DOI: 10.5433/1679-0359.2020v41n6p2695

Development of a qPCR to diagnose the genus *Eimeria* in bovines

Desenvolvimento de uma qPCR para o diagnóstico do Gênero *Eimeria* em bovinos

Sérgio Tosi Cardim[1]*; Stefani Lino Cardim[2]; Mércia de Seixas[1]; Alessandra Taroda[3]; Priscilla Gomes Carneiro[3]; Dauton Luiz Zulpo[4]; Luiz Daniel de Barros[5]; Guilherme Luis Bortolloti[6]; João Luis Garcia[5]

Highlights:
Fastest diagnosis of genus *Eimeria* in cattle.
High sensitivity of qPCR.
Faster prognosis with decreased animal injuries.

Abstract

Bovine coccidiosis is caused by protozoa in the genus *Eimeria*. These protozoa mainly affect young animals, causing a decrease in production and consequent economic losses. The routine diagnosis is made through morphological observation of the oocysts, which has several limitations. The objective of the present study was to develop a real-time PCR (qPCR) technique for the diagnosis of *Eimeria* spp. in cattle. For this purpose, the 18S rRNA region of the DNA of these parasites was selected because it is a region with low variability among the species. The qPCR technique was developed using SYBR Green, resulting in a PCR with high sensitivity, and the ability to amplify samples containing only one oocyst of an *Eimeria* spp. in bovines. The feasibility of using qPCR in the diagnosis of the genus *Eimeria* was demonstrated in this study. This technique is less laborious and requires less skill and diagnostic training compared to the technique conventionally used for this diagnosis (micromorphometry).
Key words: 18S rRNA. Cattle. Coccidiosis. *Eimeria.* qPCR.

Resumo

A coccidiose em bovinos é causada por protozoários do gênero *Eimeria.* Estes protozoários acometem principalmente animais jovens, causando diminuição de produção e consequente perdas econômicas. O diagnóstico de rotina é realizado através de observação morfológica dos oocistos, que possui várias

[1] Profs. Drs., Universidade Norte do Paraná, UNOPAR, Arapongas, PR, Brasil. E-mail: stcardim@hotmail.com; mercia_seixas@yahoo.com.br
[2] Discente do Curso de Doutorado do Programa de Pós-Graduação em Fisiopatologia Clínica e Laboratorial, Universidade Estadual de Londrina, UEL, Londrina, PR, Brasil. E-mail: stefanilino@hotmail.com
[3] Discentes do Curso de Doutorado do Programa de Pós-Graduação em Ciência Animal, UEL, Londrina, PR, Brasil. E-mail: aletaroda@gmail.com; pirt1987@hotmail.com
[4] Prof. Dr., Faculdade de Medicina Veterinária, Pontifícia Universidade Católica do Paraná, PUC, Toledo, PR, Brasil. E-mail: dau_zulpo@yahoo.com.br
[5] Profs. Drs., Departamento de Medicina Veterinária Preventiva, Centro de Ciências Agrárias UEL, Londrina, PR, Brasil. E-mail: ldbarros@uel.br; joaoluisgarcia10@gmail.com
[6] Discente do Curso de Mestrado do Programa de Pós-Graduação em Saúde e Produção Animal, UNOPAR, Arapongas, PR, Brasil. E-mail: guilherme.bortolloti@hotmail.com
* Author for correspondence

Received: Nov. 18, 2019 - Approved: Aug, 18, 2020

limitações. O objetivo do presente trabalho foi desenvolver uma técnica de qPCR para diagnóstico de *Eimeria* spp. em bovinos. Para isto foi selecionada a região 18S rRNA do DNA destes parasitas, pois a mesma é uma região com pouca variabilidade entre as espécies. A qPCR foi desenvolvida com a utilização de SYBR Green, tendo como resultado uma PCR com uma alta sensibilidade, capaz de amplificar amostras contendo apenas um oocisto de *Eimeria* spp de bovinos. Demonstra-se nesse estudo a viabilidade na utilização da qPCR no diagnóstico do gênero *Eimeria*, sendo esta técnica menos laboriosa e com menos necessidade de treinamento para diagnóstico quando comparada com a técnica convencionalmente utilizada em rotina (micromorfometria).
Palavras-chave: qPCR. *Eimeria.* Coccidiose. 18S rRNA. Bovinos.

Introduction

Bovine coccidiosis is a disease caused by a protozoan from the class Coccidia, family Eimeriidae, and genus *Eimeria* (Bruhn et al., 2011). This disease is one of the most common among bovines worldwide, and primarily occurs in animals less than 1 year of age (Daugschies, Imarom, Ganter, & Bollwahn, 2004; Bruhn et al., 2011).

Over 20 species of *Eimeria* have been described in cattle, with *Eimeria bovis* and *Eimeria zuernii* considered pathogenic because they have been associated with clinical coccidiosis cases in young animals (Bangoura, Mundt, Schmäschke, Westphal, & Daugschies, 2011; Jonsson, Piper, Gray, Deniz, & Constantinoiu, 2011; Florião, Lopes, Berto, & Lopes, 2016). *Eimeria alabamensis* has also been associated with mild diarrhea in animals raised on pasture (Svensson, Hooshmand-Rad, Pehrson, Törnquist, & Uggla, 1993, Svensson, Uggla, & Pehrson, 1994; Marshall, Catchpole, Green, & Webster, 1998; Svensson, 2000). This disease causes substantial economic losses associated with decreased animal production, increasing susceptibility to other illnesses, and consequently, expenses associated with medicines (Abebe, Wossene, & Kumsa, 2008). It has had an estimated 400 million dollar impact on the American market with over 3.8 million dollars spent on treatment in Canada (Matjila & Penzhorn, 2002; Rehman et al., 2011). The economic impact of this illness in Brazil is unknown.

The occurrence of this parasite varies from 10 to 100% (Lentze, Hofer, Gottstein, Gaillard, & Busato, 1999; Stewart, Smith, & Ellis-Iversen, 2008; Almeida, Magalhães, Muniz, & Munhoz, 2011; Bruhn et al., 2011, 2012; Koutny, Joachim, Tichy, & Baumgartner, 2012). According to studies evaluating different countries, the prevalence in Brazil varies from 33.33% to 48.2% (Rebouças et al., 1994; Almeida et al., 2011; Bruhn et al., 2011, 2012; Hillesheim & Freitas, 2016).

A few studies have been conducted to develop PCR assays to diagnose *Eimeria* spp. Kawahara et al. (2010) developed a PCR technique using primers designed to begin annealing at the 18S rRNA region and finish at the 5.8S region, running through the ITS-1 region, to diagnose the genus and to determine the applicability of the primer design to diagnose species of this parasite in bovines. In another study, a primer was obtained for the genus *Eimeria* based on the 18S rRNA site, targeting the construction of a phylogenetic tree consisting of the main species in cattle (Kokozawa, Ichikawa-Seki, & Itagaki, 2013).

Concerning technological advancements, new assays, such as real-time PCR (qPCR) are now available and capable of developing a more sensitive and specific diagnosis compared to that of conventional PCR. Hence, the goal of the present study was to develop and standardize a qPCR technique to diagnose *Eimeria* spp. in bovines.

Material and Methods

Samples

Fecal samples from bovines were sent to the Veterinary Parasitology Laboratory of the State

University of Londrina. Ten positive samples for *Eimeria* spp. from naturally infected animals were selected and stored in 2.0 mL microtubes at -20°C.

To evaluate the sensitivity of the test, a single oocyst from each species of *Eimeria* spp. (*E. bovis*, *E. alabamensis,* and *E. zuernii)* were collected for DNA extraction. The oocysts were collected using a Pasteur glass pipette under an optical microscope, and each oocyst was placed in a 2.0-mL microtube and diluted with 300 µL of sterile ultrapure water. The samples were stored at -20°C for further extraction.

For the same assay, two additional samples were used, including a positive fecal sample for *Giardia* spp. and a positive sample for *Cryptosporidium* spp. These samples were used to evaluate the specificity of the primers developed in this study.

DNA extraction

For DNA extraction, 25 mg of stool was diluted in 300 µL of sterile ultrapure water, followed by three cycles of freezing at -80°C for 10 min and thawing at 55°C for 10 min. Next, 15 µL of proteinase K (1 mg mL^{-1}) and 30 µL of 0.1% SDS were added and the solution was incubated overnight at 55°C. Subsequently, 300 µL of UltraPure™ Phenol (Invitrogen, USA) was added and centrifuged at 13,000× *g* for 5 min. The resulting liquid was transferred to another microtube and 300 µL of a mixture of UltraPure™ Phenol: chloroform: isoamyl alcohol (25:24:1, v/v) (Invitrogen, USA) was added and centrifuged at 13,000× *g* for 5 min. The DNA was precipitated with sodium acetate and ethanol according to the method described by Sambrook, Fritshc and Maniatis (1989). The microtubes with extracted DNA were stored at -20°C until use.

The DNA from the single oocyst samples for each species were individually obtained following the same freeze and thaw procedures and the DNA extraction process described above.

Primers design

The primers were designed from the 18S rRNA gene sequences of *Eimeria* spp. and deposited at GeneBank with the following accession numbers: KU641163.1; KU641162.1; KU641161.1; KU641160.1; KU641159.1; KU641158.1; KU641157.1; KU641156.1; KU641155.1; KU641154.1; KU641153.1; KU641153.1; KU351737.1; KU351736.1; KU351735.1; KU351734.1; KU351733.1; KU351732.1; KU351731.1; KU351730.1; KU351729.1; KU351728.1; KU351727.1; KU351726.1; KU351725.1; KU351719.1; KU351718.1; KU351717.1; AY876932.1; AY876931.1; AY876930.1; AY876929.1; AY876928.1; AY876927.1; and AY876926.1.

After the selection of the sequences, the primers were aligned using the BioEdit program. Following the alignment, a specific site was selected and used for primer design, which was performed with Primer Express 3.0.1 (Thermo Fisher Scientific, USA). The primers obtained were named Eim18SF (AGC TTT CGA CGG TAG GGT ATT G) and Eim18SR (CGA ACC CTA ATT CCC CGT TAC) having an amplicon of 62 bp.

PCR amplification

DNA amplification was performed with 10 µL of the total reaction and contained 5 µL of SYBR® Select Master Mix (Thermo Fisher Scientific, USA), 200 nM of each primer (Eim18SF and Eim18R), 0.4 µL of BSA (1 mg mL^{-1}), and 1 µL of DNA. The reaction cycle was run with an initial denaturation at 95 °C for 10 min, followed by 40 cycles of 94°C for 15 s, 57°C for 30 s, and 72°C for 30 s (the step when the reading was conducted). To obtain the melting curve, the reaction was continued at 94°C for 15 s and 57°C for 1 min, and then the temperature was increased to 95°C, being read every 0.3°C.

Results and Discussion

In the present study, the 18S rRNA site of the DNA from several species of *Eimeria* spp. of bovines was used to design primers, because this is a highly conserved region among species (Ogedengbe, Hanner, & Barta, 2011).

The results showed it was possible to find samples containing a single oocyst of *Eimeria* spp., as seen in Figure 1. This demonstrated that the assay showed high sensitivity and could be a useful tool for routine diagnosis. These sensitive results resemble those obtained by Kokozawa et al. (2013) who used a conventional PCR, which was also based on the 18S rRNA site, to amplify a single oocyst of this protozoan, with the goal of subsequent sequencing and evaluation of genetic variability among oocysts from the same species.

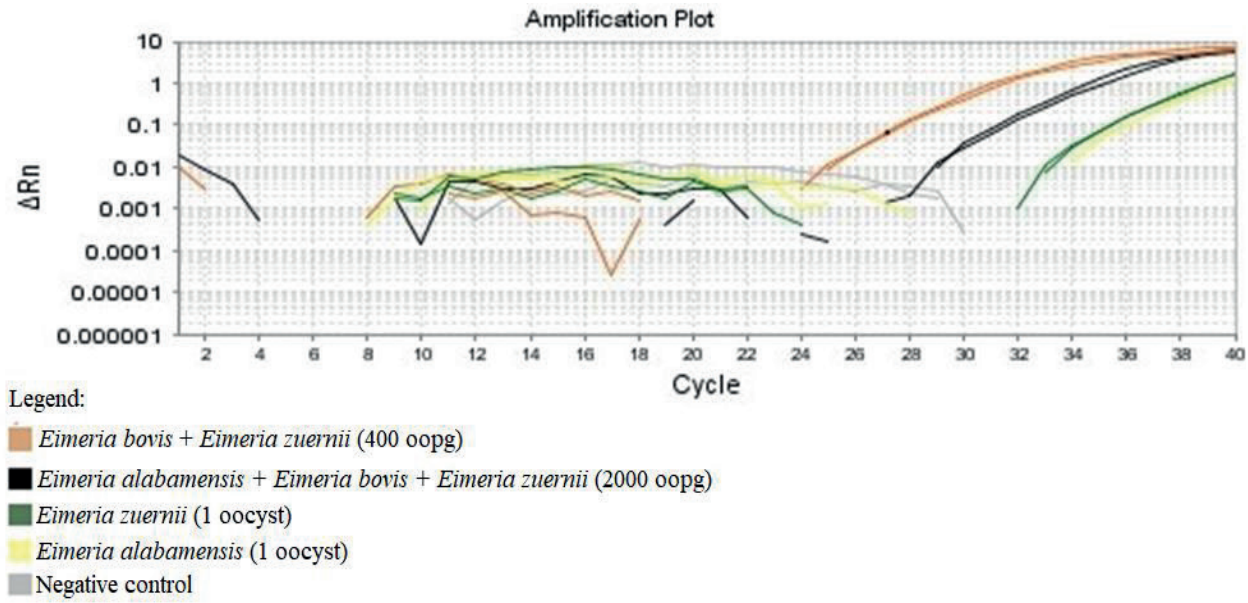

Figure 1. qPCR amplification curve for *Eimeria* spp. based on the 18S rRNA region.

PCR using *Cryptosporidium* spp. and *Giardia* spp. samples showed negative results for both genera, which demonstrated the specificity of the primers used for this *Eimeria* study.

Once the SYBR Green was used in this qPCR, the melting curve was generated to look for primer dimers or inespecific reactions. A small amount of primer dimers was noted in addition to a significant decrease in primer concentration (Figure 2).

A few studies have used the melting curve for parasitic species differentiation by examining the differences between the dissociation temperatures (Nicolas, Milon, & Prina, 2002; Khademvatan, Neisi, Maraghi, & Saki, 2011). This could not be performed in the present study because of similar dissociation temperatures.

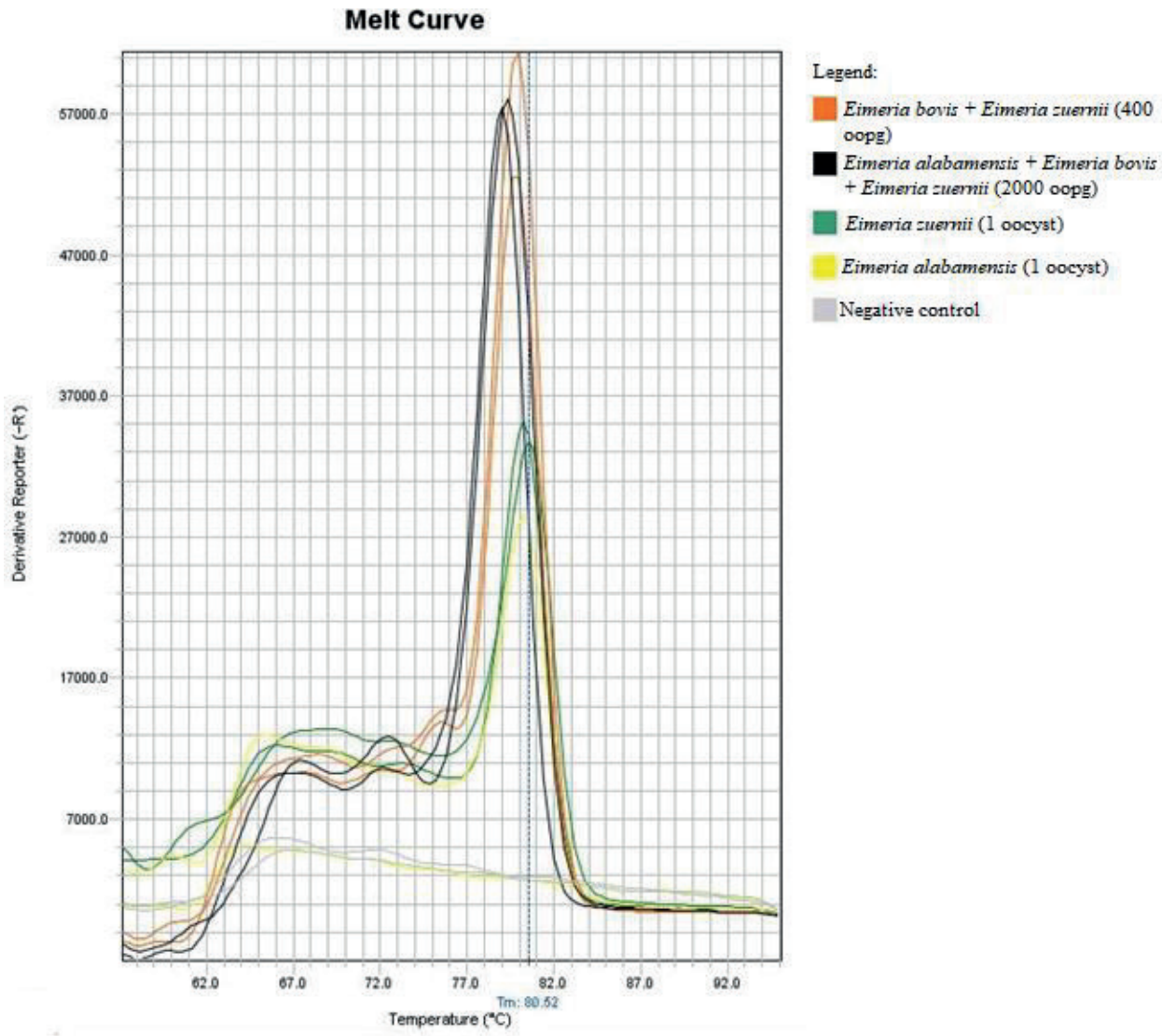

Figure 2. qPCR melting curve for *Eimeria* spp. based on the 18S rRNA region.

Conclusion

The qPCR developed in this study could be used as a more sensitive alternative for routine diagnosis of *Eimeria* spp. in cattle fecal samples. In addition to being more sensitive than routine morphometry, this technique is less laborious and produces faster results. The development of a probe for TaqMan qPCR will be performed in the future.

Acknowledgments

This study recived financial support from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, INCT-Leite CAPES 016/2014). J.L. Garcia is recipient of CNPq fellowships.

References

Abebe, R., Wossene, A., & Kumsa, B. (2008). Epidemiology of *Eimeria* infections in calves in Addis Ababa and Debre Zeit dairy farms, Ethiopia. *Journal of Applied Research in Veterinary Medicine, 6*(1), 24-30.

Almeida, V. A., Magalhães, V. C. S., Muniz, E. S., Neta, & Munhoz, A. D. (2011). Frequency of species of the genus *Eimeria* in naturally infected cattle in Southern Bahia, Northeast Brazil. *Revista Brasileira de Parasitologia Veterinária, 20*(1), 78-81. doi: 10.1590/S1984-29612011000100017

Bangoura, B., Mundt, H. C., Schmäschke, R., Westphal, B., & Daugschies, A. (2011). Prevalence of *Eimeria bovis* and *Eimeria zuernii* in German cattle herds and factors influencing oocyst excretion. *Parasitology Research, 109*(2), 129-138. doi: 10.1007/s00436-011-2569-z

Bruhn, F. R. P., Lopes, M. A., Demeu, F. A., Perazza, C. A., Pedrosa, M. F., & Guimarães, A. M. (2011). Frequency of species of *Eimeria* in females of the Holstein Friesian breed at the post-weaning stage during autumn and winter. *Revista Brasileira de Parasitologia Veterinária, 20*(4), 303-307. doi: 10.1590/S1984-29612011000400008

Bruhn, F. R. P., Silva, F. A., Jr., Carvalho, A. H. O., Orlando, D. R., Rocha, C. M. B. M., & Guimarães, A. M. (2012). Ocorrences of *Eimeria* spp. and gastrointestinal nematodes in dairy calves in southern Minas Gerais, Brazil. *Revista Brasileira de Parasitologia Veterinária, 21*(2), 171-175. doi: 10.1590/S1984-296 12012000200019

Daugschies, A., Imarom, S., Ganter, M., & Bollwahn, W. (2004). Prevalence of *Eimeria* spp. in sows at piglet producing farms in Germany. *Journal of Veterinary Medicine, 51*(3), 135-139. doi: 10.1111/j. 1439-0450.2004.00734.x

Florião, M. M., Lopes, B. B., Berto, B. P., & Lopes, C. W. G. (2016). New approaches for morphological diagnosis of bovine *Eimeria* species: a study on a subtropical organic dairy farm in Brazil. *Tropical Animal Health and Production, 48*(3), 577-584. doi: 10.1007/s11250-016-0998-5

Hillesheim, L. O., & Freitas, F. L. C. (2016). Ocorrência de eimeriose em bezerros criados em propriedades de agricultura familiar - nota científica. *Ciência Animal Brasileira, 17*(3), 472-481. doi: 10.1590/1089-6891v17i333327

Jonsson, N. N., Piper, E. K., Gray, C. P., Deniz, A., & Constantinoiu, C. C. (2011). Efficacy of toltrazuril 5% suspension against *Eimeria bovis* and *Eimeria zuernii* in calves and observations on the associated immunopathology. *Parasitology Research, 109*(1), 113-128. doi: 10.1007/s00436-011-2408-2

Kawahara, F., Zhang, G., Mingala, C. N., Tamura, Y., Koiwa, M., Onuma, M., & Nunoya, T. (2010). Genetic analysis and development of species-specific PCR assays based on ITS1 region of rRNA in bovine *Eimeria* parasites. *Veterinary Parasitology, 174*(1-2), 49-57. doi: 10.1016/j.vetpar.2010.08.001

Khademvatan, S., Neisi, N., Maraghi, S., & Saki, J. (2011). Diagnosis and identification of *Leishmania* spp. from Giemsa-stained slides, by real-time PCR and melting curve analysis in south-west of Iran. *Annals of Tropics Medicine & Parasitology, 105*(8), 559-565. doi: 10.1179/2047773211Y.0000000014

Kokozawa, T., Ichikawa-Seki, M., & Itagaki, T. (2013). Determination of phylogenetic relationships among *Eimeria* species, which parasitize cattle, on the basis of nuclear 18S rDNA sequence. *The Journal of Veterinary Medicine Science, 75*(11), 1427-1431. doi: 10.1292/jvms.13-0273

Koutny, H., Joachim, A., Tichy, A., & Baumgartner, W. (2012). Bovine *Eimeria* species in Austria. *Parasitology Research, 110*(5), 1893-1901. doi: 10.1007/s00436-011-2715-7

Lentze, T., Hofer, D., Gottstein, B., Gaillard, C., & Busato, A. (1999). Prevalence and importance of endoparasites in calves raised in Swiss cow-calf farms (Article in German.) *Deutsche Tierärztliche Wochenschrift*, *106*(7), 275-281.

Marshall, R. N., Catchpole, J., Green, J. A., & Webster, K. A. (1998). Bovine coccidiosis in calves following turnout. *Veterinary Record, 143*(13), 366-367. doi: 10.1136/vr.143.13.366

Matjila, P. T., & Penzhorn, B. L. (2002). Occurrence and diversity of bovine coccidia at three localities in South Africa. *Veterinary Parasitology, 104*(2), 93-102. doi: 10.1016/s0304-4017(01)00605-7

Nicolas, L., Milon, G., & Prina, E. (2002). Rapid differentiation of Old World *Leishmania* species by LightCycle polymerase chain reaction and melting curve analysis. *Journal of Microbiological Methods, 51*(3), 295-299. doi: 10.1016/s0167-7012(02)00099-4

Ogedengbe, J. D., Hanner, R. H., & Barta, J. R. (2011). DNA barcoding identifies *Eimeria* species and contributes to the phylogenetics of coccidian parasites (Eimeriorina, Apicomplexa, Alveolata). *International Journal for Parasitology, 41*(8), 843-850. doi: 10.1016/j.ijpara.2011.03.007

Rebouças, M. M., Grasso, L. M. P. S., Spósito, E. Fª, Amaral, V., Santos, S. M., & Silva, D. M. (1994). Prevalência e distribuição de protozoários do gênero *Eimeria* (apicomplexa: Eimeriidae) em bovinos nos municípios de Altinópolis, Taquaritinga, São Carlos, Guaíra - Estado de São Paulo, Brasil. *Revista Brasileira de Parasitologia Veterinária, 3*(2), 125-130.

Rehman, T. U., Khan, M. N., Sajid, M. S., Abbas, R. Z., Arshad, M., Iqbal, Z., & Iqbal, A. (2011). Epidemiology of *Eimeria* and associated risk factors in cattle of district Toba Tek Singh, Pakistan**.** *Parasitology Research, 108*(5), 1171-1177. doi: 10.1007/s00436-010-2159-5

Sambrook, J., Fritshc, E. F., & Maniatis, T. (1989). *Molecular cloning: a laboratory manual* (2a ed.). New York: Cold Spring Harbor Laboratory Press.

Stewart, I. D., Smith, R. P., & Ellis-Iversen, J. (2008). *Eimeria* species in cattle on farms in England and Wales. *Veterinary Record, 162*(15), 482-483. doi: 10.1136/vr.162.15.482

Svensson, C. (2000). Excretion of *Eimeria alabamensis* oocysts in grazing calves and young stock. *Journal of Veterinary Medicine, 47*(2), 105-110. doi: 10.1046/j.1439-0450.2000.00324.x

Svensson, C., Hooshmand-Rad, P., Pehrson, B., Törnquist, M., & Uggla, A. (1993). Excretion of *Eimeria* oocysts in calves during their first three weeks after turn-out to pasture. *Acta Veterinaria Scandinavica, 34*(2), 175-182.

Svensson, C., Uggla, A., & Pehrson, B. (1994). *Eimeria alabamensis* infection as a cause of diarrhoea in calves at pasture. *Veterinary Parasitology, 53*(1-2), 33-43. doi: 10.1016/0304-4017(94)90014-0