UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
CAMPUS DE ARAÇATUBA

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Ocorrência e caracterização molecular de *Cryptosporidium* spp. em suínos no município de Araçatuba, estado de São Paulo, Brasil

Araçatuba – SP

2019

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Dissertação apresentada à Faculdade de Medicina Veterinária – Unesp, Campus de Araçatuba, como parte das exigências para a obtenção do título de Mestre em Ciência Animal (Medicina Veterinária Preventiva e Produção Animal).

Orientador: Prof. Associado Marcelo Vasconcelos Meireles

Araçatuba – SP

2019

N244o

Nascimento, Isabela Garcia do
Ocorrência e caracterização molecular de Cryptosporidium spp. em suínos no município de Araçatuba, estado de São Paulo, Brasil / Isabela Garcia do Nascimento. -- Araçatuba, 2019
43 p. + 1 CD-ROM

Dissertação (mestrado) - Universidade Estadual Paulista (Unesp), Faculdade de Medicina Veterinária, Araçatuba
Orientador: Marcelo Vasconcelos Meireles

1. Suínos. 2. Brasil. 3. Criptosporidiose. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca da Faculdade de Medicina Veterinária, Araçatuba. Dados fornecidos pelo autor(a).

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UNIVERSIDADE ESTADUAL PAULISTA

Câmpus de Araçatuba

CERTIFICADO DE APROVAÇÃO

Título: Ocorrência e caracterização molecular de *Cryptosporidium* ssp. em suínos no município de Araçatuba, estado de São Paulo, Brasil.

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Aprovada como parte das exigências para obtenção do Título de Mestra em CIÊNCIA ANIMAL, área: Medicina Veterinária Preventiva e Produção Animal pela Comissão Examinadora:

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Araçatuba, 23 de julho de 2019.

À minha mãe, irmão e avós que sempre estiveram ao meu lado.

AGRADECIMENTOS

A Deus, por tudo que sou, que venho me tornando e por permitir a conclusão de mais um ciclo da minha vida.

Ao Prof. Dr. Marcelo Vasconcelos Meireles por me orientar pelos caminhos da ciência com paciência e dedicação.

À minha mãe, Sílvia, que sempre acreditou nos meus sonhos, me apoiando, incentivando e aconselhando.

Ao meu irmão, Vagner Luiz, que sempre me deu forças para querer ser melhor por ele.

Aos meus avós, Ovídio e Maria, que sempre estiveram comigo nas etapas mais difíceis e, com amor e paciência, sempre me apoiaram, incentivaram e auxiliaram, direta e indiretamente.

A todos aqueles a quem posso verdadeiramente chamar de amigos, dentro e fora do ambiente de trabalho, os quais estiveram comigo em todos os momentos dessa trajetória, fazendo o caminho mais leve.

A todos os colegas e colaboradores do laboratório que, mesmo em pequenos detalhes, fizeram grande diferença para o resultado final deste trabalho.

À Faculdade de Medicina Veterinária de Araçatuba – FMVA /UNESP, pela estrutura e suporte.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão da bolsa de mestrado.

“Descobrir consiste em olhar para o que todo mundo está vendo e pensar uma coisa diferente”

Roger Von Oech

NASCIMENTO, I. G. **Ocorrência e caracterização molecular de Cryptosporidium spp. em suínos no município de Araçatuba, estado de São Paulo, Brasil.** Dissertação (Mestrado) - Faculdade de Medicina Veterinária, Universidade Estadual Paulista, Araçatuba, 2019.

RESUMO

Existem poucos estudos relacionados à epidemiologia e aspectos clínicos da infecção por *Cryptosporidium* spp. em suínos, especialmente no Brasil. Os suínos apresentam infecções clínicas ou subclínicas e são mais comumente infectados por *Cryptosporidium suis* e *Cryptosporidium scrofarum*. O objetivo deste trabalho é determinar a ocorrência e realizar a caracterização molecular de *Cryptosporidium* spp. em suínos originados de quatro fazendas com sistemas de produção semi-intensivos no Estado de São Paulo, Brasil. Duzentas amostras fecais foram coletadas de suínos, de março a agosto de 2018, e analisadas para verificar a ocorrência de *Cryptosporidium* spp. por nested PCR visando a amplificação de fragmento parcial do gene da subunidade 18S do rRNA (18S rRNA) e sequenciamento genético. Resultados positivos para *Cryptosporidium* spp. foram detectados em 17% (34/200) das amostras. O sequenciamento dos fragmentos amplificados identificou *C. scrofarum* em 10 das 11 amostras sequenciadas. Dessa forma, verificamos que a espécie zoonótica *C. scrofarum* está presente em fazendas de suínos com sistemas de produção semi-intensivos no Estado de São Paulo e que não houve diferença significativa entre as taxas de prevalência entre suínos de <3 meses e >3 meses de idade.

Palavras-chave: Suínos. Brasil. Criptosporidiose.

NASCIMENTO, I. G. **OCCURRENCE AND MOLECULAR CHARACTERIZATION OF *Cryptosporidium* spp. IN PIGS IN THE MUNICIPALITY OF ARAÇATUBA, STATE OF SÃO PAULO, BRAZIL.** Dissertação (Mestrado) - Faculdade de Medicina Veterinária, Universidade Estadual Paulista, Araçatuba, 2019.

ABSTRACT

There are few studies related to the epidemiology and clinical aspects of infection by *Cryptosporidium* spp. in pigs, particularly in Brazil. Pigs present clinical or subclinical infections and are infected most commonly by *Cryptosporidium suis* and *Cryptosporidium scrofarum*. The objective of this study is to determine the occurrence and perform the molecular characterization of *Cryptosporidium* spp. in pigs originated from four farms with semi-intensive production systems in the State of São Paulo, Brazil. Two hundred fecal samples were collected from pigs from March to August 2018 and screened for *Cryptosporidium* spp. by nested PCR targeting the 18S rRNA gene and genetic sequencing. Positive results for *Cryptosporidium* spp. were detected in 17% (34/200) of the samples. Sequencing of amplified fragments identified *C. scrofarum* in 10 of the 11 samples. In conclusion, the zoonotic species *C. scrofarum* is present in pigs raised in pig farms with semi-intensive production systems in the State of São Paulo. There was no significant difference between prevalence rates from < 3 months old and > 3 months old pigs.

Keywords: Pig. Brazil. Cryptosporidiosis.

SUMÁRIO

1. INTRODUÇÃO GERAL

Cryptosporidium pertence ao filo Apicomplexa, classe Gregarinomorphea, subclasse Cryptogregaria e família Cryptosporidiidae (Cavalier-Smith, 2014; Ryan et. al., 2016) e infecta anfíbios, aves, peixes, répteis e mamíferos. *Cryptosporidium* não é um parasito epicelular obrigatório e apresenta seu ciclo biológico na superfície das células epiteliais dos tratos gastrointestinal, respiratório e urinário (Thompson et al., 2005) e, também, no espaço extracelular (Ryan et al., 2016).

A coevolução parasito-hospedeiro é comum no gênero *Cryptosporidium*. Estudos usando análise filogenética sugeriram que *Cryptosporidium parvum* e *Cryptosporidium meleagridis* são originalmente parasitos de roedores e de várias espécies de mamíferos, respectivamente, mas posteriormente expandiram sua gama de hospedeiros, incluindo humanos. Compreender a evolução das espécies de *Cryptosporidium* é importante não só para elucidar a taxonomia do parasito, mas também para avaliar a importância da infecção em animais para a saúde pública (Xiao et al., 2002).

No Brasil, a suinocultura é uma atividade de suma importância para a economia nacional. De acordo com a Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), atualmente, o país ocupa a posição de quarto maior produtor de carne suína no mundo, com 3,97 milhões de toneladas no ano de 2018, ficando atrás apenas da China, União Europeia e dos Estados Unidos, além de ser o quarto maior exportador de carne suína, com 646 mil toneladas (EMBRAPA, 2018).

Até o momento, há apenas dois relatos de caracterização molecular de *Cryptosporidium* em suínos no Brasil, com prevalência de 2,2% (2/91) no estado

do Rio de Janeiro e identificação de uma amostra como *C. scrofarum* (Fiuza et al., 2011), e prevalência de 2,1% (1/47), no estado de São Paulo, com identificação de *C. parvum* (Coelho et al., 2016).

1.1 Ciclo Biológico

O ciclo biológico do gênero *Cryptosporidium* é uma das principais características que o aproxima dos gregarinos, sendo iniciado pelos oocistos, que são eliminados já esporulados no ambiente, portanto, hábeis a infectar um novo hospedeiro que os ingerir ou, em alguns casos, os inalar. Adentrando o novo organismo, haverá a excistação, que permitirá a liberação dos quatro esporozoítos de cada oocisto, os quais farão adesão às células epiteliais de predileção. Uma vez aderidos, eles serão englobados pelas microvilosidades para que haja a formação do vacúolo parasitóforo, permitindo que o parasito assuma a posição epicelular. A partir dessa etapa, os trofozoítos podem iniciar a merogonia e se reproduzir assexuadamente ou iniciar a reprodução sexuada (macrogamontes e microgamontes), que dará origem aos oocistos esporulados (Barta; Thompson, 2006; Clode et al., 2015). Outras particularidades são os estágios extracelulares do ciclo, particularmente trofozoítos e estruturas similares aos gamontes, que são capazes de se desenvolver fora das células hospedeiras, fazendo com que este protozoário não seja um microrganismo de localização obrigatoriamente epicelular, quando em condições ideais para seu desenvolvimento (Barta; Thompson, 2006)

Os oocistos podem apresentar parede fina ou, em sua maioria, parede espessa. Os de parede fina podem excistar ainda no hospedeiro, portanto,

causando reinfecção, e os de parede espessa são eliminados no ambiente (Current et al., 1986). Os oocistos eliminados nas fezes permanecem infectantes por vários meses, dependendo das condições climáticas, sendo até seis meses em temperatura média de 20ºC, três meses a 25 a 30ºC e sete meses a 15ºC (Anderson, 1985; Fayer et al. 1998; Jenkins et al., 2003).

1.2 Epidemiologia E Sinais Clínicos

Há poucos estudos sobre a epidemiologia e características clínicas da criptosporidiose em suínos, que são comumente infectados com *C. suis* (Ryan et al., 2004) e *C. scropharum* (Kvác et al., 2012), com infecção subclínica ou diarreia associada a outros agentes etiológicos (Xiao et al., 2002).

Em estudos realizados em diversos países, incluindo o Brasil, a prevalência de infecção por *Cryptosporidium* spp. foi de 1,4% na Alemanha (Wieler et al., 2001), 7,1% nos Estados Unidos (Xiao et al., 1994), 7,6% no Brasil (Calderaro et al., 2001), 9,7% no Canadá (Olson et al., 1997), 21,9% na Espanha (Quilez et al., 1996) e 33,2 % no Japão (Izumiyama et al., 2001).

1.3 Diagnóstico

As técnicas de coloração para visualização por microscopia são amplamente utilizadas, possuem baixo custo e são rápidas e fáceis de executar. Pela coloração negativa com verde malaquita, os oocistos de *Cryptosporidium* são observados ao microscópio como estruturas redondas a ovaladas, brilhantes e envoltas por um fundo verde (Elliot et al., 1999).

As técnicas imunológicas, como o ensaio imunoenzimático (ELISA) e a reação de imunofluorescência indireta (RIFI), apresentam maior sensibilidade

que as técnicas de coloração, no entanto, os antígenos da parede dos oocistos apresentam reatividade cruzada entre diferentes espécies de *Cryptosporidium*, não sendo possível o diagnóstico espécie-específico (Fayer et al., 2000; Jex et al., 2008).

O único método de identificação das diferentes espécies de *Cryptosporidium* é a caracterização molecular (Ryan et al., 2014). Embora seja uma técnica de alto custo, a reação em cadeia pela polimerase (PCR) seguida de sequenciamento dos fragmentos amplificados detecta baixas quantidades de DNA nas amostras e permite a identificação de *Cryptosporidium* em nível de espécie. O gene mais utilizado para realização de caracterização molecular do parasito é o 18S rRNA. A análise da sequência dos fragmentos amplificados permite a identificação de todas as espécies e genótipos de *Cryptosporidium* (Ryan et al., 2014).

O tratamento das infecções causadas pelas diversas espécies de *Cryptosporidium* possui resultados clínicos insatisfatórios (Rossle; Latif, 2013), ou seja, ainda não há um tratamento efetivo para a criptosporidiose (Ryan, 2010).

Os oocistos de *Cryptosporidium* são altamente resistentes à maioria dos desinfetantes; quando são sensíveis, necessitam de períodos maiores que uma hora de contato para que 90% deles sejam inativados. O único desinfetante efetivo que pode ser usado em água potável é o ozônio (Korich et al. , 1990). A temperatura parece ser um fator importante para a viabilidade dos oocistos, sendo a inativação relacionada a maiores temperaturas (Jenkins et al. , 2003).

1. 4 Objetivo

O objetivo deste trabalho foi determinar a ocorrência e realizar a caracterização molecular de *Cryptosporidium* spp. em suínos originados de quatro fazendas com sistemas de produção semi-intensivos no Estado de São Paulo, Brasil.

CAPÍTULO 1 - OCCURRENCE AND MOLECULAR CHARACTERIZATION OF *Cryptosporidium scrofarum* IN PIGS IN THE MUNICIPALITY OF ARAÇATUBA, STATE OF SÃO PAULO, BRAZIL

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2.1 Resumo

Existem poucos estudos relacionados à epidemiologia e aspectos clínicos da infecção por *Cryptosporidium* spp. em suínos, especialmente no Brasil. Os suínos apresentam infecções clínicas ou subclínicas e são mais comumente infectados por *Cryptosporidium suis* e *Cryptosporidium scrofarum*. O objetivo deste trabalho é determinar a ocorrência e realizar a caracterização molecular de *Cryptosporidium* spp. em suínos originados de quatro fazendas com sistemas de produção semi-intensivos no Estado de São Paulo, Brasil. Duzentas amostras fecais foram coletadas de suínos, de março a agosto de 2018, e analisadas para verificar a ocorrência de *Cryptosporidium* spp. por nested PCR visando a amplificação de fragmento parcial do gene da subunidade 18S do rRNA (18S rRNA) e sequenciamento genético. Resultados positivos para *Cryptosporidium* spp. foram detectados em 17% (34/200) das amostras. O sequenciamento dos fragmentos amplificados identificou *C. scrofarum* em 10 das 11 amostras sequenciadas. Dessa forma, verificamos que a espécie zoonótica *C. scrofarum*

está presente em fazendas de suínos com sistemas de produção semi-intensivos no Estado de São Paulo e que não houve diferença significativa entre as taxas de prevalência entre suínos de <3 meses e >3 meses de idade.

Palavras-chave: Suínos. Brasil. Criptosporidiose.

2.2 Abstract

There are few studies related to the epidemiology and clinical aspects of infection by *Cryptosporidium* spp. in pigs, particularly in Brazil. Pigs present clinical or subclinical infections and are infected most commonly by *Cryptosporidium suis* and *Cryptosporidium scrofarum*. The objective of this study is to determine the occurrence and perform the molecular characterization of *Cryptosporidium* spp. in pigs originated from four farms with semi-intensive production systems in the State of São Paulo, Brazil. Two hundred fecal samples were collected from pigs, from March to August 2018, and screened for *Cryptosporidium* spp. by nested PCR targeting the 18S rRNA gene and genetic sequencing. Positive results for *Cryptosporidium* spp. were detected in 17% (34/200) of the samples. Sequencing of amplified fragments identified *C. scrofarum* in 10 out of 11 samples sequenced. In conclusion, the zoonotic species *C. scrofarum* is present in pig raised in pig farms with semi-intensive production systems in the State of São Paulo. There was no significant difference between prevalence rates from < 3 months old and > 3 months old pigs.

Keywords: Pigs. Brazil. Cryptosporidiosis.

2.3 Introduction

Cryptosporidium belongs to the phylum Apicomplexa, class Gregarinomorphea, subclass Cryptogregaria, family Cryptosporidiidae (Cavalier-Smith, 2014; Ryan, 2016). *Cryptosporidium* is a parasite that infects amphibians, birds, fishes, reptiles, and mammals. Studies have shown that *Cryptosporidium* is not a mandatory epicellular parasite, and not only completes its biological cycle on the surface of epithelial cells of the gastrointestinal, respiratory and urinary tracts of its hosts (Thompson et al., 2005), but also can complete its life cycle extracellularly (Ryan et al., 2016).

The biological cycle of *Cryptosporidium* begins with shedding of sporulated oocysts by an infected host, usually in feces or in respiratory secretions. The susceptible hosts are infected by the ingestion of oocysts present in water, food and, to a lesser extent, by inhaling oocysts in suspension (Current et al., 1986; Smith et al., 2007; Xiao et al., 2008).

Host-parasite coevolution is common in the genera *Cryptosporidium*. Studies using phylogenetic analysis suggested that *Cryptosporidium parvum* and *Cryptosporidium meleagridis* are originally parasites of rodents and various mammalian species, respectively, but later expanded their range of hosts, including humans. Understanding the evolution of *Cryptosporidium* species is important not only to elucidate the parasite's taxonomy, but also to evaluate the public health importance of infection in animals (Xiao et al., 2002).

In Brazil, a pig farming is an activity of paramount importance to the national economy. According to the Brazilian Agricultural Research Corporation (EMBRAPA) currently, the country occupies the position of fourth largest producer of pork in the world, with 3.97 million tons in the year 2018, behind only China, the European Union and the United States, and the fourth largest exporter of pork, with 646 thousand tons (EMBRAPA, 2018).

There are few studies on the epidemiology and clinical characteristics of cryptosporidiosis in pigs, which are commonly infected with *Cryptosporidium suis* (Ryan et al., 2004) and *Cryptosporidium scropharum* (Kváč et al., 2012), with subclinical infection or diarrhea associated with other etiological agents (Xiao et al., 2002).

Studies carried out in several countries reported prevalence rates of *Cryptosporidium* spp. infection in pigs of 1.4 % in Germany (Wieler et al., 2001), 7.1% in the United States (Xiao et al., 1994), 7.6% in Brazil (Calderaro et al., 2001), 9.7% Canada (Olson et al., 1997), 21.9% in Spain (Quilez et al., 1996), and 33.2% in Japan (Izumiyama et al., 2001).

2.4 Material And Methods

2.4.1 Fecal samples

This study was approved by the Animal Use Ethics Committee (CEUA) of the São Paulo State University (Unesp), School of Veterinary Medicine, Araçatuba, process FOA 2018-00280.

Two hundred fecal samples were collected from four pig farms (fifty in each farm) using a convenience sampling, in 2018 between March and August, from

crossbred pigs originated from four small-holders pig farms with semi-intensive production systems in the municipality of Araçatuba, latitude 21º 12'32"south and a longitude 50º 25'58" west in the State of São Paulo, Brazil (Fig. 1). Samples of freshly discarded feces was collected from the floor, without prior clinical examination of the animals, immediately after defecation, using a disposable wooden spatula, and stored in labeled plastic tubes at 4^0 C until an aliquot of 150 mg of feces was stored in a 2 ml microtube and kept at -20º C for genomic DNA extraction.

Pigs were kept in stalls made of masonry in farms II, III, and IV. In farm I, pigs had direct contact with the ground. In farms II and III, pigs were released in a common area during cleaning of the stalls. Farm I had the highest amount of exchange of pigs with nearby pig farms. Feeding on farms IV was composed only of balanced ration prepared on the farms while the other farms had a mix of leftover human food and rations composed of corn and soybean.

According to availability of samples from the four pig farms and taking into consideration the management practices adopted at the pig farms, fecal samples were divided in two age groups: (< 3 months old and > 3 months old).

2.4.2 Molecular characterization

The extraction of DNA from 150 mg of feces was performed using the ZR Fecal DNA Kit® (Zymo Research) following the manufacturer's guidelines.

Nested PCR targeting the 18S rRNA gene was performed using the primers 5'-TTCTAGAGCTAATACATGCG-3' and 5'-CCCATTTCCTTCGAAACAGGA 3' for the primary reaction (1325 bp) and 5' GGAAGGGTTGTATTTATTAGATAAAG-3' and 5' AAGGAGTAAGGAACAACC

TCCA-3' for the secondary reaction (826-840 bp) (Xiao et al., 2002), using the Platinum® PCR Supermix (Life Technologies), 1.5mM $MgCl_2$, 200nM of each primer, and 2.5 µl of target DNA. Samples were subjected to initial denaturation of the DNA at 94º C for 2 minutes, followed by 39 cycles, each consisting of denaturation at 94º C for 45 s, 30 s of annealing at 55º C and 60 s of extension at 72º C, with final extension at 72º C for 7 min. Positive and negative controls of the reaction were genomic DNA of *Cryptosporidium serpentis* and ultra-pure water, respectively. The amplified fragments were visualized by GelRed® (Biotium) stained gel electrophoresis.

2.4.3 DNA sequence analysis

DNA sequences were assembled with Codoncode Aligner version 7.1.1 software (CodonCode Corporation). The homology of amplified products to sequences from GenBank was assessed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) searches. Homologous sequences were aligned with consensus sequences using Clustal W (Thompson et al. 1997) and BioEdit Sequence Alignment Editor (Hall, 1999).

2.4.4 Statistical analysis

Prevalence rates with 95% confidence intervals were calculated using Wilson (score) intervals (Sergeant, 2017). Chi-Square test was performed to verify the association between the positivity for *Cryptosporidium* spp. and the age of the pigs. Results were considered significant when $p < 0.05$.

2.5 Results And Discussion

The present study showed a higher prevalence rate of *Cryptosporidium* infection in pigs than previous studies have indicated in Brazil. The rate of results positive for *Cryptosporidium* spp. obtained by nested PCR was 17% (34/200) (Table 1). In Brazil, there are reports of prevalence of *Cryptosporidium* spp. infection in pigs ranging from 1.2% (2/174) to 7.6% (32/423) in the States of Minas Gerais and São Paulo (Nishi et al., 2000; Calderaro et al., 2001).

The prevalence rate among < 3 months old (12.5%) and > 3 months old (19.5%) pigs did not differ significantly by the Chi-Square test (p=0.283) (Table 1). Kvác et al. (2014) found that *C. scrofarum* infects pig older than 5 weeks. In contrast, *C. suis* infections are commonly detected in young or pre-weaned piglets (Schubnell et al., 2016).

Owing to a low DNA yield in nested PCRs, viewed as faint DNA bands by electrophoresis, 24 out of 34 samples positive by nested PCR were not sequenced. Sequencing were successful in 10 samples identified as *C. scrofarum*. Up to date, there are only two reports on the molecular characterization of *Cryptosporidium* in pigs in Brazil, one with the reported prevalence of 2.2% (2/91) in pig from the state of Rio de Janeiro, and identification of *C. scrofarum* (Fiuza et al., 2011). Another study reported prevalence of 2.1% (1/47) in pigs from the state of São Paulo, with identification of *C. parvum* (Coelho et al. 2016).

Farm I presented the highest positivity (42%; 21/50) for *Cryptosporidium,* which may be a consequence of poor sanitary management of the property since the stalls are difficult to clean. In addition, pigs from other properties are constantly introduced to the property. The absence of positive samples on the IV

property may be a result of a better management as well as a greater care with the hygiene of the pig stalls.

Although there are a significant number of studies on cryptosporidiosis in Brazil related to many animal species, the molecular epidemiology of the *Cryptosporidium* is still little explored, particularly in swines (Meireles, 2010). This study showed a higher prevalence rate in pigs from Brazil than previously reported. However, there are few publications on *Cryptosporidium* prevalence in pigs in Brazil and additional studies must be performed to determine the importance of *Cryptosporidium* infection for the health of pigs in Brazil and its role in the epidemiology of human cryptosporidiosis.

2.6 Conclusions

Cryptosporidium scrofarum infection is present in pig raised in pig farms with semi-intensive production systems in the State of São Paulo. There was no significant difference between prevalence rates from < 3 months old and > 3 months old pigs.

2.7 Funding Sources

This work was supported by the Coordination for the Improvement of Higher Education Personnel (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES) for a master's degree scholarship awarded to I. G. Nascimento.

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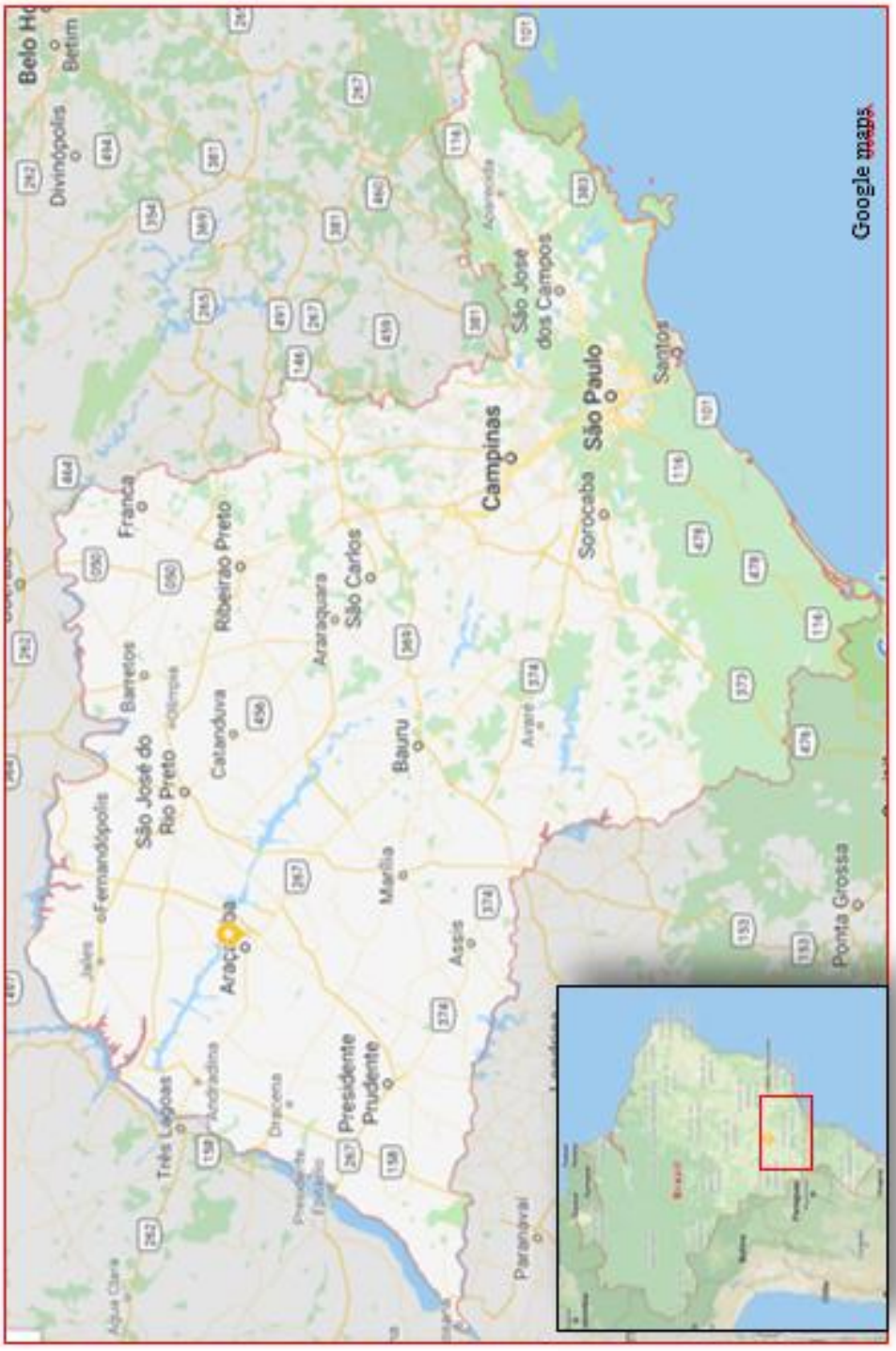
ANEXOS

ANEXO A - FIG. 1 – LOCATION OF THE MUNICIPALITY OF ARAÇATUBA, STATE OF SÃO PAULO, BRAZIL.



ANEXO B - TABLE 1 – RATE OF RESULTS POSITIVE FOR *CRYPTOSPORIDIUM* SPP. IN PIG FECAL SAMPLES.

Pigs age	N° positive/n° sampled (% positive; 95% CI[1])				
	Farm I	Farm II	Farm III	Farm IV	Total[2]
<3 months	5/17 (29.4; 13.3-53.1)	1/16 (6.2; 1.1-28.3)	3/27 (11.1; 3.9-28.1)	0/12 (0-24.2)	9/72 (12.5; 6.7-22.1)[a]
>3 months	16/33 (48.5; 32.5-64.8)	1/16 (6.2; 1.1-28.3)	1/23 (4.3; 0.8-21)	0/38 (0-9.2)	25/128 (19.5; 13.6-27.2)[a]
TOTAL	21/50 (42.29; 4-55.8)	9/50 (18; 9.8-30.8)	4/50 (8.3; 2-18.8)	0/50 (0-7.1)	34/200 (17; 12.4-22.8)

[1] 95% Confidence Interval.

[2] Lower case letters in a column do not differ significantly by the Chi-Square test ($p < 0.05$).

ANEXO C - NORMAS DE PUBLICAÇÃO DO PERIÓDICO ACTA TROPICA

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