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JANAINA RIBEIRO

RESPOSTA IMUNOLÓGICA A ANTÍGENOS DE *Hysterothylacium deardorffoverstreetorum* DE PEIXES TELEÓSTEOS

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2016

RESPOSTA IMUNOLÓGICA A ANTÍGENOS DE *Hysterothylacium deardorffoverstreetorum* DE PEIXES TELEÓSTEOS

Tese apresentada ao Programa de Pós-graduação em Medicina Veterinária da Universidade Federal Fluminense, como requisito parcial para obtenção do grau de Doutora. Área de concentração: Higiene Veterinária e Processamento Tecnológico de Produtos de Origem Animal.

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“Em algum lugar, pra relaxar
Eu vou pedir pros anjos cantarem por mim
Pra quem tem fé
A vida nunca tem fim
Não tem fim”
(Anjos - Marcelo Falcão e Tom Saboia)

RESUMO

O consumo de pescado aumenta progressivamente pois as pessoas estão cada vez mais em busca de alimentos mais nutritivos. O pescado torna-se cada vez mais apreciado no Brasil e em outros países. Outro motivo que tem contribuído para o aumento deste consumo é a incorporação de culinárias, antes exóticas, ao nosso país, como a culinária Japonesa. O hábito de consumir o pescado cru sem prévio congelamento, insuficientemente cozido ou inadequadamente salgado, traz risco à saúde coletiva, pois viabiliza a ingestão acidental de nematóides da família Anisakidae e Raphidascarididae. Esses parasitos são agentes etiológicos da Anisakidose, uma doença cujas manifestações se apresentam nas formas gastrintestinais, alérgicas e mais raramente extraintestinais. *Anisakis simplex* e *Pseudoterranova decipiens* são os principais causadores de reações alérgicas dentre nematóides que causam a anisakidose, entretanto ainda não foi estabelecido o potencial alergênico dos demais membros. O presente estudo teve como objetivo avaliar experimentalmente, em modelo murino, o potencial alergênico e a reatividade dos anticorpos induzidos por larvas de terceiro estágio de *Hysterothylacium deardorffoverstreetorum* (HD), e ainda a reatividade cruzada de anticorpos oriundos de camundongos sensibilizados com antígenos de HD frente a antígenos do parasito *Anisakis simplex*. Foram utilizadas larvas coletadas de peixes comercializados nos municípios de Niterói e Rio de Janeiro. Esses nematóides foram identificados por microscopia ótica e após processamento foram determinadas três preparações antigênicas. O extrato bruto total, extrato secretado/excretado extraído em meio ácido e extrato bruto após excreção/secreção. Esses antígenos foram utilizados para imunização de camundongos da linhagem BALB/c que foram divididos em três grupos experimentais. Amostras séricas foram obtidas em diferentes dias após imunização para determinação dos níveis de anticorpos específicos pelo ensaio imunoenzimático (ELISA). Os resultados demonstram aumento na produção de imunoglobulina G após a segunda imunização. Em relação à imunoglobulina E, a reatividade foi mais tardia, demonstrando aumento progressivo após a terceira imunização. Foi avaliada a imunidade celular através da intradermorreação com resultado estatisticamente significativo em relação ao controle utilizado. A avaliação da reatividade cruzada com antígenos de *Anisakis simplex*, aponta reação positiva para os antígenos deste nematóide. Muito embora, a resposta tenha sido inferior a

apresentada frente a antígenos homólogos (HD), os resultados são estatisticamente significativos para antígenos heterólogos (*A. simplex*). Este experimento é a primeira descrição do potencial imunogênico deste parasito em mamíferos e descreve pela primeira vez a reatividade cruzada entre antígenos de *Anisakis simplex* com *H. deardorffoverstreetorum* e, ainda que experimental, representa um avanço no diagnóstico da Anisakidose.

Palavras-chave: Anisakidose; Raphidascarididae, *Anisakis simplex*, Reatividade cruzada, Infecção experimental, Modelo murino.

ABSTRACT

The fish consumption gradually increases as people are increasingly looking for more nutritious foods. The fish becomes increasingly appreciated in Brazil and other countries. Another reason that has contributed to the growth of consumption is the incorporation of cuisines, exotic before, to our country, as the Japanese cuisine. The habit of consuming raw fish without freezing, insufficiently cooked or improperly salty, brings risk to public health because it enables accidental ingestion of nematodes of Anisakidae and Raphidascarididae family. These parasites are etiological agents of Anisakidosis, a disease whose manifestations are present in the gastrointestinal forms, allergic and more rarely extraintestinal. *Anisakis simplex* and *Pseudoterranova decipiens* are the main cause of allergic reactions among nematodes that cause anisakidose, but has not yet established the allergenic potential of the other members. This study aimed to evaluate experimentally in mice, the allergenic potential and the reactivity of antibodies induced by larvae of the third stage of *Hysterothylacium deardorffoverstreetorum* (HD), and also the cross-reactivity of antibodies derived from mice sensitized with HD front antigens the antigens of *Anisakis simplex* parasite. Collected fish larvae were used marketed in the cities of Niterói and Rio de Janeiro. These nematodes were identified by optical microscopy and after processing were determined three antigenic preparations. The total crude extract, extract secreted / excreted extracted in acid and crude extract after excretion / secretion. These antigens were used for immunization of mice BALB / c were divided into three experimental groups. Serum samples were obtained on different days after immunization to determine the levels of specific antibodies by enzyme-linked immunosorbent assay (ELISA). The results showed increased IgG production after the second immunization. Regarding immunoglobulin E, the reactivity was later, demonstrating a progressive increase after the third immunization. cellular immunity was assessed by intradermal with statistically significant results in relation to the control used. The evaluation of cross-reactivity with *Anisakis simplex* antigens, had positive reaction to the antigens of this nematode. Although the response has been less than shown against homologous antigens (HD), the results are statistically significant for heterologous antigens (A. simplex). This experiment is the first description of the immunogenic potential of this parasite in mammals and for the first

time describes the cross-reactivity between *Anisakis simplex* antigens to H*. deardorffoverstreetorum* and still experimental, it represents an advance in the diagnosis of Anisakidosis.

Keywords: Anisakidosis; Raphidascarididae, *Anisakis simplex*, Cross-reactivity, Experimental infection, Murine Model.

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1 INTRODUÇÃO

Pesca e aquicultura são importantes fontes de alimentação, nutrição, renda e subsistência para centenas de milhões de pessoas em todo o planeta. O consumo de pescado aumentou em todo mundo pois o peixe é considerado como um alimento altamente nutritivo, uma vez que é fonte de aminoácidos essenciais, vitaminas e minerais em boa quantidade, alem de possuir pouca gordura, ainda fornece ácidos graxos, configurando uma importante fonte de Ômegas 3 e 6. Consolidando-o assim como importante fonte de alimentação e economia de diversos países (FAO, 2016; MENEZES, 2006; SUÁREZ-MAHECHA, H. et al. 2002).

Apesar de todas as características positivas do pescado, assim como todo alimento, este apresenta alguns riscos e um deles é a presença de parasitos antropozoonóticos. A ingestão acidental desses parasitos mostra-se de extrema importância para saúde coletiva. Larvas de nematóides das famílias Anisakidae e Raphidascarididae apresentam-se potencialmente patogênicas para humanos. Essas larvas são responsáveis por provocar uma doença denominada Anisakidose, que pode se manifestar tanto na forma gastrointestinal, quanto na alérgica. A espécie de maior potencial patogênico em humanos é *Anisakis simplex*, sendo responsável por inúmeros casos relatados por todo o mundo (AUDICANA; KENNEDY, 2008).

A forma alérgica da anisakidose é amplamente estudada, visto que os principais alérgenos, os mecanismos de ação e interações imunológicas são conhecidos. Porém, embora o *Anisakis simplex* seja realmente o parasito mais patogênico dessa família, outros parasitos como *Pseudoterranova decipiens, Contracaecum multipapilatum e Hysterothylacium aduncum*, já foram descritos como agente causal da doença em sua forma intestinal. Larvas desses parasitos são comumente descritos nas necropsias de peixes realizadas no Brasil, sendo sua prevalência conhecida na costa brasileira (FERNANDEZ, 2010; KNOFF et al., 2007; MERCADO et al., 2001; YAGI et al., 1996).

Dentre essas espécies, encontra-se o *Hysterothylacium deardorffoverstreetorum*, parasito recentemente descrito como espécie, porém citado com diferentes nomes para descrição de suas larvas em trabalhos anteriores (FELIZARDO et al. 2009; KNOFF et al. 2012; RIBEIRO et al. 2014). Deste não há

conhecimento algum sobre potencial alergênico ou alérgenos mais expressivos, por esse motivo uma investigação mais aprofundada se torna necessária.

O objetivo do presente estudo foi a avaliação do potencial alergênico de larvas de terceiro estagio, com diferentes preparações antigênicas, do parasito da espécie *Hysterothylacium deardorffoverstreetorum* em modelo murino.

2 REVISÃO DE LITERATURA

2.1 Pescado

A denominação “pescado” é um termo genérico e compreendem peixes, crustáceos, moluscos, anfíbios, quelônios e mamíferos de água doce ou salgada que são utilizados para alimentação (BRASIL, 1952; FAO, 2016).

Devido à perceptível relação entre a dieta e boa saúde o consumo de produtos da pesca aumenta progressivamente em todo mundo. Os consumidores reconhecem que o pescado é um alimento nutritivo e saudável e o consideram como sendo uma excelente fonte de proteína de alta qualidade, com um baixo conteúdo de gordura saturada e uma boa fonte de muitos minerais e vitaminas importantes (AHMED; ANDERSON, 1994; FAO, 2016).

A proteína do peixe é considerada de alta qualidade por conter todos os aminoácidos essenciais (histidina, isoleucina, leucina, lisina, metionina, fenilalanina, treonina, triptofano e valina) distribuídos de forma balanceada e bastante semelhante entre as espécies de água doce e água salgada (MENEZES, 2006; OGAWA; MAIA, 1999).

Em relação a fibra muscular dos peixes, sabe-se que a miosina é rica em ácido glutâmico 22,5%, ácido aspártico, lisina, leucina e isoleucina, que juntos perfazem cerca de 55,0% dos aminoácidos totais e pode variar em função da espécie, tamanho, gênero, habitat e estação do ano, compreendendo, geralmente, cerca de 20,0% de proteína total (MENEZES, 2006; OGAWA; MAIA, 1999).

O conteúdo de lipídeos nos peixes é divido em dois grupos: peixes magros e peixes gordos, que variam conforme a idade, estado biológico, tipo de alimentação e estado de nutrição do peixe, como também, da temperatura da água (SANCHEZ, 1989).

Segundo Badolato et al. (1994), os óleos de peixes contêm uma grande variedade de ácidos graxos com 20 a 22 átomos de carbono, altamente insaturados, destacando-se o eicosapentaenóico (EPA-C20:5 ω-3) e o docosaexaenóico (DHA-C22:6 ω-3), da série ômega-3, os quais não ocorrem em outros animais. Estes ácidos graxos têm a capacidade de reduzir o risco de doenças coronarianas, além de serem atribuídos outros efeitos imunológicos e antiinflamatórios, principalmente no caso de asma e artrite reumatóide (MENEZES, 2006).

Com relação aos minerais, a carne de peixe é considerada uma fonte valiosa de cálcio e fósforo, em particular, apresentando quantidades razoáveis de sódio, potássio, manganês, cobre, cobalto, zinco, ferro e iodo, no músculo dos peixes também encontram-se magnésio, cloro, enxofre, selênio, cromo e níquel, entre outros (CONTRERAS-GUSMÁN, 1994; MENEZES, 2006; OGAWA; MAIA,1999).

A produção pesqueira extrativista mundial no ano 2014 foi de aproximadamente 93,4 milhões de toneladas, porém acredita-se que esse número seja muito superior, uma vez que muitos países não possuem informações sobre a real situação da pesca em seu território (FAO, 2016). A China apresenta-se como maior produtor do planeta, sendo responsável pela extração de 843 mil toneladas de pescado no ano de 2014. Junto com a China, Indonésia, Estados Unidos, Rússia e Japão compõem os cinco maiores produtores do mundo (FAO, 2016). O Brasil ocupa a 19º posição no ranking de produção mundial. Potencial subexplorado, uma vez que possuem uma extensão de 8500 km^2 de costa maritime (FAO, 2016; MPA, 2013).

2.2 Parasitos de pescado de importância sanitária

Diante da diversidade de parasitos de pescado, algumas espécies ganham destaque pelo seu potencial zoonótico. Na classe Cestoda, o *Diphyllobothrium* sp, popularmente conhecido como a Taenia do peixe, é endêmico em boa parte do mundo, incluindo o continente americano, com casos já registrados no Brasil. Com um ciclo biológico bastante complexo, a infecção em humanos está associada a ingestão de diferentes tipos de peixes consumidos sem cozimento, destacando-se o Salmão. (KNOFF; FONSECA, 2012). As espécies mais comuns são *Diphyllobothrium latum, Diphyllobothrium pacificum e Diphyllobothrium dendriticum*. São agentes causadores da doença denominada Difilobotríase, que pode apresentar-se desde assintomática, ou em infecções mais severas, com sintomas de fadiga, desconforto intestinal, diarréia, obstrução intestinal, entre outros (ACHA SZYFRES, 2003; KNOFF; FONSECA, 2012).

Dentre os trematódeos, *Ascocotyle* (*Phagicola) longa* (Digenea: Heterophyidae), provoca Fagicolose que está associada ao consumo de Tainha (*Mugil curema*). A sintomatologia é típica de parasitoses intestinais, incluindo vômitos, diarréias. Ainda da classe Trematoda, os parasitos *Clonorchis sinensis* e *Opisthorchis* sp. provocam sintomatologia hepática, podendo levar a insuficiência

hepática em casos mais graves (FRIED et al., 2004; OKUMURA et al., 1999).

O *Eustrongylides* sp é um nematódeo que possuem potencial patogênico ao homem e pretence à família Dioctophymatoidae. O parasito possui um grande poder invasivo, podendo ocasionar dor abdominal aguda após ingestão de peixes dulcícolas sem cozimento (OKUMURA et al, 1999).

Outra família de nematódeos que possuem grande potencial zoonótico é denominada Anisakidae, onde *Anisakis simplex* e *Pseudoterranova decipiens* são os mais frequentemente descritos (AUDICANA, 2008).

2.2.1 Familia Anisakidae

A família Anisakidae é a mais numerosa dentro da superfamília Ascaridoidea, e incluem espécies que parasitam peixes, répteis, mamíferos e aves piscívoras. Esses nematóides necessitam do ambiente aquático para o desenvolvimento de seu ciclo biológico e comumente envolvem invertebrados e peixes como hospedeiros intermediários ou paratênicos (ANDERSON, 2000).

Esses nematóides são parasitos heteroxenos e habitam o estômago e o intestino de mamíferos marinhos (baleias, focas, leões marinhos, entre outros) ou aves, que atuam como hospedeiros definitivos dessas espécies. Esses animais eliminam juntamente às suas fezes ovos com larvas de primeiro estágio, que evoluem ao segundo estágio ainda dentro do ovo, após essa mudança de estágio, as larvas saem do ovo e são ingeridas por pequenos crustáceos presentes no zooplâncton, que servem de alimentos para peixes teleósteos e cefalópodes, dando continuidade ao ciclo evolutivo dos anisaquídeos. Ao serem ingeridas já se encontram no terceiro estágio evolutivo. Então as larvas migram do trato gastrointestinal desses peixes para outros órgãos como fígado, cecos pilóricos, gônadas e musculatura. Evoluem ao quarto estágio larvar onde juntamente com o peixe são ingeridas por seus hospedeiros definitivos, onde completam seu ciclo tornando-se adultos. Quando o homem ingere o peixe parasitado não há continuidade do ciclo evolutivo e essas larvas não atingem a maturidade (ANDERSON, 2000; FERREIRA, 2008; NUNES et al., 2003; VALLS et al., 2005).

As larvas de terceiro estágio realizam migração para a musculatura e enrolam-se em forma de espiral, podendo medir de dois a três milímetros de diâmetro. Acredita-se que estas tenham capacidade de infecção por três anos ou

mais. Esses parasitos presentes no peixe não são capazes de ocasionar transtornos significativos nesses animais, exceto em infecções abundantes, onde podem proporcionar edemas localizados (SABATER; SABATER, 2000).

Os indivíduos da família Anisakidae possuem cutícula com ou sem cerdas ou estruturas acessórias ctenóides. Esôfago com ventrículo posterior, apêndice ventricular presente ou ausente; ceco intestinal presente ou ausente. Sistema excretor assimétrico, restrito ao cordão lateral esquerdo. Poro excretor situado próximo à base dos lábios subventrais ou ao nível do anel nervoso (FERREIRA, 2008).

Anisakis Dujardin, 1845, *Pseudoterranova* Mozgovoy, 1951, *Hysterothylacium* Ward e Margath, 1917 e *Contracaecum* Railliet e Henry, 1912 são os gêneros envolvidos no aparecimento da anisakidose, nas quais as espécies *Anisakis simplex* (Rudolphi, 1809) e *Pseudoterranova decipiens* (Krabbe, 1878) ganham destaque por serem responsáveis pelo maior número de casos relatados (FERNANDEZ, 2010; KNOFF et al., 2007; MERCADO et al., 2001).

2.2.2 Hysterothylacium Ward e Margath, 1917

Fagerholm (1991) observou mudanças sistemáticas que ocorreram na super família Ascaridoidea constatadas através da morfologia da cauda dos machos de anisaquideos, e assim, alguns gêneros, até então pertencentes à família Anisakidae e a subfamília Raphidascaridinae foram elevados em nível de família, tendo sua nomenclatura modificada para Raphidascarididae Hartwich, 1954 *sensu* Fagerholm 1991.

Outra diferença entre o *Hysterothylacium* sp. e os indivíduos da família Anisakidae, é que segundo Navone et al. (1998) os adultos do gênero *Hysterothylacium* também podem parasitar peixes teleósteos e moluscos.

Muito embora o gênero *Hysterothylacium* atualmente seja classificado taxonomicamente em outra família, Lopes et al. (2011) sugerem frequente confusão entre os gêneros *Hysterothylacium* e *Contracaecum*. Isso se deve ao posicionamento do poro excretor que no gênero *Hysterothylacium* está próximo ao anel nervoso e no gênero *Contracaecum* na região do interlábio ventral, nem sempre conspícuo em ambos os gêneros.

Segundo Felizardo et al. (2009) *Paralichthys isoceles* apresentou uma

prevalência de 100% por *Hysterothylacium* sp. Ribeiro et al. (2014) descreveram presença de *Hysterothylacium* sp. em Pampo (*Trachinotus carolinus*) e Enxada (*Chaetodipterus faber*). Larvas de *Hysterothylacium* sp. já foram encontradas parasitando mais de 30 espécies de peixes teleósteos comercializados no Estado do Rio de Janeiro, no Estado do Rio Grande do Sul e no litoral da região Nordeste do Brasil (FELIZARDO et al. 2009).

2.2.3 *Hysterothylacium deardorffoverstreetorum* Knoff, Felizardo, Iñiguez, Maldonado Jr, Torres, Pinto & Gomes, 2012

Knoff e colaboradores (2012), em estudo taxonômico molecular realizaram a descrição de uma nova espécie pertencente ao gênero *Hysterothylacium*, taxonomicamente semelhante aos descritos anteriormente como *Hysterothylacium* sp. nº 2 (PETTER; MAILLARD,1988), *Hysterothylacium* MD (DEARDORFF; OVERSTREET,1981), *Hysterothylacium* KB (PETTER; SEY, 1997) e *Hysterothylacium* MD (PEREIRA JR et al., 2004). Onde foram descritas larvas que morfologicamente possuem cutícula com extensão lateral ao longo do corpo desprovida de extensão basal. Extremidade anterior com um lábio dorsal e dois lábios ventro-laterais pouco desenvolvidos. Nove papilas cefálicas, dois pares no lábio dorsal junto a uma grande papila e um par junto ao lábio ventro-lateral. Dente ausente. Abertura do poro excretor abaixo do anel nervoso. Ventrículo levemente esférico. Apêndice ventricular duas vezes o tamanho do esôfago. Ceco intestinal presente. Quatro glândulas retais subesféricas. Cauda cônica. Mucron presente.

Após a primeira descrição, gradativamente os trabalhos tem mostrado a prevalência desta espécie em peixes comercializados no Brasil. Fontenelle e colaboradores (2013) descreve prevalência de 83% em pescada Maria-mole (*Cynoscion guatucupa)*. Já no peixe olho de cão (*Priacanthus arenatus*) houve prevalência acima de 65% de H. deardorffoverstreetorum (KURAIEM et al., 2016). Fonseca et al. (2016) descreve a presença de *Hysterothylacium* sp. em linguado (*Paralichthys patagonicus* e *Xystreurys rasile*).

2.3 Anisakidose

A primeira descrição de parasitose por nematóide da família Anisakidae em

humanos ocorreu 1876, por Leucckart. O caso ocorreu em uma criança na Groelândia. Apesar desta, somente a partir de 1960, Van Thiel estabeleceu uma relação causa-efeito do parasito com a doença humana (TORRES, 2000).

A anisakidose é uma antropozoonose cosmopolita, que ocorre principalmente em regiões próximas ao litoral, devido à facilidade de consumo de produtos do mar. É causada pela ingestão acidental de larvas de nematóides da família Anisakidae, possui maior prevalência nos países cuja culinária tradicional envolva pratos crus, mal cozidos, defumados a frio, inadequadamente salgados e refrigerados. É considerada endêmica no Japão, Espanha, Chile e no Peru (CABRERA; OGNIO, 2002; LÓPEZ SERRANO et al., 2000; SÃO CLEMENTE et al., 1995; VALLS et al., 2005).

No Brasil, até o presente momento, há um possível relato sobre a anisakidose humana, diagnosticada através de endoscopia digestiva. No estudo evidencia-se a larva e lesões na mucosa duodenal. Porém não foi possível a identificação da larva através de microscopia para definição da espécie envolvida (CRUZ et al., 2010).

É importante considerar a subnotificação ou diagnóstico incorreto de casos de anisakidose devido a semelhança de sintomas de outras doenças com quadros gastrintestinais como a obstrução intestinal, apendicite, peritonite ou doença de Crohn (BARROS et al., 2006; MCCARTHY; MOORE, 2000; SABATER; SABATER, 2000).

A anisakidose é descrita nas formas gastrointestinal, extra-intestinal e alérgica. Pacientes podem apresentar dor abdominal posteriores à ingestão de peixes e/ou mariscos crus. A forma gastrointestinal apresenta-se de diferentes formas. Quando há apenas aderência do parasita à mucosa digestiva denomina-se luminal. Esta forma é quase sempre assintomática, sendo a larva expelida em fezes ou vômitos. Quando há a penetração da larva na mucosa, a sintomatologia é variada, provocando náuseas, vômitos e epigastralgias que surgem 24-48 horas após a ingestão do pescado cru (NUNES et al., 2003; TORRES, 2000).

A extraintestinal é de raríssima ocorrência e sua sintomatologia se apresentara de acordo com o orgão atingido pela migração do parasito (TORRES, 2000).

A forma alérgica é provocada pela sensibilização do sistema imunológico em contato com as larvas, seus produtos de secreção ou excreção induzindo uma

reação alérgica mediada por imunoglobulina E (IgE). Os antígenos podem ser liberados durante a fixação das larvas, durante a migração nos órgãos, no encapsulamento das larvas e na sua desintegração. A hipersensibilidade imediata é caracterizada por manifestações clínicas como urticária, angioedema, anafilaxia e excepcionalmente, a asma e dermatite (AUDICANA et al., 2002; MORENO et al., 2006; VALLS et al., 2005).

Os casos de hipersensibilidade ocorrem em consequência ao consumo do pescado parasitado por larvas vivas ou mortas de anisaquídeos em indivíduos previamente sensibilizados. Alguns antígenos de *A. simplex* são extremamente resistentes a aplicação do calor ou de congelamento não havendo alteração no seu potencial alergênico. Adicionalmente, a larva de terceiro estágio do *A. simplex* possui um amplo número de moléculas alergênicas, e a reatividade a estes alérgenos ocorre de forma exagerada (AUDICANA et al., 2002; LÓPEZ SERRANO et al., 2000; SABATER; SABATER, 2000).

Alguns indivíduos apresentam sinais clínicos como urticária e angioedema associado à dor abdominal e vômito, descrito como anisaquiose gastro-alérgica. Neste caso, os sintomas de hipersensibilidade após o contato com o parasito, são mais intensos e severos do que os gástricos (LÓPEZ SERRANO et al., 2000; VALLS et al., 2005). As manifestações clínicas surgem após um período de latência da ingestão do pescado cru ou insuficientemente cozido, que geralmente é de uma a doze horas para a afecção gástrica e reações alérgicas, e a partir de doze horas para afecções intestinais Acredita-se que os e antígenos provenientes de larvas de anisaquideos se dispersam na musculatura do peixe infectado e podem causar reações alérgicas em indivíduos sensibilizados mesmo que o nematóide se encontre morto quando consumido (SOLAS et al., 2008).

Kasuya et al. (1990) observaram que larvas de *Anisakis* sp foram os verdadeiros responsáveis por quadros de urticária em pacientes sem dor abdominal e sem suspeita clínica de anisaquiose. O *Anisakis simplex* é considerado como um agente etiológico capaz de originar alergias alimentares (DASCHNER et al., 1997).

A ingestão de peixe com larvas de nematóides da família Anisakidae pode desencadear tanto reações mediadas por Imunoglobulina E (IgE), como também pode provocar hipersensibilidade do tipo IV (reação tardia), e essa reação se caracteriza pela formação de um granuloma eosinofílico com ou sem a presença dessas larvas. Adicionado a esses fatores, pode ainda acarretar alergia

gastrointestinal, urticária, eczemas, vômitos, conjuntivite, dermatite de contato, úlceras gástrica e/ou intestinal, podendo determinar um choque anafilático e morte (AUDICANA; KENNEDY, 2008)

A anisakidose provocada por parasitos do gênero *Hysterothylacium* é de rara ocorrência e foi relatada por Yagi et al. (1996), onde um paciente relata dor abdominal e diarréia durante a passagem completa do parasito pelo tubo gastrointestinal, sendo expelido ainda vivo pelas fezes do individuo.

2.3.1 Prevenção e controle da Anisakidose

A evisceração a bordo logo após a captura tem sido apontada por diversos autores como uma medida de controle da anisakidose humana, uma vez que a presença dessas larvas na musculatura ocorre, em sua maioria, por migração das larvas nas vísceras para a musculatura durante os períodos de espera nos barcos e entrepostos (DIAS et al., 2010; VALLS et al. 2005). Além disso, como ressaltado por Sabater e Sabater (2000), não se deve descartar diretamente ao mar essas vísceras, pois os órgãos parasitados seriam ingeridos por mamíferos marinhos e completariam seu ciclo, evitando o aumento da disponibilidade do parasito em seu habitat. Para prevenção da anisakidose, recomenda-se a não ingestão do peixe cru, e a cocção deverá atingir 70^{o} C por um minuto. O congelamento na temperatura de -20^{o}C por, no mínimo, 72 horas ou -35^{o}C por 15 horas e a salga, desde que em altas concentrações de sal sejam distribuídas uniformemente em todo o peixe, são capazes de matar os parasitos (ACHA; SZYFRES, 2003).

Porém, a morte das larvas não inviabiliza os antígenos alergênicos, assim para minimizar a ocorrencia de alergia, indica-se a retirada da porção ventral da musculatura, visto que essa região, por estar mais próximo das vísceras é, em geral, a mais parasitada (SABATER E SABATER, 2000).

Outra medida importante para a prevenção é a conscientização. Alguns autores sugerem que as autoridades sanitárias realizem campanhas educacionais por folhetos explicativos, informes, reportagens, debates e propagandas. Através de uma abordagem correta, clara e didática evitando-se alarme social desnecessário, conduzindo à conscientização e educação sobre o assunto (DIAS et al., 2010; SABATER;SABATER,2000).

2.3.2 Reação cruzada entre antígenos de *Anisakis simplex* e *Hysterothylacium sp.*

Embora os relatos de anisakidose alérgica apontem o *Anisakis simplex* como principal responsável pelas reações de hipersensibilidade, diversos autores sugerem que há reação cruzada entre antígenos de *Anisakis simplex e Hysterothylacium* spp. esses parasitos possuem alguns antígenos comuns e outros que são espécies-específicos (FERNANDEZ-CALDAS et al., 1998; IGLESIAS et al., 1996; LOZANO-MALDONADO et al., 2004; MARAÑON et al., 1998).

Iglesias et al. (1996), avaliou a reatividade cruzada entre *Anisakis simplex* e outros nematóides, incluindo o *Hysterothylacium aduncum* (Rudolphi, 1802), através de diferentes antígenos extraídos. Foram utilizados os antígenos totais, antígenos secretado-excretados, antígenos do pseudoceloma e antígenos cuticulares. Observou-se uma reação cruzada moderada em relação aos antígenos somáticos de *A. simplex* e *H. aduncum*. Já em relação aos antígenos secretado-excretados, que são considerados os mais imunogênicos e são utilizados para o diagnóstico da alergia, houve uma reação considerável entre esses dois nematóides. Os outros antígenos também reagiram entre ambos nematóides.

Lozano Maldonado et al. (2004), também avaliando reatividade cruzada de *A. simplex* com outros nematóides, utilizou duas espécies diferentes, o *Hysterothylacium aduncum* e o *H. fabri* (Rudolphi, 1819), foram extraídos os antígenos somáticos e os antígenos secretado-excretados. Os autores apontam reatividade cruzada de ambas as espécies com *A. simplex*.

Já em estudos realizados por Fernandez-Caldas et al. 1998 e Marañon et al. 1998, foram testados soros de pacientes positivos para anisaquiose alérgica com antígenos de *H. aduncum* revelando uma reatividade cruzada em ambos.

3 DESENVOLVIMENTO

Foram realizados estudos experimentais para avaliação do potencial imunogênico/alergênico de larvas de *Hysterothylacium deardorffoverstreetorum*. Paralelamente foi elaborado o capítulo de livro sobre *Anisakis*.

A metodologia utilizada na experimentação, assim como os resultados obtidos, estão expressos em dois manuscritos submetidos para publicação em periódicos científicos. A formatação dos textos estão de acordo com as normas exigidas pelos editores de cada periódico.

3.1 Chapter 42 – ANISAKIS

Laboratory Models for Foodborne Infections by CRC Press: Food Microbiology Series- ISBN 9781498721677 - CAT# K25596. Edited by Dongyou Liu.

3.2 Cross-reactivity between Anisakidae antigens of commercial fish in Brazil

Artigo enviado a revista Ciência Rural (ISSN 0103-8478 – Qualis B1/ CAPES 2014 – Ciências Agrárias I). Foi aceito em 16 de agosto de 2016.

3.3 Resposta imunológica a antígenos de *Hysterothylacium deardorffoverstreetorum* de peixes teleósteos

Artigo enviado ao Arquivo Brasileiro de Medicina Veterinária e Zootecnia (ABMVZ – ISSN 0102-0935 - Qualis B2/ CAPES 2014 – Ciências Agrárias I). Submetido em 26 de agosto de 2016.

3.1 chapter 42

42.Anisakis

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42.1 Introduction

Parasites from the marine environment have historically been overlooked as a risk for human disease and are thus not in the main stream of basic or clinical investigation, although they can infect humans, causing anthropozoonosis, therefore a public health risk. Within the marine worms with clinical importance are those pertaining to the Anisakidaefamily (*Anisakis*, *Pseudoterranova* and *Contracaecum)* and Raphidascarididae family (*Hysterothylacium*) causing Anisakidosis.[1] Anisakids are nematodes whose definitive hosts are marine mammals; intermediate hosts are crustaceans (L2), fish and cephalopods (L3) and have a worldwide distribution. Humans become accidental hosts after ingestion of raw or undercooked infected seafood.[2]

There is an estimate of 20,000 human cases of anisakidosis with an annual registration of 2,000 new cases. The highest incidence with approximately 90% of all reported cases occurs in Japan[3] probably due to the routine habit of eating raw fish in dishes like sushi and sashimi.[4] Other countries that habitually consume raw or undercooked seafood also record an expressive number of cases of the disease. This is the case of European countries, mainly in the coastal areas of Germany, Netherlands and Scandinavian countries that consume salted, pickled and smoked herring, or Spain where typical appetizers are ceviche (fresh seafood marinated in lemon juice) and *Boquerón'senvinegar* (pickled anchovies).[1] In the Americas, there has been an increase in the number of reported anisakid cases, probably because of the popularization of oriental cuisine and the consumption of dishes like Lomi-lomi salmon and Ceviche [1,4,5] The improvement of diagnostic methods is probably another explanation for the increase in the report of new cases.

Since the first descriptions of human cases, the number of researchers that investigate actual and potential human marine infections has increased and several animal models have

been developed in order to understand the host-parasite relationship associated with the sensitization of individuals who accidentally ingest anisakid larvae. In this chapter, we will contextualize several *in vitro* and *in vivo* experimental models that are employed to reproduce and understand the natural history of human disease and explore the molecular and biological aspects of these parasites.

42.2 Taxonomy, life cycle and world distribution of *Anisakis* species

The taxonomic classification of Anisakids[6] consists of:

⇨ Kingdom: Animalia

⇨ Phylum: Nematoda

⇨ Class: Rabdititia (= Secernentea)

⇨ Subclass: Rabditia (= Phasmidea)

⇨ Order: Ascarida

⇨ Superfamily: Ascaridoidea

⇨ Family: Anisakidae

⇨ Genus:*Anisakis*

Pseudoterranova

Contracaecum

⇨ Family: Raphidascarididae

⇨ Genus: *Hysterothylacium*

Within the Ascaridoidea superfamily, the Anisakidae family is considered the largest and includes species that can parasitize fish, reptiles, mammals and fish-eating birds. The representatives of this family are dependent on the aquatic environment for the development

of their biological cycle and usually involve invertebrates and fish as intermediate or paratenic hosts.

Among the Anisakidae family, the species of the genus *Anisakis* have low specificity for the definitive host, but in general, live in the stomach of cetaceans such as whales, dolphins and porpoises. Those of the *Pseudoterranova* genus are more specific having the pinnipeds (seals, walruses and sea lions) as definitive hosts. The species belonging to the *Contracaecum* genus have as definitive hosts fish-eating birds and pinnipeds, and unlike the other genera,*Contracaecum* larvae can parasitize both marine and fresh water fish. Finally, the definite hosts of the species belonging to the *Hysterothylacium* genus of the Raphidascarididae family are pinnipeds, fish and shellfish. [7-10] As the biological cycles of the four genera are similar, and the aim here is the experimental approach to study these worms, we will only depict the *Anisakis* life cycle.

Adult worms release their eggs in the gut of the definite host. Through the feces the eggs gain access to the seawater, where they embryonate and form the first larval stage (L1) and progress to the second stage (L2). The L2 are eaten by small crustaceans such as krill, (first intermediate host), where they progress to the third stage larvae (L3) the infective stage for the definitive host. Second intermediate hosts, (fish or shellfish) ingest the crustaceans, which in turn are eaten by bigger fish transferring L3 are through the food chain and resulting in their accumulation in the larger fish until eaten by sea mammals, their definite hosts.[11,12] Once L3 have been eaten by their definite hosts they progress to the fourth larval stage (L4) and finally become adults.[13,14] (Figure 42.1)

When fish are captured, soon after their death L3 migrate to the viscera, peritoneal cavity and muscles. The degree of migration depends on environmental conditions, the parasite and fish species. When humans consume raw or undercooked infected fish or shellfish they may become accidental hosts. As the parasites are not adapted to humans, they

do not reach sexual maturity although they may cause from mild irritation to anaphylactic shock.[5,15,16]

At least 200 fish and 25 cephalopods species have been described as being infected with Anisakid larvae.[17-19] Within all Anisakids, species pertaining to the *Anisakis* genus are considered the most pathogenic and cause the largest number of human occurrences.[20,21] Although morphologically very similar, the genus has nine species that have been identified by molecular technologies, and have distinct definitive host distribution worldwide.[22-24] As depicted in Figure 42.2 Larvae are classified by their morphology and genetic characteristics in "clades (I and II)". Clade I contains the *Anisakis simplex* complex, which includes *A. simplex* (*strict sense*), *A. pegreffii*, *A. simplex* (complex), the other sister species in this clade are *Anisakis typica*, *Anisakisziphidarum* and *Anisakis nascettii*. The definitive host of the clade I species are mainly distributed in the Atlantic and Pacific oceans. *A. simplex* (ss) and *A. Pegreffii* are also found in the Mediterranean, Arctic and AntarcticaSeas.[2,12]*Anisakis* species pertaining to Clade II are classified as *Anisakis physeteris* complex, which includes *A. physeteris*, *A. brevispiculata* and *A. paggiae* species. Although considered to have a cosmopolitan distribution they are mainly found in the Atlantic Ocean.[2,25]

The *Pseudoterranova decipiens* complex consists of species that include the *P.decipiens* (*sensustricto*) or *P. decipiens B*; *P. krabbei*, *P.bulbous*, *P. azarasi* and *P. cattani*. They are considered cosmopolitan, and very abundant in the Atlantic Ocean, occurring from the Arctic to Antarctica.[26]

The *Contracaecum* genus has species that are able to parasitize both freshwater and marine organisms. From this genus the species that most frequently cause Anisakidosis pertain to the *Contracaecum osculatum* complex which is a set of five members *C. osculatum* types A, B, C, D and E where *Cosculatum sensu stricto* corresponds to type C.[27] The most

frequent geographic distribution of these species is the Alaskan and Japanese waters, the Baltic Sea and Antarctic and Atlantic Ocean.[7,28]

Although the *Hysterothylacium* genus has a worldwide distribution, it is described as a rare occurring causative agent of Anisakidosis. Apparently the first human case caused by the *H.aduncum* was only registered in 1996 in Japan[29]. Molecular identification of *Anisakis* and *Hysterothylacium* larvae from marine fish of the East China Sea and the Pacific coast of central Japan showed that approximately 10% of the larvae pertained to the *Hysterothylacium* genus, (*H.amoyense* - 5.0%, *H.aduncum* - 1.6%, *H.fabri* - 3.4% and *Hysterothylacium.* spp. - 2.9%) while the majority of the remaining Anisakidae nematodes belong to the *Anisakis* genus.[30] This result correlates well to the clinical finding.

Using classical techniques (Morphological taxonomy) Human Anisakidosis is most frequently described as being caused by *Anisakis* and *Pseudoterranova* genus.[29,31-33] Among the *Anisakis* species, *A. simplex* (ss) is reported as responsible for the highest number of human cases. However, after the introduction of molecular biology in taxonomy, *A. pegreffii* has been more frequently described as the agent responsible for anisakiosis in some countries, *e.g.*Italy.[18,34,35]

42.3 Allergen nomenclature

The abbreviation of the name of the gender (first three letters) and of the species (first letter) followed by a number indicating the chronology of the allergen purification was adopted as the systematic nomenclature of Allergens, implemented by the Nomenclature Sub-Committee of the World Health Organization (WHO) and International Union of Immunological Societies. So the *Anisakis simplex* allergens are called "Ani s #" e.g. Ani S1.[36]*Anisakis simplex* (ss) has 14 allergens characterized by origin and molecular aspects. The immunoreactivity pattern for these allergens has been studied both with human sera and with

experimental animals. A synthesis of the structural classification of *Anisakis* allergens is presented in Figure 42.3 based mainly on the allergen database AllFam[37] which can be accessed on the web at http://www.meduniwien.ac.at/allergens/allfam. The data was complemented from other published literature. For example, data from the Conserved domain database (CDD) and from the domain of unknown function (PF; DUF, Pfam) were used.

42.4 Pathogenesis, immunological response and clinical signs and symptoms

In humans, the ingestion of the live anisakid larvae causes distinct clinical forms of illness: gastric, intestinal and/or ectopic anisakidosis and/or allergic reactions, which may vary from mild to severe reactions. Although not a very common finding, gastro-allergic anisakiosis (GAA) is a well-established clinical entity, characterized by acute IgE-mediated urticaria, angioedema or anaphylaxis shortly after an *A. simplex* acute infection. The immunologic response that accompanies this parasite presents a significant polyclonal stimulation of different immunoglobulin isotypes comprising a mixed Th1 and Th2-mediated reaction.[38] The ingestion of dead Anisakid larvae or proteins derived from the larvae may also trigger mild to severe allergic reactions. Therefore anisakid extracts should be included in the standard sets of allergens used to investigate undefined allergies and anaphylactic reactions.[39]

The insertion of the cephalic portion of the larva in the mucosal wall and the secretion of proteases that permits its fixation results in a local inflammatory reaction that leads to the clinical symptoms such as epigastric pain, nausea, diarrhea, vomiting and fever.[40] Furthermore, when larvae penetrate the submucosa it may sensitize the host with its excretory-secretory (ES) products by stimulating the development of a predominantly Th2 immune response, which favors the production of IgE antibodies, responsible for allergies.[41] Persistence of larvae in the tissue can result in direct damage, and as a result the development of a eosinophilic granuloma, characterized by an inflammatory infiltrate of eosinophils and

neutrophils associated with a diffuse interstitial edema and proliferation of connective tissue around the body of the larva.[42-46]

There is evidence in the literature that the continued exposure to *Anisakis* antigens by fish factory workers, anglers and their families can sensitize them through inhalation or direct contact.[47,48] Farmers are another group of workers that can become sensitized to *Anisakis* antigens when in direct contact with the corresponding allergens in, e.g. fish meal.[47] Gastrointestinal conditions, asthma, conjunctivitis and occupational contact dermatitis have been frequently described in *Anisakis* sensitized patients.[47,49-53] Signs and symptoms can range from discreet allergic symptoms, urticaria up to Angioedema and fatal anaphylactic reactions with or without gastrointestinal symptoms. [54,55]

The human immune response to *Anisakis* sp. antigens is highly heterogeneous varying both in quantity and in quality between individuals.[56] Studies in patients showed that infection with *Anisakis* larvae induces a strong immune response with the production of specific antibodies reaching maximum titers within the first month of infection.[57] Infections with low numbers of larvae and continuous exposure frequently results in the production of high levels of IgE while the exposure to high numbers of larvae frequently results in the production of IgG. [58,59]The analysis of the cytokine profile obtained from the peripheral blood and intestinal biopsy samples of newly infected patients reinforces the concept that the Th2 response plays an important role in the immunopathogenesis of anisakiosis.[38] Further detail pertaining to the immune response shall be presented during the experimental section.

42.5 Laboratorial diagnosis

Initially specific IgG was used for to diagnose anisakiosis, however as the IgG titers persist elevated for a relatively long period, it is not a good parameter to differentiate current

from previous *A. simplex* infections. Another observation is that anisakid allergy is frequently associated with high levels of specific IgG4.[60-62]

A good diagnostic tool can be the use of the proportion of specific IgE and IgG4 titers. This strategy as has been used to evaluate allergic disease caused by a variety of other nematodes even if the nematode is not observed by a gastroscopy.[60,63,64] Thus, the serological diagnosis of a gastro-allergic anisakiosis can be a good alternative.[57,60,63,65] Chronic urticaria (CU) associated to anisakiosis is another clinical setting in which IgG4 can be used for diagnosis and follow-up. found that unlike those patients who continue their exposure to the fish, those that are subjected to a fish free diet experience a reduction CU symptoms improving significantly accompanied by significant reduction of IgG4 levels.[65] The comparison of the levels of IgE, IgG and IgG4 to *A. simplex* in CU and GAA patients showed that the latter presented significantly higher levels of all tested immunoglobulins.[66]

42.6 Experimental models

42.6.1 General considerations

Even if the conditions that are used in animal experimentation do not exactly match those that occur in the natural history of disease this is a widely used method for acquiring knowledge of various diseases in human and veterinary medicine. Through *in vivo* experimentation, it is possible to answer specific questions about the pathophysiology of diseases generating information that can then be extrapolated to the clinical setting permitting a better understanding of the disease, leading to better prevention and better treatment.

Since the discovery of the first human anisakiosis cases in the 1960's, many animal species have been used as a model for this disease. The first studies used rabbits and guinea pigs to understand the migration trajectory of the larvae to the tissues and granuloma formation. However, to study the allergic reactions induced by *Anisakis* larvae most

researchers prefer to use rats and mice. We chose to present the animal models by species and route of infection / sensitization.

42.6.2 Guinea pigs

Intradermic route. In order to evaluate the *in vivo* chemotactic effect of *A. simplex* larvae extract Tanaka and Torisu [67] used Guinea pigs as experimental animals. These researchers found that a few hours after intradermal injection of crude larvae extract (CE) a dose dependent accumulation of eosinophils occurred at the site of injection. To confirm this effect these authors carried out *in vitro* chemotaxis assays using Boyden chambers.[68] Using the same concentration of the extract with which eosinophil chemotaxis was observed no chemotactic activity was found for neutrophils supporting the idea that the CE plays an important role in the development of eosinophilia in anisakiosis.

Intraperitoneal route. Early in the 1980's, in the attempt to determine the etiologic mechanism of the allergic reactions associated to anisakiosis, guinea pigs were sensitized by implanting live *Anisakis* sp. larvae in the peritoneal cavity.[69] The Schultz-Dale[70,71] reaction was used to determine the presence of type I reactivity. In short, intestinal fragments of intraperitoneal-sensitized guinea pigs with live *Anisakis* larvae responded intensely when stimulated with *Anisakis* larvae hemoglobin and with less intensity when stimulated with CE from other Anisakids (*Contracaecum* and *Pseudoterranova*) while no response was observed when *Toxocara canis* or *Ascaris suum* extracts were used. These results confirm the IgE mediated etiology of the allergic reactions associated to anisakiosis

Intragastric route. To determine the migratory pattern and viability of live *Anisakis* larvae these were delivered to the gastric cavity. Larvae gained different organs and tissues passing the stomach wall through an active migration mechanism without a pre-established migratory pattern. Live larvae without any morphological changes were recovered up to the 5th day after administration. These were able to re-infect another guinea pig maintaining the

same migration capability. However, as of the 6th day, post infection, all larvae disappeared leaving no hint of its presence in any part of the body.

Guinea pigs experimentally infected with *A.simplex* have also been used to test drugs.[72] For example oral treatment with ivermectin or albendazole was tested presented high *in vivo* efficacy against the larvae present in different organs of the guinea pigs.[73]

42.6.3 Pigs

Oral route *Anisakis* larvae infection in pigs was studied by feeding the animals with fish contaminated with L3. In these studies, researchers observed that the severity of injury was proportional to the number of larvae ingested. Histological alterations due to larvae interaction with the mucosa included primary mechanical damage accompanied with bleeding, ulceration of the mucosa and submucosa, intense cellular infiltration with connective tissue proliferation around the larva.[74] The histological changes of the stomach mucosa from experimentally infected pigs with *Anisakis* sp. and *Pseudoterranova* sp. larvae involved intense inflammatory reaction around the larva with the presence of numerous eosinophilic cells.[75] After almost 3 decades, studies using the pig as an experimental study were resumed several larvae feeding pigs L3 *C. osculatum* and observed the same histopathological findings that corresponded to findings in infections caused by other pathogens.[76]

42.6.4 Rabbit

The histological aspects of intestinal sections of experimentally infected rabbits resemble those of accidentally infected human the suggesting a similarity of the pathogenesis. Thus rabbits were successfully introduced as experimental anisakiosis models soon after the publication of the first human anisakiosis cases.[77]

Intragastric route. In the early 1970's the experimental determination of the pathogenesis of anisakiosis was performed by administrating live larvae to the stomach of rabbits and semi-quantitatively grading the inflammatory reaction of the surrounding tissue where larvae penetrated.[78] Three days after the oral administration of 40 *Anisakis* larvae, only a very small number entered the stomach wall, many of which were still alive and the degree of the inflammatory reactions of the gastric mucosa surrounding the distinct larvae varied between mild, moderate or severe in an individual animal and between individuals.

Necrosis, massive amounts of granulocytes, including eosinophils, were the main findings on day three after infection. On day five, the larval viability declined and an infiltrate of plasma cells and immunoblasts was observed along with the granulocytes in the center of the reaction while in the periphery fibroblasts were already present. After seven days, the fibroblast infiltrate became more intense, by ten days, granulation tissue is observed and by a month, the necrotic tissue was substituted by new connective tissue surrounded predominantly by mononuclear cells with moderate amounts of eosinophils.

The serological reactivity in association to the histopathological pattern was also studied in rabbits infected with 30 live *Anisakis simplex* larvae through the oral rout. Although most larvae were recovered in the stomach, some migrated from the gastrointestinal tract and reached extra-gastric tissues resulting in the formation of abscess that contained dead larvae. By 30 days, the reactions progressed to granulomatous abscesses followed by calcification of the larvae.[79]

From the serological point of view, IgG peaked by 30 days coinciding with the granuloma resolution and calcification of the larva followed by an abrupt decline. Another study that infected rabbits with 10 larvae showed a peak of IgM on the 11th day while IgG peaked approximately a month later. [80]

Intragastric sensitization of rabbits with *Anisakis* larvae was also employed to assess the recognition pattern of somatic and secreted antigens of infective *Anisakis* larvae comparing possible relationships with antigens from other nematodes of ascaroidea family using radioimmunoprecipitation techniques.[81] Such as in serum derived from *Anisakis* infected patients, infected rabbits preferentially respond to somatic antigens and that the recognition sequence occurs to different components of secreted antigens. The differences in the recognition of secreted / excreted antigens and somatic components may be due to the duration of sensitization and the degree of penetration by nematodes in the tissues. Kennedy, et al. [81] also demonstrated that a 14 kDa component derived from *A. simplex* cross-reacts with a homologues component derived from *Ascaris suum, Ascaris lumbricoides, and Toxocara canis*, species from the Ascaroidea family.

Subcutaneous route A chemotactic factor selectively attractive for eosinophils found in the extract from *Anisakis* larva was termed eosinophil chemotactic factor of parasites (ECF-P).[67] To determine if the eosinophilicphlegmonous inflammation typically observed in human anisakiosis could be experimentally reproduced normal and subcutaneously immunized rabbits received intraserosal injection of ECF-P into the ileum of rabbits. All rabbits developed a significant eosinophilic inflammation at the injection site in a dose-dependent manner. Although immunized rabbits presented high anti-ECF-P antibody titers while normal animals had no detectable antibody there was no significant histological difference between the lesions observed in either group of rabbits. These results support the argument that, in especially in the early phase of primary infection with anisakiosis, ECF-P may contribute to the development of eosinophilicphlegmonous inflammation without any immunologic intervention.[67,82]

Intramuscular route One of the experimental protocols involves the intramuscular route to investigate if larval antigens of *Anisakis simplex* present molecular similarity to

interleukin IL-4. The resulting rabbit anti-mouse IL-4 antibodies were tested against *A. simplex* ES and CE antigens in ELISA. The anti-IL-4 antibodies showed a strong cross reactivity, which was confirmed by western blot analysis. A complementary assay, the absorption of the anti-IL-4 sera with *A. simplex* antigen, demonstrated a 70-80% inhibition of antigen binding when retested in ELISA. These results support the hypothesis that *A. simplex* proteins, share several epitopes with IL-4, or conversely that *Anisakis simplex* larval excretory-secretory and somatic products present IL-4-like molecules. This finding implies that the parasite may control and modulate the mucosal Th1-Th2 dichotomy for its own benefit in an attempt to avoid its expelling.[83]

Currently experimental *Anisakis* research has not used rabbits as a model to study allergic reactions caused by this nematode. However intramuscular inoculation with *Anisakis* antigens has been employed when the aim is to characterize allergens and to produce laboratory reagents.[84-88]

42.6.5- Rats

Rats have been used extensively to investigate the immune response to *Anisakis* larvae. Although the oral route is the natural form of infection, in the experimental scenario investigators have shown a limited usefulness of *per os* administration due to the difficulty in accurately determining the parasite load since many larvae are expelled through the anus hampering the establishment of the relationship between parasite load and immune response.[89] Although the surgical implant may appear to be an inadequate route of infection, the argument used to validate this technique and to expect that the antibody production profile would be the same regardless of the route, is that orally administered larvae pass from the intestinal lumen into the peritoneal cavity after infection [90,91]. Another observation that supports to this hypothesis is that extra-gastrointestinal anisakiosis has also been observed in humans that are infected [92].

Intraperitoneal larval Implant Immunization of rats by intraperitoneal L3 larvae implantation was used to determine the immune response to SE and CE. In contrast to oral infection in rabbits, intraperitoneal implantation of live larvae in rats induced a strong response to both SE and CE antigens. After 63 days of implantation no larva was found alive, thus the hypothesis is that the immune response was due to the release of somatic antigens in the peritoneal cavity.[81]

ES-specific IgM and IgG titers of rats inoculated with increasing *Anisakis simplex* L3 load (1, 5, or 20 larvae) show a positive correlation after the primary inoculum but not to the secondary inoculum. IgM and IgG titers of animals inoculated with 20 larvae did not further increase. However, after the second inoculation, those animals that received 1 or 5 larvae presented antibody titers comparable to the 20 L3 inoculation.

The primary inoculation induced low ES-specific IgE antibody titers in all groups and in the secondary inoculation, a negative correlation was obtained. In other words, rats receiving 1 larva developed higher IgE titers than rats receiving larger inoculums. IgE titers of single larvae-inoculated rats peaked at 3-5 days after secondary inoculation and disappeared by day 14, which is consistent with the duration of infection. Thus, monitoring ES-specific IgE may be a useful diagnostic tool for human intestinal anisakiosis since in the natural scenario infections typically course with low larvae loads.[93]

Intragastric infection Let us return to the intragastric/intraperitoneal duel. Authors argue that despite the importance of the live larvae intraperitoneal inoculum studies, the human natural history of gastroalergic anisakiosis is given orally, so experiments using this pathway are important.[59] Rats were infected with L3 *Anisakis* by the oral route twice with an interval of 9 weeks to investigate the kinetics of isotype-specific antibody expression, and found that IgM's peak with similar titers after primary and reinfection presenting the same antigenic recognition. After reinfection, as expected, IgG1 and IgG2a levels were higher and

showed accelerated kinetics, however, IgG2b level was substantially lower. The biological allergy state peaked earlier (1 week) than the immunochemical allergy state (2 weeks). Since no meaningful correlation between specific IgE avidity and biological allergy state was found and elevated IgM levels at reinfection occurred, the hypothesis is that the allergic response induced by oral L3 infection might not be related to specific IgE avidity.[94]

A procedure developed recently to deliver live larvae directly to the stomach of mice by an esophageal catheterization [95] was adapted to perform live *Anisakis* spp. infection in rats.[96] The aim of this study was to understand the histopathological effects of acute (single) and chronic (multiple reinfections – 24, 48, 72, and 96 h intervals) *Anisakis* infection. Live larvae were found anchored to the mucosa at different locations (whose milieu varied from a very acid to basic pH gradient), passing through the stomach wall and in organs out of the gastrointestinal tract. The histopathology showed an acute inflammatory reaction, with eosinophil predominance and a mild fibrotic reaction. Even though not all larvae were recovered, as previously placed as an obstacle to the oral route this protocol can be considered a good experimental model because the histopathological alterations are similar to those described in human anisakiosis.[97]

Although there are reported cases of allergic reactions due to the ingestion of cooked and frozen seafood, there is also evidence that only live larvae trigger the allergic reactions. Consequently, the debate on the risk of *Anisakis*-associated hypersensitivity by ingestion of properly cooked and frozen fish remains. To elucidate this fact an experimental model was designed to study the antibody production kinetics in after oral inoculation with live or dead *Anisakis* L3. The results show that animals produce specific IgM, IgG, and IgE to ES antigens after primary and secondary inoculation with live L3 but not after dead L3 (frozen, heated, cut, or homogenized). These results suggest that the ingestion cooked or frozen seafood containing *Anisakis* L3 is safe even for allergic individuals.[98]

***In vivo* L3-L4 transformation model in rats** To study the morphological transformations of L3 to L4 *Anisakis* type I, *P. decipiens*, *Contracaecum* type B and *Hysterothylacium* L3, recovered after experimental infection in rats, and *Anisakis* type I L4 derived from humans were examined with the aid of scanning electron microscopy to examine the anterior and posterior extremities and the cuticular structures of the larvae. Rats were sacrificed at different times after oral administration and a careful search in the digestive tract, abdominal cavity, muscles, and viscera was performed. Molting from L3 to L4 was observed as of the third day onwards in rats that received *Anisakis* type I and *P. decipiens*. *Anisakis* larvae penetrated the stomach and the intestinal wall, a single larva of *Pseudoterranova* penetrated to muscularis mucosa of the stomach. No *Contracaecum* larvae were recovered. Electron microscopy revealed that L4 of *Anisakis* type I from rat and man were similar, while the L4 of *Anisakis* type I and *P. decipiens* showed ultrastructural differences. which might be of clinical value for the identification of fragments recovered during endoscopy in man.[99]

42.6.6 Mice

Because of the accumulated data in the last decades, in special concerning IgE synthesis the antibody associated to allergic reactions, mice are considered better animal models, than other species, to investigate allergic reactions.[100-102] In food-associated allergies, it is still unclear what conditions make certain foods strong IgE inductors. There are reports of over 170 foods causing food allergies, but only eight (peanut, tree nuts, milk, egg, wheat, soy, fish and shellfish) account for 90% of all food-allergic reactions.[103] It is known that the major reaction to food proteins when ingested in physiological conditions usually is a phenomenon called oral tolerance while the parenteral administration of the same food proteins in experimental models induces sensitization.[104-110] This intriguing dichotomy has interested immunologists.

It is also known that in natural helminthic infections IgE and eosinophilia are major hallmarks of the immunological response as the consequence of a Th2 lymphocyte profile activation by helminths. Among other interleukins, Th2 cells secrete IL-4 and IL-5, the first, promotes immunoglobulin class switching to IgE, and the latter stimulates eosinophil development and activation. Furthermore, in IgE experimental models, animals are immunized with the antigenic preparations mixed with adjuvants such as aluminum hydroxide [102] or pertussis toxin [111,112] and commonly administered by a parenteral route. In experimental models where the aim is to develop oral sensitization and food allergy, antigens are associated to cholera toxin.[113] Complete or incomplete Freund's adjuvant is another commonly used adjuvant which is considered a good IgG inducer.[114]

The humoral and cellular immune responses to live *Anisakis simplex* larvae observed in mice models share similarities with those observed in human disease. However due to the difficulty in introducing live larva into the gastric cavity of mice, the majority of the experiments have been conducted by immunizing the animals with CE or with ES antigens of cultivated larvae. Thus, indicating the relevance of the investigation of the immune mechanisms that control the allergic responses to live and dead *Anisakis* spp. larvae.[115-118]

Intraperitoneal larva implant *Anisakis simplex* L3 were surgically implanted into the abdominal cavity of mice to investigate histopathological alterations.[119] Necropsy performed at 7, 14, or 21 days post infection evidenced that larvae were mostly found embedded in the gut mesentery and only rarely invaded the viscera. On day 7, adjacent to viable parasites, an intense neutrophil aggregation characterizing an acute inflammatory reaction was observed, by Day 14, this reaction evolved to a mature eosinophilic granuloma with large numbers of fibroblasts and associated collagen. Granulocytes and occasionally multinucleate giant cells were observed at the still viable host-parasite interface. By day 21 the L3 were dead, invaded by inflammatory cells and the lesions displayed the predominance of connective tissue.

Multinucleate giant cells and eosinophils adjacent to parasite remnants or scattered within the walls of the granulomata was frequent. Hematological findings, regardless of the number of implanted worms showed that on Days 7 and 14 mice presented neutrophilia of varying magnitude accompanied with an eosinopenia that began to return to normal values by day 21. Both hematological and histological findings are consistent with those seen in human anisakiosis.

Intraperitoneal immunization To help understand some of the unknown immune interactions between helminth infection and allergy mice were intraperitoneally sensitized to develop a hypersensitivity reaction with *A. simplex* proteins, by followed by an intravenous or oral *A. simplex* challenge. The sensitized mice presented as of the 3rd week specific IgE, IgG1 and IgG2a to numerous *A. simplex* allergens, some of which were similar to those found in human serum. When challenged with intravenous *A. simplex* antigens but not after an oral antigen challenge anaphylaxis and plasma histamine release was observed. The cellular and molecular profile showed that *A. simplex* stimulated splenocytes to release IL-10, IFN-γ, IL-4, IL-13 and IL-5 thus a mixed Th1/Th2 pattern.[120] This seems a good model to investigate the peculiar allergic reactions to parasitic proteins.

Intragastric infection Live *Anisakis*L3, were orally inoculated in C57BL/10 and BALB/c mice to investigate isotype-specific immune responses to ES and CE products. The C57BL mouse strains typically produces a Th1-Type cytokine profile while BALB/c mice produce a Th2-Type cytokine profile. Both ES and CE antigens stimulated similar antibody patterns however CE stimulated the production of higher antibody levels. BALB/c mice produced a faster IgM response than C57BL/10 mice while the latter produced higher IgG1 and IgG2b antibodies with practically undetectable IgG2a levels.[121] Further anisakiosis studies using BALB/c mice, showed that after multiple immunizations using Freund's adjuvant mice presented a has a single maximum peak of IL-4 between weeks 8 -14 while animals

inoculated with a single larva *peros* showed two IL-4 peaks. The first with moderate levels, between days 6 - 12 p.i. and the second maintained from week 3 to 9.[122]After Perteguer and Cuellar papers showing the consequences of natural sensitization [121,122] the authors of this chapter proposed a simplified method to introduce live larvae with an intragastric tube. This technique results in similar data as those published in the literature.[95,118]

Epicutaneous immunization As cited before contact dermatitis is one of the consequences of antigen exposure to *Anisakis* proteins in seafood-processing workers. Thus to understand the basic mechanisms in the development of allergic sensitization through the skin repeated epicutaneous exposure of *Anisakis* proteins in wild-type (WT), IL-4, IL-4Rα, IL-13 and IL-4 / IL-13 deficient mice were evaluated by following the systemic signs and symptoms. Epicutaneous sensitization with *Anisakis* larval antigens induced in the WT localized inflammation, epidermal hyperplasia, production of TH2 cytokines, antigen-specific IgE and IgG1 and anaphylactic shock after intravenous challenge. IL-13 deficient mice failed to develop epidermal hyperplasia and inflammation, and in IL-4, IL-4 / IL-13 and IL-4Rα deficient mice anaphylaxis was reduced. These results suggest that interleukin-13 plays a central role in contact dermatitis development whereas IL-4 drives the Th2 profile and resultant anaphylactic reactions.[48]

Subcutaneous immunization The subcutaneous route is a technique frequently utilized in immunological studies. The footpad is a very often-used location since the draining lymph nodes are easily removed making it possible to study the local immunological response. Taking the advantages of this strategy the cellular immune response to *Anisakis simplex* L3 antigens was compared in mice that were infected either after a pre-sensitization with a homologous CE antigen or not. The immunization protocol induced an increase in the size and weight of the popliteal lymph nodes (PLN) after footpad injection. A high proportion of systemic $CD4^+$, $TCR\alpha\beta^+$ T cells in both groups. A reduction in B cells accompanied by a

decrease of CD8α$^+$ T cells was observed in pre-immunized and infected mice while those only exposed to infection present the greatest increase in CD8α+ and TCRαβ- T cells.[117]Histological analysis showed that the most prominent lesions were gastric and intestinal in animals infected orally with one larva.

Intranasal immunization. To examine the immunological mechanisms underlying the development of allergic airway inflammation Wild-type (WT) and interleukin-4 receptor alpha (IL-4Rα)-deficient mice were sensitized to *Anisakis* antigens through different routes.[123] Live or heat-killed *Anisakis* larvae were administered intraperitoneally while *Anisakis* extract was administered by the intranasal rout. Subsequently all animals were challenged intranasally with an *Anisakis* extract. Allergen-specific antibodies developed only in intraperitoneally sensitized mice however, both routes of sensitization induced IL-4Rα-dependent allergic airway responses in WT mice thus an IL-4/IL-13 dependent pathway. Unexpectedly, infection with live *Anisakis* larvae induced Airway hyper responsiveness that was abrogated when IFN-γ was neutralized *in vivo*. Thus, infection leads to IL-4/IL-13 independent, IFN-γ dependent airway hyper responsiveness. Together, these results demonstrate that both infection with larvae and inhalational exposure to *Anisakis* proteins are potent routes of allergic sensitization, explaining food- and work-related allergies in humans, which can involve either IL-4/IL-13 or IFN-γ. Importantly for diagnosis, detectable *Anisakis*-specific antibodies may not accompany allergic airway inflammation.

In vitro studies demonstrated that a 24 kDa protein (22U homologous; As22U) derived from *Anisakis simplex* larva elicits several Th2-related chemokine gene expression meaning that it may be one of the important allergens for the clinical setting. In order examine their hypothesis 6 intra-nasal applications of ovalbumin (OVA) or recombinant As22U (rAs22U) and OVA was performed. When compared to the group that only received OVA, the animals challenged with rAs22U associated to OVA, presented severe airway inflammation, immune

cell recruitment, in special, eosinophils, increased levels of IL-4, IL-5, and IL-13 in the BALF, significantly increased airway hyper responsiveness, significantly higher anti-OVA specific IgE and IgG1. After receiving rAs22U, the GRO-α (CXCL1) gene expression increased immediately while eotaxin (CCL11) and TARC (CCL17) gene expressions increased significantly at 6 hr. Thus rAs22U may be responsible for a Th2/Th17 mediated airway allergic inflammation.[124]Using the same experimental protocol two other *Anisakis* antigens (Ani s 1 Ani s 9) were tested eliciting similar results expressing Th2 (IL-4, IL-5, IL-13, e IL-25) and Th17 (IL-6 e IL-17) cytokines because of the intranasal exposure.[125]

Nematode molecules as immunoregulators In the last decades, a variety of immunoregulatory molecules has been isolated from a number of nematodes. The identified biological activities include actions equivalent to cytokines, protease inhibitors, macrophage migration inhibitory factor-like protein (MIF), proteins as poison expressed sequence tags (ESTs) and allergen.[126-132] The *Anisakis simplex* macrophage migration inhibitory factor like protein obtained from third stage larvae of *A. simplex* was cloned (rAs-MIF) and tested in a murine OVA/Alum induced asthma model.[129] The rAs-MIF treatment coupled with OVA/alum induced a complete inhibition of eosinophilia, reduced lung goblet cell hyperplasia, profoundly improved lung hyperactivity, reduced the quantity of Th2-related cytokines (IL-4, IL-5, and IL-13) in the BALF and allergen-specific IgG2a in sera. Conversely, the BALF of the rAs-MIF-treated group contained significantly higher of IL-10 and TGF-β than controls. Additionally, rAs-MIF recruited regulatory T cells ($CD4^+CD25^+Foxp3^+$) to the spleen and lungs.

These authors evaluated the function of rAs-MIF on a dextran sodium sulphate (DSS) induced intestinal inflammation. Mice treated with rAs-MIF recovered weight loss and the disease activity index (DAI) value. The cytokine profile evaluation showed that rAs-MIF-treated mice presented higher levels of splenic and mesenteric lymph nodes (MLN) TGF-β

and IL-10 with lower levels of IFN-γ, IL-6 and IL-13. Additionally, Treg were greatly increased in the MLNs of the rAs-MIF-treated mice. *In vitro* experiments showed that rAs-MIF stimulated IL-10 production via toll-like receptor 2.[133]

Further studies on rAs-MIF also showed that TLR2 gene expression was significantly increased following rAs-MIF treatment. To father understand the relation between TLR2 and the amelioration mechanisms of rAs-MIF, the OVA/Alum allergic airway inflammation protocol was induced with or without rAs-MIF associated or not to anti-TLR2-specific antibody and comparing WT and TLR2 knockout mice. As a result, the amelioration effects of rAs-MIF in allergic airway inflammation model as previously described were diminished under two of the TLR2 blocking model. The expression of TLR2 on the surface of lung epithelial cell was significantly elevated by rAs-MIF or Pam3CSK (TLR2-specific agonist) treatment.[134] While α-mTLR2 Ab or Pam3CSK pretreatment inhibited the elevation of IL-10 gene expression by rAs-MIF suggesting that the anti-inflammatory effects of rAs-MIF might be closely related to TLR2.

42.6.7 Fish

Many marine fish are infected with third-stage larvae of *Anisakis simplex (stricto sensu).* To ensure food safety, it is important to determine whether these larvae are present in the flesh of commercial fish species. However, there is little information regarding the tissue specificity of Anisakid species. Thus, the rationale for the use of fish as an experimental model to study Anisakidae nematode is to understand the infective capacity in commercially relevant fish species, the parasite mechanisms of aggression, and the host's immunological response.

Oral infection. Rainbow trout (*Oncorhynchus mykiss*), and olive flounder (*Paralichthys olivaceus*) received L3 larvae of two sibling species of *A.simplex per os* and were accompanied for 5 weeks. In the rainbow trout, *A.simplex s.s.* predominantly migrated into the

body muscle while a small number of freely moving *A. pegreffii* larvae were recovered within the body cavity. In the olive flounder, *A. simplex s.s.* larvae were found in both in the body cavity and the muscle, while *A. pegreffii* larvae were only found in the body cavity encapsulated in lumps.[135]

In another set of *in vivo* investigations, *A. simplex* was used to experimentally infect Rainbow trout (*Oncorhynchus mykiss),* Baltic salmon (*Salmo salar*) and brown trout (*Salmo trutta*). Of the three species, Baltic salmon was the most susceptible presenting the highest number of successfully established nematodes, whereas brown and rainbow trout had a higher natural resistance. The preferred *A.simplex* larvae microhabitat in the brown trout was the stomach, pyloric caeca, and intestine, while the majority of larvae found in rainbow trout were located at the pyloric caeca. In the Baltic salmon, the most susceptible fish species, nematodes were dispersed in and on the spleen, head kidney, liver, swim bladder and musculature. $CD8^+$ cells were present while IgM^+-bearing cells were absent in the inflammatory tissue around the nematodes of all three fish species. MHCII-bearing cells were present in the encapsulated *A. simplex* in rainbow and the brown trout, but not in Baltic salmon.[136]

Yet, another set of recent experiments show that closely related salmonids differ in their susceptibility towards different anisakid larvae and agree that parasites select different microhabitats in the hosts.[137]Orally infected Rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*), and Atlantic salmon (*Salmo salar*) with larval stages of *H. aduncum, C. osculatum,* or *A. simplex* were studied to determine parasite survival and location up to14 days post infection (dpi). Although the most prevalent and numerous nematode in brown trout at 2 dpi was *H. aduncum,* a large proportion of the worms were already recovered dead with no tissue penetration. This fish species exhibited the highest natural resistance to *A. simplex.* Rainbow trout exhibited the highest susceptibility to *C. osculatum* larvae at 2, 7, and 14 dpi with eventual pyloric cecum penetration. *A. simplex* larvae established a more successful

infection in salmon compared to rainbow trout although at 2 and 7 dpi this fish showed the highest intensity and abundance of larvae, but not after 14 days. Although the pyloric ceca was the preferred microhabitat for *Anisakis* in both rainbow trout and salmon larval penetration into muscle and liver were found.

Intraperitoneal Since hydrolytic enzymes play an important role, in the nematode host tissue penetration, determination of which enzymes are present within the ES proteins seems important. Lipase, esterase/lipase, valine and cysteine arylamidases, naphthol-AS-BI-phosphohydrolase and α-galactosidase activities were found. To further elucidate the influence of intraperitoneally injected ES substances on the immune system of fish specific gene expression in spleen and liver of the rainbow trout (*Oncorhynchus mykiss*) was measured. The results demonstrate a generalized down-regulation of immune related gene expression suggesting a suppressive immunomodulatory role for ES proteins. Form the ecological point of view this makes biological sense. One can argue that when worm enzymes directly target the host's immune molecules a decreased immune response with an increased worm survival is the consequence.[138]

42.6.8 *In vitro* cultivation

The *in vitro* cultivation of nematodes has been for long a goal of the field of parasitology. These techniques permit the understanding of parasite behavior, physiology and metabolism as well as the molecular nature of the ES products and their relationship with the host. This in turn, permits more adequate vaccines production designs, vaccine efficacy testing, and antigen-production for serological reagents, detection of drug-resistance, screening of potential therapeutic agents and conducting epidemiological studies. However, the complexity of the parasite's life cycle involving different host species for their developmental stages frequently makes their cultivation a difficult task. Each parasite requires different cultivation conditions with specific nutrients, temperature and incubation conditions.

A search in biological data bases indicate that the first papers regarding parasite cultivation, in general, were published in the 1910's[139] and the first *Anisakis* cultivation papers in the 1970's.[140] An important systematization of the developed technique was performed by Silverman [141] which has been updated in a diversity of technical books.[142,143]

For many clinically important parasites, *in vitro* cultivation is an important diagnosis tool. An array of commercial systems, which have been developed, such as the Harada-Mori culture technique for larval-stage nematodes, permit rapid diagnosis. In comparison although *in vitro* cultivation techniques are used more often than *in vivo* techniques, the *in vivo*techniques are sometimes used for diagnosing parasitic infections such as trypanosomiasis and toxoplasmosis. Parasite cultivation continues to be a challenging diagnostic option. Thus, an overview of intricacies of parasitic culture and an update on popular methods used for cultivating parasites are presented

Culture media The first description of Anisakidae nematode cultivation occurred in the early 1960's. *Pseudoterranova decipiens* larvae removed from the flesh of fresh fish were immediately transferred to 199 culture media enriched with glucose, beef embryo extract, beef liver extract and antibiotics. In this study, the authors obtained larvae that reached morphological changes consistent with adult worms.[144] Subsequently, with adjustments of the initial conditions *A. marina* developed successfully to adult worms. The first larval developmental changes were observed within four days with the release of cuticles in the medium. The development of gonadal tissue characterizing the pre-adult stage occurred between 26 to 98 days. The complete maturation characterized by the worm wall thickening, and gonadal maturation can be distinguished *in vitro*. The first free larvae were observed after 4-8 days at a temperature of 13-18°C and after 20-27 days, at a temperature of 5-7°C. The larvae are very active and their mobility has no fixed direction. In seawater they can live for 3-4 weeks at temperatures of 13-18°C, for 6-7 weeks at 5-7°C, temperatures above 20°C lead

to increased mortality, and a temperature of 34°C was absolutely inadequate, indicating that the first intermediate host must be cold-blooded.[145]

L3-L4 transformation model Improvements of *in vitro Anisakis* L3 culture conditions were introduced in 1976, these allowed to explore of the formation of cuticles and ecdysis.[140]Different culture media (199, Krebs-Ringer), carbon dioxide concentration, temperature, storage conditions were tested. Among the tested conditions culture media 199 gave the best results, with the highest number of molts and viability. The carbon dioxide concentration of 5% in low concentrations is more efficient in the first 40 hours of cultivation. Using fluorescent tracers it was determined that larvae do not feed (salt and glucose) until their digestive tract is complete in other words when they enter the fourth stage of development (L4).

To simulate the natural conditions of the fish's body, where the larvae remain for long periods in anabiose and determine the temporal resistance, *Anisakis* L3, were collected from herring and kept in saline solution culture (0.65% NaCl) at about 5° C. The mortality of *Anisakis* in culture presented three phases. Phase 1 (months 1-2): low mortality. Phase 2 (month 3-5): significant increase in mortality rate. Phase 3 (month 6-8): only the strongest survive larvae. Thus the larvae kept in saline solution survived for about 35 weeks.[146]

CO_2 fixation is an important metabolic process for many organisms. In anisakid nematodes, CO_2 has been shown to be required for its development, at least *in vitro*. Comparing culture conditions, molting to L4 was reduced to 1/3, after a 30 day culture in air which corresponds to a 1/3 of the survival of L3 cultivated in air + 5% CO_2. Thus, at suitable temperatures, a high pCO_2 is vital for the optimum development of L3 to adult (M3). Regarding the activity of the CO_2-fixing enzymes, Phosphoenolpyruvate Carboxykinase (PEPck) activity (305nmol/min.mg protein) was much higher than that of PEPC (6.8 nmol/min.mg protein). The activity of these enzymes in the worms cultivated in air + 5% CO_2

was highest during M3, and in general was higher than that of those cultivated in air only, especially during molting from L3 to L4. The presence of CO_2 stimulates the molting from L3 to L4 and prolongs the survival at least *in vitro*.[147-153]

A. simplex L3-larvae tend to prefer fish tissues with high lipid content.[154]*In vitro* tests were carried out to study the behavior of *A. simplex* L3 in response to different concentrations of cod liver oil lipids. Larvae were placed into culture dishes containing agar separated into three segments, containing 0,2 to 7% of cod liver oil. The results demonstrate that although L3 move randomly they do not stop randomly. The tendency to move out of a certain area was inversely correlated with lipid concentration. A second observation indicates that the intentional migration range of larvae is short. In conclusion, L3 prefer high-fat content and seek it over short distances. These *in vitro* data agree with previous observations that *A. simplex* L3, randomly tend to migrate out of the fish gut into the flesh.[155]

42.6.9 Larvicida lmodels

With the growing number of human anisakiosis cases, an alternative was the search for active larvicidal compounds. *In vitro* and *in vivo* assays were undertaken to evaluate herbs used to season fish based on epidemiological observations that prevalence of Anisakidosis in the Chinese regions where raw fish is often seasoned with ginger (rhizome of *Zingiber officinale*) and /or "perilla mint", "Chinese basil", or "wild basil" common names for *Perilla frutesceans* (Lamiaceae) is smaller [156,157]

In vitro studies showed that [6]-Shogaol and [6]-gingerol components derived from ginger rhizome, induced an important reduction in larvae mobility and altered both their cuticle and digestive tract.[158] Further studies revealed that [10]-gingerol, [10]-shogoal, other compounds derived from ginger also has a very effective larvicidal effect.[159]

In vivo protocols where rats were infected by delivering larvae directly to the stomach through the use of a gavage was used to evaluate the action of essential oils on *Anisakis* L3

Simultaneously or 2 h after infection each rat received one of five monoterpenes. To determine the localization and viability of the larvae and determine gastrointestinal histopathological changes rats were sacrificed at various times points. The order of *in vivo* larvicidal activity was peril aldehyde >citral>citronellol>cuminaldehyde>carvacrol. When peril aldehyde, citral and citronellol, were given together with the nematodes no hemorrhages were observed leading to the conclusion that these monoterpenes, somehow inhibit the fixation and/or penetration capacity of the larvae. The time gap of 2h between the infestation and the administration of any of the tested compounds is sufficient for the larvae to develop their pathogenicity in the rats.[160]

Three sesquiterpenic derivatives (nerolidol, farnesol and elemolto) were studied to determine their *in vivo* larvicidal activity. The order of *in vivo* larvicidal activity wasnerolidol>farnesol>elemolto; the first two caused the death of all nematodes, which showed cuticle changes and intestinal wall rupture. Only 20% of infected rats treated with nerolidol or farnesol showed gastric wall lesions in comparison to 86.6% of control animals suggesting that nerolidol and farnesol are good candidates for further research as biocidal agents against L(3) larvae of *Anisakis* type I.[161]

The histological parameters to evaluate the effect of potentially larvicidal compounds were the analysis of the cuticle and intestinal wall structure. Fixed formalin *A. simplex* L3 was assessed by optical microscopy study of transverse thin sections (0.5-1μm) stained with hematoxylin eosin, Masson's trichromic dyes or toluidine blue.

Knowing that essential oils can irritate the mucosa, gut inflammatory reaction was studied after oral administration of the tested compounds.[161]A marker of neutrophilic infiltration is the titration of myeloperoxidase activity (MPO), determined by solubilization of myeloperoxidase with hexadecyltrimethylammonium bromide and measured with a dianisidine-H_2O_2 assay.[162]

42.7 Conclusions

A range of laboratory models are available to investigate foodborne infectious diseases, including those due to *Anisakis* nematodes. As presented in the a short epidemiological and taxonomical review of the anisakid family along with the review of laboratory models used to study anisakiosis there are still many open questions regarding the life cycle, host-pathogen interaction, pathogenesis and immune response of the anisakid family larvae. These questions should be addressed because these nematodes are more frequently contaminating foods due to the diffusion of oriental and Spanish cuisine making this an emerging anthropozoonosis thus of clinical importance

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Figures

Fig 42.1

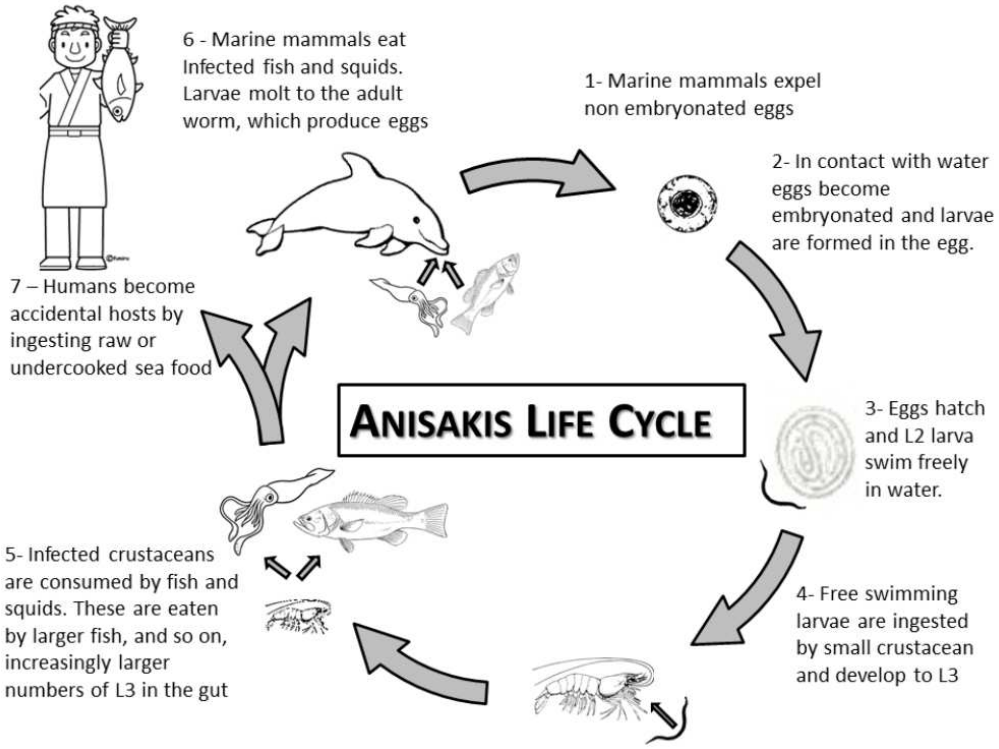

Fig 42.2

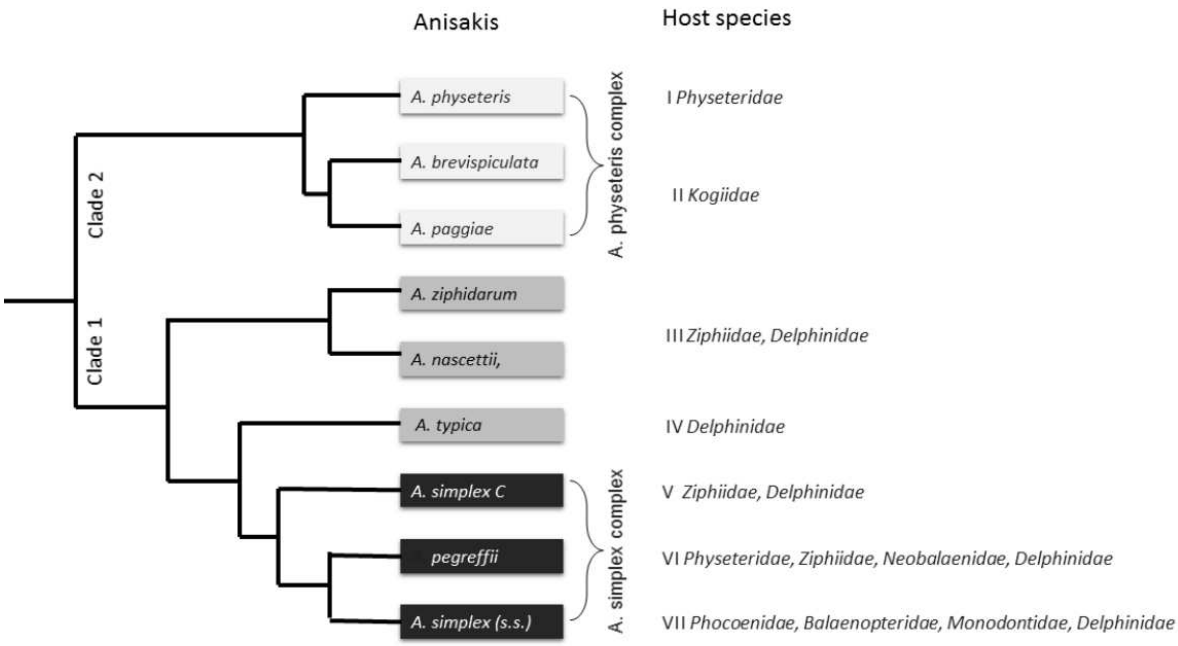

Fig 42.3

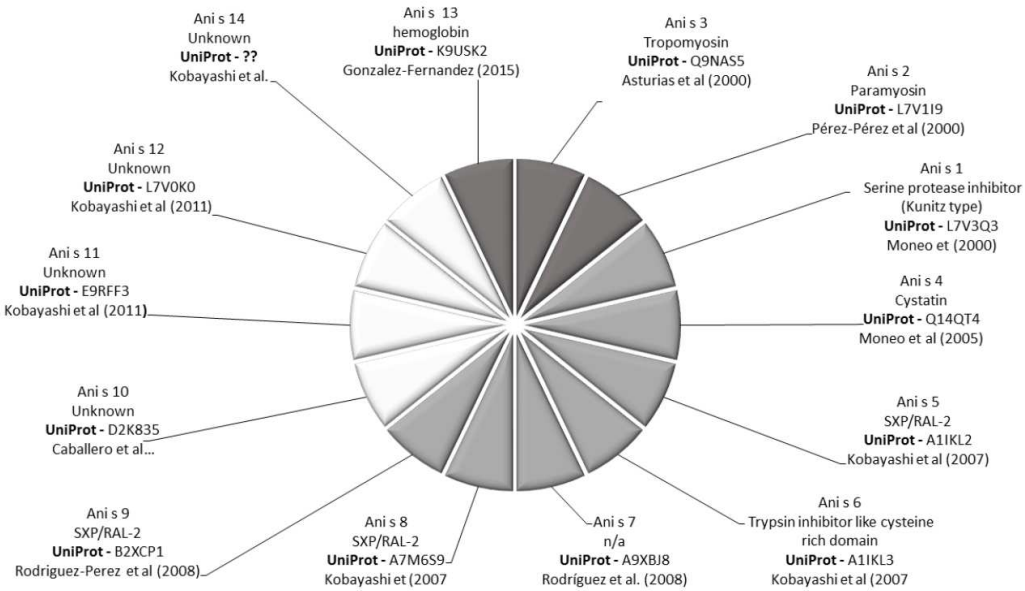

Figure legends

Figure 42.1 Life cycle of anisakids

Figure 42.2 Cladistic distribution of anisakid larvae

Figure 42.3 Updated *Anisakis* allergencompiled mainly from data extracted from the Allergome database in combination with published literature. The colors of the wedges indicate the origin of the antigens: Dark grey - Somatic antigens, Medium grey -Excretory-secretory antigens; light grey – unknown origin http://www.allergen.org/treeview.php

3.2 Resposta imunológica a antígenos de *Hysterothylacium deardorffoverstreetorum* de peixes teleósteos

RESPOSTA IMUNOLÓGICA A ANTÍGENOS DE *HYSTEROTHYLACIUM DEARDORFFOVERSTREETORUM* DE PEIXES TELEÓSTEOS

[Imunne response against *Hysterothylacium deardorffoverstreetorum* from teleost fish]

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RESUMO

Anisakidose é uma doença provocada por parasitos da família Anisakidae e se caracteriza por manifestações gastrointestinais e alérgicas. O *Anisakis simplex* é o parasito mais patogênico ao homem e altamente alergênico. Porém, outros Anisaquídeos também são danosos aos humanos, mas é desconhecida a imunogenicidade dessas larvas. O objetivo deste trabalho foi avaliar o potencial imunogênico do parasito *Hysterothylacium deardorffoverstreetorum* (HD) em modelo murino. Camundongos da linhagem BALB/c foram divididos em três grupos experimentais e receberam as preparações antigênicas obtidas de larvas de HD. Extrato bruto de larvas (E.B.H), extrato secretado/ excretado de larvas (ESH) e extrato bruto de larvas após excreção/secreção (EEH). Amostras séricas foram obtidas em diferentes dias após imunização para determinação dos níveis de anticorpos específicos pelo ensaio imunoenzimático (ELISA). Os resultados demonstram aumento na produção de IgG após a segunda imunização com aumento progressivo. Já em relação à IgE, a reatividade foi mais tardia, demonstrando aumento progressivo após a terceira imunização. Foi avaliada a imunidade celular através da intradermorreação, como resultado estatisticamente significativo em relação ao controle

utilizado. Este experimento é a primeira descrição da potencialidade patogênica deste parasito em mamíferos e representa um avanço no diagnóstico da Anisakidose humana.

Palavras-Chave: Anisakidose; *Hysterothylacium*, Infecção experimental, Modelo murino

ABSTRACT

Anisaquidosis is a disease caused by parasites of Anisakidae family and is characterized by gastrointestinal and allergic reactions. The *Anisakis simplex* is Anisakidae more pathogenic to humans and highly allergenic. However, other species of this family also have characteristics harmful to humans, but little is known about the immunogenicity least described parasites. The objective of this study was to experimentally assess the immunogenic potential of the parasite *Hysterothylacium deardorffoverstreetorum* (HD) in mice. Mice of inbred BALB/c strain were divided into three groups and received three immunizations one of the following antigenic preparations obtained from L3 larvae HD: Crude larval extract of HD (CEH) Extract secreted / excreted larvae HD. (ESH) and crude extract of larvae after excretion / secretion (EEH). Serum samples were obtained on different days after immunization to determine the levels of circulating specific antibodies by enzyme-linked immunosorbent assay (ELISA). The results show increased production of IgG after the second immunization with a gradual increase. Regarding the IgE reactivity was later, demonstrating a progressive increase only after the third immunization. Also it evaluated the cellular immunity by intradermal, statistically significant result compared to the control used. This experiment is the first description of the pathogenic potential of this parasite in mammals and represents a breakthrough in the diagnosis of human Anisakidosis.

Keywords: Anisakidosis; *Hysterothylacium*, Experimental infection, Murine model

INTRODUÇÃO

Os peixes teleósteos capturados e comercializados na costa brasileira são comumente parasitados por nematóides das famílias Anisakidae e Raphidascarididae. Parasitos membros destas famílias utilizam os peixes como hospedeiros intermediários e podem ser encontrados em vísceras e musculatura (CRUZ et al. 2010). O homem atua como hospedeiro acidental ao consumir o pescado cru, insuficientemente cozido, defumado a frio ou inadequadamente salgado contendo larvas de terceiro estágio desses nematoides (SABATER; SABATER, 2000). Desta forma, a ingestão acidental de larvas da família Anisakidae pode ocasionar uma doença conhecida como Anisakidose. Atualmente sabe-se que nematóide *Anisakis simplex* é a espécie mais importante para saúde publica, seguido de *Pseudoterranova decipiens*

(HOCHBERG, & HAMER, 2010). Larvas de *A. simplex* possuem um grande poder de sensibilização do sistema imunológico, o que pode resultar em manifestações alérgicas e gastrointestinais severas (HOCHBERG, & HAMER, 2010, CHO et al. 2014). No entanto, com rara ocorrência outros membros são citados como causadores de Anisakidose, como larvas de *Contracaecum* sp.e de *Hysterothylacium aduncum*. (YAGI et al., 1996; BARROS et al. 2006; HOCHBERG, & HAMER, 2010; PISCAGLIA et al. 2014). Com relação a estas duas ultimas espécies, há poucos estudos sobre a resposta imunológica e o potencial alergênico das larvas para seres humanos. Com as larvas dos parasitos do gênero *Hysterothylacium*, foram realizados somente estudos experimentais com o objetivo de avaliar reatividade cruzada com antígenos de *Anisakis simplex* (FERNANDES-CALDAS et al. 1998). Esse trabalho tem por objetivo a investigação experimental sobre o potencial alergênico de larvas de terceiro estágio do parasito *Hysterothylacium deardorffoverstreetorum* com utilização de modelo murino.

MATERIAL E METODOS

Parasitos e antígenos - No presente estudo, os parasitos foram coletados de peixes das espécies *Cynoscion guatucupa* (Pescada Maria mole); *Priacanthus arenatus* (Olho de cão); *Paralichthys isococeles* (Linguado), comercializados nos mercados de Niterói e Rio de Janeiro no período de agosto de 2012 a novembro de 2014. As larvas foram então identificadas em microscópio ótico, seguindo as descrições realizadas por Knoff et al. (2012). Depois de identificados, os parasitos foram processados com três preparações diferentes. Extrato bruto de larvas de *Hysterothylacium deardorffoverstreetorum* (E.B.H), extrato secretado/ excretado de larvas de *Hysterothylacium deardorffoverstreetorum* (ESH) e extrato bruto de larvas após secreção/excreção - esgotado- (EEH). O CEH foi obtido através da maceração do parasito íntegro. Já o ESH foi obtido através da imersão de larvas vivas em meio ácido, a fim de induzir a excreção dos antígenos desejados. Já o EEH foi obtido através das larvas que passaram pelo processo de extração do antígeno secretado/excretado mediante a incubação de larvas L3 vivas em meio ácido, com uma concentração de ácido clorídrico entre 50 e 100 mMol/L em temperatura de 37°C segundo protocolo citado por Valls et al. (2003). Os antígenos foram obtidos segundo protocolo descrito por Perteguer et al. (1996). Em um homogeneizador de Potter (Thomas Phila U. S. A.) as larvas, em solução de cloreto de sódio a 0,9%, foram desintegradas na presença de fluoreto fenil-metil-sulfonil (PMSF). Posteriormente, foi centrifugada a 8500 x *g* em temperatura 4 º C por um período de 30 minutos na centrífuga (Internacional portable refrigerated centrifuga Model PR). A

quantificação protéica dos extratos foi realizada pelo método de Lowry et al. (1951), foi utilizada albumina sérica bovina (BSA) 1mg/mL como padrão.

Animais - Para o experimento utilizou-se camundongos BALB/c de 8-10 semanas de idade de ambos os sexos, criados e mantidos no biotério local (Núcleo de Animais de Laboratório-NAL-UFF), Esses animais foram alojados em ambientes com exaustão de ar, temperatura ambiente a 23-25 ºC, alimentados Nuvilab CR-1 Chow (Nuvital Nutrientes S/A)e água destilada tratada com 0,1% HCL ad libitum.. Todos os procedimentos foram aprovados pelo Comitê de Ética da Universidade Federal Fluminense sob o numero 00137/09.

Protocolo de imunização – Foram utilizados três diferentes grupos, cada um com seis camundongos. Os animais foram imunizados pela via intraperitoneal com 10 ug de cada preparação antigênica e associado a 2mg de hidróxido de alumínio no volume de 0,2ml nos dias 0, 21 e 42 do experimento. Como controle, foi utilizado o soro coletado no dia 0 antes da primeira imunização. A contenção química para inoculação e sangria foi realizada com xilazina 200 µg/kg, associada a ketamina 10mg/kg.

Obtenção e preparo do soro - Amostras de sangue foram colhidas do plexo retro-orbitário em um volume de 0,1 ml nos dias 0 (controle antes da imunização), 14^o, 21^o,28^o,35^o,42^o,49^o,56^o,e 70^o do experimento.O material foi inicialmente diluídos 1:10 em salina fisiológica e então foi centrifugado a 800 x *g* por 10 minutos e separado o soro.

Determinação dos níveis de anticorpos IgG e IgE específicos -A presença de anticorpos dos isotipos IgE e IgG, anti-*Hysterothylacium* nos soros dos camundongos foi determinada através do método imunoenzimático ELISA. Em placas com poços de fundo chato (Maxi - Sorp - Nunc) foram colocados antígenos de *Hysterothylacium deardorffoverstreetorum* reparados segundo o método descrito anteriormente. O antígeno foi colocado na concentração de 20 µg proteína/mL em tampão contendo bicarbonato 0.05M, pH 9.6. As placas foram incubadas durante duas horas a 37 ºC, e foram lavadas em tampão fosfato salina (PBS) pH 7.2. Os sítios livres foram bloqueados com solução de gelatina a 1% em PBS (PBS-G) durante duas horas a temperatura ambiente. Em seguida foram lavadas três vezes em PBS contendo 0,05 % de Tween 20 (PBS- T). Os soros foram diluídos em PBS-G de forma seriada na base 3 a partir de 1:40 e incubados por duas horas a 37ºC. Os anticorpos conjugados a peroxidase: anti-IgE (cadeia ε), (RatAnti-Mouse IgE – Invitrogen) e anti- IgG total (L e H) (1:10000), (RabbitAnti-MouseIgG, wholemolecule - Sigma), diluídos na solução de bloqueio foram acrescidos a reação (50 µL/ poço) e as placas foram mantidas a 37 ºC durante uma hora. Após lavagem das placas com PBS-T a reação com o substrato e cromógeno se fez pela adição de 50 µL/ poço de solução coletada 10 µL de peróxido de hidrogênio a 30% diluídos

em 25 mL de tampão citrato a 0,1 M em presença de 10 mg de OPD (ortofenilenodiamina). As placas foram incubadas durante 5 minutos a temperatura ambiente. A reação enzimática foi interrompida com adição de solução de ácido sulfúrico 4N. A leitura de densidade óptica (DO) foi realizada na leitora de microplaca (Thermo Plate TP-READER) em comprimento de onda de 492 nm. O resultado foi expresso através das médias aritméticas do somatório das DO das diluições. A análise dos resultados foi realizada pela comparação do somatório das DO de cada soro.

Avaliação da Imunidade celular- Grupos de camundongos BALB/c imunizados com antígenos secretados e somáticos HD, receberam na 8ª semana após a imunização secundária, uma injeção intradérmica no pavilhão auricular de 20 µL da solução antígeno somático com salina fisiológica a 1 mg/mL. A espessura do pavilhão auricular foi feita antes da inoculação e 24, 48 e 72 horas após a injeção com micrômetro de mostrador (Mitutoyo nº 7301).

Análise estatística - A analise estatística foi realizada por análise de variância com pós teste de Tukey com o programa GraphPadInStat – versão 4.10 for windows XP, GraphPad Software, San Diego Califórnia USA, www.graphpad.com Copyright 1992-1998. Na análise estatística dos dados experimentais, foram considerados que os valores significativos a partir de $p<0,05$ (Rodrigues, 1996).

RESULTADOS

Os resultados obtidos no presente estudo demonstram que após a primeira imunização nos três grupos experimentais não houve elevação dos níveis de anticorpos IgG, no entanto após a segunda e a terceira imunização (realizadas nos dias 21 e 42 dia, respectivamente) houve uma gradativa elevação dos níveis de anticorpos até o 49º dia e esses níveis foram estatisticamente significativos em comparação as amostras séricas obtidas após a primeira imunização. Conforme demonstrado na Fig.1, Analisando os níveis de anticorpos produzidos ao longo do experimento, nenhuma diferença significativa foi observada entre dos três grupos experimentais (EBH, ESH e EEH). Na figura 2 verifica-se os grupos imunizados com EBH e ESH responderam após a segunda imunização 35º dia, uma discreta, porem significativa elevação dos níveis de IgE ($p<0.05$). Entretanto, a partir do 42º dia da terceira imunização realizada, os três grupos responderam com uma marcante elevação de anticorpos IgE específicos atingindo ao nível máximo no 56º dia seguido de uma diminuição no 70º dia. Diferente dos níveis de IgG houve diferença estatística nas diferentes preparações nos dias 14, 28 e 35 entre o EEH que ficou um pouco abaixo dos demais antígenos. A avaliação da imunidade celular (figura 03) mensurada pela intradermorreação revelou que as três

preparações antigênicas apresentaram um aumento do espessamento auricular nos três tempos avaliados (24, 48 e 72 horas). No entanto verificou-se no tempo de 48 para 72 horas que o grupo sensibilizado com EBH aumentou, diferente dos grupos ESH e EEH que mantiveram no mesmo patamar da leitura anterior. Os resultados obtidos indicam que as preparações antigênicas do HD apresentaram potencial imunogênico desencadeantes da produção de anticorpos IgG e IgE específicos em camundongos BALB/c após a segunda imunização. Os animais imunizados apresentaram uma positividade na intradermorreação, principalmente para EBH, sugerindo um forte envolvimento da imunidade celular no processo imunológico estudado.

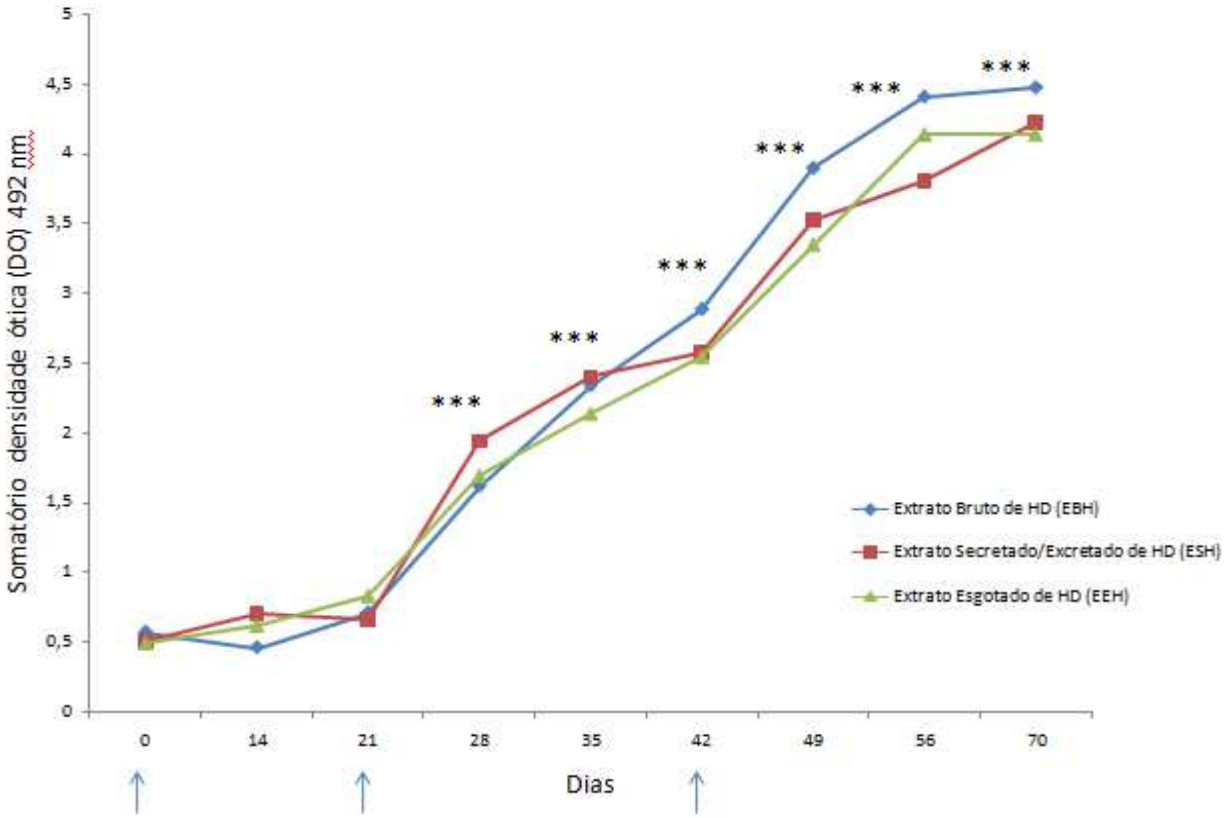

Figura 01 - Níveis de anticorpos IgG anti larvas de *Hysterothylacium deardorffoverstreetorum*. Camundongos foram imunizados com diferentes preparações antigênicas de larvas de *H. deardorffoverstreetorum*. As setas indicam as imunizações (0, 21 e 42 dias). Os valores indicam as médias do somatório das DO. +/- erro padrão da média de cada grupo. A análise estatística foi realizada através de ANOVA, com *** p>0.001comparado ao dia zero.

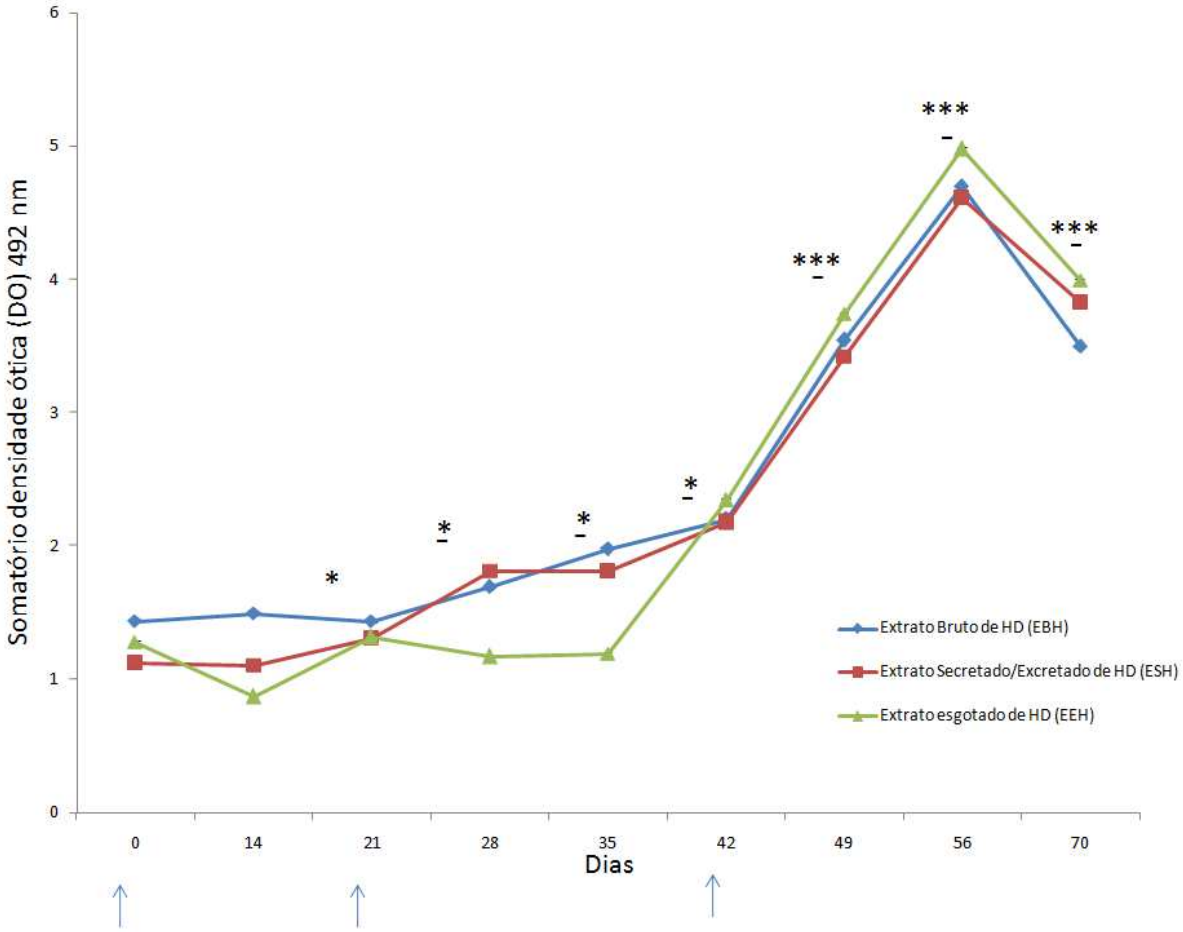

Figura 2- Níveis de anticorpos IgE anti larvas de *Hysterothylacium deardorffoverstreetorum* . Camundongos foram imunizados com diferentes preparações antigênicas de larvas *de H. deardorffoverstreetorum*. As setas indicam as imunizações (0, 21 e 42 dias). Os valores indicam as médias do somatório das DO. +/- erro padrão da média de cada grupo. A análise estatística foi realizada através de ANOVA, com *p>0.05 e ***p>0.001comparado ao dia zero.

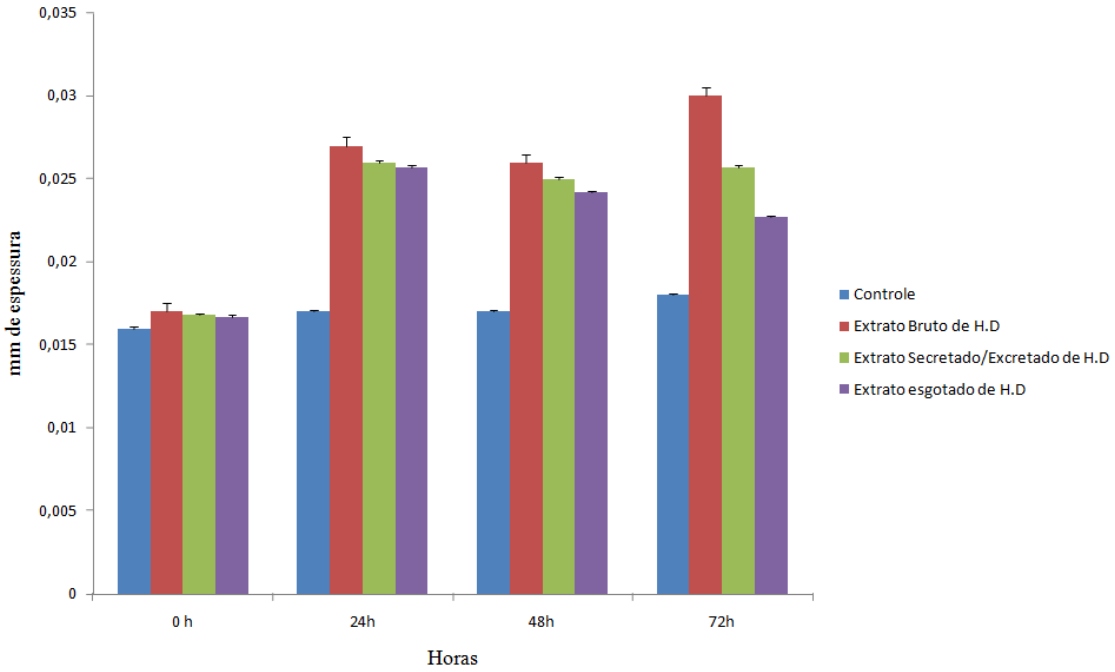

Figura 3- Mensuração da espessura do pavilhão auricular dos camundongos BALB/c sensibilizados com antígenos EBH, ESH e EEH. Camundongos foram imunizados com diferentes preparações antigênicas de larvas de *H. deardorffoverstreetorum*. As setas indicam as imunizações (0,21 e 42 diasOs resultados estão expressos em mm de espessura +/- erro padrão da média de cada grupo. A análise estatística foi realizada através de ANOVA, com **p*<0.05 e ****p*<0.001 comparado a 0 h.

DISCUSSÃO

Nas infecções por helmintos a produção de IgE é um marco da resposta imunológica. Esta é consequência da ativação de um perfil linfocitário Th2 pelos helmintos na qual predomina a secreção de IL-4 que por sua vez promove a mudança de classe de imunoglobulinas secretadas pelos linfócitos B para IgE. Apesar de este ser um conhecimento de longa data a investigação inicial da resposta imunológica a larvas de terceiro estágio (L3) de *A. simplex* foi conduzida através da identificação da produção de anticorpos IgG específicos, mais abundantes no soro e portanto mais acessíveis à pesquisa (CHO & LEE, 2006). Embora controverso, a produção de IgG 4 específica tem sido utilizada por investigadores para avaliação de doenças alérgicas,uma vez que esta imunoglobulina também se liga a epítopos reconhecidos pela IgE específica, sendo assim considerada como indicadora de estados de

doença alérgica (CHO & LEE, 2006). Na forma alérgica de anisakidose, o achado mais relevante é o aumento dos níveis de IgE total e específica. Em humanos as respostas contra antígenos de *A. simplex*, tanto com anticorpos IgE quanto com IgG são altamente heterogêneas variando muito entre os indivíduos tanto quantitativa quanto qualitativamente (AUDICANA & KENNEDY, 2008). Esta heterogeneidade de resposta pode ser observada em modelo experimental murino, onde a imunização com extrato bruto de larvas L3 de *A. simplex* na cavidade peritoneal resulta na maior produção de anticorpos específicos da classe IgG2a do que IgG 1, o que indica um predomínio da resposta imunológica com padrão Th1 sobre a resposta Th2 (CHO & LEE, 2006). Em estudo realizado por Baeza et al. (2004) com o objetivo de avaliar as diferentes preparações antigênicas do *A. simplex* comprovou que o produto provenientes da secreção/excreção do parasito mostrou-se mais alergênico que a preparação com antígeno somático. Esse resultado difere parcialmente do presente estudo, uma vez que em relação ao HD, não houve diferença entre as preparações antigênicas na produção de IgG. Nos níveis avaliados de IgE, houve diferença apenas no grupo imunizado com larvas após excreção (EEH), cujo resultado é abaixo dos demais antígenos, sugerindo que o antígeno mais alergênico está presente na secreção/excreção da larva ou na larva antes do tratamento em meio ácido, pois na avaliação da imunidade celular, o antígeno bruto obteve resultado mais expressivo que os demais. Estudos alergênicos realizados anteriormente com larvas de *Hysterothylacium* spp. apontam o objetivo de avaliação de reatividade com outros nematóides, sobretudo com larvas de *Anisakis* sp. Em avaliação da reação cruzada testando-se soro de pacientes com anisakidose alérgica com antígenos de *H. aduncum*, evidenciou-se reação positiva, confirmando a reação cruzada entre esses dois nematóides. (FERNANDEZ-CALDAS et al. 1998 e MARAÑON et al. 1998). Em estudo realizado por IGLESIAS et al. (1996), com objetivo de avaliar a reatividade cruzada de *Anisakis simplex* e outros nematóides, incluindo o *Hysterothylacium aduncum* foram fracionados diferentes antígenos. Utilizaram-se antígenos totais, antígenos secretado-excretados, antígenos do pseudoceloma e antígenos cuticulares. Observou-se uma reação cruzada moderada em relação aos antígenos somáticos de *A. simplex* e *H. aduncum*. Outro estudo que comparou a reatividade cruzada de *Anisakis simplex* com diferentes antígenos, incluindo duas espécies diferentes, o *Hysterothylacium aduncum* e o *H. fabri* foi realizado por LOZANO MALDONADO et al. (2004). Foi evidenciada a reação cruzada em ambos os antígenos testados, tanto somáticos, quanto secretados.

CONCLUSÃO

O presente estudo é o primeiro relato do potencial patogênico em mamíferos, desse parasito que está amplamente distribuído no continente americano. Esses resultados mostram que essas larvas são capazes de ativar o sistema imunológico tanto celular quanto humoral. Estudos posteriores serão necessários para melhorar o esclarecimento sobre o mecanismo de ação do parasito, frações antigênicas mais importantes e reatividade cruzada.

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3.3 Cross-reactivity between anisakidae antigens of commercial fish in Brazil

Cross-reactivity between Anisakidae antigens of commercial fish in Brazil

Reatividade cruzada entre antígenos de Anisaquídeos de peixes comercializados no Brasil

Ribeiro, J.[I]; Knoff, M.[II]; Felizardo N.[III]; Vericimo M. A.[IV]; São Clemente, S. C.[III]

RESUMO

Anisakidose é uma enfermidade provocada pela ingestão acidental de parasitos da família Anisakidae. As manifestações incluem distúrbios gastrointestinais e alérgicos e para seu diagnóstico, são utilizados métodos sorológicos, a fim de identificar anticorpos específicos. Porém, pode haver reação cruzada com nematóides da mesma família. O presente estudo teve como objetivo avaliar a reatividade de anticorpos oriundos de camundongos sensibilizados com antígenos de *Hysterothylacium deardorffoverstreetorum* (HD) frente a antígenos do parasito *Anisakis simplex* (A.S). Foram utilizadas larvas de *Anisakis simplex* e *Hysterothylacium deardorffoverstreetorum* coletadas de peixes comercializados nos municípios de Niterói e Rio de Janeiro. Esses nematóides foram identificados por microscopia ótica e processados a fim de se obter extrato parasitário (Crude extratct - CE). Esse extrato foi utilizado para imunização de camundongos Balb/C e após obtenção do soro, foram realizados ELISAs para determinação de anticorpos das classes IgG e IgE. Os resultados demonstram que houve reação a antígenos homólogos e heterólogos e embora a reação tenha sido maior contra antígenos de HD, a reatividade foi significativa para antígenos A.S.

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Palavras-chave *Anisakis simplex, Hysterothylacium deardorffoverstreetorum,* Anticorpos, Reatividade cruzada, Infecção experimental, Modelo murino

ABSTRACT

Anisakidosis is a disease caused by accidental ingestion of parasites belonging to *Anisakidae*. Its manifestations include gastrointestinal and allergic disorders, and serological methods are used to diagnose it and to identify specific antibodies. However, there may be cross reaction with nematodes of the same family. This study aimed to evaluate the reactivity of antibodies derived from mice sensitized with *Hysterothylacium deardorffoverstreetorum* (*HD*) antigens against *Anisakis simplex* (*AS*) parasite antigens. Larvae of *Anisakis simplex* and *H. deardorffoverstreetorum* were used and collected from fish market in the municipalities of Niterói and Rio de Janeiro, Brazil. These nematodes were identified by optical microscopy and processed to obtain parasitic extract [Crude extratct (CE)]. This extract was used for immunization of Balb/c mice and, after obtaining serum, enzyme-linked immunosorbent assays (ELISA) were performed for determining antibodies of the Imunoglobulina G (IgG) and Imunoglobulina E (IgE) classes. Results show that there was reaction to homologous and heterologous antigens and, although the response was higher against *H. deardorffoverstreetorum* antigens, the reactivity was significant to *A. simplex* antigen.

Keywords - *Anisakis simplex, Hysterothylacium deardorffoverstreetorum,* Antibodies, Cross-reactivity, Experimental infection, Murine Model

INTRODUCTION

Anisakidae family parasites have worldwide distribution and are commonly found in fish market on Brazilian coast (DI AZEVEDO et al., 2015). These nematodes have zoonotic character and are responsible for causing a disease denominated Anisakiadosis. *Anisakis*

simplex is the main member of this family. Manifestations caused by accidental ingestion of these parasites include gastrointestinal disorders and allergic reactions with different symptomatologies. Existing diagnostic methods involve careful medical history, endoscopies, and serologic tests, which are especially useful as the allergic aspect (CHUNG; LEE, 2015). Serologic methods aim to identify antibodies present in the individual, which are specific to *A. simplex*; however, some studies describe the cross-reactivity of nematodes of this genus with others of the different family, such as the *Hysterothylacium* genus (LOZANO-MALDONADO et al., 2004). This parasite is commonly found on Brazilian coast, and the *Hysterothylacium deardorffoverstreetorum* is the most reported in more recent studies (FONTENELLE et al., 2015). This species was firstly described in molecular taxonomic study in 2012 (KNOFF et al., 2012), but already it was being reported by many authors around the world with other denominations as *Hysterothylacium* 2 (PETTER; MAILLARD, 1988), *Hysterothylacium* MD (DEARDORFF; OVERSTREET, 1981; PEREIRA JÚNIOR et al., 2004), and *Hysterothylacium* KB (PETTER; SEY, 1997). This study aimed to evaluate the reactivity of antibodies derived from mice sensitized with *H. deardorffoverstreetorum* antigens against *A. simplex* parasitic antigens.

MATERIALS AND METHODS

PARASITES AND ANTIGEN

In this study, the parasites were collected from fish of *Cynoscion guatucupa* (Maria Mole Fish), *Priacanthus arenatus* (Dog Eye), and *Paralichthys isocoeles* (Linguado) species, traded in the markets of Niteroi and Rio de Janeiro, Brazil, from August 2012 to November 2014. Larvae are identified by optical microscope following the descriptions made by TIMI et al. (2001) and KNOFF et al. (2012). Once identified, the parasites were processed for the parasitic extract preparation of *H. deardorffoverstreetorum* (CEH.) and *A. simplex* (CEA.).

The CEH and CEA were obtained according to the protocol described by Perteguer et al. (2003). In a Potter homogenizer (Thomas phila USA), larvae in 0.9% sodium chloride solution were disintegrated in presence of phenyl methyl sulfonyl fluoride (PMSF). Subsequently, they were centrifuged at 8,500 g at 4 °C temperature for 30 minutes by the International portable refrigerated centrifuge Model PR. Protein quantification of the extracts was performed by the LOWRY et al. (1951) method; bovine serum albumin (BSA) 1 mg/mL was used as standard.

ANIMALS

BALB/c mice 8-10 weeks old, created and maintained on local biotherium (Laboratory-Animal Center of Fluminense Federal University), were used for the experiment. These animals were housed in rooms with air exhaust at 23-25 °C temperature, fed with Nuvilab CR-1 Chow (Nuvital Nutrients S/A) and distilled water treated with 0.1% HCL ad libitum. All procedures were approved by the Ethics Committee of Fluminense Federal University under the number 00137/09.

IMMUNIZATION PROTOCOL

Animals were immunized via intraperitoneal injection with 10 µg CEH associated to 2 mg aluminum hydroxide in a volume of 0.2 ml at zero; 21st; and 42nd days of the experiment. As control, animals received 2 mg of aluminum hydroxide by the same inoculation via. The chemical containment for inoculation and bleeding was performed with xylazine 200 µg/kg and Ketamine 10 mg/kg.

SERUM COLLECTION AND PREPARATION

Blood samples were collected from the retro-orbital plexus in a volume of 0.1 ml at zero (control before immunization), 14th; 21st; 28th; 35th; 42nd; 49th; 56th; and 70th days of the experiment. The material was initially diluted in 1:10 in physiological saline solution and then centrifuged at 800 *g* for 10 minutes and the serum was separated.

DETERMINATION OF LEVELS OF SPECIFIC IgG AND IgE ANTIBODIES

The antibodies presence of IgE and IgG, anti-*Hysterothylacium,* and anti-*Anisakis* isotypes in the mice sera was determined by immunoenzymatic ELISA method. *Hysterothylacium deardorffoverstreetorum* and *Anisakis simplex* antigens were placed on plates with flat bottom pools (Nunc MaxiSorp) prepared according to the previously described method. The antigen was placed in the concentration of 20 µL protein/mL in 0.05 M sodium carbonate-bicarbonate buffer solution, pH 9.6. The plates were incubated for two hours at 37 °C and were washed in phosphate buffered saline solution (PBS), pH 7.2. Free sites were blocked with gelatin solution at 1% in PBS (PBS-G) for two hours at room temperature. They were then washed three times in PBS containing 0.05% Tween 20 (PBS-T). Sera were diluted in PBS-G serially on the base 3 from 1:40 and incubated for two hours at 37 °C. The antibodies were conjugated with peroxidase:anti-IgE (ε-chain) [(rabbit anti-mouse IgG (Invitrogen)], and total anti-IgG (L and H) (1:10000), [(Rabbit anti-Mouse IgG (whole molecule - Sigma)], diluted in blocking solution, added to the reaction (50 µL/pool); and plates were maintained at 37°C for one hour. After washing the plates with PBS-T, the reaction with the substrate and chromogen was made by adding 50 µL/pool of solution, collected 10 µL of hydrogen peroxide at 30%, diluted in 25 mL of citrate buffer at 0.1 M in presence of 10 mg of orthophenylenediamine (OPD). The plates were incubated for 5 minutes at room temperature. The enzymatic reaction was stopped by adding Sulfuric Acid Solution (4N). The reading of optical density (OD) was performed by a microplate reader (Thermo

Plate TP-READER) at a wavelength of 492 nm. Result was expressed by arithmetic average of the OD summation of dilutions. The analysis was performed by comparing the OD summation of each serum.

STATISTICAL ANALYSIS

Statistical analysis was performed by analysis of variance with Tukey post-test with GraphPadInStat program - Version 4.10 for Windows XP, GraphPadSoftware, San Diego California USA, www.graphpad.com Copyright 1992-1998. In the statistical analysis of experimental data, it was considered that values were significant from $p<0.05$ (RODRIGUES, 1996).

RESULTS

Results of this study show that antibodies derived from mice sensitized with CEH are reactive to homologous antigens and heterologous antigens. Figure 1 shows the IgG antibody levels of the animals that were immunized at zero; 21st; and 42nd days with 10 µg CEH and evaluated by ELISA reaction in plates adsorbed with homologous antigens to immunizations or sensitized with CEA (non-homologous antigen to immunizations). Immunization of mice with CEH gradually increased the levels of specific IgG antibodies and reached the maximum after the third immunization, i.e., at the 56th day. These levels were statistically significant when compared to zero day ($p<0.001$). Comparing antibody levels at the 56th day with the ones at 70th day, it is observed slight decrease ($p<0.01$). These same serum samples, when evaluated in sensitized plates with CEA, also showed positive reactions and reached the maximum level at the 49th day and remained so during the observed period; however, these reactions were significantly lower than the reactions performed with the sensitized plates with CEH ($p<0.001$).

Figure 2 shows the levels of IgE antibodies of animals immunized at the zero; 21st; and 42nd days with 10 µg CEH and evaluated by ELISA reaction in plates adsorbed with homologous antigens to immunizations or sensitized with CEA (non-homologous antigen to immunizations). Immunization of mice with CEH gradually increased the levels of specific IgE antibodies and reached the maximum in the last evaluation at the 70th day. These levels were statistically significant when compared to the zero day ($p<0.001$). Comparing the antibody levels at the 56th day with the ones at the 70th day, it was observed a statistically significant increase ($p<0.001$). These same serum samples, when evaluated in sensitized plates with CEA, also showed positive reactions and reached the maximum levels at the 70th day; however, these values do not show significant differences when compared to the ones of the 56th day. Serum samples tested with CEA antigens to IgE, as observed with IgG, obtained positive reactions, but statistically lower ($p<0.001$) than the reactions performed with the sensitized plates with the homologous antigen to immunization.

DISCUSSION

Human infection by *Anisakis simplex* larvae has been known for many years in places where the seafood is important food source (FAO/WHO, 2014). However, in recent decades, infection with *A. simplex* has received major emphasis on public health because of its association with allergic reactions frames, ranging from localized to generalized reactions (NIEUWENHUIZEN; LOPATA, 2014; AUDICANA; KENNEDY, 2008; DASCHNER et al., 1997). The allergenic potential deriving from *Anisakis simplex* larvae and its interaction with the immune system are massively studied, since their manifestations are common in many parts of the world. In relation to other parasites of *Anisakidae* family, there is still little knowledge about this immunological interaction and it is still not clear about the extent of cross-reactivity among members. Experimental studies carried out previously showed that

Anisakis sp. larvae antigens show cross-reactions with antigens of *Toxocara canis* and *Ascaris suum* (PERTEGUER et al., 2003).

Some authors have developed studies to evaluate the cross-reactivity with other parasites, suggesting that there is cross-reactivity between *Anisakis simplex* and *Hysterothylacium* sp. antigens. These parasites have some common antigens and others that are specific of this species (FERNANDEZ-CALDAS et al., 1998; IGLESIAS et al., 1996; LOZANO-MALDONADO et al., 2004; MARAÑON et al., 1998).

Iglesias et al. (1996) evaluated the cross-reactivity among *Anisakis simplex* and other nematodes, including *Hysterothylacium aduncum*, using different extracted antigens. In their studies, the total antigens, secreted-excreted antigens, pseudocoelom antigens, and cuticular antigens were used. It was observed a moderate cross-reactivity compared to somatic antigens of A. *simplex* and *H. aduncum*. In relation to secreted-excreted antigens, which are considered the most immunogenic and are used for allergy diagnosis, there was significant reaction between these two nematodes. The other antigens also reacted with both nematodes.

LOZANO MALDONADO et al. (2004), also evaluating cross-reactivity of *A. simplex* with other nematodes, used two different species, *Hysterothylacium aduncum* and *H. fabri*, from which somatic antigens and excreted-secreted antigens were extracted. The authors point out cross-reactivity of both species with *A. simplex*.

In studies by FERNANDEZ-CALDAS et al. (1998) and MARAÑON et al. (1998), positive patients' sera were tested for allergic anisakiasis with *H. aduncum* antigens, showing cross-reactivity for both.

In this study, the immunogenic potential of L3 larvae of *H. deardorffoverstreetorum* was evaluated, immunizing isogenic mice of BALB/c line with 10 µg of crude antigen. After three immunizations, high specific antibody levels of the IgG and IgE classes were observed. Also, in this study, antigenic relationship with antigens of *Anisakis* sp. larvae was evaluated.

Thus, it was observed that sera from animals immunized with *H. deardorffoverstreetorum* crude antigens are able to react by ELISA reaction with crude antigens of *Anisakis* sp. This set of data is highly suggestive that somatic antigens of *H.deardorffoverstreetorum* confer cross-reactivity with total antigens of *Anisakis* sp. larvae. These data are also important under hygienic-sanitary aspect of interest for public health, because there are reports of significant parasitic indices of these *anisakid* in teleost fish, as recorded by Dias et al. (2011).

The seroepidemiological survey recently performed by FIGUEIREDO et al. (2013) shows a high prevalence of individuals with reactivity to antigens of *Anisakis* and *Contracaecum* sp. larvae. These data confirm the seriousness of fish intake parasitized by *Hysterothylacium deardorffoverstreetorum* by humans that may cause allergic medical conditions.

Reacting individuals to anisakid antigens are some concern to humans, since the prevalence of these parasites in teleost fish is significant. This fact draws attention because individuals previously sensitized with *Anisakis* sp. larvae could develop allergic reactions when exposed to fish intake contaminated with *H. deardorffoverstreetorum* antigens.

CONCLUSION

This paper describes for the first time the cross-reactivity between antigens of *A. simplex* and *H. deardorffoverstreetorum* and, although experimental, it is a big step, since these nematodes are often described in necropsies of Brazilian commercial fish. Future studies may discriminate whether this activity occurs in patients with allergic anisakiasis and its degree of importance for the diagnosis of this disease.

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ETHICS AND BIOSAFETY COMMITTEE

This research was approved by the Ethics Committee on Animal Research, under the N. 00137/09 protocol.

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FIGURE CAPTIONS

Figure 1 - Comparison of the levels of IgG antibodies in plates sensitized with CEH and tested for CAE/CEH. BALB/c (n=6) mice were immunized at zero; 28th; and 42nd days with 10 µg ABT *H. deardorffoverstreetorum* larvae, associated with 2 mg of aluminum hydroxide + magnesium hydroxide via intraperitoneal. Values indicate the means of summation of the OD +/- standard error of the mean for each group. Statistical analysis was performed by ANOVA, with ***p<0.001 compared to zero day. The # symbol represents a significant difference with ***p<0.001 between CEH and CAE.

Figure 2 - Comparison of the levels of IgE antibodies in plates sensitized with CEH, tested for CAE/CEH. BALB/c mice (n=5) were immunized at zero; 28th; and 42nd days sensitized with 10 µg ABT of *H. deardorffoverstreetorum* larvae, associated with 2 mg of aluminum hydroxide + magnesium hydroxide via intraperitoneal. Values indicate the means of summation of the OD +/- standard error of the mean for each group. Statistical analysis was performed using ANOVA, ***p<0.001 compared to zero day. The # symbol represents a significant difference with ***p<0.001 between CEH and CAE.

FIGURE 01

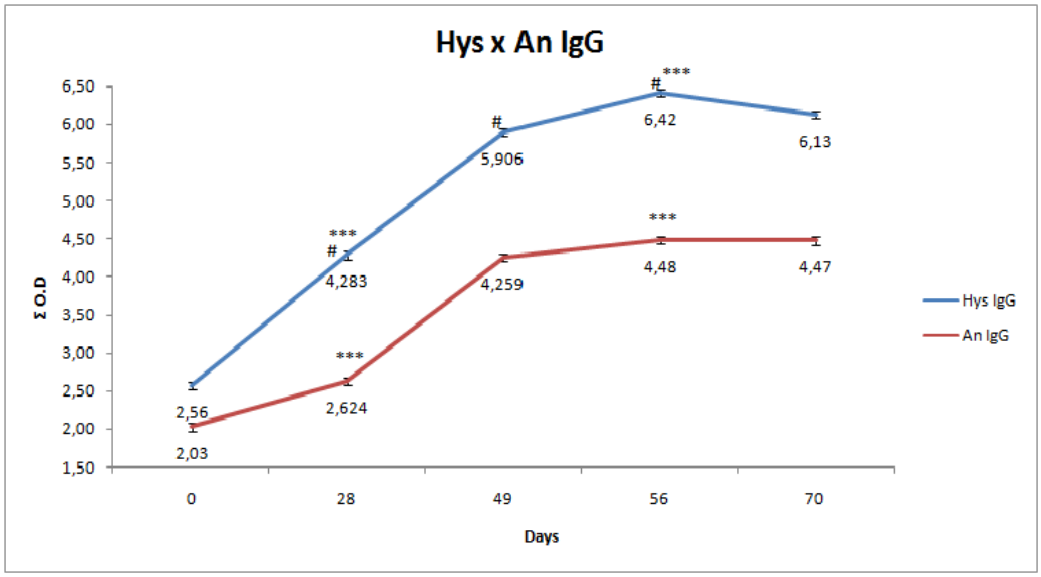
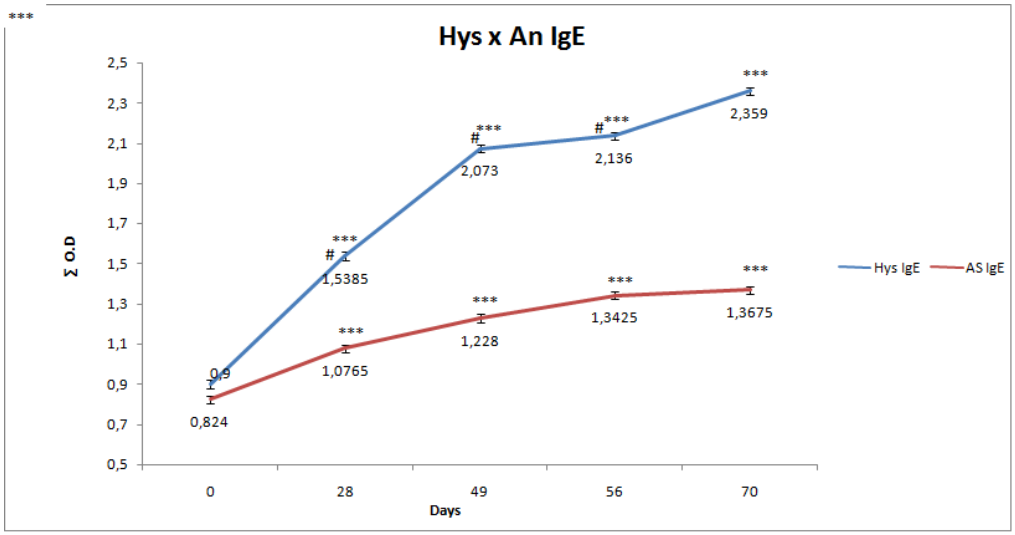


FIGURE 02



4 CONSIDERAÇÕES FINAIS

O presente estudo é a primeira descrição da interação de larvas de terceiro estágio de *Hysterothylacium deardorffoverstreetorum* com o sistema imunológico de mamíferos. Foi comprovado que os antígenos oriundos dessas larvas são capazes de ativar o sistema imunológico humoral, com produção de anticorpos das classes IgG e IgE em níveis detectáveis, e ainda realizar a ativação da imunidade celular.

Outra constatação de extrema importância é a detecção da reatividade cruzada com antígenos de *Anisakis simplex*. Estudos mais aprofundados deverão ser realizados para avaliação de quais antígenos são comuns às duas espécies, e principalmente, qual o impacto dessa interatividade em humanos.

Esses nematóides são rotineiramente detectados em necropsias de peixes em nosso país, demonstrando alta prevalência nas espécies consumidas pelos brasileiros. E considerando a incorporação de diferentes culturas em nossa culinária, introduzindo o hábito do consumo do pescado cru, a classe médica deverá ser sensibilizada para essa problemática e estar preparada para detectar quaisquer manifestações relacionadas à Anisakidose. Além disso, o investimento em pesquisa deverá ser continuo, uma vez que ainda restam inúmeras lacunas a serem elucidadas.

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