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Oocysts of *Cryptosporidium* Tyzzer, 1907 (Apicomplexa: Cryptosporidiidae) in brown mussels *Perna perna* L., 1758 (Mollusca: Bivalviae) in Ponta do Tinguí, Mangaratiba, RJ: A biomarker of environmental contamination

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Abstract Cardozo SV, Berto BP, Cardozo TSF, Mesquisa EFM, Lopes CWG. 2013. **Oocysts of *Cryptosporidium* Tyzzer, 1907 (Apicomplexa: Cryptosporidiidae) in brown mussels *Perna perna* L., 1758 (Mollusca: Bivalviae) in Ponta do Tinguí, Mangaratiba, RJ: A biomarker of environmental contamination** [Oocistos de *Cryptosporidium* Tyzzer, 1907 (Apicomplexa: Cryptosporidiidae) em mexilhões *Perna perna* L., 1758 (Mollusca: Bivalviae) na Ponta do Tinguí, Mangaratiba, RJ: Um biomarcador de contaminação ambiental.] *Coccidia* 1, 32-38. Curso de Especialização em Ciências do Laboratório Clínico e Diagnóstico *in vitro*, Escola de Ciências da Saúde, Universidade do Grande Rio. Rua Prof. José de Souza Herdy, 1160, 25071-202, Duque de Caxias, RJ, Brasil. E-mail: sergian.cardozo@unigranrio. com.br

Discharge of treated or untreated sewage into rivers and seas undermines the quality of the water, besides introducing human or animal enteric pathogens and contribute to higher levels of organic nutrients in these areas. The aim of this work was to use oocysts of *Cryptosporidium* Tyzzer, 1907 as a biomarker of environmental contamination in bivalve molluscs *Perna perna* L., 1758 collected in Ponta do Tingüi, Mangaratiba, RJ. For this, the mussels collected were processed in the laboratory, being detached from their shells and macerated. The biomass was diluted in distilled water and filtered to obtain a water-biomass solution, which was subjected to formalin-ether sedimentation technique, with observation in bright field, and safranin-methylene blue staining technique. All samples were positive for *Cryptosporidium* oocysts. Forty oocysts were measured and compared with *Cryptosporidium hominis* Morgan-Ryan, Fall, Ward, Hijjawi, Sulaiman, Fayer, Thompson,Olson, Lal, Xiao, 2002 oocysts by using histograms and linear regressions. Morphome-

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tric differences were observed when both oocysts samples were compared; however, this statistical morphometric evaluation was efficient to determine the presence of *Cryptosporidium* oocysts in samples of mussels, although inconclusive in determining the specific identification. In conclusion, this study reported the presence of *Cryptosporidium* sp. from biomass of mussels *P. perna* and, consequently, environmental contamination, whether by human or animal waste, in Ponta do Tingüi, Mangaratiba, RJ.

Keywords coccidium, morphometry, mussels, environmental contamination, sewage, biomarker, bioaccumulation.

Resumo O despejo de esgoto sem tratamento ou tratado em rios e mares compromete a qualidade destes corpos hídricos, pois pode introduzir patógenos entéricos humanos ou animais além de contribuir para a elevação dos níveis de nutrientes orgânicos nestas áreas. O objetivo do presente trabalho foi utilizar oocistos de *Cryptosporidium* Tyzzer, 1907 como biomarcador de contaminação ambiental em moluscos bivalves *Perna perna* L., 1758 coletados na Ponta do Tinguí, Mangaratiba, RJ. Para isso, os mexilhões coletados foram processados em laboratório, sendo destacados de suas conchas e macerados manualmente em gral e pistilo. O macerado foi diluído em água destilada e filtrado obtendo-se uma solução água-biomassa que foi submetida à técnica de centrífugo-sedimentação formaldeido-éter com observação em campo brilhante, e à técnica de coloração de safranina-azul de metileno. Todas as amostras foram positivas para os oocistos de *Cryptosporidium*. Quarenta oocistos foram medidos e comparados com oocistos de uma amostra positiva para *Cryptosporidium hominis* Morgan-Ryan, Fall, Ward, Hijjawi, Sulaiman, Fayer, Thompson, Olson, Lal, Xiao, 2002, através de histogramas e regressões lineares. Diferenças morfométricas foram observados quando ambas as amostras de oocistos foram comparadas; no entanto, esta avaliação morfométrica estatística foi eficiente para determinar a presença de oocistos de *Cryptosporidium* em amostras de mexilhões, embora inconclusiva na identificação específica. Neste sentido, conclui-se que foi identificada a presença de oocistos de *Cryptosporidium* na biomassa dos mexilhões *P. perna* e, conseqüentemente, a contaminação ambiental, seja por dejetos humanos ou animais, na Ponta do Tinguí, Mangaratiba, RJ.

Palavras-chave coccidium, morfometria, mexilhões, contaminação ambiental, esgoto, biomarcador, bioacumulação.

Introduction

The domestic and industrial waste discharge, which contaminates rivers, lakes and seas, is responsible for the degradation of water resources in Brazil and most countries of the world (Valença 2003).

Marine bivalves are distributed along the coastal environment and, due to their filter-feeding habits, can accumulate large numbers of bacteria, parasites and heavy metals (Carvalho 2001, Leal & Franco 2008). These mollusks filter 19 to 50 liters per hour with little or no selective capability (Galvão 2004). In oysters, for example, several bacteria survives the digestive process due to the adaptation to the marine environment, mechanisms of enzymatic resistance and use of the intestinal environment of the host as a nutritional source (Silva et al. 2004).

In humans, many food outbreaks are associated to fresh consumption of seafood contaminated with gastrointestinal pathogens (Croci et al. 2000 Pereira et al 2007). Shellfish are the biggest bioaccumulators of pollutants from the environment, and thus they are bioindicators of unsanitary water (Leal & Franco 2008). The main biomarkers detected and quantified in these bioindicators are fecal coliforms (Sande et al. 2010) and protozoa of the genera *Cryptosporidium* Tyzzer, 1907 and *Giardia* Kunstler, 1882 (Leal & Franco 2008, Cardozo et al. 2012).

Worldwide, few studies are aimed to detect oocysts of *Cryptosporidium* and cysts of *Giardia* from marine ecosystems, although, both protozoa already have been reported from beaches and estuarine regions of some countries, including Brazil (Johnson et al. 1995, Cardozo et al. 2012).

The aim of this study was to detect contamination by waste of human and/ or animals in the natural banks of Ponta do Tingüi, Man-

garatiba, RJ, by isolation of oocysts of *Cryptosporidium* from biomass of brown mussels *Perna perna* L., 1758. Secondly, these oocysts, which are biomarkers of environmental contamination, were morphometrically compared with oocysts of *Cryptosporidium hominis* Morgan-Ryan, Fall, Ward, Hijjawi, Sulaiman, Fayer, Thompson,Olson, Lal, Xiao, 2002, searching for similarities that would suppose the species isolated from the mussels.

Materials and methods

Study area

Brown mussels *P. perna* were directly collected from natural banks located in Ponta do Tingüi, in the coastal region of Sepetiba Bay. The Sepetiba bay is a partially mixed estuarine system located at latitude 23º S and longitude 44º W, in the southeastern coast of the state of Rio de Janeiro covering the municipalities of Rio de Janeiro, Itaguaí and Mangaratiba. Sepetiba bay has been a site of industrial and domestic waste discharge, which constituents are accumulated within the bay due to the reduced current dynamics.

Experimentation sites

The samples of brown mussels were analyzed at the Laboratório de Coccídios e Coccidioses in the Universidade Federal do Rio de Janeiro, Seropédica, RJ and at the Scientific Research Center (SRC) in the Universidade do Grande Rio, Duque de Caxias, RJ.

Samples processing

At the SRC, the biomass of the mussels was removed from the shell, sectioned and macerated. Then, this macerate was diluted in distilled water and filtered to produce a biomass-water solution. This solution was processed according to formalin-ether sedimentation technique (Ritchie 1948). An aliquot of the pellet of each sample was observed in bright field microscopy, being the positive samples stained by safranin-methylene blue technique.

Measurement and photomicrography

The oocysts of *Cryptosporidium* recovered from the biomass of the mussels were measured at the Laboratório de Coccídios e Coccidioses using a Carl Zeiss binocular microscope with an apochromatic oil immersion objective lens and an ocular micrometer (K-15X PZO, Poland). Size ranges are shown in parenthesis followed by average and shape index (L/W ratio). Photomicrographs were taken using a digital camera (Evolution MP 5.0 Colled).

Statistical evaluation

Two statistical methods were employed for morphometric comparison of the oocysts of *Cryptosporidium* isolated from brown mussels in the current study, compared to oocysts of *C. hominis* from a sample previously identified by molecular methods: (1) Histograms were prepared to plot the values of the length, width and shape-index of the oocysts, as well as their relative frequencies, according to Sampaio (2002) and Berto et al. (2008); (2) Linear regression was prepared to compare the distribution of the samples of *Cryptosporidium* using methods proposed by Norton & Joyner (1981) and modified by Sampaio (2002) and Berto et al. (2011). The graphic and coefficient of regression line was obtained using the software Microsoft Excel 2007®.

Results and discussion

Oocysts of *Cryptosporidium* (Figure 1a) were recovered and identified from all samples of biomass of brown mussels *P. perna*. Fourty of these oocysts were morphometrically compared with oocysts of *C. hominis* (Figure 1b), resulting in the graphs of the Figures 2 and 3.

The histograms (Figure 2), showed a regular distribution when it was observed each sample separately; in other words, the frequencies of the different classes increased and declined gradually, indicating only one species in each sample. The histogram of shape-index (Figure 2c) revealed a higher frequency in the range of values from 1.1 to 1.3. This observation indicates a tendency for the oocysts to adopt a ellipsoidal shape.

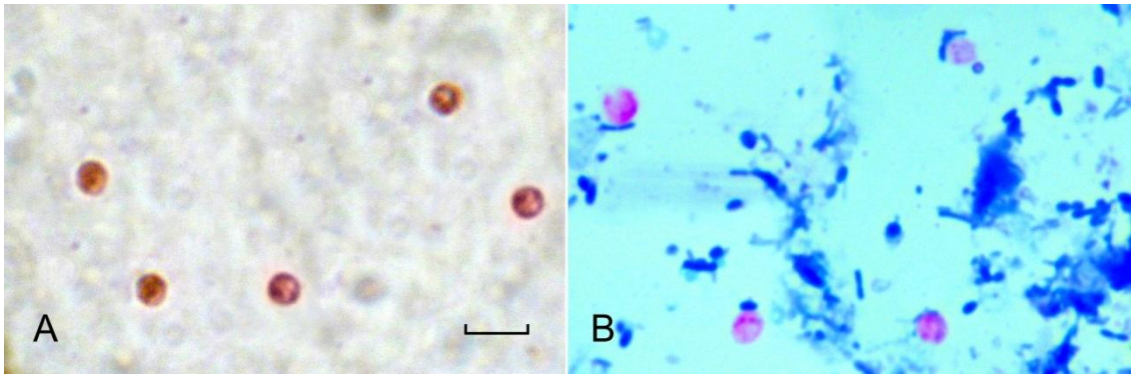

Fig. 1. Oocysts of *Cryptosporidium* recovered from brown mussels in Ponta do Tingüi, Mangaratiba, RJ (A) and oocysts of *Cryptosporidium hominis* (B). Safranin-methylene blue staining. Scale: 10µm.

In a comparative evaluation between samples, it was observed the uniformity of frequencies in the length classes (Figure 2a), which would suggest that the oocysts recovered from brown mussels would be *C. hominis*; in contrast, in the histograms of width and shape-index (Figure 2b-c) it were observed widely divergent frequencies in several classes.

The linear regression (Figure 3) confirms the morphometric differences between the samples. The regression lines were not superimposed and the different inclinations indicate different shapes of oocysts, which can also be observed in the histograms of shape-index. The regression line more inclined for the sample from brown mussels indicates that these oocysts were more spherical than oocysts of *C. hominis*. In addition, the oocysts recovered from brown mussels were distributed in a regular regression, with R^2 near 0.7, while the oocysts of *C. hominis* were irregular in its distribution.

Importantly, these morphometric differences do not necessarily indicate that the oocysts recovered from brown mussels in the current study are of a different species than *C. hominis*. There are environmental factors associated with host-parasite interaction and, particularly, to methods of isolation and identification, which may become polymorphic oocysts. Ramirez et al. (2008) concluded that the oocysts of *Cryptosporidium* become more polymorphic by Ziehl-Neelsen staining technique compared to flotation in Sheather's sugar solution. Therefore, this statistical morphometric evaluation was efficient in the characterization and morphometric comparison of samples evidencing the presence of *Cryptosporidium* sp. in samples of mussels; however, it is inconclusive in specific identification.

In another aspect, the presence of oocysts of *Cryptosporidium* in the biomass of brown mussels revealed environmental contamination by human and/ or animal waste in the study area. According to Leal & Franco (2008) the location at which occur the production and extraction of different species of bivalve molluscs intended for human consumption needs to be extensively studied so that they can be traded safely and free of any pathogenic microorganisms. These observations support this study, since that these brown mussels contaminated by *Cryptosporidium* are widely traded and consumed by the surroundings of Ponta do Tingüi.

Outbreaks associated with the consumption of bivalve molluscs have been reported worldwide, especially in North America, Asia, Europe and Oceania, and in the last three decades there has been a increase in reports of outbreaks caused by eating oysters, clams and mussels (Potasman et al. 2002). The largest outbreak ever reported occurred in Shanghai, China, in 1988, where 290,000 people have contracted hepatitis A after eating clams (Tang et al. 1991).

Currently, contamination of bivalve molluscs by pathogenic protozoa has been presented as an emerging aspect with implications for public health. Although there has not been reports of outbreaks of protozoan infections related to the ingestion of seafood worldwide, the natural occurrence of *Cryptosporidium* has been reported in several countries in different species of bivalve molluscs (Leal & Franco 2008, Cardozo et al. 2012). Thus, concepts have been widely discussed

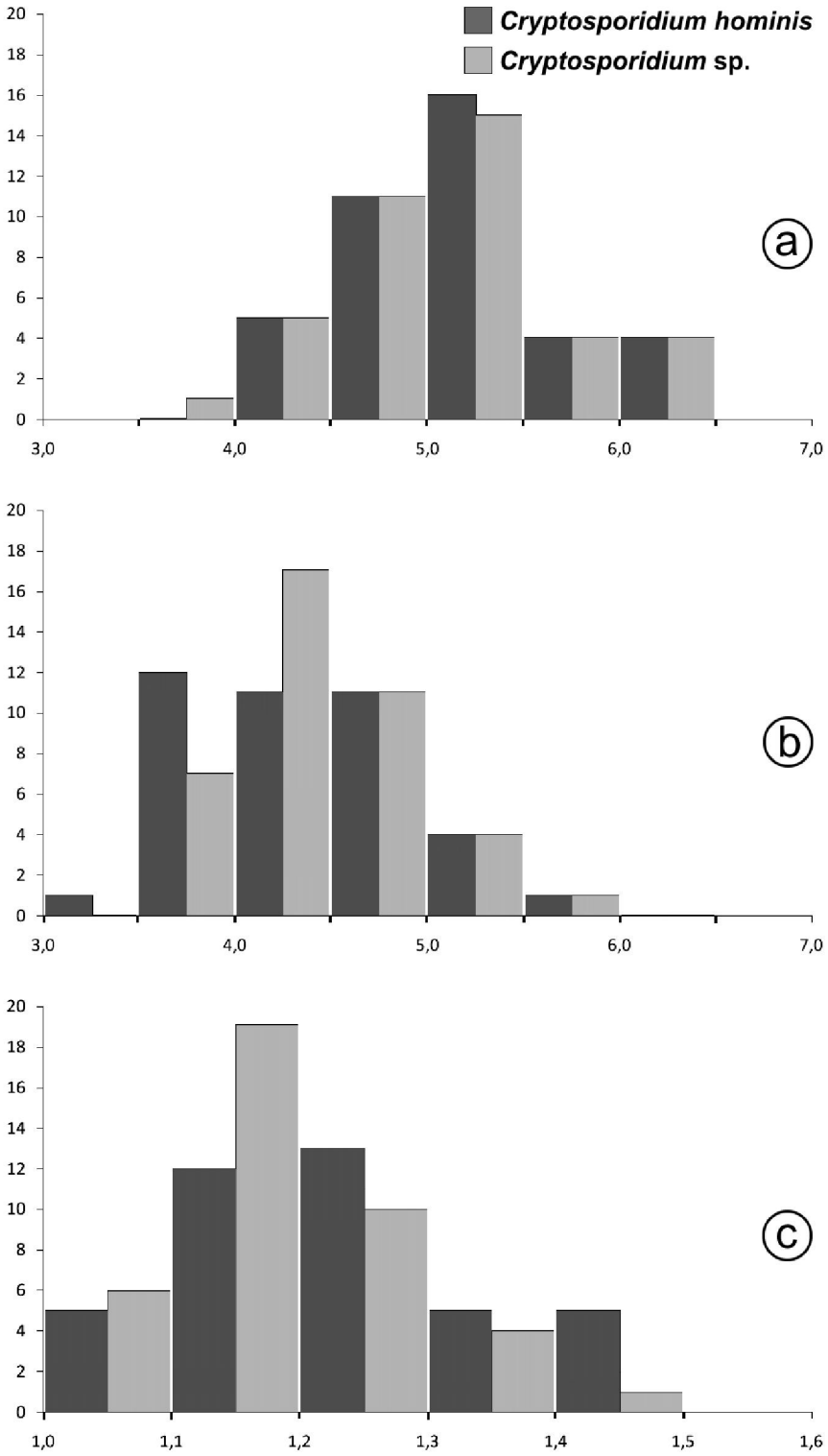

Fig. 2. Histograms of length (a), width (b), and shape-index (c) of *Cryptosporidium* sp. recovered from biomass of brown mussels *Perna perna* in Ponta do Tingüi, Mangaratiba, RJ, compared to *Cryptosporidium hominis*. Y axis: frequencies, X axis: classes of measures.

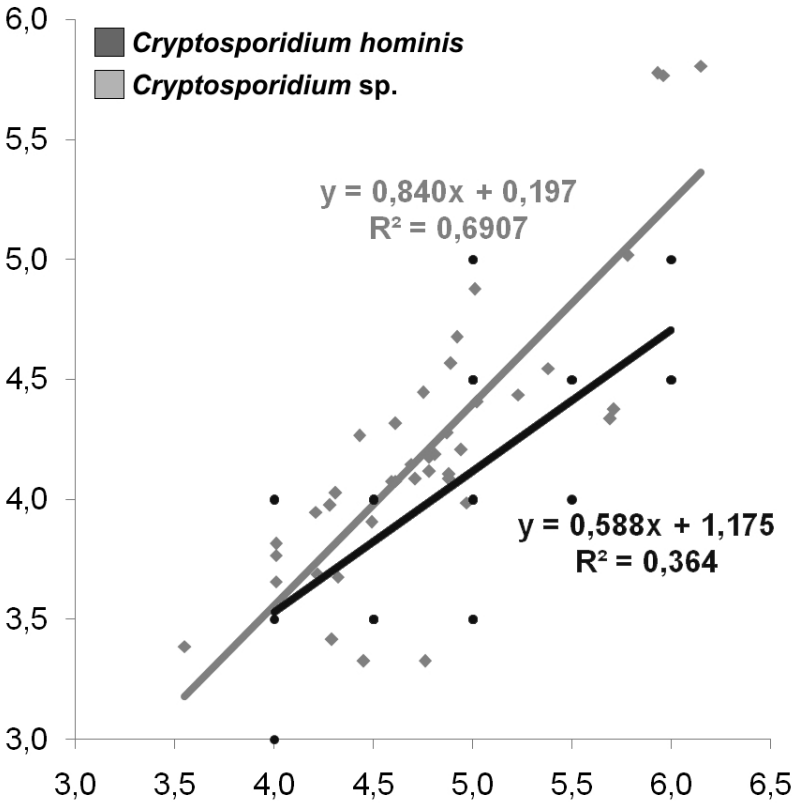

Fig. 3. Linear regression of the oocysts of *Cryptosporidium* sp. recovered from biomass of brown mussels *Perna perna* in Ponta do Tingüi, Mangaratiba, RJ, compared to *Cryptosporidium hominis*. Y axis: width, X axis: length.

and disseminated worldwide on the potential risks of acquiring protozoosis by ingestion of raw or undercooked shellfish (Rose et al. 2002).

Finally, it becomes especially relevant and necessary the parasitological monitoring of bivalve molluscs that will be traded, and also in the areas where the bivalve molluscs are found, both on mariculture farms and in natural environments available for exploratory collection.

References

Baxby D, Blundell N, Hart CA. The development and performance of a simple, sensitive method for the detection of *Cryptosporidium* oocysts in faeces. *Journal of Hygiene*, 93, 317-323, 1984.

Berto BP, Cardozo SV, Teixeira-Filho WL, Ferreira AMR, Lopes CWG. Aflatoxin effect on the oocysts morphometry and contribution on the morphology of *Eimeria bateri* Bhatia, Pandey and Pande, 1965 of the Japanese quail *Coturnix japonica*, in Brazil. *Revista Brasileira de Parasitologia Veterinária*, 17, 235-238, 2008.

Berto BP, Luz HR, Flausino W, Teixeira-Filho WL, Ferreira I, Lopes CWG. Isosporoid coccidia (Apicomplexa: Eimeriidae) parasites of tanagers (Passeriformes: Thraupidae) from the Marambaia Island, Brazil. *Pesquisa Veterinária Brasileira*, 31, 798-805, 2011.

Cardozo TSF, Santos IR, Berto BP, Cardozo SV, Mesquita EFM, Lopes CWG. Correlação entre dados biométricos e parasitológicos de mexilhões (*Perna perna*) da Ponta do Tinguí, Mangaratiba, RJ, Brasil. *Revista Brasileira de Medicina Veterinária*, 34, 9-13, 2012.

Carvalho CEV, Cavalcante MPO, Gomes MP,

Faria VV, Rezende CE. Distribuicao de metais pesados em mexilhoes (*Perna perna*, L.) da Ilha de Santana, Macae, SE, Brasil. *Ecotoxicology Environmental Rest*, 4, 1-5, 2001.

Croci L, De Medici D, Scalfaro C, Fiore A, Divizia M, Donia D, Consentino AM, Moretti P, Costantini G. Determination of enteroviruses, hepatitis A virus, bacteriophages and Escherichia coli in Adriatic Sea mussels. *Journal of Applied Microbiology,* 88, 293-298, 2000.

Galvão JA. *Qualidade microbiológica da água de cultivo e de mexilhões Perna perna (Linnaeus, 1758) comercializados em Ubatuba, SP*. Dissertacao do Mestrado em Ciencia e Tecnologia de Alimentos da Escola Superior de Agricultura Luiz de Queiroz, Piracicaba. 2004. http://www.teses.usp.br/teses/disponiveis/11/11141/tde-16092004-164101/publico/juliana.pdf [20-10-2013]

Johnson DC, Reynolds KA, Gerba CP, Pepper IL, Rose JB. Detection of *Giardia* and *Cryptosporidium* in marine waters. *Water Science and Technology*, 31, 439-442, 1995.

Leal DAG, Franco RMB. Moluscos bivalves destinados ao consumo humano como vetores de protozoários patogênicos: metodologias de detecção e normas de controle. *Revista Panamericana de Infectologia*, 10, 48-57, 2008.

Norton CC, Joyner LP. *Eimeria acervulina* and *E. mivati*: oocysts, life-cycle and ability to develop in the chiken embryo. *Parasitology*, 83, 269-279, 1981.

Pereira CHS, Rodrigues DP, Viana CM. Isolamento de *Vibrio* spp. em mexilhões (*Perna perna*) coletados na Região de Ponta de Itaipu, Niterói, RJ. *Revista Higiene Alimentar*, 21, 157, 94-97, 2007.

Potasman I, Paz A, Odeh M. Infectious outbreaks associated with bivalve shellfish consumption: a worldwide perspective. *Clinical Infectious Diseases*, 35, 921-928, 2002.

Ramirez L, Teixeira-Filho WL, Flausino W, Berto BP, Almeida CRR. Importância das técnicas diagnósticas para oocistos do gênero *Cryptosporidium* Tyzzer, 1907 (Apicomplexa: Cryptosporidiidae) de suínos do Vale do Paraíba, Sul Fluminense, RJ. *Revista Brasileira de Medicina Veterinária*, 30, 111-114, 2008

Ritchie LS. An ether sedimentation technique for routine stool examination. *Bulletin of the United State Armed, Medical Department*, 8, 326, 1948.

Rose JB, Huffman DE, Gennaccaro A. Risk and control of waterborne cryptosporidiosis. *FEMS Microbiology Reviews*, 26, 113-123, 2002.

Sampaio IBM. *Estatística Aplicada à Experimentação Animal*. Belo Horizonte: FEPMVZ, 2002.

Sande D, Melo TA, Oliveira GSA, Barreto L, Talbot T, Boehs G, Andrioli JL. Prospecção de moluscos bivalves no estudo da poluição dos rios Cachoeira e Santana em Ilhéus, Bahia, Brasil. *Brazilian Journal of Veterinary Research and Animal Science,* 47, 190-196, 2010.

Silva AIM, Vieira RHSM, Menezes FGR, Fonteles-Filho AA, Torres RCO, Sant'anna ES. Bacteria of fecal origin in mangrove oysters (*Crassostrea rhizophorae*) in the Coco river estuary, Ceara State, Brazil. *Brazilian Journal of Microbiology*, 35, 126-130, 2004.

Tang YW, Wang JX, Xu ZY, Guo YF, Qian WH, Xu JX. A serologically confirmed, case-control study, of a large outbreak of hepatitis A in China, associated with consumption of clam. *Epidemiology and Infection*,107, 651-657, 1991.

Valença JFS. *Rio Salgado: agente de agravos a saúde das populações ribeirinhas*. Dissertação do Mestrado em Desenvolvimento Regional e Meio Ambiente da Universidade Estadual de Santa Cruz, Ilhéus. 2003. http://www.uesc.br/cursos/pos_graduacao/mestrado/mdrma/teses/dissertacao_joana.pdf [20-10-2013]