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Chimeric Protein Designed by Genome-Scale Immunoinformatics Enhances Serodiagnosis of Bovine Neosporosis

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ABSTRACT Neosporosis has become a concern since it is associated with abortion in cattle. Currently, *in situ* diagnosis is determined through anamnesis, evaluation of the history, and perception of the clinical signs of the herd. There is no practical and noninvasive test adapted to a large number of samples, which represents a gap for the use of new approaches that provide information about infections and the risks of herds. Here, we performed a search in the *Neospora caninum* genome by linear B-cell epitopes using immunoinformatic tools aiming to develop a chimeric protein with high potential to bind specifically to antibodies from infected cattle samples. An enzyme-linked immunosorbent assay with the new chimeric antigen was developed and tested with sera from natural field *N. caninum*-infected bovines. The cross-reactivity of the new antigen was also evaluated using sera from bovines infected by other abortive pathogens, including *Trypanosoma vivax*, *Leptospira* sp., *Mycobacterium bovis*, and *Brucella abortus*, and enzootic bovine leucosis caused by bovine leukemia virus, as well as with samples of animals infected with *Toxoplasma gondii*. The assay using the chimeric protein showed 96.6% ± 3.4% of sensitivity in comparison to healthy animal sera. Meanwhile, in relation to false-positive results provided by cross-reactivity with others pathogens, the specificity value was 97.0% ± 2.9%. In conclusion, immunoinformatic tools provide an efficient platform to build an accurate protein to diagnose bovine neosporosis based on serum samples.

KEYWORDS immunoinformatics, *Neospora caninum*, chimeric protein, serodiagnosis

Neosporosis is a parasitic disease caused by the *Neospora caninum* protozoan that affects farm animals worldwide. In the past, neosporosis was wrongly detected as toxoplasmosis due to morphological similarities with the *Toxoplasma gondii* parasite (1). Since the first report in Norwegian dogs (2), neosporosis has also become a major concern in beef and dairy cattle (3). A review study detected anti-*Neospora caninum* antibodies in dairy cattle serum samples from at least 35 countries spread across all continents (4).

Abortion is the main clinical manifestation of the disease, and it may occur in endemic or epidemic form between the third and ninth gestational months (1). However, complete understanding of determinant aspects of infection that are associated with abortion remains a challenge (5). Infected calves with no apparent sign maintain the pathogen, and they are important to disease transmission and epidemi-

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ology. These animals are clinically healthy, seropositive, and harbor the encysted parasite in several tissues. The continuous presence of dogs in farms is directly related to transmission of the parasite (6).

Currently, the promotion of epidemiologic analysis within a noninvasive examination adjusted to multiple samples is a challenge, requiring innovative mechanisms able to provide information about the routes of infections and herd risks (3, 7, 8). Farmers use anamnesis, historical evaluation of animals, and perception of clinical signs to make *in loco* diagnoses (9, 10). Nevertheless, in chronic infections, clinical signs are almost undetectable, hampering disease disclosure and enhancing its spread (6, 11). PCR is a sensitive tool to detect DNA parasites in the abortive fetus or tissue samples (12). However, *Neospora caninum* genetic variability, difficulty in perceiving the correct stages of infection, and the need to have specific equipment limits this technique (13). Serological tests that identify IgG and IgM within a few days postinfection may enable a clear perception of each infection phase (12). Among the common serological techniques, enzyme-linked immunosorbent assay (ELISA) can be easily automated to evaluate multiple samples at low cost.

Some recombinant antigens for serodiagnosis bovine neosporosis have already been described, including rNcGRA7 and rNcSAG1 (5), NcGRA7 (7), NcSRS2 (14), NcMIC10 (15), NcSAG1 (16), NcSAG4 (17), surface antigen p40 (18), and subtilisin-like serine protease 1 (19). Overall, these antigens exhibit a high potential to recognize *Neospora caninum* antibodies in infected animals, thereby differentiating healthy and toxoplasmosis-infected samples. On the other hand, these antigens do not detect cross-reactions other than abortive bovine infections, such as leptospirosis, brucellosis, and pestivirus infections, which yield a high specificity for neosporosis investigation (11). Recombinant antigens are produced on a large scale and are standardized for diagnostic tests to decrease the risks of cross-reaction (16). The use of chimeric proteins increases in an attempt to enhance diagnostic power in a combination of multiple antigens (20). In addition, synthetic antigens are easily manufactured, reaching high levels of purity in the absence of any living organism (21).

Finding an efficient, affordable, and specific method to diagnose the animal in time to avoid infection's spread among cattle is a bottleneck to controlling the disease. The production of a synthetic and highly antigenic protein that meaningfully interacts with *N. canum*-infected sera represents an improvement in diagnostic agents. The purpose of our work was to map and select antigenic epitopes based on an immunoinformatic approach to design a chimeric protein to specifically serodiagnose bovine neosporosis.

MATERIALS AND METHODS

Animals and ethics statements. Veterinary Hospital of Uberaba and Animal Science Department of Universidade Federal de Viçosa provided all serum samples used at this work. The samples were collected from Minas Gerais and Santa Catarina state, Brazil, from 2015 to 2019. Approval to use the samples was given by the Animal Experimentation Ethical Committee of the Universidade de Uberaba, Uberaba, Minas Gerais, Brazil (CEEA/UNIUBE, protocol 001/2013). The samples were collected according to the ethical principles of cattle experimentation procedures.

The bovine serum panel consisted of samples from an area of endemicity for neosporosis and other abortive cattle infectious diseases, including a neosporosis group ($n = 29$), a brucellosis group ($n = 7$), tuberculosis group ($n = 7$), trypanosomiasis group ($n = 8$), leptospirosis group ($n = 7$), and a leucosis group ($n = 5$). The neosporosis samples were from animals from Uberaba ($n = 7$, 19°44′54″S and 47°55′55″W), Viçosa ($n = 10$, 20°45′14″S and 42°52′54″W), and Lages ($n = 12$, 27°49′0″S and 50°19′35″W). The samples of others abortive infectious diseases were collected from Uberaba. A total of 8 samples of bovines infected with *T. gondii* and validated by parasite isolation from Santa Catarina state were also used (22). The samples of animals infected with *Besnoitia besnoiti* ($n = 6$) and *Sarcocystis* spp. ($n = 7$) were collected from slaughterers in Sete Lagoas (19°28′04″S and 44°14′52″W), Marília (22°13′15″S and 49°56′55″W), São José do Rio Preto (20°49′13″S and 49°22′47″W), and Petrópolis (22°30′17″S and 43°10′56″W), and the infection was confirmed by PCR using DNA extracted from brain, kidney, muscle, and lung tissues using primers previously described (23, 24). The infection by *N. caninum* was identified by using an indirect fluorescence antibody test with a cutoff of 1:100 (13) and confirmed by tissue immunohistochemistry of aborted fetuses. Identifications of animals with brucellosis and tuberculosis were performed, respectively, by using a serum (tube) agglutination test and an intradermal tuberculin test, with both followed by PCR for pathogen confirmation. Infection by *T. vivax* was confirmed by both parasitological and serological test through microscopic identification of pathogens in blood smears and detection of anti-*T. vivax* IgG by using an indirect immunofluorescence assay, respectively. Infection by

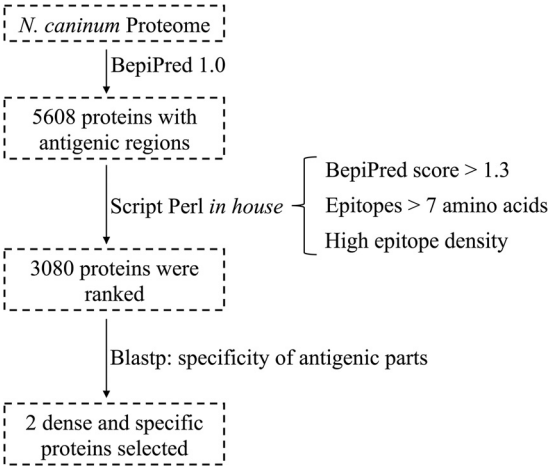

FIG 1 Workflow schematic for informatic selection of potential antigenic proteins containing a high density of linear B-cell epitopes in predicted *N. caninum* proteins from the genome sequence.

Leptospira sp. was confirmed by using a microscopic agglutination test. Bovine leucosis virus was identified by agar gel immunodiffusion. Sera of healthy animals, confirmed by negative results in parasitological tests, were also used as a control group (noninfected group, $n = 34$). Molecular techniques based on PCR were used to confirm previous histopathological tests and determine bovine monoinfection using primers previously validated and published (22–29).

Epitope identification by immunoinformatic tools. Figure 1 shows a workflow schematic that summarizes the process used for *in silico* genome-wide screening of the epitopes and design of the chimeric protein. Initially, the NCBI public database (28) provided *Neospora caninum* proteins (NCBI taxid572307) based on the genome sequence (30). A total of 21,357 proteins were submitted to the BepiPred 1.0 web server (31) to screen *Neospora caninum* linear B cell epitopes. A total of 5,608 proteins had at least a predicted epitope. Next, a Perl in-house script ranked selected proteins by (i) the average predicted BepiPred 1.0 score higher than 1.3 (the threshold value was fixed to get a confidence value higher than 96% for true epitopes), (ii) epitopes with at least seven sequentially amino acids, and (iii) the percentage of protein associated with epitopes (epitope density). Epitope density ordered more than 3,000 proteins in descendant order. Then, BLASTp (32) evaluated antigen specificity against predicted proteins of the genome sequence from pathogens belonging to the bovine environment and with symptoms similar to neosporosis, including *Trypanosoma vivax* (33), *Cryptosporidium parvum* (34), *Leptospira* spp. (35), *Mycobacterium bovis* (36), *Brucella abortus* (37), *Babesia bovis* (38), *Anaplasma marginale* (39), *Toxoplasma gondii* (40), *Sarcocystis* sp. (NCBI taxid59669), and *Besnoitia besnoiti* (NCBI taxid94643). Two proteins with high epitope density and specific to *Neospora caninum* were selected. A chimeric protein was designed by connection of the antigenic regions afforded by two chosen proteins. Flexible linkers (GSGSGS) were included within epitopes to enhance protein solubility and to prevent neighboring epitope interaction from disrupting antibody recognition (41). After the chimeric protein designed, BLASTP was used again to compare the final protein sequence to the same pathogens tested previously.

In order to produce the antigen in *Escherichia coli*, the sequence was submitted to a web server codon optimization tool (https://www.idtdna.com/CodonOpt) to increase frequent codons based on codon usage metrics and to reduce the presence of intramolecular interactions inside the mRNA molecule.

Construction and expression of chimeric protein in a bacterial system. A DNA sequence that encodes the chimeric protein was chemically synthesized in the pET28-a(+) expression system (GenScript, USA) according to the manufacturer's instructions (42). The plasmid containing the synthetic sequence was added to 50 μl of electrocompetent *E. coli* BL21 Arctic Express (DE3) cells (Agilent Technologies) by using an electroporation cuvette (Bio-Rad, Hercules, CA) and submitted to a 2.50-kV pulse using MicroPulser electroporation equipment (Bio-Rad). Bacterial transformants were grown overnight on Luria-Bertani agar plates containing 50 μg/ml of kanamycin and 20 μg/ml of gentamicin (Neoquímica, Brazil). PCRs using T7 primers were carried out to confirm all transformants before protein expression. Recombinant protein expression was induced by using 0.4 mM IPTG (isopropyl-D-thiogalactopyranoside), and the flasks were incubated with shaking for 24 h at 12°C. Sonication disrupted induced cells, and centrifugation removed all insoluble components. His-tagged chimeric protein was purified from the soluble fraction by affinity chromatography by using a His-Trap column (GE Healthcare Life Sciences). The column was washed with the buffer A containing 20 mM Na_2HPO_4, 500 mM NaCl, and 30 mM imidazole. The buffer B, containing 20 mM Na_2HPO_4, 500 mM NaCl, and 500 mM imidazole, was used to elute the protein on a gradient ranging from 0 to 100%.

Purified protein fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Initially, bacterial cells were harvested, and the pellet was resuspended with phosphate-buffered saline (PBS). Proteins were mixed in sample buffer containing

2-mercaptoethanol and heated at 95°C for 10 min. The samples were subjected to electrophoresis on a 14% polyacrylamide gel and stained using Coomassie brilliant blue R-250. The proteins were transferred to a nitrocellulose membrane, which was blocked with 5% bovine serum albumin (BSA) protein in PBS at room temperature for 1 h. The nitrocellulose membrane was washed three times in PBS buffer supplemented with 0.5% Tween 20 (PBS-T). Mouse anti-His antibody at 1:5,000 was added, followed by incubation and shaking for 2 h at room temperature. Another wash step was performed. The membrane was then incubated in peroxidase-conjugated secondary antibody at 1:15,000 for 3 h at room temperature. Finally, membrane reacted with substrate 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich) and H_2O_2 for 10 min. This reaction was carried out in the dark.

The samples of purified chimeric protein were dosed by the bicinchoninic acid colorimetric method (BCA; Thermo Scientific, Waltham, MA), according to the manufacturer's recommendations, and the reading performed using SpectraMax M reader (Molecular Devices, San Jose, CA) with a wavelength of 562 nm.

ELISA and statistical analysis. ELISA was performed in MaxiSorp multiwell plates (Nunc, Roskilde, Denmark). First, the plates were coated with 1 μg of chimeric protein per well, diluted in 1 M carbonate buffer (pH 7.3). After overnight incubation at 4°C, the plates were washed five times with a washing buffer containing PBS (pH 7.2) and 0.05% Tween 20 (Sigma-Aldrich). Blocking buffer (PBS with 5% [vol/vol] BSA protein) was added, and the wells were incubated at 37°C for 1 h. An additional wash step was carried out to remove residual blocking buffer. Bovine sera were diluted 1:300 in washing buffer, and the plates were incubated at 37°C for 1 h. Another wash step was performed. Anti-bovine IgG antibody at 1:5,000, coupled with horseradish peroxidase, was then added, followed by incubation at 37°C for 1 h. OPD (*o*-phenylenediamine dihydrochloride; Sigma-Aldrich), the substrate of the reaction, was diluted in a pH 5.0 buffer containing 0.1 M citric acid, 0.2 M Na_2PO_4, and 0.02% H_2O_2, and the solution was added. The plates were kept at room temperature for 30 min in the dark. Spectrophotometric data were obtained at 492 nm using a SpectraMax M reader.

Statistical analyses were performed using GraphPad Prism (version 5.0, for Windows) and GraphPad QuickCals software. A cutoff value, based on the reactivity of sera at a lower limit of positivity, was established for optimal accuracy using the receiver operating characteristics (ROC) curve. The selected cutoff was based on the maximum sensitivity and specificity values supported by maximum-likelihood statistical metric. The performance of each test was evaluated according to the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy. A one-sample Kolmogorov-Smirnoff test was used to determine whether a variable was normally distributed. A nonparametric one-way analysis of variance (ANOVA) test was used to compare the means of absorbance between noninfected and infected groups. The degree of agreement between the ELISAs using chimeric protein with the direct diagnosis test was determined by kappa index (κ) values with 95% confidence intervals (CI) and interpreted according to the following Fleiss scale: 0.00 to 0.20, poor; 0.21 to 0.40, fair; 0.41 to 0.60, moderate; 0.61 to 0.80, good; 0.81 to 0.99, very good; and 1.00, perfect. The differences were considered statistically significant at $P < 0.001$.

RESULTS

Screening of *N. caninum* epitopes *in silico*. NCBI database provides the *Neospora caninum* proteome with 21,357 sequences. Immunoinformatic tools allowed a strict protein screening to select potential antigenic sequences, and the applied pipeline reduces the number of predict candidates for two proteins (Fig. 1). The proteins XP_003885442.1 (Fig. 2A) and XP_003884964.1 (Fig. 2B) were predicted to have more than 20% of their sequences associated with epitopes. Epitope sequences were selected according to average of BepiPred's score (Table 1) and *in silico* specificity to *N. caninum* evaluated by BLAST analysis (Table 2). In order to predict the potential specificity of epitopes, each sequence was aligned with bovine pathogens that are described in Table 2. A total of eight selected epitopes were joined using the linker sequence to produce the chimeric protein (Fig. 2C).

Validation of the expression system. The synthetic gene encodes the chimeric protein was inserted into the bacterial expression system pET-28a(+). PCR with T7 primers that anneal in pET-28a(+) vector was performed to confirm plasmid construction through amplification of a 700-bp amplicon (Fig. 3A). The amplicon size refers to the sum of a 250-bp vector amplification plus 450 bp of coding DNA.

SDS-PAGE was performed to evaluate the production of the protein by *E. coli* containing the expression system. A comparison of the protein profile was established between wild-type *E. coli* Arctic Express and the noninduced built system (Fig. 3B). It should be noted that a band at 25 kDa, even in basal expression, is associated with the expected protein, as confirmed in the purified protein lane. Western blotting was performed to validate the expression and purification of protein containing the His tag that was added to chimeric protein (Fig. 3C).

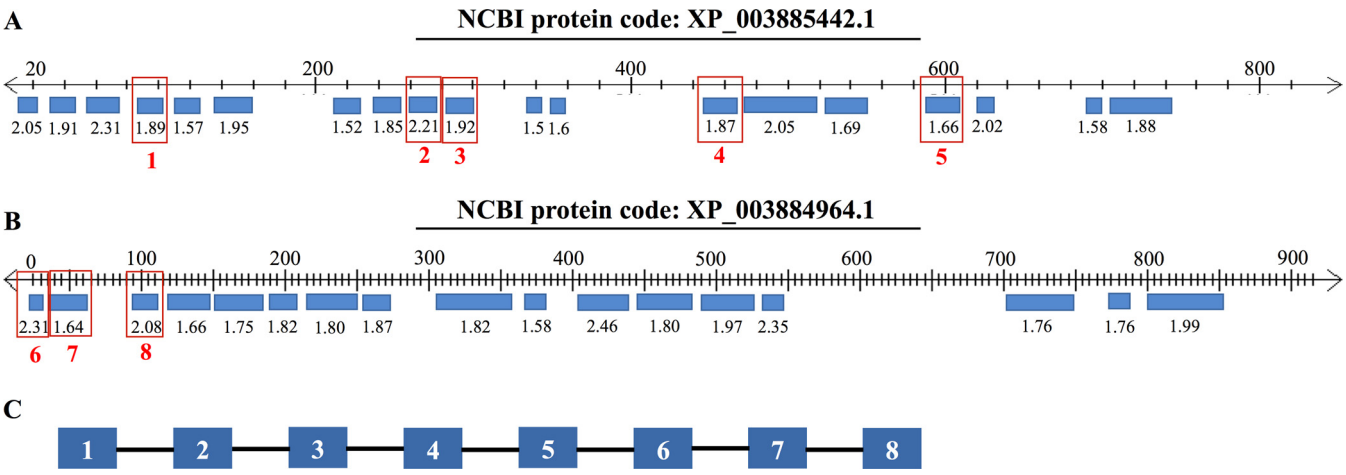

FIG 2 Epitope density of each selected protein. (A and B) Proteins XP_003885442.1 and XP_003884964.1, respectively. Blue boxes represent the predicted epitopes on protein sequence, and the average BepiPred score for each peptide is shown below the blue box. Red outlines indicate selected epitopes, and the red numbers indicate the order of peptides in the chimera protein. (C) Schematic showing the chimera sequence, with the numbered peptides representing each selected epitope from the original sequence. Black lines represent the GSGSGS linkers added between each epitope.

Evaluation of chimeric protein serodiagnostic potential. An ELISA was performed to validate and characterize protein reactivity against field-infected animal sera. The serum antibody form *N. caninum*-infected bovine showed strong reactivity with the chimeric protein (Fig. 4A). Statistical analysis was performed using an optimized cutoff value of 1.089 (Table 3). The chimeric protein showed 96.6% sensitivity compared to healthy animal sera. Meanwhile, a specificity value of 97.0% was calculated based on the relation to false-positive reactivity afforded to bovine pathogens and noninfected sera. Furthermore, 96.5 and 96.9%, respectively, are the probabilities that the disease is present when the test is positive and not present when the test is negative. A kappa index of 0.935 represents an almost perfect agreement within results provided for histopathological and molecular tests compared to those obtained using chimeric protein. A nonparametric one-way ANOVA test with a P value of <0.001 compared to neosporosis and settled groups gave statistical support to the data. The ROC curve showed that the selected threshold yielded high specificity and sensitivity values (Fig. 4B).

DISCUSSION

In summary, the present study applied immunoinformatic tools to screen antigenic proteins in a predicted proteome of the *N. caninum* genome sequence. Based on this screen, we built a chimeric protein to serodiagnose bovine neosporosis by combining the predicted antigenic regions with high potential specificity. For this, the sequences of 21,357 proteins were evaluated to provide eight epitopes as a cast for the synthetic molecule. Our data suggest that an *in vitro*-assembled protein could be easily expressed

TABLE 1 Selected potential epitopes of *N. caninum* proteins to compose the chimeric protein

NCBI protein code	Epitope	Sequence	Length (aa)[a]	BepiPred score
XP_003885442.1	1	YSPPGAPAGQNME	13	1.89
	2	SRDSPPSPAGGAT	13	2.21
	3	NPRRGHPTGEPRG	13	1.92
	4	GRPPRGGYEPHRGRPEPEAM	20	1.87
	5	SLGPSGPSLSSPE	13	1.66
XP_003884964.1	6	MGPPPTAASGA	11	2.31
	7	SSGVPSSANASPSSSATA	18	1.64
	8	PPPGRFTGNPPASPGGRQ	18	2.08

[a]aa, amino acids.

TABLE 2 Similarity of each selected *N. caninum* epitope with others bovine pathogens accessed by BLASTp

Epitope	Similarity (%)									
	Toxoplasma gondii	*Trypanosoma vivax*	*Cryptosporidium parvum*	*Leptospira* spp.	*Mycobacterium bovis*	*Brucella abortus*	*Babesia bovis*	*Anaplasma marginale*	*Sarcocystis* spp.	*Besnoitia besnoiti*
1	53.8	53.8	53.8	61.5	69.2	53.8	53.8	46.1	22.5	58.3
2	76.9	46	61.5	23	46	46	48.4	46	30.7	83.3
3	61.5	46	46	53.8	46	0	53.8	30.7	0	70
4	60	40	35	40	0	40	30	25	20	61.1
5	61.5	61.5	61.5	61.5	53.8	61.5	53.8	53.8	30.3	90
6	100	54.5	63.6	63.6	54.5	63.6	36.3	54.5	36	72.7
7	67	55.5	55.5	50	55.5	44.4	27.7	11	27.5	76.9
8	67	22	38.8	55.5	27.7	22	27.7	0	21.8	58.8

in a bacterial system, and it can highly discriminate *N. caninum*-infected sera from other sera.

Mapping and identification of specific linear B-cell epitopes that bind to antibodies induced by pathogens represents a challenging step in immunological investigation. Epitope mapping is quite useful in vaccine development, antibody production, and the rational design of therapeutic protein and provides clues to improve peptide immunogenicity (21). Experimental B-cell epitope determination is costly and difficult work that allows an accurate investigation of stagger informatic approaches (43). Initially, proteome-wide screening required *N. caninum*-specific and antigenic proteins with epitope regions higher than 10% of total sequence length (Fig. 2). Determination of linear B-cell epitopes represents the first step in mapping antigens against specific pathogens, and it should be based on reliable and simple bioinformatics tools to rating in genomic scale (31). The BepiPred 1.0 web server made a refined search employing a threshold of 1.3, which supports the specificity (96%) of prediction regarding sensitivity, increasing the chance of being a true epitope. The amino acid linear segment may vary from 2 to 9 when the entire sequence is considered, and the epitope could reach more than 17 amino acids when nonepitopes are considered (44). Hence, selected epitopes are sized within 11 to 20 amino acids and have a medium score higher than 1.3 (Table 1). The percentages of identity values indicate an average that is smaller than 70% versus other bovine pathogens, with higher diversity values for epitopes 4 and 8 (Table 2). As an example of the importance of the joined epitope sequence, a study fragmented antigenic elements of the NcGRA7 protein and detected a decrease in antibody recognition (7). Aiming to construct a robust and all-antigenic molecule, the GSGSGS linker combined chosen epitopes to form a chimeric protein.

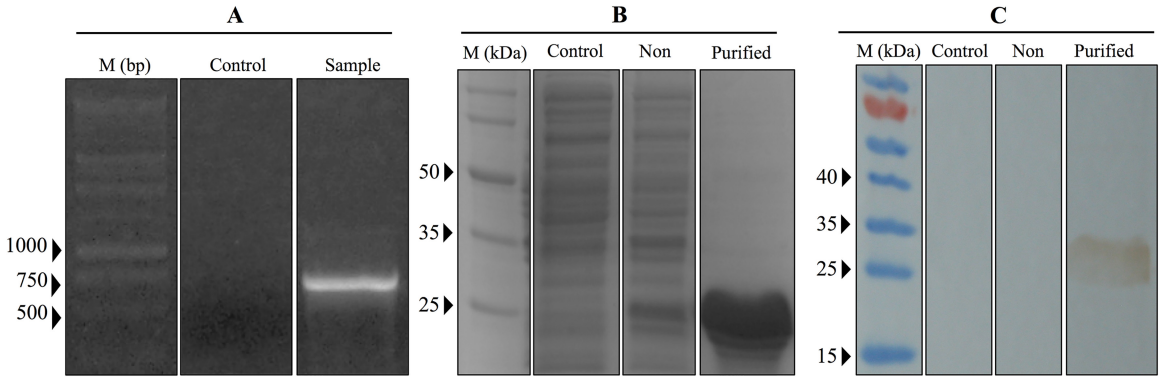

FIG 3 Confirmation of chimera expression in *E. coli*. (A) PCR confirmation of *E. coli* Arctic Express containing the chimeric gene transformation protocol with DNA plasmid. (B) SDS-PAGE protein profile in a wild-type system (Control), a non-IPTG-induced system (Non), and after protein purification (Purified). (C) Western blot analysis with anti-His antibody was performed to evaluate the expression of chimeric protein containing His tag in a wild-type system (Control), a non-IPTG-induced system (Non), and after protein purification (Purified).

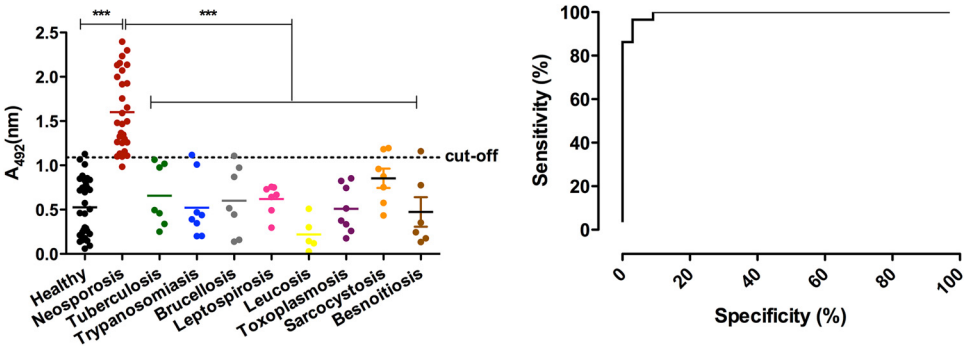

FIG 4 Evaluation of the diagnostic potential of chimeric proteins. (A) ELISA reactivity of healthy samples ($n = 34$, black circles), neosporosis samples ($n = 29$, red circles), tuberculosis samples ($n = 7$, green circles), trypanosomiasis samples ($n = 8$, blue circles), brucellosis samples ($n = 7$, gray circles), leptospirosis samples ($n = 7$, pink circles), leucosis samples ($n = 5$, yellow circles), toxoplasmosis samples ($n = 8$, purple circles), sarcocystosis samples ($n = 7$, orange circles), and besnoitiosis samples ($n = 6$, brown circles) against the chimeric antigen. The neosporosis samples are statistically distinct from the other values with a P of <0.05 as determined in a one-way ANOVA analysis. (B) ROC curve showing the relationship between assay sensitivity and specificity.

Glycine-rich linkers are flexible, preserve individual domain function, afford structural stability, and smooth protein folding (41).

The SDS-PAGE protein pattern ensures that the concentrations of all tested samples were equivalent (Fig. 3B). In addition, should be noted that in a 25 kDa that there was a differential pattern among control and noninduced lanes which indicates a system basal expression. Also, it should be noted in the purified lane that the 25-kDa protein concentration suggests a high protein purification level sufficient for ELISA. In Western blot analyses, an anti-His antibody recognized the purified chimeric protein sample instead of *E. coli* Arctic Express control cells and noninduced lanes (Fig. 3C). In general, these results imply that the bacterial expression system was effective in chimeric protein expression with a preserved sequence considering that the molecule was marked in expected size. Antigen prediction was looking for linear epitopes, so the preserved protein sequence increases the affinity with antibodies anti-*N. caninum* from infected samples.

ELISA is a high-throughput, fast, and cost-effective method that can be used to investigate a multitude of samples in fewer reactions and has been well characterized in serological diagnosis (45). The indirect fluorescent antibody test is a reference technique used to diagnosis neosporosis, although it is expensive, laborious, and less accurate than ELISA (14). An indirect ELISA with chimeric protein as the antigen was performed to measure diagnostic potential in serum samples (Fig. 4A). Multiepitope or chimeric proteins demonstrate better performance in serodiagnosis compared to isolated antigens (46). The results show that the chimeric protein associates strongly with infected animals anti-*N. caninum* IgG antibody. The generated data yielded an ROC curve that presents the relationship between specificity and sensitivity (Fig. 4B). For a diagnostic approach, the chosen cutoff value provided 96.5% sensitivity, picking 28 of

TABLE 3 Statistical analysis of ELISA results

Parameter[a]	Result
% (95% CI)	
Sensitivity	96.5 (±3.3)
Specificity	92.9 (±5.0)
PPV	94.5 (±2.9)
NPV	98.7 (±1.1)
Accuracy	94.3 (±4.1)
Kappa index	0.91 (±0.08)
P	$2.71e^{-14}$

[a]PPV, positive predictive value; NPV, negative predictive value.

29 true-positive sera. On the other hand, the cutoff value differentiated 74 to 80 false-negative results (92.9% specificity) (Table 3). By comparing the sensitivity and specificity parameters to the average diagnostic accuracy, we found that the chimeric protein afforded equivalent or better values than those presented in the literature (5, 7, 14–19). Moreover, the chimeric protein can discriminate infected samples from abortive diseases such as brucellosis, leptospirosis, and trypanosomiasis, which were not tested in previous articles, as well as from phylogenetically related pathogens, including *T. gondii*, *Sarcocystis* spp., and *Besnoitia besnoiti*. ELISA achieved numbers that were all predicted in each epitope immunoinformatic study (Table 2). It should be noted that after combining epitope pieces, the predicted sequence differences were enhanced. For further analysis, testing sera collected from non-South American animals would broaden the genetic diversity of *N. caninum* strains, increasing the diagnostic power of the chimeric protein worldwide.

N. caninum infections are commonly confused with those caused by *T. gondii* in cross-reactions (7). Since 1986, *T. gondii* has been reported as a nonabortive protozoan in cattle because the pathogen is quickly eliminated from animal tissue (47). More recently, the presence of *T. gondii* in aborted fetuses is not confirmed, and parasite identification in naturally infected cattle is rare (48). In a Brazilian case report, the incidence of toxoplasmosis in sheep was 80 times higher than in infected cattle (49). A United Kingdom research group studied the prevalence of *T. gondii* naturally infected cattle and found that only 1.79%, in a group of 305 animals, carried the disease (50). Another study indicates the low tissue presence of viable *T. gondii* parasite, even in experimentally infected animals (51). Therefore, bovine toxoplasmosis is a rare phenomenon in naturally infected cattle, and usually the infection is not associated with abortion. However, Table 2 shows differences within predicted epitopes and *T. gondii* proteins, which implies different intensity reactions with toxoplasmosis sera, as confirmed in other bovine infections. Nevertheless, we performed PCR screening of the samples to confirm that the infection was caused by *N. caninum*, and none of them were amplified to a *T. gondii*-specific primer. In addition, we included rare samples of *T. gondii*-infected cattle that showed no cross-reaction with the antigen. There have been many studies that indicate similarities within infections induced by *N. caninum*, *Sarcocystis* spp., and *Besnoitia besnoiti* (47, 48, 52, 53). Protein sequence similarities were assessed among eight selected epitopes with *Sarcocystis* spp. (taxid 59669) and the *Besnoitia besnoiti* (taxid 94643) proteome utilizing BLASTP tool. Lower identity values seen in every epitope concerning *Sarcocystis* sp. suggests that there is no cross-reaction with this pathogen, which was confirmed by ELISA with sera from animals infected with this pathogen. On the other hand, these epitopes have a high identity compared to the *B. besnoiti* proteome. However, ELISA using a small set of samples ($n = 6$) demonstrated low cross-reactivity with the chimeric antigen. To date, a single study has been intended to evaluate the frequency of anti-*B. besnoiti* antibodies in ruminants throughout South America (49). According to the authors of that study, the low titers detected do not match those already described in Africa and Europe, and more investigations are needed to characterize the disease epidemiology and immunological response to infection in Brazilian territory. It is also worth mentioning that reports observed in the literature indicate that infections caused by this organism lead to biochemical alterations in the blood (50), a drop in milk production (51), and reproductive failures (54), with no reports indicating the involvement of bovine besnoitiosis to abortion.

Statistical analysis was performed to better understand the collected data. The positive and negative predictive values indicate that the detected individual is correctly grouped. Consequently, the accuracy in classification of samples shows a significative value with a low error margin (Table 3). The kappa index suggests an agreement among gold standard techniques, such as molecular and histopathological tests and serological assay. The closer the kappa index is to 1, the higher the test agreement. According to Landis and Koch (55), a value of 0.91 indicates an almost perfect correlation.

To our knowledge, no study has evaluated chimeric protein potential in bovine

neosporosis diagnosis. In the present study, immunoinformatic tools provided valuable information in antigenic molecule functional screening, allowing us to develop a synthetic protein that was effective in definitive disease diagnosis. Moreover, the applied procedure facilitates the construction of powerful molecules to recognize a diversity of diseases.

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