Seroreactivity to *Anisakis* spp. in the perinatal period

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Abstract

Background: This study had sought to assess the seroreactivity to the fish nematode *Anisakis* spp. in a puerperal population, as well as to ascertain whether a correlation exists between maternal and cord blood levels.

Methods: Blood samples were obtained from puerperal women and cord blood to measure specific anti-*Anisakis* antigen IgG and IgE by ELISA. Non-parametric tests were used to compare two or more independent and related samples.

Results: Of the 99 maternal serum samples assessed, 21 were positive on ELISA (21.2%). There were no significant differences in the mean ranks of IgG optical density levels between women who ate fish and those who did not ($p = 0.456$), those who ate raw fish and those who did not ($p = 0.479$), or between those who had allergic complaints and those who did not ($p = 0.431$).

Conclusion: Transplacental passage of antibodies occurred, leading to moderate correlation between maternal and cord blood serum levels.

Keywords

Parasitology, nutrition, maternal–fetal medicine, immunology

Date received: 7 May 2016; accepted: 13 December 2016

Introduction

Fish intake during pregnancy has been linked to suspected fetal problems, such as fetal growth restriction,¹ potential adverse stimuli regarding allergies,² and infection with various pathogenic agents, including nematodes.³ Th1 and Th2 responses are often triggered by ingestion of an aggressor pathogen, as may a specific immune response, at any period of life – including pregnancy. The fetal immune system has also been the subject of investigation, including research into the presence of specific antibodies⁴ triggered by maternal exposures during pregnancy.

Anisakis simplex, a white, cylindrical nematode approximately 3 cm in length, is a parasite of fish species which also follows an incomplete life cycle in man, where the larvae cannot reach adulthood.⁵ It is found in a large variety of fish worldwide, including those caught off the shore of Rio de Janeiro, Brazil.^{6,7} When ingested by humans, the parasite may produce initial sensitization followed or not by a range of allergic manifestations.⁵ Acute intestinal illness, associated with the effects of live larvae and their potential migration, has also been reported, especially among raw fish eaters.⁸

The influence of exposure to this nematode in pregnancy and the puerperal period, including fetal serology profile and sensitization markers in cord blood (CB), is unknown.

Therefore, the objectives of this study were to assess the seroreactivity to *Anisakis* spp. in a puerperal population, including puerperal women who ate raw fish and with allergy complaints, as well as to ascertain whether a correlation existed between maternal and CB levels of anti-*Anisakis* IgG and IgE.

Methods

Study design

This was a prospective and non-interventional study. Blood samples were collected routinely from healthy female volunteers in the puerperal period and CB samples were obtained from their neonates. A structured questionnaire was also administered to collect general information on the participants and their neonates and data on potential sensitization by *Anisakis* spp. The project was approved by the Ethics

Committee of the Universidade Federal Fluminense School of Medicine (CAAE 0167.0.258.000-08).

Setting

Puerperal women were recruited from two birthing centers in the city of Niterói, metropolitan area of Rio de Janeiro, Brazil, between January 2009 and December 2010. One was a tertiary referral unit for high-risk pregnancies, and the other, a unit for low-risk deliveries.

Participants

The eligibility criterion was the presence of the woman and her newborn at either of the two study units at the time the investigator attended the unit for data collection, which could occur randomly on any day of the week. Women were selected from the study population using a numbered list generated by a statistical software package. Volunteers who had received information on anisakiasis and provided written informed consent for participation were included. Maternal and CB samples with any issues regarding blood collection or from subjects who had not provided informed consent were excluded, as were women receiving chemotherapeutic and/or immunosuppressant agents and those with

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acute illnesses and/or chronic systemic diseases. Overall, 211 serum samples from puerperal women and 161 CB samples from neonates were validated for analysis. Of these, 99 had no issues, no missing data, and were paired, and were thus selected for analysis.

Data sources/measurement

Blood samples and interviews. Samples were picked up at the clinical laboratories of the study units after completion of the structured questionnaire. Potential biases regarding observation and data collection were eliminated by joint administration of 30 questionnaires by the study investigator and academic advisor. Blood was collected aseptically from mothers and neonates for serum analysis. CB was obtained from an umbilical cord vein after visual identification. Maternal blood (5 mL) was drawn from the cubital vein as per routine practice at the study units. Samples were centrifuged and the resulting serum stored at -20°C until the time of testing.

Anisakis larvae and specific antigens. *Anisakis* spp. larvae were obtained from the gut of fresh fish acquired at a local fish market in the municipality. After identification and classification at the Universidade Federal Fluminense School of Veterinary Medicine, larvae were homogenized to produced total body antigens.⁹

ELISA

The occurrence of specific anti-*Anisakis* IgG and IgE antibodies in blood samples was determined by enzyme-linked immunosorbent assay (ELISA). Briefly, 50 μL of the *Anisakis* total body antigen solution, containing 20 μg protein/mL, was added to each well of a 96-well microplate (Nunc[®] MaxiSorpTM, Thermo Fisher Scientific) and incubated overnight at 4°C . After rinsing with PBS-T, the plates were blocked with PBS-1% gelatin (PBS-G) for 2 h at room temperature. Then, 50- μL duplicates of each serum sample, diluted 1:50 v/v in PBS-G, were added to each well. After 2 h of incubation at 37°C , the plates were once again washed with PBS-T and 50 μL of HRP-mouse anti-human IgG and IgE (ϵ chain-specific) (Invitrogen, Camarillo, CA, USA) was added to each well according to manufacturer instructions, followed by another hour of incubation at 37°C . Finally, after a last rinse, the reaction was revealed by adding *o*-phenylenediamine and H_2O_2 diluted in citrate-phosphate buffer (pH 5.0). The reaction was stopped after 20 min with 2 N sulfuric acid. The individual optical density (OD) of each well was read with an automatic plate reader (Anthos 2010) at 490 nm. The results are expressed as the mean of each duplicate. The cutoff level for positive reactivity was established by calculating three times the average OD of 20 wells of the ELISA reaction described, substituting human sera with PBS-T.¹⁰

Variables

Data regarding personal identification, place of residence, medical history, gestational history, history of present pregnancy, events at birth, neonatal vitality at birth, handling of fish, fish intake, and presence of fish-related allergies were collected. As noted above, the presence of IgG and IgE was determined by ELISA of maternal and CB samples. Immunoglobulins were analyzed separately and were also evaluated by applying a cutoff level and stratifying participants into groups: seroreactive and non-seroreactive to total *Anisakis* spp. antigen.

Statistical methods

Data were entered into spreadsheets and analyzed in SPSS[®] 17.0 (SPSS Inc., Chicago). Descriptive statistics were expressed as absolute frequencies. Means, standard deviations, and confidence intervals were

calculated for maternal and CB IgG and IgE antibody levels. Multiple response analysis was used for the variables "site of fish acquisition" and "fish consumed."

Non-parametric tests were chosen after the assumption of normality was rejected by the Shapiro–Wilk test and due to the size of the sample. The Mann–Whitney *U* test was used to investigate the difference in mean IgG levels in postpartum women in the presence vs. absence of allergic complaints, consumption vs. non-consumption of any fish, and consumption vs. non-consumption of raw fish. Spearman correlation coefficients were used to test the measure of association between two variables, postpartum maternal IgG and CB serum IgG, while the Wilcoxon test for two related samples was used to ascertain whether the median of the differences between the two samples was equal to zero. For the sake of clarity, a curve was plotted after analysis of variance with $p < 0.05$, where CB IgG was considered the dependent variable and maternal IgG the independent variable. The trend equation for the study group was also calculated. A *p*-value of < 0.05 was considered significant for rejection of the null hypothesis.

Results

Paired samples of puerperal maternal blood and CB were validated for analysis ($n = 99$). Most mothers were from the city of Niterói ($n = 84$). Mean age was 25.9 years (range, 15–39 years), and the median number of prenatal visits was 6.5 (range, 2–13). There was a predominance of vaginal delivery ($n = 62$). Mean gestational age was 39.2 weeks. The majority of neonates were female ($n = 51$) and the mean birth weight was 3.206 g. Overall, 83 newborns did not require resuscitation; the mean length of hospital stay was three days, predominantly in co-rooming arrangements ($n = 91$).

Most women in the sample did not handle fish or seafood ($n = 61$). Those who reported contact ($n = 38$) claimed it occurred only sporadically ($n = 21$), after acquisition of products, which were mostly purchased from supermarkets ($n = 65$). Only seven women denied eating fish; most reported doing so sporadically ($n = 34$), followed by monthly ($n = 15$) and weekly ($n = 12$). Raw fish was consumed by only four women. The vast majority of participants had no known allergies ($n = 78$). Among those who reported allergic symptoms ($n = 21$), the gastrointestinal tract was the most common site of allergic manifestations. There was no association of allergic complaints with overall fish intake ($p = 1.000$) or raw fish intake ($p = 0.742$) on binary regression. The main fish species consumed were the Brazilian Sardine (*Sardinella brasiliensis*) ($n = 69$), Whitemouth Croaker (*Micropogonias furnieri*) ($n = 54$), and Cutlass fish (*Trichiurus lepturus*) ($n = 37$), as determined by multiple response analysis.

The mean OD of IgG in puerperal maternal blood was 0.2330 (SD, 0.0799; 95% CI, 0.2170–0.2489) vs. 0.2306 in CB (SD, 0.1164; 95% CI, 0.2134–0.2598). Regarding IgE, the mean OD was 0.1217 (SD, 0.0289; 95% CI, 0.1158–0.1277) in maternal and 0.1461 (SD, 0.0426; 95% CI, 0.1374–0.1549) in neonatal samples. There were no significant differences in mean ranks of IgG levels as determined by OD between women who ate fish vs. those who did not ($p = 0.456$), those who ate raw fish vs. those who did not ($p = 0.479$), or between those who had allergic complaints vs. those who did not ($p = 0.431$). Using the cutoff described, IgG reactivity was considered positive in 21 mothers (21.2%). No maternal or CB samples were considered positive for IgE using the aforementioned criterion.

The Spearman test revealed moderate correlation between postpartum maternal IgG and CB serum IgG ($r = 0.349$; $p < 0.0001$). Curve estimation with a positive linear model, validated by ANOVA ($p = 0.003$), showed that as the OD of puerperal maternal blood IgG increases, the OD of CB samples also increases. The following equation can be used to estimate the expected CB IgG level (study group): $y = 0.589252 \cdot x + 0.105777$, where y is the CB IgG level and x is the maternal IgG level. A paired samples test showed that mean CB antibody levels were lower than maternal levels, although the difference was not statistically significant ($p = 0.763$).

Discussion

Antibody-mediated adaptive immunity is characterized by passive transfer of IgG through the placenta during the fetal period, starting at 12–14 weeks.¹¹ Particularly in the third trimester, transplacental transfer provides a substantial amount to antibodies to the fetus, facilitating extrauterine survival and protecting against infections.¹² The present study found a behavior similar to conventional maternal–fetal immunoglobulin transfer and determined that specific anti-*Anisakis* antibodies are detectable in CB and maternal blood in the puerperal period. It also demonstrated a clear correlation between maternal and neonatal IgG levels, suggesting that the greater the OD in maternal serum, the higher the serum levels CB drawn from full term, appropriate-for-gestational-age newborns.

The fetal immune system begins to form concomitantly with the onset of hematopoiesis, and the fetus may begin to produce IgE as early as the first trimester, although there is no consensus as to whether it occurs from the 11th week¹³ or from the 20th week.¹⁴ The ability of the fetus or neonate to respond to antigenic stimuli changes gradually with maturation of the immune system. For example, at birth, total and specific IgE levels measured in CB are low; excessive IgE values may be predictors of atopy.¹⁵

One of the limitations of the present study was the lack of concomitant assessment of IgA in CB samples, which could confirm the absence of contamination by maternal blood and the presence of a false-positive result for anti-*Anisakis* IgG and IgE. To mitigate this potential influence, we provided prior guidance on CB collection procedures. Another issue was the risk of cross-reactivity with some antigens.^{16–18} After exhaustive repetition of the methods, careful work with the antigens, fine plate preparation, and assessment of the sensitivity and specificity of the antibody, we reached a consensus that reactivity on ELISA would be attributable to contact with *Anisakis* spp.

Despite numerous studies on the development of allergy in response to intrauterine stimuli, we were unable to find any reports of the influence of fish intake (and, consequently, intake of fish parasites) during pregnancy, although fish is the first food of choice – even for children – in many communities worldwide. Anti-*Anisakis* IgE was detected both in maternal serum and in CB, although levels were considered to be within the normal range. However, to the best of our knowledge, no serological surveys of CB to test for transplacental passage of specific anti-*Anisakis* IgE had ever been conducted before the present study.

Conclusions

In conclusion, this investigation established that the rate of reactivity for anti-*Anisakis* spp. antibodies in the puerperal period is similar to that seen in a normal adult population and that transplacental passage of anti-*Anisakis* antibodies did occur, leading to moderate correlation between levels in neonatal CB and maternal serum, with CB levels below those measured in maternal peripheral blood.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

Guarantor

IF Jr.

Ethical approval

The Ethics Committee of the Federal Fluminense University approved the study. The patients gave their consents for the study and the investigations were carried out to a high ethical standard.

Contributorship

LS: Collecting data, analysis of results, initial draft manuscript; MV: Help with data collection, discussion; GT: Supervision of project; SSC: Revising manuscript; IFJ: Conceived idea for project, final version of manuscript. All authors have participated in the concept and design, analysis and interpretation of data, drafting or revising of the manuscript and they have approved the manuscript as submitted.

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