

Association between immunoreactivity to *Anisakis* spp. antigens and high-risk pregnancy

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Abstract

Numerous factors contribute to perinatal risk, many of which remain undefined. This study sought to determine the frequency of fish intake in postpartum women, and to establish a relationship between the rates of immunoreactivity for antigens from *Anisakis* spp. and high-risk pregnancy. In this prospective noninterventional study, a structured questionnaire was administered and serum was collected from postpartum women at two perinatal centers (a high-risk birth unit [HRBU] and a low-risk birth unit [LRBU]) in the Niterói municipality of Brazil. *Anisakis* species-specific IgG and IgE were measured by ELISA. The chi-squared test was performed, and odds ratios (ORs) with their 95% confidence intervals were estimated. The *t*-test or Mann-Whitney test was applied to continuous, normally distributed variables. In total, 309 women (170 from HRBU, 139 from LRBU) between 24.8 and 26.7 years old with a median of 6 to 8 prenatal visits were enrolled. Women in the two units exhibited differences in some variables, including prenatal care ($p = 0.01$), maternal and fetal risk ($p = 0.00$; OR = 6.17), and gestational age ($p = 0.00$), but no differences in fish consumption ($p = 0.29$), frequency of fish intake ($p = 0.40$), allergic symptoms ($p = 0.51$), or frequency of anti-*Anisakis* reactivity ($p = 0.22$). Logistic regression analysis revealed that only age was independently associated with postpartum anti-*Anisakis* reactivity. This study confirmed a low prevalence of fish intake and suggested that *Anisakis* spp. had no impact on high-risk pregnancies among this postpartum study population.

Keywords

Anisakis spp., fish, puerperium, ELISA

Introduction

Health issues most commonly associated with fetal risks during pregnancy include hypertensive disorders, prepartum hemorrhage, fetal growth restriction, premature rupture of the membrane, hypoxia, perinatal infection, maternal conditions, congenital abnormalities, and specific perinatal conditions (Andriamandimbison *et al.* 2013). Over the past 20 to 25 years, the incidence of preterm births has increased by 33%, resulting in an increase in neonatal mortality due to respiratory infections and neurologic complications (Shapiro-Mendoza *et al.* 2012). However, approximately 19.4% of fetal deaths are due to unknown causes (Helgadottir *et al.* 2013). Moreover, although maternal mortality is generally due to preventable causes, the cause of maternal death cannot be defined in at least 7–10% of cases (Maria 2001; Buschmann *et al.* 2013).

Characteristics used by the World Health Organization (WHO) to categorize the perinatal risk include a maternal history of problems during previous pregnancies or births, need for neonatal resuscitation, and presence of neonatal risk factors (Brasil -2012).

Anisakis spp. are parasitic fish nematodes that can infect humans after the accidental ingestion of larva-containing seafood. Elicitation of anti-*Anisakis* immune responses is affected by the integrity of the ingested *Anisakis* antigens (*i.e.*, consumption of live vs. dead larvae) (Daschner *et al.* 2012). For example, mechanical lesions in the gut mucosa often result from the immune response after ingestion of live larvae (Audicana *et al.* 2008).

Rates of infection with *Anisakis* spp. are increasing around the world. Despite evidence that several species of fish living off the coast of Rio de Janeiro are infected with *Anisakidae*

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larvae (Knoff *et al.* 2012), the serum epidemiological and clinical features associated with *Anisakis* spp. infections in Brazil are poorly characterized. Therefore, the aim of this study was to characterize fish ingestion rates by pregnant woman and to define the relationship between the rates of immunoreactivity to *Anisakis* spp. antigens and high-risk pregnancy.

Materials and Methods

Study design

In this prospective cross-sectional study, a questionnaire was administered to and blood samples were collected from postpartum women at two perinatal centers in Brazil. The study was explained and informed consent was obtained before any subject participated in the study. The project was approved by the Research Ethics Committee of the Universidade Federal Fluminense (CAAE 0167.0.258.000-08).

Participants and recruitment sites

All participants received information regarding anisakidosis and were enrolled from the metropolitan area of Rio de Janeiro, Brazil between January 2009 and December 2010. The metropolitan area comprises 2 million people and 7 municipalities (Niterói, São Gonçalo, Itaboraí, Maricá, Rio Bonito, Silva Jardim, and Tanguá). Visits were scheduled randomly at two perinatal centers. Computer-generated numerical records were used to choose subjects from among hospitalized postpartum women. Participants who did not provide informed consent or who were unable to provide blood samples were excluded from the study. The two distinct perinatal centers consisted of a high-risk birth unit (HRBU), designed for women who were hospitalized for high-risk pregnancies, and a low-risk birth unit (LRBU), designed for women with low-risk deliveries. The characteristics used to define high-risk perinatal conditions/neonates were established by the World Health Organization (Brasil – 2012).

Data sources and measurements

A structured questionnaire was used to collect the following information from each participant: age; current residence; birth unit (LRBU or HRBU); medical history; histories of any prior pregnancy and the present pregnancy; any birth events; vitality of the newborn at birth; frequencies of handling and consuming fresh, frozen, raw, or slightly cooked fish; and allergic events (skin, respiratory, or gastrointestinal manifestations).

Blood samples (5 ml) were obtained aseptically from the cubital vein without anticoagulant. Sera collected from the mother and newborn were stored at -20°C until analysis. The presence of immunoglobulin (Ig) was measured by ELISA. The optical densities (ODs) of the IgG and IgE were analyzed.

Anisakis larvae and specific antigens

Anisakis simplex larvae were obtained from the gut of fresh fish of a variety of species, mainly from the pink cusk-eel *Genypterus brasiliensis* Regan, 1903, cutlassfish *Trichiurus lepturus* Linnaeus, 1758 and red porgy *Pagrus pagrus* Linnaeus, 1758. These were acquired at a local fish market in the municipality of Niterói, Brazil, and were transported to the laboratory of fish research at the School of Veterinary at UFF (Universidade Federal Fluminense) for the removal and taxonomical identification of the parasites.

Once classified, to produce somatic antigens of the parasites were disintegrated in a Potter homogenizer to obtain total body antigens. The resultant material was centrifuged and the supernatant was collected and stored. In order to obtain secretory/excretory antigens, live larvae were incubated in physiological saline containing 50 mM of HCl at 37°C for 24 hours (Caballero *et al.* 2008). After that, the culture medium was collected. Protein concentrations were determined (Lowry *et al.* 1951) and all antigens were stored at -70°C until being used.

ELISA

The presence of specific anti *Anisakis* IgG and IgE antibodies in the blood of the volunteers was determined by ELISA. In short, 50 μL of the *Anisakis* antigen solution containing 20mg of protein/mL was added to each well of a 96-well micro plate (Maxi – Sorp – Nunc) and was incubated overnight at 4°C . After a wash with PBS-T, the plates were blocked with PBS- 1% gelatin (PBS-G) for two hours at room temperature. The next step consisted in adding duplicates of 50 μL of each serum diluted 1:50 v/v in PBS-G to each well. After a two hour incubation period at 37°C , plates were once again washed with PBS-T and 50 μL of HRP-mouse anti-IgG and anti-IgE (ϵ chain specific) (Invitrogen, Camarillo CA USA) was added to each well according to the manufacturers' instruction and were then incubated at 37°C for one hour. Finally, after a last wash the reaction was revealed by adding o-Phenylenediamine, H_2O_2 diluted in Citrate Phosphate buffer pH5.0. The reaction was interrupted after 20 minutes with 2N Sulfuric acid. The individual optical density of each well was read with an automatic plate reader (Anthos 2010) at 490 nm. The results are expressed as a mean of each duplicate.

The cut off level for positive reactivity was established by calculating 3 fold the average optical densities of 20 wells of the ELISA reaction described above substituting human sera with PBS-T (Figueiredo Junior *et al.* 2013).

Quantitative and qualitative variables

Binary categorical variables were used to indicate birth unit (0 for HRBU, 1 for LRBU), serum reactivity to anti-*Anisakis* spp. antigens (0 for nonreactive, 1 for reactive), and allergic complaints (0 for not present, 1 for present). The principal continuous variables were age, number of prenatal visits, gestational age, birth weight, frequency of exposure to fish, consumption of fish, and ODs of anti-*Anisakis* IgG and IgE.

Table I. Binary logistic regression analysis, in which the dependent variable is women with specific IgG anti-*Anisakis* reactivity (0/1)

								95% C.I.for EXP(B)	
		B	S.E.	Wald	df	Sig.	Exp(B)	Lower	Upper
Step 1 ^a	Age<30(1)	0.993	0.426	5.421	1	0.020	2.699	1.170	6.225
	Municipality	0.107	0.118	0.833	1	0.362	1.113	.884	1.401
	Perinatal centers(1)	−0.012	0.329	0.001	1	0.970	0.988	.519	1.882
	fish handling(1)	−0.140	0.341	0.168	1	0.682	0.870	.445	1.698
	fish intake>2xweek(1)	−0.137	0.417	0.107	1	0.743	0.872	.385	1.975
	Raw fish(1)	0.580	0.778	0.556	1	0.456	1.786	.389	8.203
	Constant	−1.933	0.649	8.869	1	0.003	0.145		

a. Variable(s) entered on step 1: Age, Municipality, Perinatal centers, Handling, Fish intake, Raw.

Statistical methods

Data were processed with the SPSS® 17.0 statistical package (SPSS Inc., Chicago, IL, USA). Continuous variables were evaluated by the mean, median, standard deviation, and variance. The presence or absence of a normal distribution was verified by the Kolmogorov-Smirnov and Shapiro-Wilk tests. On the basis of this analysis, either the parametric (*t*-test) or nonparametric (Mann-Whitney) test was used.

Contingency tables were developed to assess the association between birth unit (0/1) and the presence of reactive IgG anti-*Anisakis* responses (0/1), high fish intake (≥ 2 times/week) (Corres *et al.* 2001) (0/1), ingestion of raw fish (0/1), and presence of allergic manifestations. The relationship between categorical variables was tested by chi-squared analysis and adjusted by Fisher's exact test. When calculated, the odds ratio (OR) was considered statistically significant if the 95% confidence interval did not include 1. Analysis of multiple responses was used to explore the existence of significant differences between the types of fish consumed by women in the two perinatal units. A binary logistic regression model was used to analyze relationships between categorical variables (possible factors of infection). Sensitization to *Anisakis* spp. was the dependent variable, while current residence, age (<30 years), presence of prenatal and perinatal problems, frequency of fish intake, eating raw fish, presence of allergic complaints, and birth unit were independent variables. For all hypotheses, the tested significance level was set at 95%.

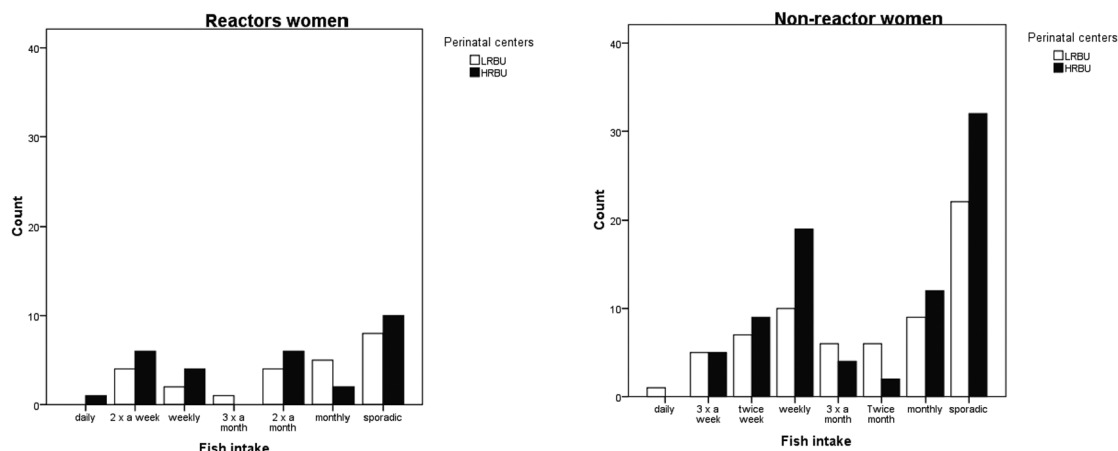
Results

Questionnaire responses and blood samples were collected from 309 mother-newborn pairs at the two perinatal centers (LRBU = 139, HRBU = 170). Mothers enrolled at both units were predominantly from the municipality of Niterói (LRBU

= 129 women, HRBU = 75 women) and were between 15 and 42 years old (mean age: LRBU = 24.80 years, HRBU = 26.19 years). The number of prenatal visits ranged from 1 to 13 (median: LRBU = 6, HRBU = 8).

Only 97 women (LRBU = 74 women, HRBU = 23 women) described having no problems during the prenatal or perinatal period. The most commonly cited complication was infections (LRBU = 53, HRBU = 39), primarily those of the urinary tract. Vaginal deliveries were predominant at the LRBU ($n = 140$), whereas cesarean sections were more common at the HRBU ($n = 80$). The median gestational age at both units was 39 weeks. Newborns were predominantly male at the HRBU ($n = 133$). The average birth weight at both birth centers was 3,280 grams. Most babies ($n = 224$) did not require resuscitation. The average hospital stay of neonates was 3.87 days in the HRBU group and 2.61 in the LRBU group. Differences between the LRBU and HRBU groups were observed in the number of prenatal visits ($p = 0.01$), gestational age ($p = 0.00$), problems during fetal gestation ($p = 0.00$; OR = 6.17), and hospital stay duration ($p = 0.00$). The HRBU group exhibited a predominance of males ($p = 0.03$), lower birth weight ($p = 0.000$), and greater incidence of neonatal problems in the nursery ($p = 0.00$ and OR = 14.61).

There were no differences between the LRBU and HRBU groups in terms of complaints of allergic symptoms ($p = 0.21$), fish consumption ($p = 0.29$), or frequency of fish consumption ($p = 0.48$; Fig. 1). The two groups showed similar ELISA OD levels of IgG ($p = 0.225$). Only 25 women reported not eating fish or seafood, with no difference in age between women who did or did not eat fish ($p = 0.664$). Women who ate fish did not exhibit more allergic symptoms compared to women who did not eat fish ($p = 0.337$). The most frequently consumed fish species in the LRBU and HRBU groups were sardine (*Sardinella brasiliensis*; 25.4% and 23.5% of responses, respectively), white mouth croaker (*Micropogonias furnieri*; 18.6% and 22.2% of responses, respectively), and



Most women consumed fish only sporadically during pregnancy. There were no significant differences in fish consumption between the two perinatal centers among reactive women ($p = 0.451$) or nonreactive women ($p = 0.682$).

Fig. 1. Bar charts describing fish consumption frequencies in postpartum women from the two perinatal centers studied

swordfish (*Xiphias gladius*; 10.9% and 9.8% of responses, respectively).

On the ELISA assay, a value of more than 3 times the control response was considered to be a positive response for IgG reactivity to crude *Anisakis* larva antigens. In total, 60 mothers (19.41%) tested positive for IgG reactivity by this definition (HRBU = 32 women, LRBU = 28 women; $p = 0.42$). There was no difference in the mean rank of the ELISA OD between the two groups ($p = 0.39$). According to the binary logistic regression analysis, only younger age (<30 years) was associated with the presence of anti-*Anisakis* antibodies in postpartum women ($p = 0.02$, OR = 2.69; Table I).

Discussion

Distinct differences in perinatal risk, gestational age, and neonatal problems in the nursery were observed between the perinatal centers. However, no relationship was found between *Anisakis*-positive serum responses and pregnancy risk.

Despite the increased overall longevity of women in today's society (Abouzahr 2014), there remain many undetermined factors related to maternal death (Mhyre 2012) and fetal loss in various stages of development in the uterus. At the same time, there is an absence of knowledge regarding many risk factors and environmental influences (Stingone *et al.* 2014) during pregnancy (Boyon *et al.* 2011; Renou *et al.* 2012). Numerous studies have been conducted on the development of allergic symptoms during pregnancy (with *in utero* stimuli) (Rowe 2007; Joerink *et al.* 2009; Shah 2009), which have reported the influence of infections and consuming certain foods in the peripartum period. *Anisakis* spp. are parasites that infect various fish worldwide, including fish inhabiting the coast of Rio de Janeiro (Melo *et al.* 2006; Knoff *et al.* 2007). Participants in both study groups con-

sumed similar types of fish; however, no data exist describing *Anisakis* infection rates in sardines from the Rio de Janeiro coast.

The most surprising observation in this study regarded the reduced amounts of fish consumed by the study populations (Figure 1), even though fish are rich in essential nutrients (Leventakou *et al.* 2014) and have been linked with longer life (Qin *et al.* 2014; Zaribaf *et al.* 2014). There were no significant differences in fish intake and reactivity to *Anisakis* antigens between women at the two perinatal centers, and the logistic model confirmed that there was no direct relationship between the independent variables and reactivity to *Anisakis* antigens. Only younger age (<30 years) was significantly associated with *Anisakis* reactivity. This observation may be explained by the fact that younger people were more likely to consume raw or semiprepared fish.

Currently, there is no evidence supporting the possibility that the transfer of anti-*Anisakis* species-specific antigens across the placenta would cause protection or harm to the fetus or newborn. The presence of anti-*Anisakis* IgE reactivity has been observed in approximately 20% of ordinary people without clinical manifestations (Figueiredo Junior *et al.* 2013). The absence of a positive IgE response in this study may be explained by the low intake of fish by the study population.

In conclusion, fish consumption in pregnant women was low, regardless of the birth unit. Immunoreactivity to *Anisakis* antigens was not greater in high-risk pregnancies, suggesting that this parasite is unlikely to pose a perinatal risk.

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