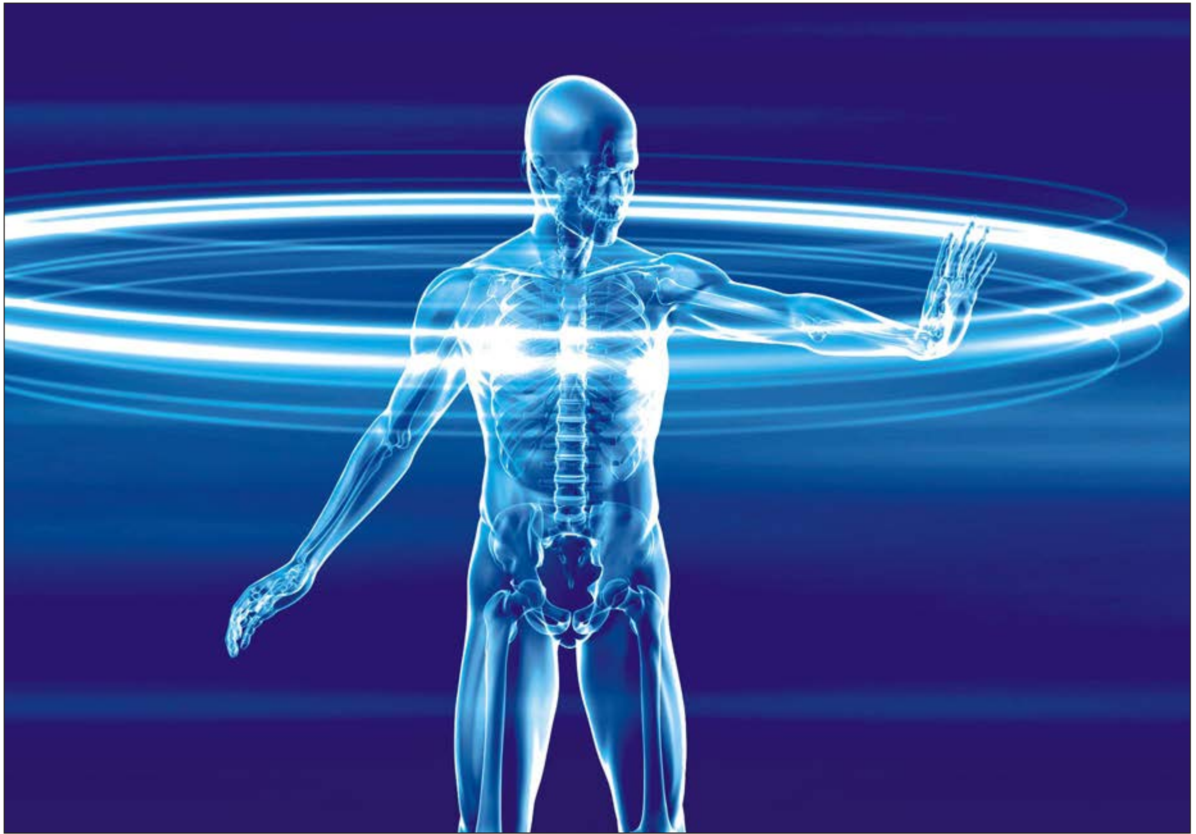

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Use of Phage Displayed Peptides Libraries for Epitope Mapping of Bovine Viral Diarrhea Virus E2 Protein

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Abstract: In this study, it was attempted to map immunogenic epitopes contained within the E2 glycoprotein of Bovine Viral Diarrhea Virus (BVDV) using a random phage display peptide library and serum against a recombinant BVDV carrying the glycoprotein E2 of a close relative virus, Border disease was used. This approach was hypothesized to favor the later selection of BVDV E2 specific mimotopes after positive pannings with serum from a bovine infected with wild type BVDV. Phage clones obtained after three rounds of selection were sequenced and analyzed by informatics tools. Four sequences were chosen to synthesize peptides with different modifications to be utilized in a peptide ELISA test and as immunogens in guinea pigs and bovines. Moreover, groups of mice were immunized directly with phages displaying the selected four mimotopes. Mice and guinea pigs produced antibodies against the immunogenic peptides as seen by the ELISA test, but these antibodies failed to neutralize the virus. However, serum with high neutralizing titer from an infected calf recognized some of the selected peptides in an ELISA test. Present results suggest that the use of a recombinant virus does not help in the selection of mimotopes from a random phage display peptide library and that all selected sequences need to be tested for their immunogenic potential.

Key words: Mimotopes, BVDV, phage display, peptides, immunogen

INTRODUCTION

Bovine Viral Diarrhea Virus (BVDV) belongs to the genus Pestivirus within the Flaviviridae family. Pestiviruses consist of four species: Bovine Viral Diarrhea Virus (BVDV) genotypes 1 and 2, Border Disease Virus (BDV) and Classical Swine Fever Virus (CSFV).

BVDV is a worldwide distributed virus that produces important economic losses to livestock industry (Houe, 2003). Economic losses on cattle industry due to BVDV infection could be prevented through vaccination programs accompanying measures to eliminate immunotolerant animals.

E2, known previously as gp53, is a structural glycoprotein of the BVDV envelope and the major target of the virus neutralizing response (Donis *et al*., 1988; Paton *et al*., 1992). Neutralizing antibodies against E2 can efficiently protect from infection with homologous strain (Bolin *et al*., 1988; Bolin and Ridpath, 1995). However, analysis of antigenic variants

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revealed an extensive variability on E2 among different BVDV strains (Corapi *et al*., 1990), which is considered to cause vaccination failure (Bolin *et al*., 1991).

Epitope mapping of E2 has been mainly accomplished using monoclonal antibodies (mAbs) in competition experiments and studies on escape mutants to neutralization by mAbs, altogether with sequence comparison analysis (Paton *et al*., 1992; Deregt *et al*., 1998). However, differences in epitope recognition between bovine and mouse antibodies have been reported by Zamorano *et al*. (1994). On the other hand, computer programs were developed for predicting B epitopes (Larsen *et al*., 2006); however, they cannot distinguish residues within the epitope that interact with the antibody molecule. As epitope-antibody interaction depends on both molecules, we considered interesting to study B epitopes of the protein E2 with natural host antibodies, using a random peptide phage display library as the mapping strategy. Also, we used a BVDV recombinant virus as a novel approach to favor specific selections from the library. Successful epitope mapping with phage display technology has been reported for several pathogens, including HIV-1 (Ferrer *et al*., 1999), Taenia solium (Gazarian *et al*., 2000), PRRSV (Ostrowski *et al*., 2002), CSFV (Zhang *et al*., 2006), among others. Moreover, the use of phage display libraries to obtain mimotopes-conformational variants that resemble the epitope to which a specific antibody is produced-shows an additional advantage: immunization with phages displaying the peptides of interest is effective, because mimotopes are exposed to the immune system in their native conformation (Delmastro *et al*., 1997).

There are no references on the use of phage display as an approach to find immunogenic epitopes for BVDV. The closest to this strategy was attempted by Kalaycioglu *et al.* (2007), who successfully located two mimotopes of BVDV gpE using an 8-mer-solid phase peptide library and a monoclonal antibody.

Our overall strategy involved a panning procedure that consisted on the subtraction from the library of those phages that display peptides corresponding to BVDV epitopes/mimotopes-except E2-, followed by positive pannings of phages displaying peptides that resemble the E2 protein. For library depletion, antibodies against a recombinant virus BVDV-$E2_{BDV}$ were produced by oronasal and intramuscular inoculation of a negative calf previously treated with dexametasone. Serum was collected when seroneutralization (SN) titer against the homologous virus reached 1:512. On the other hand, positive pannings required affinity purified antibodies against BVDV strain NADL which were obtained by oronasal infection of a negative adult calf. On day 64, the calf was superinfected and bled when the SN titer reached 1:2048. Antibodies were precipitated and concentrated from serum by saturated ammonium sulfate treatment and later affinity purified in accordance to Harlow and Lane (1998). Columns for affinity purification of antibodies were prepared with 3.4 mg of purified BVDV strain NADL produced in MDBK cells (Magar and Lecomte, 1987) and coupled overnight at 4°C to CNBr-activated Sepharose 4B (Amersham Biosciences).

Immunoaffinity selection of phages from the previously depleted library was done using antibodies against the BVDV-$E2_{BDV}$ recombinant virus. Ten micro liters of the phage library (Ph.D.12; New England Biolabs), were incubated for 20 min at room temperature with 10 μL of serum against BVDV-$E2_{BDV}$. After pelleting of the phage-antibody complexes with protein G-agarose, the supernatant was used for positive selections of phages using the affinity purified antibodies as referred in the instruction manual.

Enrichment during pannings was monitored as eluates titers increased from 9.4×10^5 PFU mL^{-1} in the first round, to 1×10^7 PFU mL^{-1} in the third. After the third round of selection, nonamplified eluates were plated and 30 single phage plaques were individually amplified and sequenced. The first approach to study the selected phage clones was to

compare their sequences using bioinformatics tools in order to define putative groups of mimotopes. Selected peptide sequences were aligned using the multiple sequence alignment (msa) algorithm CLUSTALX in conjunction with the Tudos matrix for scoring amino acid substitutions (Tudos *et al*., 1990). The MSA analyses included peptides as many times as they occurred and a low penalty of one was applied for the introduction of gaps between aligned sequences (Davies *et al*., 1999). Based on msa analysis, peptide hidrophobicity/hidrophilicity profiles and aminoacidic residues occurrence within the 12-mer PhD library, four peptides were selected and synthesized with modifications: 1. AHMDNQRRIPGEGGC, 2. KNLMETFDKPMTC, 3. TGSKLTTIYQSRC and 4. TNMPVELINNRKGGC. Cysteine residues were added to C terminal end of all peptides to direct binding to Reacti-Bind maleimide-activated plates (Pierce). A neck of glycines was added to peptides one and four in order to increase their flexibility and peptides one and two were acetylated at the N terminal end. To test immunogenicity, peptides were used in a peptide ELISA format and for immunization purposes (coupled to Keyhole Lympet Hemocyanin (KLH) as carrier protein). Peptide ELISAs were performed following standard protocols (Pierce). Two adult calf and groups of four guinea pigs were inoculated with synthesized peptides coupled to KLH. In parallel, groups of mice were vaccinated with phages displaying the selected peptides (1×10^{12} phages/animal in Incomplete Freund´s Adjuvant).

In this study we evaluated the use of a recombinant virus with a replaced protein as a strategy to favor the selection of immunogenic epitopes in a phage display approach. For that purpose we selected the E2 from BVDV and we used sera raised against two viruses: a wild type BVDV strain NADL and a recombinant version of the same virus containing the homologue E2 protein from a highly similar relative, Border Disease Virus (BVDV-$E2_{BDV}$). We assumed this approach would favor for the later selection of specific BVDV E2 mimotopes from a random phage peptide library. Thus, exposure of the serum with antibodies against the recombinant virus to the phage display library, would allow the capture of phages against BVDV except E2. This approach was expected to clean the library from unwanted epitopes from the whole virus and narrow down the selection of E2 mimotopes through positive pannings with policlonal antibodies from the calf infected with BVDV strain NADL. We later applied bioinformatics tools to select those peptides with the highest scores as a prediction of their in vivo behavior and we rationally designed four immunogenic peptides.

Mice and guinea pigs inoculated with phages displaying the selected peptides or synthetic peptides, respectively, produced antibody responses against all of them, as shown by peptide ELISAs (Fig. 1, 2). Sera from the calves vaccinated several times with synthetic peptides were analyzed by indirect ELISA and peptide ELISA and results were not conclusive due to high background levels. We also performed SN assays to sera from mice, guinea pigs and bovines and found no neutralizing antibodies. However, serum from an experimentally infected calf analyzed by peptide ELISA (Fig. 3), showed antibodies that recognized peptides two and three.

These results raise the question whether these peptides are related to E2, as they are not neutralizing and may not be immunodominant. A limiting factor in this study of mimotopes has been the lack of knowledge about the tertiary structure of this glycoprotein and the use of polyclonal antibodies added complexity to the analyses of sequences. Epitope mapping with mAbs has shown conformational epitopes within E2, making the finding of these mimotopes very difficult. This situation led to rely on bioinformatics tools or previous investigations for the selection and design of immunogenic peptides. Another approach to map mimotopes has been recently described which is based on the use of an insoluble solid

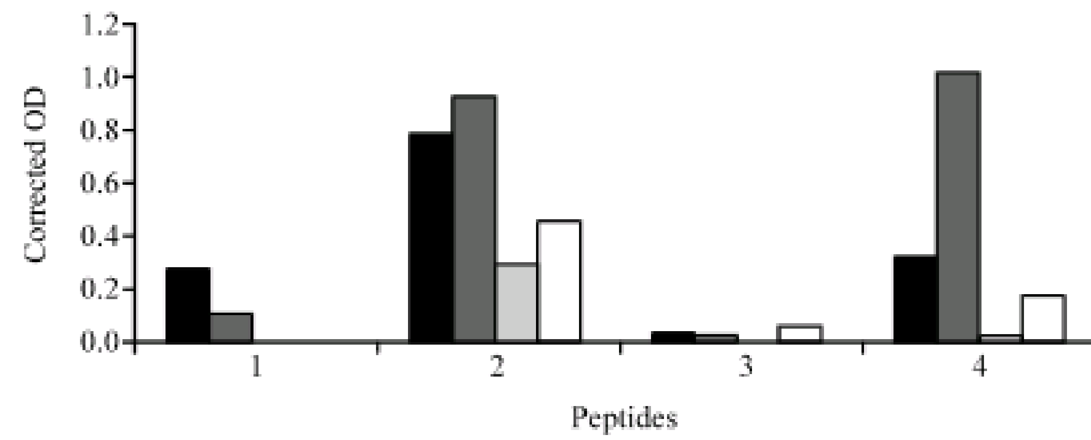

Fig. 1: Peptide ELISA of sera from guinea pigs vaccinated with synthetic peptides coupled to KLH. Maleimide activated plates were coated with synthetic peptides and sera from immunized guinea pigs were analyzed against its corresponding peptide. Each bar represents one individual (4 guinea pigs per peptide). Optical densities (OD) were corrected by subtracting values from the same animals prior to vaccination

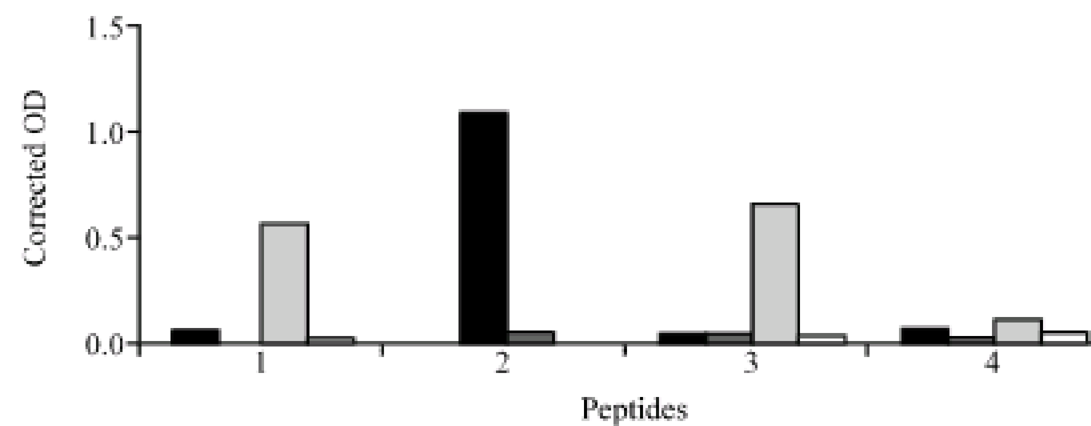

Fig. 2: Peptide ELISA of sera from mice vaccinated with phages displaying peptides. Maleimide activated plates were coated with peptides 1 to 4, separately and sera from mice vaccinated with phages displaying peptides were analyzed. Each bar represents one individual (4 mice per peptide). Optical densities (OD) were corrected by subtracting values from the same animals prior to vaccination

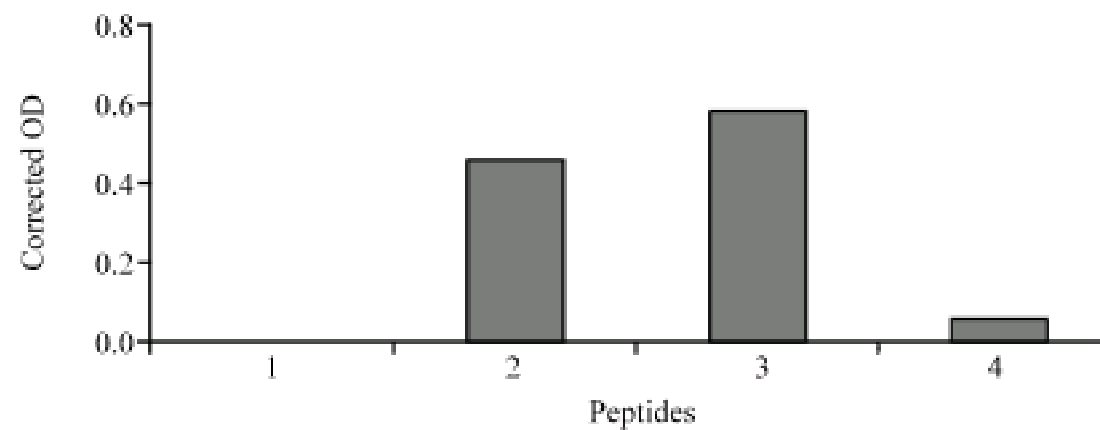

Fig. 3: Peptide ELISA of a serum with high seroneutralization titer (1:2048) from an experimentally infected adult calf with BVDV strain NADL. Maleimide activated plates were coated with synthetic peptides and specific recognition against each peptide is shown by bars. Optical densities (OD) were corrected by subtracting values from the same animals prior to vaccination

phase to detect peptides reactive with a BVDV E2 mAb. Two peptides with very low structural o sequence match with E2 were recognized by the mAb (Kalaycioglu *et al*., 2007). The solid phase approach identified less potential peptides than our phage display approach.

Furthermore, the four peptides the authors identified were all recognized by the mAb in spite of not having good scores with the program used (PROASD).

Looking at the difficulties that bioinformatics tools have predicting the *in vivo* immunogenic potential of in vitro selected mimotopes, we attempted to see in our work whether a different approach could improve these deficiencies. This approach was based on the use of sera produced against viruses differing only on the target immunogenic protein. Selection and enrichment lead to a final group of peptides which, in spite of good scores by computer analysis, did not induced antibodies with the ability to recognize the native viral protein. These results suggest that the alternative approach analyzed in this work does not contribute to a better selection of immunogenic peptides produced through a phage display technique and that probably all peptides selected need to be tested in vivo independently of their scores by computer analysis.

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