infections, as only a single stool sample per child was examined. A previous study has shown that a single sample identifies only 75% of the parasitic infections that are detected using three stool samples [39]. Nevertheless, no direct evidence was found in this study to support the hypothesis that nematode infections explain the high levels of total IgE among school-age children from Greenland [13]. Although *Trichinella* seropositivity was associated with elevated total IgE, only 11 (1.1%) of 1012 children were seropositive for *Trichinella*. No association was observed between Anisakidae seroprevalence and total IgE. Although other parasitic infections might explain the high total levels of IgE among children in Greenland, this seems unlikely, as Anisakidae and *Trichinella* are the most obvious candidates for such infections in an Arctic population, and there is no indication of a high prevalence of intestinal parasitic infections. Other possibilities are that intestinal Anisakidae exposure elicits an IgE response without inducing measurable Anisakidae-associated IgG antibodies, or that the tests for IgG were insufficiently sensitive to detect infection. With no indication of either allergic disease or other parasitic infections, the phenomenon of high IgE levels currently remains unexplained.

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