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Evaluation of a synthetic peptide from the *Taenia saginata* 18 kDa surface/secreted oncospheral adhesion protein for serological diagnosis of bovine cysticercosis

Rafaella Paola Meneguete Guimarães-Peixoto[a,*], Paulo Sérgio Arruda Pinto[a], Marcus Rebouças Santos[b], Marcelo Depólo Polêto[c], Letícia Ferreira Silva[a], Abelardo Silva-Júnior[b]

[a] *Universidade Federal de Viçosa, Departamento de Veterinária, Laboratório de Inspeção de Produtos de Origem Animal, Campus Universitário, Viçosa, Minas Gerais, Brazil*
[b] *Universidade Federal de Viçosa, Departamento de Veterinária, Laboratório de Virologia Animal, Campus Universitário, Viçosa, Minas Gerais, Brazil*
[c] *Universidade Federal do Rio Grande do Sul, Centro de Biotecnologia, Porto Alegre, Rio Grande do Sul, Brazil*

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ABSTRACT

Bovine cysticercosis is a zoonotic infection widely spread throughout Brazil, creating a burden on hygiene maintenance and the economy. Diagnosis of cysticercosis usually relies on *post mortem* inspection of carcasses in slaughterhouses. This detection method provides only low sensitivity. Recent advancements have improved the performance of serologic tests, such as ELISA, providing greater sensitivity and specificity. The objective of the current study was to identify and evaluate a synthetic peptide derived from the *Taenia saginata* 18 kDa oncospheric surface protein for the diagnosis of bovine cysticercosis in ELISA. Test performance of the identified peptide was compared to an ELISA based on a heterologous crude *Taenia crassiceps* antigen (Tcra), widely used for the sero-diagnosis of bovine cysticercosis. Based on the primary sequence of an *in silico* structural model of the 18 kDa protein, an epitope region designated EP1 was selected (46-WDTKDMAGYGVKKIEV-61). The peptide derived from this region yielded 91.6% (CI = 80–96%) sensitivity and 90% (CI = 82–95%) specificity when used in an ELISA, whereas the crude antigen yielded 70% (CI = 56–8%) sensitivity and 82% (CI = 73–89%) specificity. Thus, we conclude that EP1 has higher diagnostic potential for detecting bovine cysticercosis than the crude antigen Tcra.

1. Introduction

Bovine cysticercosis is a parasitic infection. It's caused by the larval stage of the human intestinal parasite *Taenia saginata*. It is rather a food hygienic and economic problem than a health problem with severe impact, and is widely spread throughout Brazil, causing concern in meatpacking depots as well as among rural livestock producers. In Brazil, the reported average prevalence is 1.05%, which varies among Federal States (Dutra et al., 2012).

Usually, bovine cysticercosis is diagnosed during a routine *post mortem* examination of carcasses at official meat inspection in slaughterhouses. The examination consists of standard cuts through the muscle tissue for the detection of cysticerci (Brasil, 1971). However, some infected carcasses are missed during this procedure, as demonstrated by a probabilistic model developed by Kyvsgaard et al. (1990), which showed that over 85% of infected animals may be missed during routine meat inspection. The low sensitivity of routine *post-mortem* examination is reported by several authors (Dorny et al., 2000; Eichenberger et al., 2013; Guimarães-Peixoto et al., 2015), and with that comes new emerging alternatives for the diagnosis of cysticercosis, as an example, Eichenberger et al. (2011) that suggested the use of an auxiliary model by additional cuts in the muscle; Wanzala et al. (2003), recommend increasing the area and number of locations inspected; and currently, it has been employed the use of auxiliary serological tests such as ELISA identifying the disease in the animal with greater precision (Dorny et al., 2000; Pinto et al., 2006; Monteiro et al., 2004; Allepuz et al., 2012; Guimarães-Peixoto et al., 2015). It is noteworthy that, in a retrospective study of data from the Federal Inspection Service of Brazil, Souza et al. (2007) found that 94% of carcasses parasitized by cysticerci is monocysticercosis (only one cysticercus in routine inspection sites).

* Corresponding author.
E-mail address: rafaella.peixoto@ufv.br (R.P.M. Guimarães-Peixoto).

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The *Taenia crassiceps* antigen has been used in ELISA assays for the diagnosis of cysticercosis since it features proteins homologous to those of *Taenia saginata.* Another advantage of using the *Taenia crassiceps* antigen is the ease of handling of cysticerci in the laboratory and that standardized reproducible results can be obtained using low-cost materials for immunological assays (Larralde et al., 1990). However, the accuracy of the test may be compromised by the lack of species specificity. Many researchers emphasize the need to implement serologic ELISA tests as auxiliary routine inspection methods. This would require further evaluation of various laboratory protocols in order to increase the tests' accuracy and efficiency (Monteiro et al., 2006; Ogunremi and Benjamin, 2010; Eichenberger et al., 2011; Allepuz et al., 2012).

The indirect ELISA test is not yet reliable in the detection of bovine cysticercosis in naturally infected animals (Monteiro et al., 2006; Allepuz et al., 2012) or in chronic cases of the disease, due to low amount of circulating antibodies in bovine. Therefore, new technologies are being implemented and utilized, including molecular techniques, such as synthetic peptides and prediction of their production in heterologous expression systems, with the goal of improving the performance of the immunological test (Ferrer et al., 2003, 2007; Parkhouse et al., 2008).

In its larval form, *T. saginata* expresses proteins that mediate the adhesion of the parasite to host cells. These proteins present one or two fibronectin type-III domains, which confer adhesion ability (Gonzalez et al., 2007). Among the binding proteins, the 18 kDa protein is characterized as an important adhesin (Bonay et al., 2002) and potential vaccine immunogen protecting from cysticercosis (Lightowlers et al., 1996). The 18 kDa oncosphere protein (HP6-TSAG) is a secretion/excretion antigen (Ferrer et al., 2007). The use of *Taenia saginata* protein HP6 has been reported for the diagnosis of both animal and human cysticercosis (Ferrer et al., 2003, 2007; Abuseir et al., 2007).

Antigen-specific antibodies may be captured from serum samples via the substitution of short, synthetic peptides in the place of the protein antigen epitopes (Andresen and Bier, 2009). Innovative computational approaches results in the prediction of acceptable to high-precision of mapping of antigenic epitopes, facilitating the selection of peptides used for immunodiagnostics (Zimic et al., 2011; Wang et al., 2014). Within this context, the aim of the present study was to characterize and evaluate the diagnostic potential of a synthetic peptide derived from the *T. saginata* 18 kDa oncopsheric antigen in an ELISA for the detection of bovine cysticercosis.

2. Materials and methods

2.1. Computational characterization

Preliminary, assembly of structural models was performed for the *Taenia saginata* 18 kDa protein using a sequence was obtained from GenBank (Access code ADO86979.1). The structural models were generated by I-TASSER (Yang et al., 2015) and Phyre2 servers (Kelley et al., 2015) and the two best results from each server were compared in this work. Additionally, transmembrane regions of the proteins were predicted by using the TMHMM server (Krogh et al., 2001). Possible *N*- and *O*-glycosylation sites from the primary amino acid sequence were predicted by using the NetNGIyc 1.0 (Gupta et al., 2004) and NetOGIyc 4.0 (Steentoft et al., 2013) servers.

2.2. Selection and preparation of antigenic peptide

An epitope map was assembled based on the three-dimensional model predicted for 18 kDa protein and its primary structure. Four methods were utilized: Bepipred® (Larsen et al., 2006), which uses a combination of Parker's hydrophobicity scales (Parker et al., 1986), Levitt's secondary structure (Levitt, 1978), and the hidden Marcov model; ABCpred®, which utilizes an artificial neural network (Saha and Raghava, 2004); AAPpred®, which utilizes an amino acid pair antigenicity scale (Chen et al., 2007); and ELIPRO®, which predicts discontinuous epitopes (conformational) by analyzing regions accessible to solvents and flexibility in a 3D structure (Ponomarenko et al., 2008). Ultimately, the resulting data converged in epitope EP1 (46-WDTKDMAGYGVKKIEV-61), which was subsequently synthesized (Genscript®, USA) with >95% purity.

2.3. Serum samples

The samples of bovine serum used in our study constituted five distinct groups, as follows: Group 1 (G1, n = 60) was composed of experimentally infected bovine blood samples, which were previously collected at different post-infection periods from an establishment without a history of cysticercosis. Nine male bovines, livestock of mixed Dutch-Zebu breeds, were inoculated with a parasitic load of 120.000 eggs of *Taenia saginata*. The tapeworm sample was previously obtained by a human donor, and tapeworm species was confirmed by direct microscopy. Cysticercosis was confirmed in all animals belonging to G1 at meat-inspection in a slaughterhouse. Group 2 (G2, n = 60) consisted of samples from naturally infected animals whose cysticercosis diagnosis had been made during routine *post-mortem* surveillance conducted in slaughterhouses supervised by the official inspectional service. Group 3 (G3, n = 60) constituted of samples from livestock slaughtered in commercial slaughterhouses that tested negative for cysticercosis and other diseases during routine *post-mortem* inspection. Lastly, Group 4 (G4, n = 28) consisted of samples from livestock that tested negative for cysticercosis, but presented with other diseases (tuberculosis (n = 8), hydatidosis (n = 8), and fasciolosis (n = 12)), to check possible cross-reaction with cysticercosis.

2.4. Indirect ELISA

2.4.1. Synthetic peptide-based ELISA

The optimal dilutions for the reagents used in the ELISA assays were determined via block titration. The immunoassay plates (Thermo. Nunc, Maxisorp) were initial treated with Polypep® 2% (Sigma P5163) for 10 h at 25 °C, followed by three washes with PBS, pH 7.4. The peptides were diluted with 0.5 M (1 μg/mL) carbonate-bicarbonate buffer, pH 9.4, and incubated in the plates overnight at 4° C. After three washes (with 0.15 M saline solution, pH 7.4, containing 0.05% Tween 20), the plates were blocked (with 1% Molico® denatured powdered milk in PBS) and incubated for 1 h at 37° C. The livestock serum samples were diluted 1:25 in the blocking solution with 0,05% Tween 20, for 1 h at 37° C. Following three times washing, an rabbit anti-bovine IgG antibody conjugated to peroxidase (A5295, Sigma Chemical Co., St Louis, MO, USA) was added to the plates at a 1:1.250 dilution, followed by incubation and washing steps as described above. The spectrophotometric detection was initiated via incubation with a solution of 0.1% *o*-phenylenediamine dihydrochloride (OPD) and 0.003% H_2O_2 in 0.2 M citrate-phosphate buffer, pH 5.0, for 30 min 37° C. The reaction was stopped with H_2SO_4 (4N), and the plates were read in a spectrophotometer (Biotek Instruments) at a 492 nm wavelength. All reagents were added to the plates at a volume of 100 μL/well, except the blocking solution, at 200 μL/well.

2.4.2. Taenia crassiceps *antigen-based ELISA*

Taenia crassiceps larval antigens were obtained via intraperitoneal inoculation of BALB/c female mice, following the methodology according to Vaz et al. (1997).

After collection, the cysticerci were immediately frozen (−20° C). Posteriorly, the cysticerci were lyophilized at 25 °C and after that, homogenizer and mixed with a 0.15 M saline solution, resulting in a final concentration of 6.5–10%. The diluted cysticerci were then homogenized on ice using a tissue homogenizer (Potter) and then centrifuged at 17.400*g* for 30 min at 4° C. A protease inhibitor (PMSF, Sigma Chemical Co, St. Louis, MO, USA, P7626, 0.25 M – 10 μL/mL) was added to the supernatant and the antigen was stored (−20° C) until used.

The polystyrene plates were sensitized with diluted antigens (40 μg/mL) in a 0.5 M buffered solution of carbonate-bicarbonate, pH 9.6. The ELISA plate was coated for 1 h at 37° C. After three washes in saline solution containing 0.05% Tween 20, the reactive sites were blocked (using 5% denatured milk in PBS, pH 7.4) for 1 h at 37 °C. After additional three washes, the samples were diluted in 1% denatured milk in PBS, pH 7.4. The plates were then incubated for 30 min at 37 °C. After washing, peroxidase-conjugated anti-IgG antibody (A5295, Sigma Chemical Co., St Louis, MO, USA) (1:5000) was added to each well, and the incubation and wash procedures were repeated. The chemiluminescent reaction was initiated using a solution of 0.1% *o*-Phenylenediamine dihydrochloride (OPD) P8287 (Sigma Chemical Co.; St Louis, MO, USA) and 0.003% H2O2 in 0.2 M citrate-phosphate buffer, pH 5.0. After a 5-min-incubation, the reaction was stopped using 4N H2SO4, and the plates were read in a spectrophotometer at a wavelength of 492 nm. All reagents applied to the plates were applied at a volume of 100 μL/well, with the exception of the 200 μL blocking solution.

2.5. Determination of test performance

The reactivity of the peptides was evaluated based on the results obtained from the ELISA tests Each ELISA reaction was performed in triplicate and the mean optical densities (OD) were calculated. The OD values were normalized to a standard reference plate, and the correction factor was calculated according to Passos (1993). The cut-off point was selected based on the mean OD obtained from analyses of five negative serum-control samples collected from livestock raised in isolation, kept under controlled conditions and slaughtered under rigorous inspection, including two standard deviations.

To determine the performance of the ELISA test, were used serum-control groups distributed as follows: analysis of antibody kinetics (G1) to evaluate the diagnosis performance; to calculate the sensitivity (G2), specificity (G3-G4) considering all the negative samples for cysticercosis after inspection and potential cross-reactions. The tests' sensitivity, specificity, positive and negative predictive values and accuracy were calculated based on the recommendations of Altman, Bland (1994) and Akobeng (2006). To test the performance rates it was taken into account a confidence interval of (CI) 95%.

2.6. Ethics committee approval

The norms of conduct for the use of animals in research from the Ethics Committee for Animal Experimentation of the Federal University of Viçosa were followed according to the Process reference 20/2011 CEUA/UFV.

3. Results

Structural models of 18 kDa protein were generated using I-TASSER and Phyre2 servers through comparative modeling, fold recognition and *ab initio* algorithms. Among the resulting models, the ones with higher scores from each server were selected for more in-depth comparisons. Interestingly, the structures with the highest scores were obtained for an protein model using a fibronectin type-III domain of human neural cell adhesion molecule (PDBID 2DOC), leading to C-score of −1.99 in I-TASSER and Confidence of 95.4% in Phyre2. Both models converged to a very similar fold, with a root mean square deviation (RMSD) value of 1.167 Å (Fig. 1a). Due to the similarity between both models, all structural features of 18 kDa protein were discussed in this work using the 3D structure obtained by Phyre2.

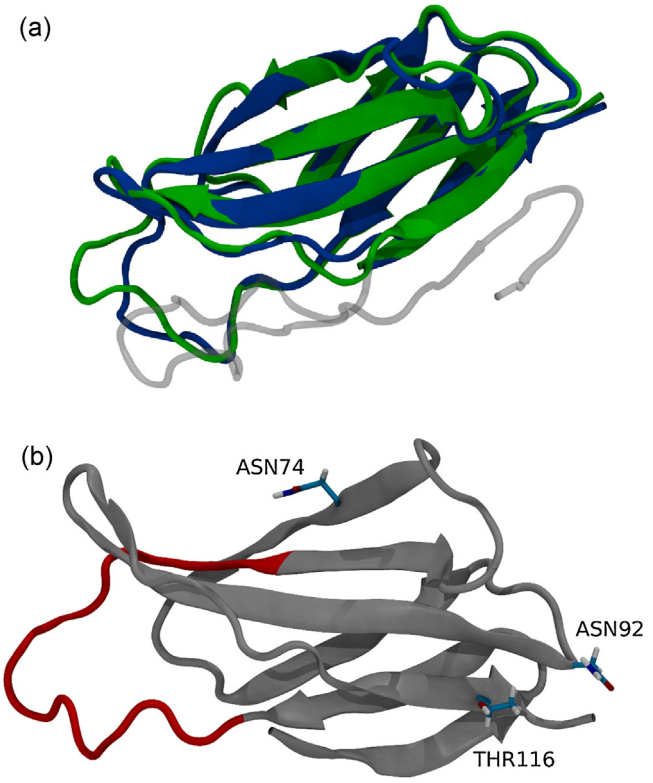

Fig. 1. Three-dimensional model of the 18 kDa protein. (a) Overlapping of predicted models by I-TASSER (in blue) and Phyre2 (in green). In translucent silver, the N-terminal structure modeled by I-TASSER and not considered in this study. (b) Localization of the predicted epitope EP1 (in red). In blue, the putative sites of *N*-glycosylation (ASN74 and ASN92) and *O*-glycosylation (THR116). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Since 18 kDa protein is known to be a membrane protein (Bonay et al., 2002), further analyses were carried out using the TMHMM server in order to predict transmembrane helical regions. Despite the highly hydrophobic region 5-FGLILLVAVVVLA-17, the server did not predict sequence as a transmembrane helix with high probability of occurrence (data not shown). However, region 1–17 was predicted to contain a signal peptide with a mean score of 94.4%, confirming the hypothesis of an N-terminal transmembrane region in 18 kDa protein. Therefore, the predicted N-terminal structure of 18 kDa was not taken into account in this study, due to the high probability that this region forms a transmembrane structure.

In addition, since 18 kDa protein is located in the outer membrane and it contains a signal peptide, it is possible that glycosilation pathways could be involved in maturation. Therefore, glycosilation sites predictions were performed using the NetNGlyc 1.0 and NetOGlyc 4.0 servers. Both servers predicted N-glycosylation sites at ASN74 and ASN92, and an *O*-glycosylation site at THR116 (Fig. 1b), with scores of 0.7303, 0.5054 and 0.7021, respectively.

The results of the bioinformatics programs used for epitope prediction of 18 kDa protein mostly converged in the region of amino acid of 46-WDTKDMAGYGVKKIEV-61. The peptide derived from this region was subsequently named EP1, being used for

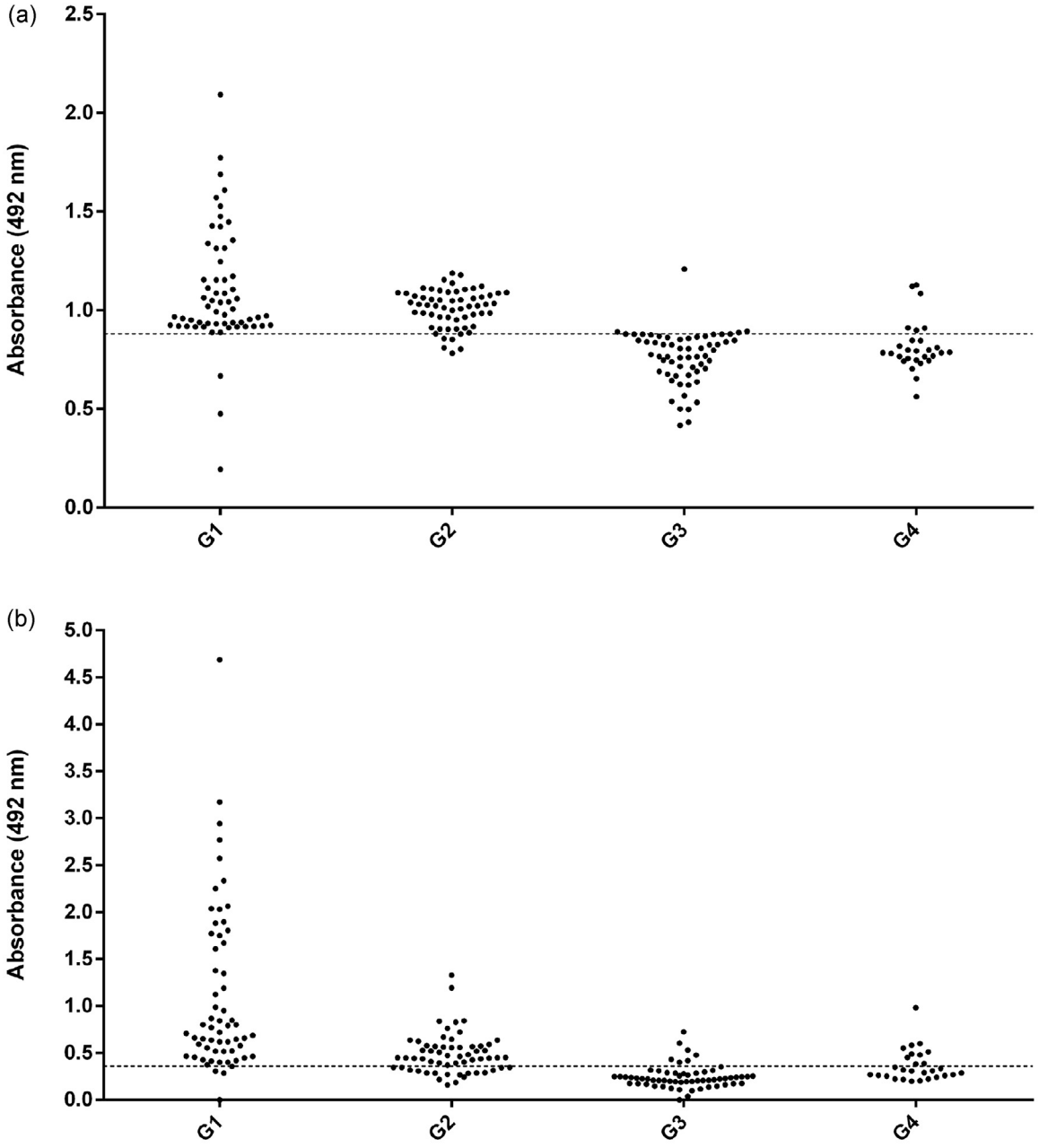

Fig. 2. Reactivity of bovine serum samples in the ELISA test and cut-off (dotted line). Assays conducted using (a) EP1 peptide as an antigen (Cut off 0,881); (b) *Taenia crassiceps* heterologous antigen (Cut off 0,359).

the posteriors analyses. EP1 in our model was exposed to a solvent in a beta-hairpin without any predicted sites of glycosylation (Fig. 1b). The antibody discrimination by the peptide and its diagnostic potential was measured by its performance in ELISA. The EP1 antigen was detected in most of the positive serum controls (n = 112/120) (animals experimentally or naturally infected) (Fig. 2), while the majority of negative animal serum samples (n = 80/88) did not react with the EP1 peptide. Therefore, these results indicate that EP1 enabled distinction between positive and negative samples in the ELISA assay. The performance rates were calculated and were obtained the following results: 91.6% sensitivity (CI = 80–96%), 90% specificity (CI = 82–95%), 85% positive predictive value (PPV) (CI = 75–93%), 93% negative predictive value (NPV) (CI = 86–97%) and 91% accuracy.

4. Discussion

The major recent improvements in biomolecular modeling techniques have greatly improved the accuracy of theoretical structural models (Figueiredo et al., 2014). Based on this background, we generated structural models of *Taenia saginata* 18 kDa oncopsheric protein, using computational approaches. Based on the fold recognition steps and *ab initio* calculations used for the creation of this model, the folding convergence and low RMSD indicate that our model is likely and accurate structural representation of 18 kDa protein. It is important to note that the final models were very similar to the one described by Kyngdon et al. (2006) for the *Taenia solium* TSOL18 protein, which has 61% identity with the *Taenia saginata* 18 kDa protein and analogous function. Furthermore, Lightowlers et al. (2003) previously described a putative ASN74 gly-

cosylation site in TSOL18, as was also predicted in our model, thus further increasing the confidence in our model.

With the aim of improving diagnostic tests against different pathogens, various studies have attempted to map B lymphocyte epitopes in a manner similar to that presented in this study (Meloen et al., 2003). The main technique used to identify conformational epitopes relies on a three dimensional structural representation of the protein of interest, as well as inferences regarding regions or amino acid residues that may interact with antibodies. Despite this attractive method, the majority of the studies describe linear and not conformational epitopes (Vita et al., 2010). The main explanation for this discrepancy is that various proteins present a challenge in plausible resolution of tertiary structure, because of either the high cost and time required for crystallography, or in computational molecular modeling. For our research, we employed four methods of epitope prediction, including one conformational epitope prediction method and the three linear prediction methods. It is important to emphasize that only linear epitopes were predicted, which were based on convergent regions predicted by the software programs and regions not described in other studies.

The immunodominant oncosphere antigens are considered immune targets and may also have preventive and diagnostic applications (Ferrer et al., 2003). The 18 kDa oncospheric protein is termed HP6 (Ferrer et al., 2003, 2007; Gonzalez et al., 2011) or TSA-18 (Lightowlers et al., 1996; Jabbar et al., 2010).

The following performance indicators were obtained from the ELISA test using the heterologous antigen (*T. crassiceps*): 70% sensitivity (CI = 56–80%), 82% specificity (CI = 73–89%), 72% PPV (CI = 60–84%), 80% NPV (CI = 70–87%), and 78% accuracy. The use of EP1 in an ELISA yielded slightly higher values than those from the heterologous antigen, indicating an improved performance of the diagnostic test. While EP1 was able to detect 91.6% of samples from the naturally infected animals, the *Taenia crassiceps* crude heterologous antigen only detected 70% of the samples. It is known that there is a homology between the 18 kDa protein of *Taenia saginata* and different species such as: *Taenia multiceps* – Tm18 (85.5%), *Taenia solium* – TSOL18 (61.5%), *Taenia ovis* – To18 (79.7%), *Taenia asiatica* TASI18 (95.5%) e *Taenia hydatigena*. A infecção em bovinos só ocorre com a *Taenia multiceps* (Avcioglu et al., 2011) e *Taenia hydatigena* (Dada and Belino, 1978), however, it is important to note that the serum control samples (G1, G2, G3, G4) are obtained from animals that have undergone *post-mortem* inspection and discard a possible co-infection with these parasites.

Ferrer et al. (2003) synthesized six peptides derived from *Taenia saginata* proteins, using the antigenic index of Jameson and Wolf (Jameson and Wolf, 1988). The two peptides (HP6-2 and HP6-3) derived from 18 kDa protein were able to differentiate serums from cysticercosis-positive and -negative animals in a smaller sample size. It is important to state, in contrast to EP1, which is located in an exposed beta-harpin, HP6-2 is mostly buried in our model, while HP6-3 is mainly located in the protein C-terminal region and, therefore, exposed to interactions (data not shown). This difference in location might reveal differences in the performance of diagnostic tests using the distinct peptides; a greater exposure of the peptide in the protein structure increases the probability of interaction with antibodies. Immunodominant peptides have a direct effect on disease, as evidenced by their high affinity for the host antibodies released during infection. Thus, a more thorough understanding of the biology of the immunodominant peptides will greatly aid the attempts to improve the performance of immunodiagnostic tests (Noya et al., 2003).

Thus, in comparison to using the heterogenic antigen, the use of the EP1 peptide may enable improved detection of cysticerci in naturally infected animals and/or in animals with monocysticercosis. Animals naturally infected have low amounts of circulating antigens, which normally hinder accurate diagnoses. According to Allepuz et al. (2012), the cost of implementing of a more sensitive tool for the diagnosis of bovine cysticercosis should be compared with the associated benefits for human health, given the great potential of limiting the transmission cycle of bovine cysticercosis. The use of an improved diagnostic tool for bovine cysticercosis would also benefit the livestock-farming sector by reducing economic loss.

The ELISA test has been used in research worldwide (Pinto et al., 2000; Ferrer et al., 2003, 2007; Monteiro et al., 2006; Allepuz et al., 2012; Guimarães-Peixoto et al., 2015). It should be noted that the ELISA should be able to detect animals at different stages of infection (viable or non-viable cysticerci), and similarly, showing satisfactory performance (Pinto et al., 2006; Monteiro et al., 2006; Minozzo et al., 2004; Guimarães-Peixoto et al., 2015). According Smith et al. (1991) low levels in low cyst-burden produced antibodies hinder the selection of a cutoff point and interpretation of findings in serological tests such as ELISA, especially in monocysticercosis (Thomaz-Soccol et al., 2010).

In the performance of an ELISA test, it is important to consider the amplitude value of the difference between the positive and negative control sera (Guimarães-Peixoto et al., 2015; Silva et al., 2015). Thus, the aim of this research was to set up a laboratory scenario using the optical density values adjusted to a standard plate to compare the performance of peptide EP1 with the antigen Tcra, thus the background are reduced.

5. Conclusion

In the present study, a computational approach was used to select a peptide with great potential for use in serologic diagnosis of bovine cysticercosis. The selected EP1 peptide enabled discrimination between samples from animals positive or negative for cysticercosis. The EP1 peptide also exhibited improved performance compared to the heterologous antigen of *Taenia crassiceps*.

The increasing use of computational tools to obtain structural data of biomolecules has significantly improved our understanding of antigen-antibody interactions. In this study, the creation of a conformational model of 18 kDa protein provided a theoretical framework for a *suitable* and rational methodological design that enabled the identification of a new epitope with potential use in the diagnosis of bovine cysticercosis.

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