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MICROBIOLOGY

Selection and characterization of peptides mimetic to *Campylobacter fetus* subsp. *venerealis* using phage display

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ABSTRACT: *Bovine genital campylobacteriosis (BGC) is a venereal and subclinical disease that affects the fertility of cattle herds, and it is caused by Campylobacter fetus subsp. venerealis . This study selected peptides mimetic to the BGC-causing agent from a phage library. Phage display is a technique that applies bacteriophage libraries that reveal peptides fused to the viral capsid in biological selections against target proteins. Biopannings were performed for biological selection in the phage library using rabbit hyperimmune serum and C. fetus subsp. venerealis protein extract. Five selected heptapeptides were considered mimetic to Cfv-NCTC 10354 based on the results of bioinformatics analysis and assays with hyperimmune serum and cervicovaginal mucus obtained from heifers. ALASLPL and LSYLFPP were the most reactive peptides and considered promising as possible mimetic immunogens for C. fetus subsp. venerealis.*
Key words: *Campylobacteriosis, phage display, peptides.*

Seleção e caracterização de peptídeos miméticos a *Campylobacter fetus* subsp. *venerealis* usando phage display

RESUMO: *Campilobacteriose Genital Bovina (CGB) é uma doença venérea e subclínica que causa problemas reprodutivos em rebanhos, causada por Campylobacter fetus subsp. venerealis. Este trabalho teve como objetivo selecionar peptídeos miméticos ao agente da CGB de uma biblioteca de fagos. Phage display é uma técnica que aplica bibliotecas de bacteriófagos que expõem peptídeos fusionados ao capsídeo viral em seleções biológicas contra proteínas alvo. Biopannings foram realizados para seleção biológica na biblioteca de fagos por meio de soro hiperimune de coelho e extrato proteico de C. fetus subsp. venerealis. Cinco heptapeptídeos selecionados foram considerados miméticos para Cfv-NCTC 10354 a partir de análises de bioinformática e ensaios com soro hiperimune e muco cérvico-vaginal de novilhas. ALASLPL e LSYLFPP foram os peptídeos mais reativos e considerados promissores como possíveis imunógenos miméticos para C. fetus subsp. venerealis.*
Palavras-chave: *Campilobacteriose, phage display, peptídeos.*

INTRODUCTION

Bovine genital campylobacteriosis (BGC) is a subclinical disease with very subtle signs in large herds, especially in the absence of zootechnical controls. It is a venereal disease that infects the female genital tract. Bulls are asymptomatic animals when they carry the etiological agent of the disease, the bacterium *Campylobacter fetus* subsp. *venerealis*. The foreskin is the habitat for the microorganism, which remains predominantly within the crypts of the epithelium and in the glands of the penis and distal urethra. *C. fetus* subsp. *venerealis* is transmitted to bovines sexually, but it can also be transmitted through contaminated semen or fomites, especially during semen collections. In heifers and cows, *C. fetus* subsp. *venerealis* infects the vagina, cervix, uterus, and oviducts, colonizing and causing inflammation in the genital tract. *C. fetus* subsp. *venerealis* causes inhibition of embryo implantation in the uterus or

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embryonic death, resulting in temporary infertility and repetition of heat at larger and irregular intervals. This subsequently develops into secondary endometritis, cervicitis, salpingitis, and abortion, resulting in economic losses (ALVES et al., 2011; BRITO et al., 2017; HAAS et al., 2019).

BGC is usually diagnosed in bulls because they are few in number in majority of the herds and are the most responsible for spreading the disease. Laboratory techniques that identify the bacterium in preputial lavage and vaginal mucus are isolation, direct immunofluorescence, and polymerase chain reaction (PCR) analysis. Serological tests such as agglutination and enzyme-linked immunosorbent assay (ELISA) have been used for the detection of antibodies against *C. fetus* subsp. *venerealis* in a vaginal mucus sample (HEWSON et al., 1985; HUM et al., 1994; REIS et al., 2018).

Strategies for BGC control include the establishment of artificial insemination programs, segregation of young animals, and vaccination. Vaccines against *C. fetus* subsp. *venerealis* are fundamental in herds in which the introduction of artificial insemination is difficult. Vaccination improves the reproductive efficiency of females with an increase in immunity, but it does not protect against infection after contact with infected bulls (FÓSCOLO et al., 2005).

Phage display technology, which involves the exposure of biomolecules in bacteriophage viruses (phages), has shown increasing use in many areas. With the help of this technology, different synthetic products have been obtained, usually by using a property mimetic to a protein structure of a living organism. This technique consists of the expression of peptides, including particles of antibodies, on the surface of phages, in which their DNA sequence is manipulated to include genes that encode for the exposed protein molecules. Peptide selection has several applications, such as recognizing interactions between proteins, mapping domains, and arrangements, identifying receptors of the cellular surface along with their ligands that may be directly used as prototypes for therapeutic targets. Antigenic and immunogenic mimeticity is the basis for discovering epitopes with applications in vaccine development and diagnostic platforms for diseases (SERGEEVA et al., 2006; FELICIANO et al., 2016; COSTA et al., 2019).

Accordingly, we proposed to identify peptides mimetic to the BGC-causing agent, and the selected peptides may present advantages, such as being simple and cheap to produce. In addition, they can focus on a particular diagnostic specificity, without cross-reactions, and they can be applied as components of synthetic vaccines (SANTOS et al., 2012b; LOPES et al., 2018). In the present study, peptides presented by phages were selected and characterized using phage display technology, immunological assays, and bioinformatics to identify *C. fetus* subsp. *venerealis* mimetopes.

MATERIALS AND METHODS

Bacterial samples

Reference sample of *C. fetus* subsp. *venerealis* NCTC 10354 (National Type Culture Collection, England) was cultivated in brain heart infusion agar (BHI,Difco, USA), supplemented with 5% of equine defibrinated blood in microaerophilic conditions (5% O_2, 5% H_2, 10% CO_2 and 80% N_2) at 37ºC for 48 h. In addition to this standard sample, the P3 sample (*Cfv*-NCTC 10354-P3) was used, isolated, and stored by the Applied Bacteriology Laboratory of the Department of Preventive Veterinary Medicine of the Veterinary School of the Federal University of Minas Gerais following experimental vaginal infection of virgin heifers with the reference sample *Cfv*-NCTC 10354 (COTTORELLO et al., 2009). Pre-inoculums of *Escherichia coli* ER2738 were cultivated in Luria Bertani broth (LB, NaCl 1%, LabSynth, Brazil; tryptone 1%, Oxoid, England; yeast extract 0.5%, BD, France), supplemented with 20 µg/mL of hydroalcoholic tetracycline solution (Sigma-Aldrich, Germany) at 37 ºC for 16 h.

Extraction of bacterial protein using sonication

A suspension of *Cfv*-NCTC 10354-P3 in 10 mL of phosphate-buffered saline solution (PBS – 0.01M NaHPO4, 0.15M NaCl, pH 7.4) and standardized in 10 McFarland standards (3.0×10^9 bacteria/mL) was sonicated via six cycles of 30 s with 1 min rest intervals and a frequency of 70 kHz. Bacterial lysis of 60% was established as optimal and assessed using colored optical microscopy with fuchsin (ALVES et al., 2012).

Production of hyperimmune serum in rabbits

Three New Zealand rabbits of about 2.5 kg were subjected to intramuscular inoculation of 1.0 mL of a suspension of *Cfv*-NCTC 10354, standardized at 10 McFarland standards, containing 50% v/v of Freund incomplete adjuvant (Sigma-Aldrich, Germany). Four inoculations of 0.5 mL, 1.0 mL, 2.0 mL, and 4.0 mL of the same suspension,

without adjuvant, at 1-week intervals, were administered intravenously.

Collection of cervicovaginal mucus

Samples of cervicovaginal mucus were collected from negative BGC heifers and a herd with a history of BGC in Nhumirim Farm, Embrapa Pantanal, Corumbá, Mato Grosso do Sul, Brazil. Feminine internal sanitary tampons (Johnson & Johnson, Brazil) were introduced into the vaginas of heifers with the aid of an applicator coupled to a metal tube. The collected mucus was diluted at a 1:10 ratio with a PBS buffer and Tween 20 at 0.05%, pH 7.4. These samples were previously tested in indirect ELISA (the technique is described below) and 12 negative and 12 positive samples were selected.

Phage library

Phage library M13 (Ph.D.-C7CTM, New England BioLabs, USA) expressing random conformational peptides of seven amino acids at the amino-terminal portion of the pIII protein (1×10^{11} UFC/µL) was employed.

Biopannings

Three cycles of phage selection were performed using the methodology summarized in figure 1. Magnetic microspheres bound with rabbit anti-IgG monoclonal antibodies (Dynabeads® - Invitrogen - USA) were used as a platform for antigen-antibody bindings. The suspension of microspheres (100 µL) was washed with PBS (pH 7.4) three times, added to 200 µL of rabbit hyperimmune serum, and incubated for 30 min at 2–8 ºC. It was washed with 1.0 mL of 0.2 M triethanolamine (Garden Chemistry, Brazil) (pH 8.2) twice, resuspended with 20 mM of dimethyl pimelimidate dihydrochloride (DMP, Sigma-Aldrich, Germany) solution dissolved in triethanolamine, and incubated for 30 min at 25 ºC under rotational agitation. This reaction was stopped with Tris buffer wash (25 mM, pH 7.5) for 15 min, washed with 5% PBS-BSA buffer twice, and incubated for 1 h at 37 ºC. At the end of the process, the microspheres were resuspended in 200 µL of PBS. For each selection cycle, 40 µL of prepared microsphere suspension was used. After magnetic sedimentation, the microspheres were resuspended in 10 µL of the phage library diluted

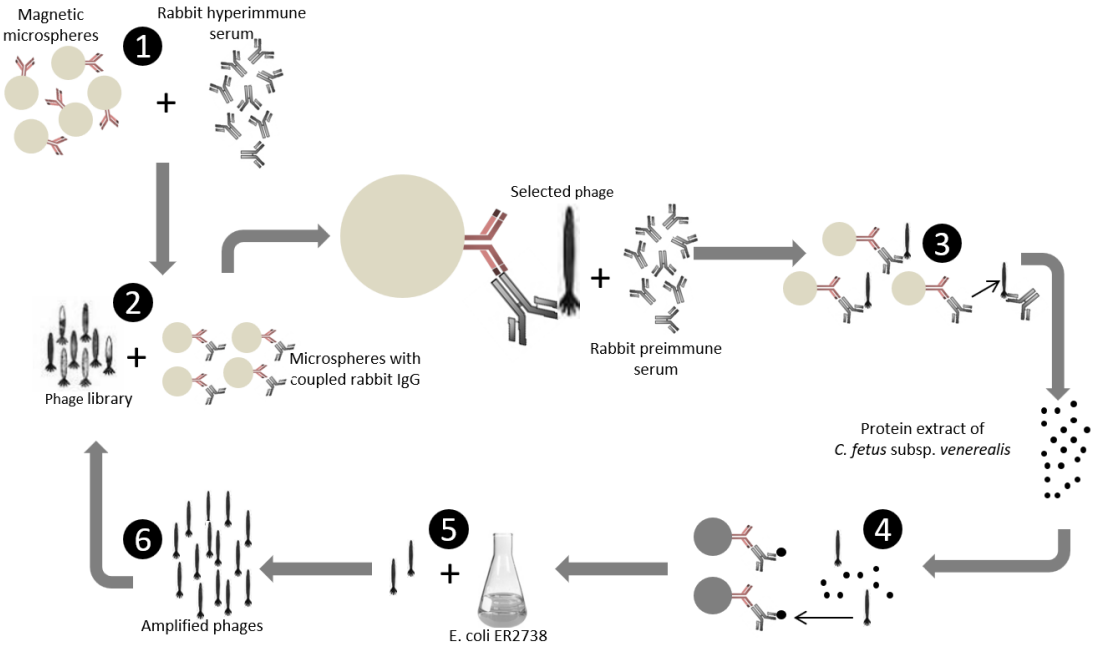

Figure 1 - Summary flowchart of complete biopanning selection cycle in six steps. 1) coupling of rabbit IgG in magnetic microspheres; 2) interaction between phage library and magnetic microspheres; 3) negative dissociation with rabbit pre-immune serum; 4) positive dissociation with bacterial proteins; 5) incubation of the selected phage in *E. coli* inoculum; 6) precipitation of the amplified phages.

in 200 µL of 0.1% TBS-T (50 mM Tris, 50 mM NaCl, 0.1% Tween 20), incubated for 30 min under slow rotational agitation at 25 ºC, and washed 10 times with 500 µL of 0.1% TBS-T. The microspheres were incubated with 200 µL of rabbit preimmune serum diluted at a ratio of 1:4 in 0.1% TBS-T for 1 h at 25 ºC under rotational agitation (negative dissociation step) and then washed 10 times with 500 µL of TBS -T 0.1%. Finally, the microspheres were resuspended in 200 µL of sonicated bacterial protein (0.1 µg/ µL) and incubated at 25 ºC for 1 h under rotational agitation (positive dissociation step). The supernatant obtained after sedimentation of the microspheres (non-amplified eluate) was used in the next stage of amplification, which follows the conventional methodology for biopanning in phage display (SMITH & PETRENKO, 1997). *E. coli* ER2738 pre-inoculum was inoculated in LB medium with 20 µg/ mL tetracycline added and incubated under agitation at 37 ºC until the early-log stage of bacterial growth (optical density, OD600 nm of 0.3). At that point, 150 µL of the non-amplified eluate was added and the incubation was followed for 5 h. Bacteria were pelleted by centrifugation twice (10000 × *g*, 10 min, 4 ºC), the supernatants were added with 1/6 of the volume of polyethylene glycol (20% PEG-NaCl 2.5 M, Promega, USA) and stocked at 4ºC for 12 h. After centrifugation (15 min, 4 ºC, and 10000 × *g*), the phage pellet was resuspended in 1 mL of TBS, centrifuged again, the supernatant was added to one-sixth of the volume of 20% PEG-NaCl and incubated for 1 h on ice. Finally, after centrifugation and resuspension of the pellet in TBS-T 0.5%, the amplified phage eluate was obtained, with which the next selection cycle was started.

Titration, isolation of colonies, and DNA amplification and extraction

Serial dilutions of non-amplified eluates (10^1–10^3) and amplified eluates (10^8–10^{11}) of the three selection cycles were added to 200 µL of *E. coli* ER2738 in the mid-log phase (OD600 nm, 0.5) and incubated for 5 min at 25 ºC. The infected bacterial cell suspension was spread in plates of solid LB medium supplemented with IPTG/X-gal (200 mg/mL, BioBasic, Canada) and tetracycline (20 µg/mL) and incubated at 37 ºC for 16 h. Blue colonies of infected *E. coli* ER2738 were counted to determine the titers of the non-amplified and amplified eluates of each cycle. Colonies by the amplified eluate from the third selection cycle were isolated and individually added to a well of the deep-well plate (Eppendorf, Brazil) containing 1.2 mL of culture medium (LB) with *E. coli* ER2738 (OD600 nm, 0.3), adding up to 96 colonies in total. The deep-well plate was incubated (at 37 ºC and 300 rpm) for 16 h. After 5 h growth, 50 µL of the aliquot was separated from each well and frozen with 50 µL of 50% glycerol at -80 °C to serve as a backup. After the growth, 800 µL of the supernatants were separated after centrifugation (10 min, 4000 × *g*, and 4 ºC), added of 200 µL of 20% PEG-NaCl, incubated for 10 min at 25 ºC, and centrifuged for 40 min, at 4000 × *g* and 20ºC. The phage sediment was resuspended in 100 µL of sodium iodine buffer (Tris-HCl 10 mM pH 8.0, EDTA 1 mM and NaI 4 M). Furthermore, 250 µL of absolute ethanol was added, and after 10 min, it was centrifuged (for 40 min at 4000 × *g* and 20 ºC). A volume of 150 µL of 70% ethanol was added to the resulting sediment and centrifuged (for 15 min at 4000 × *g* and 20 ºC). The pellets were resuspended in 20 µL of deionized water. The quantity and quality of DNA obtained were checked through electrophoresis in 0.8% agarose gel (SANTOS et al., 2012a, 2012b).

DNA Sequencing

Approximately 500 ng of DNA, 5 pmol of primer-96 gIII (5'- CCC TCA TAG TTA GCG TAA CG-3', New England Biolabs, USA) and Premix (DYEnamic ET Dye Terminator Cycle Kit, Amersham Biosciences, UK) were used. A 35-cycle reaction was performed on a plate thermal cycler (MasterCycler, Eppendorf, Brazil) with the following protocol: denaturation (95 °C/20 s); annealing (50 ºC/15 s); and extension (60 ºC/1 min). After DNA precipitation, automated sequencer MegaBace 1000 (Amersham Biosciences, UK) was used for readings according to the recommendations of the software of the equipment (Sequence Analyzer, Caller Base, Cimarron 3:12, Pred 15).

Deduction of peptide amino acid sequences

DNA2PRO12 software (http://relic.bio.anl.gov/dna2pro12.aspx) translated the DNA sequences obtained and the selected peptides were nominated with their heptapeptidic amino acid sequences.

Amplification of selected phages

A 5 µL aliquot of each selected phage back-up was added to the wells of a deep-well plate containing 1.2 mL of a culture medium of *E. coli* ER2738 grown in LB medium (OD600 nm of 0.5). After incubation (at 37 ºC and 300 rpm) for 16 h, the deep-well plate was centrifuged (at 4000 × *g* and 4 ºC) and 800 µL of the supernatant was separated and stocked at 4 ºC. Similarly, the growth of a wild-

type phage sample M13KE (New England BioLabs, USA), which does not express exogenous proteins fused to its own, was amplified (SANTOS et al., 2012a, 2012b).

Phage-ELISA

The microtitrate plates (Maxisorp, NUNC, USA) were sensitized with 100 µg/well of anti-M13 monoclonal antibody (GE Healthcare, USA) diluted in 50 µL in bicarbonate buffer (50 mM, pH 9.6) for 12 h at 4 ºC. Approximately 300 µL/well of blocking buffer (PBS with 5% skimmed milk, Nestlé Brasil, Brazil) was added to the plates and incubated for 1 h at 37 ºC. Three washings were performed with a specific buffer (200 µL of PBS-T at 0.05) and the supernatant from the cultures of the selected and wild-type phages was added for the negative control (50 µL/well, 1 h, and 37 ºC). Five washes were performed, and the plates were incubated with rabbit pre-immune and hyperimmune sera diluted at a 1:100 ratio in blocking buffer (50 µL/well, 1 h, and 37 ºC). Five washes were again performed, and the plates were incubated with anti-rabbit IgG conjugated with peroxidase (Sigma, USA) diluted at a 1:5000 ratio in blocking buffer (50 µL/well, 1 h, and 37 ºC). After five additional washes, ortho-phenylenediamine (OPD, Sigma-Aldrich, Germany) buffer (1 mg/mL) and 3% hydrogen peroxide (H_2O_2) were added. The reaction was stopped with sulfuric acid (H_2SO_4) 1 M. The reactivity was obtained with a plate reader (Titertek Multiskan Plus, Flow Laboratories, USA) by reading at 492 nm. The reactions were performed in duplicates.

Bioinformatics

Analysis of the most frequent amino acid sequences, search for similarity between peptide sequences, and multiple alignments of the most reactive peptides selected were performed using the ClustalW program, which is available online (http://www.ebi.ac.uk/clustalw/index .html). The sequences of the selected peptides were analyzed for homology with sequences of the proteins of *Cfv*-NCTC 10354 (STYNEN et al., 2011) deposited in the GenBank (Tax id 983328) through the BLAST program (Basic Local Alignment Search Tool), which is available on line (http://www.ncbi.nlm.nih.gov/BLAST/).

Synthesis of peptides

The five most reactive peptides in phage-ELISA were selected and synthesized (GenScript, USA). Each peptide contains 14 amino acids: seven from the random sequence selected, one alanine residue, two cysteine residues with a disulfide bond between them, GGGS sequence, as it is in the conformational Ph7 libraries, conjugation with the BSA, and C-terminal amidation. The lyophilized synthetic peptides were diluted in 1 mg/mL concentration according to the manufacturer's recommendation.

Reactivity of synthetic peptides with hyperimmune rabbit serum

Indirect ELISA was performed as described for phage triage; however, sensitizing the plates with 1 µg of each synthetic peptide for a well and 1 µg of bacterial extract. The reactions were performed in duplicates.

Reactivity of cervicovaginal mucus with the synthetic peptides and bacterial extract

Loaded microtitration plate were sensitized with 1 µg/well of synthetic peptides and *Cfv*-NCTC 10354-P3 bacterial extract diluted in 100 µL of bicarbonate buffer. Indirect ELISA was replicated as described for phage triage; however, the volume applied to each well was 100 µL. For the primary reaction, cervicovaginal mucus samples that are positive and negative to BGC (n=12) were used and diluted at a 1:50 ratio in a blocking buffer. For the secondary reaction, an additional step was performed by incubating with bovine anti-IgA produced in mice (Serotec, USA) and diluting at a ratio of 1:500 in a blocking buffer. After the washings, conjugated antibody anti-IgG of mice-peroxidase (Sigma-Aldrich, Germany) , diluted at a ratio of 1:5000 in a blocking buffer, was added.

Statistical analyses

The data were analyzed using the GraphPad software package 5.0 (GraphPad Software Inc., USA). Analysis of variance (ANOVA) and comparison of averages using the Student's *t*-test were employed to determine differences in peptide reactivity. Probability (P) values < 0.05 were regarded as significant and 95% confidence intervals (CI 95%) were provided (SAMPAIO, 2007).

For the ELISAs, a cut-off value was calculated (mean optical density (OD) + 2 × standard deviation) from negative controls (wild phage or BSA) and positive samples (rabbit serum hyperimmune or cervicovaginal mucus BGC+). The ELISA index (EI) was calculated for positive (EI+) and negative (EI-) samples (OD mean/cut-off) and finally the ratio (EI+/EI-) was determined.

RESULTS

From the second selection cycle, biopannings were started with amplified eluate

obtained in the previous cycle, to enrich and refine the specificity of the ligands. The phage input titers in the three selection cycles (1.0×10^{11}, 1.2×10^{10}, and 2.2 × 109 CFU/µL) were higher than the output titers, obtained by titrating the non-amplified eluates (1.5×10^{2}, 6.5×10^{3}, and 2.0×10^{4} UFC/µL) demonstrating the efficiency of the bacterial amplification process. The output titers showed a gradual increase in the number of phages from the first to the third cycle, with the maturation of the affinity of the clones during the selection steps, and demonstrating that the selection of the clones was directed to targets.

Sequencing of the extracted DNA after amplification of blue colonies isolated from the titration of the non-amplified eluate of the third cycle yielded 88 sequences that after translation showed the selection of 12 different heptapeptides from the phage library. These phages were nominated according to the sequence of the seven amino acids and had their frequencies calculated, according to table 1.

The alignment of these 12 heptapeptides by the ClustalW program revealed three repetitions of amino acid sequences: LFxP, LxSP, and APF. These repeated sequences are closely related to the most frequent amino acids in this group of peptides: serine (S) – 17.86%, proline (P) – 17.86%, leucine (L) – 16.67%, phenylalanine (F) – 10.71%, and alanine (A) – 9.52%. All of these values are above expectations, according to the manufacturer's information on the frequency of each amino acid in the phage library: leucine and serine – 9.4%, alanine and proline – 6.2%, and phenylalanine – 3.1%.

For the phage-ELISAs that employed the amplified selected phages, the comparison of absorbance means of each reaction of phage with rabbit hyperimmune and pre-immune sera indicated a significant difference for six phage sequences (Figure 2). In addition, from the positive and negative ELISA indexes, it was possible to list the most reactive ones for the EI+/EI- ratio: PFASFLS (2.34), ALASLPL (2.27), LSYLFPP (1.91), SLSPVNS (1.91), LFSPLSA (1.88), and TLPYTPQ (1.80).

The five most reactive peptides were chosen to be synthesized and used in the following immunological assays and to be evaluated using bioinformatics. All the synthetic peptides showed significantly higher absorbance values in indirect ELISA in reactions with rabbit hyperimmune serum compared to those with pre-immune serum, confirming the high reactivity shown in the phage-ELISA (Figure 3). In addition, they all had EI+/EI- ratios greater than 2.0 (Figure 5).

In the indirect ELISA employing cervicovaginal mucus, only ALASLPL and LSYLPFF peptides showed statistically significant reactivity to IgA antibodies of BGC+ heifers (Figures 4 and 5).

Homology analysis of the amino acid peptide sequences with *C. fetus* subsp. *venerealis* proteins is summarized in figure 6, in which the points of greatest similarities achieved, by at least five

Table 1 - Selected heptapeptides from phage library with *Cfv*-NCTC 10354.

Selected heptapeptides from phage library*	Frequency**
ALASLPL	2.27%
HVPGALF	2.27%
LFSPLSA	6.81%
LSYLFPP	22.72%
PAPFHVR	4.54%
PFASFLS	34.09%
QTLSRPS	1.13%
SLSPVNS	17.04%
SSAHSYF	3.40%
TLPYPTQ	2.27%
TPLLSPF	2.27%
VTTAPFT	1.13%

*Peptides were named with seven letters, each an abbreviation of an amino acid: A - Alanine; F – Phenylalanine; G – Glycine; H – Histidine; L – Leucine; N – Asparagine; P – Proline; Q – Glutamine; R – Arginine; S – Serine; T – Threonine; V –Valine; Y – Tyrosine (LEHNINGER et al., 2014):
**Frequencies were calculated from 88 selected phage clones.

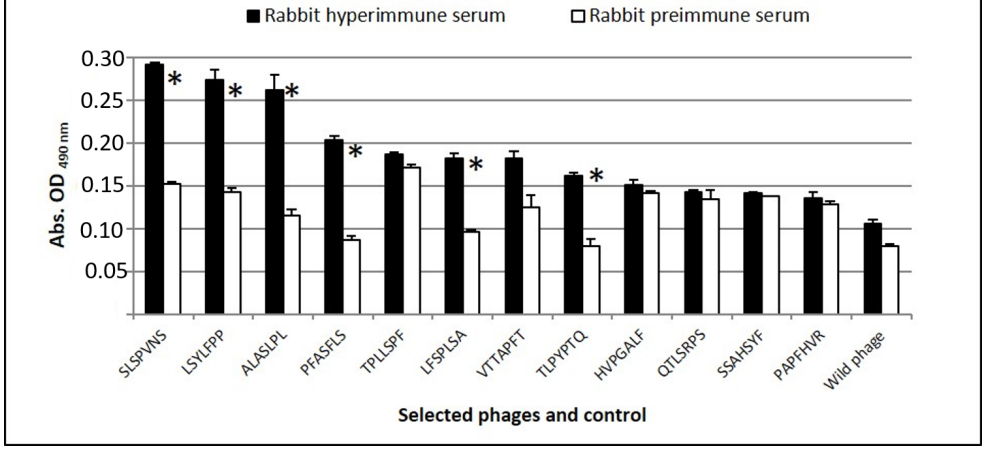

Figure 2 - Phage-ELISA involving 12 selected phages and wild phage M13 with hyperimmune rabbit serum against *C. fetus subsp. venerealis* and preimmune serum. Comparison of the absorbance (OD 490nm) means of the reactions of each phage indicate statistically significant difference (*) based on ANOVA and the Student's *t*-test ($p < 0.05$).

amino acids, are presented. Altogether, eleven *Cfv*-NCTC10354 proteins were identified in the alignments.

DISCUSSION

Phage display techniques make it possible to identify new antigens even without prior information about them. Random sequences of the selected peptides are compared to the amino acid of the natural linker and usually several of them do not show similarity with the natural linker. These peptides that specifically bind to the target receptor-binding site, mimicking the epitope of the natural linker without displaying amino acid sequence similarity,

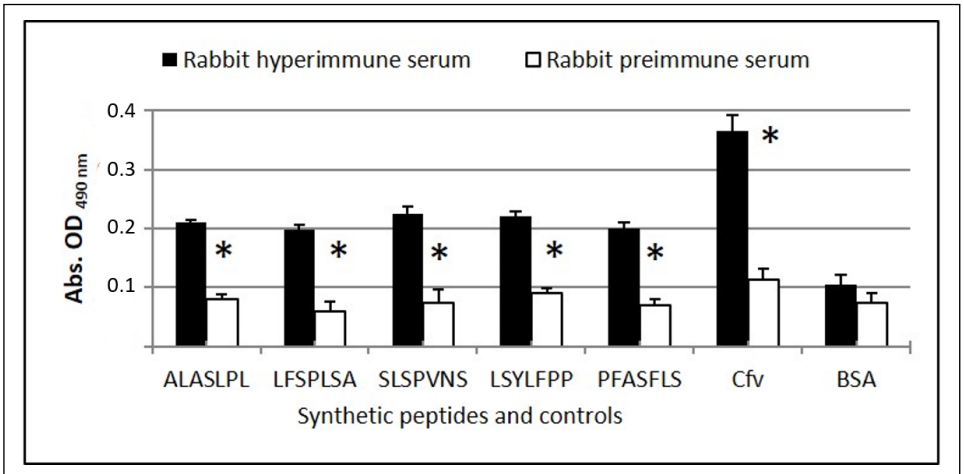

Figure 3 - Indirect ELISA involving five synthetic peptides, bacterial extract of *C. fetus subsp. venerealis* (Cfv) and negative control (BSA) with preimmune and hyperimmune rabbit serum. Comparison of the absorbance (OD 490nm) means of reactions of each peptide indicates statistically significant difference (*) based on ANOVA and Student's *t*-test ($p < 0.05$).

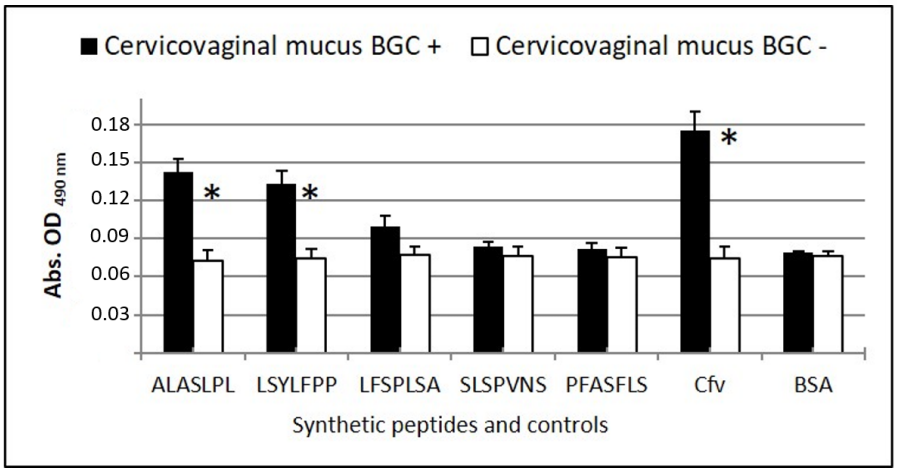

Figure 4 - Indirect ELISA involving synthetic peptides and cervicovaginal mucus positive and negative to BGC. The positive control is represented by *C. fetus subsp. venerealis* bacterial extract (Cfv) and negative control by BSA. Comparison of the absorbance (OD490 nm) means of the reactions of each peptide and controls with each type of mucus sample and within peptides indicates a statistically significant difference (*) based on ANOVA and the Student's *t*-test ($p < 0.05$).

are known as mimetopes (ARAP et al., 1998; ZHAO et al., 2012).

In this study, peptides were identified and considered antigenic mimetics using proteins of *Cfv*-NCTC 10354 from the conformational peptide library of seven amino acids. This library was chosen because it is assumed that the recognition of rabbit IgG in hyperimmune serum by conformational peptides would be better than that by linear peptides. Peptides that incorporate conformational characteristics have

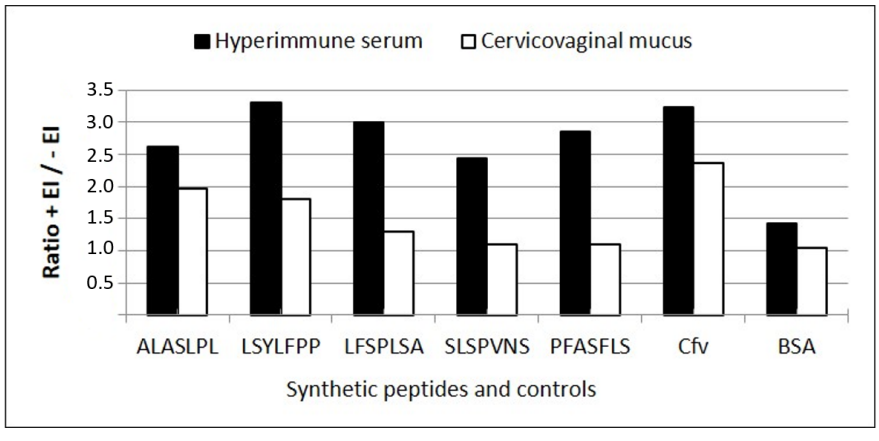

Figure 5 - Ratio between positive (EI +) and negative (EI-) ELISA indexes obtained from indirect ELISA. Synthetic peptides were tested with rabbit hyperimmune serum and cervicovaginal mucus. Control is represented by *C. fetus subsp. venerealis* bacterial extract (Cfv) and negative control by BSA.

important preferences in the development of vaccines and the recognition of ligands (SUNDARAM et al., 2004; SANTOS et al., 2012a). Peptide isolation was performed through repetition of selection cycles by immunological affinity to the biological target, in this case, IgG-positive. It was necessary to use hyperimmune serum, produced against bacterial proteins because BGC is a disease characterized by being restricted to the female genital tract without systemic infection and very low seroconversion (HUM et al., 1994). These features limit the use of field samples in the biopannings. In addition, antibodies raised by hyperimmunization would more likely have affinities for various types of epitopes, especially conformational ones.

Titration of the phages at the beginning and the end of the selection cycles inform the enrichment of the eluates during the selection process. Presumably, phages with higher affinity for positive IgG remained bound and those with lower affinity were removed in the washing or competition processes by negative dissociation. The output titers increased gradually from the first to third cycle, showing enrichment of positive clones and that the selection was directed to the targets.

Based on the selection of the 12 peptides, the highest frequency of serine, proline, leucine, phenylalanine, and alanine observed in the analysis of amino acids indicated that these were the main agents in the peptide-immunoglobulin interaction in the biological selection process. These frequencies were even above the expected frequency, according to information from the manufacturer. These amino acids are also part of the common motifs found when the five main peptides selected in this study are analyzed together. The critical amino acids in peptide sequences provide indications of specificity in the process of the recognition of the ligands, and the presence of common motifs between peptides may be related to a selection favoring the ligand, from the indication that these amino acids may be critical for peptide recognition by IgG. Through the analysis of protein alignment, it was possible to observe that selected peptides contain critical residues with identity and conservation and that could mimic the structure and role of the original epitopes existing in *C. fetus* subsp. *venerealis*. (CORTESE et al., 1995; SANTOS et al., 2012b).

Based on the values reported for absorbance and ELISA Indexes in the phage-ELISA, the five most reactive peptides were selected to be synthesized and to participate in new immunological assays. The results showed that synthetic peptides were recognized by the hyperimmune serum of rabbits and with less reactivity compared to the pre-

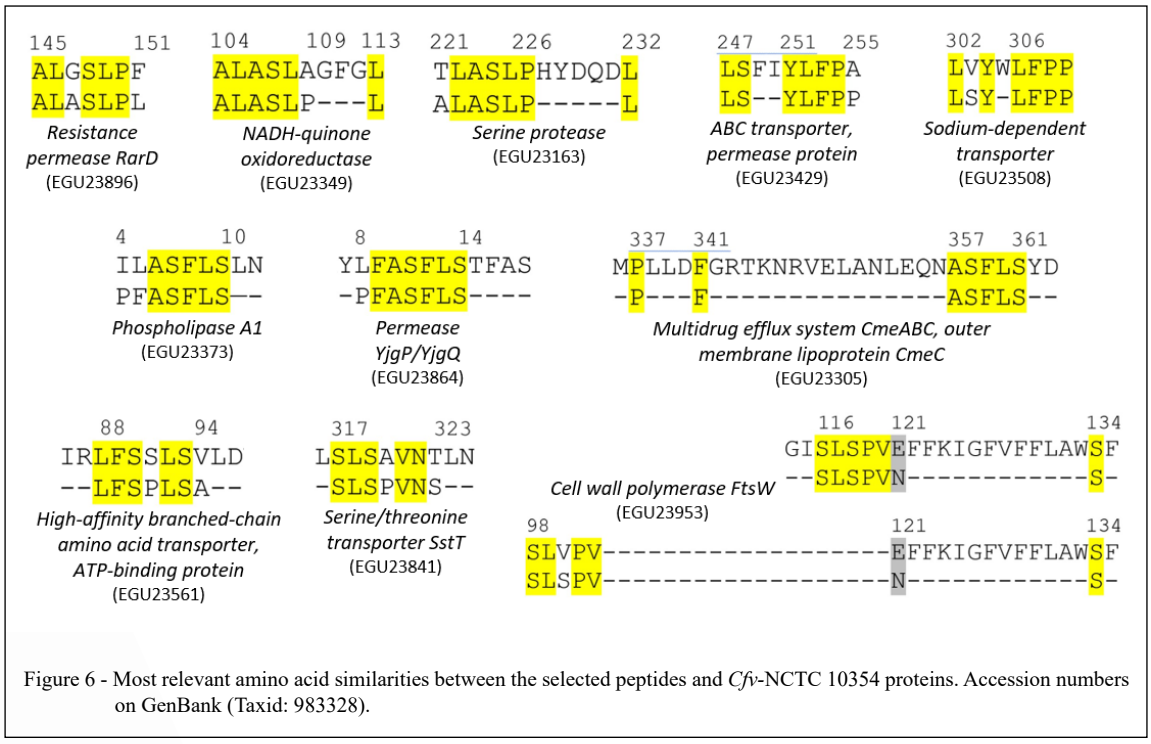

Figure 6 - Most relevant amino acid similarities between the selected peptides and *Cfv*-NCTC 10354 proteins. Accession numbers on GenBank (Taxid: 983328).

immune serum. The rabbit sera used in this assay was the same used in biopanning, from which peptides were selected. It suggested that the clones of the IgG antibodies that reacted strongly with the phages of the C7C library, again found this interaction, compared to the synthetic peptides, which have the same heptapeptide sequences linked to pIII of the phages.

The choice of the sample *Cfv*-NCTC 10354-P3 used in the biopannings is a consequence of studies that showed that there are differences in the expression of virulence factors in assays of cellular adherence, cytokine expression, proteomics analysis, and activation of innate immunity receptors, between reference samples kept in laboratory and samples activated, inoculated, and isolated from the mucus of the genital tract of cows (COTTORELLO et al., 2009). The virulence mechanisms required for the colonization of the host's mucosa are activated after the passage of the bacterial sample into the animal, such as the "S" layer, a capsule that has arrays of protein subunits, called SAP. The "S" layer covers immunogenic structures of the bacteria, including the lipopolysaccharide (LPS), making it inaccessible to the defenses of the host (GARCIA et al., 1995; ALI et al., 2012).

The greatest similarities between the heptapeptide sequences and the *Cfv*-NCTC 10354 proteome (GenBank, Tax id 983328), with the alignment of at least five amino acids, pointed to 11 proteins. Among these, eight have a subcellular transmembrane location. For function, most are involved in the transport of ions, amino acids, and ATP, which justifies transmembrane localization, but there is also an endopeptidase (*Serine protease*). Notably, there is the presence of three proteins related to the constitution of the outer cell membrane: a cell wall polymerase of transmembrane location involved in cell division processes (*Cell wall polymerase FtsW*), in addition to two proteins present in this outer membrane, one of which is a lipoprotein (*Phospholipase A1 and Lipoprotein CmeC*). The P3 sample was isolated after successive passages in the vaginal mucosa of heifers, after which it showed greater expression of virulence factors present in the bacterial outer layer, which contains LPS and lipoproteins, which could justify the greater availability of targets. Interestingly, both proteins showed similarity with the same peptide (PFASFLS), which was precisely the one with the highest frequency of occurrence in the selection (34.09%).

ZHAO et al. (2012) applied a phage display to select peptides from a linear Ph.D-12 linear library using monoclonal antibodies against the main S layer protein, SAPa, from *C. fetus* subsp. *venerealis*. They identified seven peptide sequences that individually showed similarity between up to four amino acids, but together they showed an epitope of 27 amino acids, among which 12 were homologous. There is consensus among many researchers that the use of antibodies as tools for the selection of mimetopes from phage libraries does not reach 100% homology (LI et al., 2008). In our study, we used total *C. fetus* subsp. *venerealis* proteins looking for a broader approach, trying to identify the different origins of immunogenic antigens, especially starting from a bacterial sample recognized for expressing more virulence factors, in addition to the conformational Ph. D, C7C library, aiming to involve a greater variety of epitopes.

In phage display, pre-validation of the most reactive ligands is generally followed by a more careful validation, based on field or patient sera (ARAP et al., 1998). In the case of BGC, a disease whose infection occurs in the genital tract, samples of cervicovaginal mucus would be the main inputs to be applied in immunological tests aimed at inferring the reactivity of the selected peptides. Another option would be to hyperimmunize cows using vaccination since serum samples and genital lavages of naturally infected animals would tend to present limitations in the analytically desirable antigen-antibody reactions (FÓSCOLO et al., 2005). In this study, it was possible to obtain a reasonable amount of cervicovaginal mucus samples from animals from an experimental herd known to be positive for BGC and which showed high reactivity in pre-analytical tests. Although, the diagnosis of BGC in herds is preferentially done in bulls, with agent identification, serological tests such as agglutination and ELISA have been used for the detection of antibodies against *C. fetus* subsp. *venerealis* in vaginal mucus samples (PELLEGRIN et al., 2011).

In indirect ELISAs using field samples, the peptides, ALASLPL and LSYLFPP, were prominent, presenting significant reactivity with IgA of the cervicovaginal mucus in BGC+ heifers. This result, coupled with the reactivities presented in the previous tests and findings of the bioinformatics analysis, makes the two sequences the best *C. fetus* subsp. *venerealis* mimetopes selected in this study.

CONCLUSION

The phage display-selected peptides presented similarities to *Cfv*-NCTC 10354 proteins, thus being recognized as antigens potentially mimetic to the bacteria in immunological assays.

The assessment of the field samples and prediction of mimeticity make the heptapeptides, ALASLPL and LYSLFPP, promising antigens for the development of immunogens against *C. fetus* subsp. *venerealis*.

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BIOETHICS AND BIOSSECURITY COMITTEE APPROVAL

This research project was approved by the Ethics Committee in Animal Experimentation (Comitê de Ética em Experimentação Animal, CETEA, UFMG) under the protocol 274/2010 in 02/23/2011.

DECLARTION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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