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Author Address: Year: 2008
Chapter: Pages: Book Title: Methods in Molecular Biology
Editor: Publisher: Humana Press
City: New Jersey, USA
Keywords: Epitope mapping
Linear B-cell epitope
Biotinylated peptides
ELISA
VapA
Rhodococcus equi.
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URL: http://www.humanapress.com
Linear B-Cell Epitope Mapping Using Libraries of Overlapping Synthetic Peptides based Enzyme-Linked Immunosorbent Assay

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Abbreviated title: B-Cell Epitope Mapping by Peptide-ELISA
Abstract

The aim of this chapter is to provide a strategy for mapping linear antibody epitopes of protein antigens in order to discover candidates for vaccines or diagnostic tests. A set of overlapping peptides was designed and synthesised based upon a known amino acid sequence of the target protein, VapA (virulence-associated protein A) of the bacterium Rhodococcus equi, an important pulmonary pathogen in foals.

The peptides were biotinylated and used in an ELISA to screen immune sera from foals. These biotinylated peptides were coated directly onto micro titre plates which had been pre-coated with NeutrAvidin™. A linear B-cell epitope was identified by a universal recognition of sera to the synthetic peptides which corresponded to a particular fragment of the VapA protein.

Key Words: Epitope mapping; Linear B-cell epitope; Biotinylated peptides; ELISA; VapA; Rhodococcus equi.

1. Introduction

B-cell epitope mapping using a series of overlapping synthetic peptides is a very efficient way to identify a linear antigenic determinant(s) recognised by a particular antibody through an immunoassay. This approach has successfully been used in several studies (1-5) to identify immunogenic epitopes of potential target vaccine proteins.

Mapping epitopes can also be undertaken using X-ray crystallography or by using a combination of computer-aided molecular modelling, ligand binding and spectroscopy to identify the structure of antigen-antibody complexes (6, 7). However, these methods, particularly antigen-antibody crystals are very difficult to make and impractical in the vast majority of cases. Thus, the
use of synthetic peptides of target proteins has become an alternative method in identifying individual epitopes because synthetic peptide fragments can be sufficiently similar to the native antigen, thus allowing the binding of the antibody produced by B cells. This method provides a rapid, practical and cost-effective approach in identifying linear epitopes.

In the following sections, details of procedures for detection of linear B-cell epitopes of the highly immunogenic VapA protein of *R. equi* that we have developed will be described. Importantly, the amino acid sequence of the protein antigen must be known (either via protein sequencing or Genbank database) before epitopes can be mapped using synthetic peptides. Then, overlapping peptides of a defined length and homologous with the VapA protein were synthesised and screened with a population of sera from foals that had been diagnosed with *R. equi* disease, using sera from uninfected foals as a negative control.

2. Materials

2.1 Design and Synthesis of Biotinylated Peptides

*Important: The target protein should be known to be immunogenic and be able to elicit an antibody response in the host*

1. The amino acid sequences of the *R. equi* VapA protein (Genbank accession No. D21236).

2. Biotinylated peptides (Mimotopes, Australia). The peptides are supplied as lyophilised product. Dissolve the peptides in a suitable solvent (following manufacturer’s instructions) at concentrations of 2.86 mg/ml and 28.6 µg/ml and store in small aliquots as stock solutions at -20°C (see Note 1).

3. Solvent: 0.1% (v/v) acetic acid in deionised water. This solution is used to reconstitute the peptides and can be stored at room temperature (see Note 2).
2.2 **ELISA Assay of Biotinylated Peptides**

1. **Foal sera:** Sera obtained from foals that had been diagnosed with *R. equi* disease and sera from healthy foals were used to screen the synthetic peptide bank.

2. **Micro titre plates:** Nunc-Immuno® Maxisorp F96-well micro titre plates (Nalge Nunc International, Denmark; Cat. No. 442404).

3. **NeutrAvidin** biotin-binding protein (Pierce Chemical Company, IL, USA; Cat. No. 31000). Reconstitute the protein in ultra pure water at concentration of 1 mg/ml. Aliquots of this stock solution should be stored frozen at -20ºC.

4. **Phosphate buffered saline (PBS)** (10 mM phosphate and 150 mM NaCl, pH 7.4). Dissolve 8.0 g NaCl; 0.2 g KCl; 1.44 g Na₂HPO₄ anhydrous (or 3.63 g Na₂HPO₄.12H₂O) and 0.24 g KH₂PO₄ in 900 ml of H₂O. Adjust the pH to 7.4 with either 50% (w/v) NaOH or concentrated 37% (w/w) HCl. Adjust the volume of the solution to 1 litre with H₂O and sterilise by autoclaving. Store the sterile PBS at room temperature.

5. **PBS/Tween 20 (PBST):** 0.05% (v/v) Tween 20 in PBS. This solution is used as the washing solution and the diluent of the primary antibody and peptides. It can be stored at room temperature for several weeks. Discard it if there is any sign of contamination.

6. **Blocking buffer:** 1% (w/v) sodium caseinate in PBST. This solution is the diluent for the secondary antibody and is used to block non-specific binding. It should be refrigerated and discarded if there is any sign of contamination.

7. **Secondary antibody:** Goat anti-horse IgG horseradish peroxidase-conjugated antibody (Bethyl Laboratories, TX, USA). Keep the reagent in the refrigerator or as per the manufacture's recommendations.
8. Substrate buffer: Dissolve one Phosphate-Citrate Buffer Tablet (Sigma Co., MO, USA; Cat. No. P4809) in 100 ml of deionised water with stirring to obtain 50 mM phosphate - citrate buffer, pH 5.0. Alternatively, dissolve 1.46 g Na$_2$HPO$_4$ and 1.02 g citric acid in deionised water to a final volume of 200 ml. This solution can be stored in the refrigerator and the amount required should be brought to room temperature before each use. Substrate buffer should be used within two weeks of preparation and checked for signs of contamination immediately before use.

9. Chromogenic substrate solution: Dissolve one tablet (1 mg) of 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma Co., MO, USA; Cat. No. T3405) in 10 ml substrate buffer. Prepare the substrate solution immediately before use. This chromogenic substrate is used to detect horseradish peroxidase conjugated antibodies.

10. Stop solution (0.5 N H$_2$SO$_4$). Add 15 ml of concentrated 35.6 N H$_2$SO$_4$ slowly to a final volume of 1 litre deionised water and with adequate protection from splashes. Store the solution in Pyrex glass bottles at room temperature. Caution! Sulfuric acid is extremely corrosive to skin, metals and clothing. It must always be added slowly to water when making dilutions. Avoid contact with liquid and vapour.

3. Methods

3.1 Design and Synthesis of Biotinylated Peptides

A total of 50 overlapping peptides were designed based upon the 189 amino acid sequence in length of the \textit{R. equi} VapA protein (Genbank accession No. D21236) (8), and were synthesised by Mimotopes, Vic., Australia (www.mimotopes.com). Every peptide was 11 amino acid residues in length, offset by 3 and overlapping by 8 residues (see Note 3). Each peptide was captured with biotin at the N-terminus followed by a tetrapeptide spacer sequence SGSG prior the peptide sequence of
interest, and a free acid (-OH) at the C-terminus. Please contact Mimotopes if you need help in designing peptide length and endings that are more appropriate to your project. The purity and identity of peptides were quantified by the manufacturer using reverse phase high-performance liquid chromatography and confirmed by ion-spray mass spectrometry methods, respectively.

3.2 **ELISA Assay of Biotinylated Peptides**

It is strongly recommended that concentrations of reagents such as peptides, NeutrAvidin™ biotin-binding protein and secondary antibodies be optimised for each ELISA system or when a new batch of the reagent is purchased.

1. Prepare 10 ml of 1:300 dilution of the stock solution (1 mg/ml) of NeutrAvidin™ biotin-binding protein in sterile deionised water per plate to be tested.

2. Coat Nunc-Immuno® Maxisorp F96-well micro titre plates with 100 µl (0.33 µg) per well of the diluted NeutrAvidin™ biotin-binding protein. Incubate plates overnight at 37°C or at 4°C if it is not possible to continue the test next day.

3. Add 200 µl of blocking buffer to each well to block non-binding sites, followed by incubation at room temperature for 1 hr or for 2 hrs at 4°C.

4. Flip out solution in the plates. Wash the plates five times with PBST (400 µl/well) using an automatic ELISA washer (Ultrawash Plus™, Dynex Technologies, VI, USA).

5. Remove any excess buffer by slapping the plates (well side down) against on a clean towel or absorbent paper.

6. Just before use, prepare a working strength (0.286 µg/ml) of biotinylated peptides by making 1:100 dilutions of the stock solution (28.6 µg/ml) in PBST. Then, transfer 100 µl
of each of the diluted peptide solutions into the corresponding NeutrAvidin™-coated wells (see Note 4).

7. Place the plates on a platform shaker (Adelab Scientific, SA, Australia) at a low speed (approximately 125 rpm) and allow the reaction to proceed for 1 hr at room temperature. After incubation, flick out solution and repeat the washing procedure described in steps 4 and 5 (see Note 5).

8. Dilute the horse serum to 1:250 in PBST. However, this dilution may not be ideal if other types of sera are being tested, because the optimum dilution of serum depends on the source and the amount of antibodies present in the sample.

9. Add 100 µl of the diluted serum to each of the wells containing captured peptides. The plates are then incubated overnight at 4°C for better sensitivity. Positive and negative control sera should be included in every assay.

10. Remove the incubation mixture by flicking the plate and repeat the washing procedure as described in steps 4 and 5.

11. Immediately before use, dilute 1:25,000 of the secondary antibody (goat anti-horse IgG horseradish peroxidase-conjugated antibody) in Blocking buffer (see Note 6).

12. Dispense 100 µl of the diluted secondary antibody into each well and incubate at room temperature for 1hr.

13. Repeat steps 4 and 5 plus three additional washes with PBS containing no Tween20 to remove traces of Tween.

14. Detect the presence of peroxidase by adding 100 µl of freshly prepared Chromogenic Substrate solution to each well. Place plates in a dark place for 15 minutes at room temperature to protect from light.
15. Stop reaction by adding 100 µl of 0.5 N H₂SO₄ into each well.

16. Read plates on a MR7000 ELISA plate reader (Dynatech laboratories, USA) using a test wavelength of 450 nm and a reference wavelength of 630 nm. Any positives in this assay would be due to conjugated antibodies binding directly to the peptides (see Note 7).
4. Notes

1. After lyophilisation, peptides retain significant amounts of water. Peptides are oxidised over time at -20ºC and slowly degrade. Thus, the peptide stock solution should be stored in small aliquots upon arrival to prevent degradation caused by repeated freezing and thawing.

2. A common problem with synthetic peptides (particularly peptides with a predominance of hydrophobic residues) is insolubility in aqueous solution. Other solvents recommended by Mimotopes are 30% (v/v) dimethylformamide (DMF), dimethyl sulfoxide (DMSO), or 40% (v/v) acetonitrile in water.

3. Often the terms “offset” and “overlap” are confused. Overlapping residues are those amino acids common to two peptides, while the offset is the distance (in amino acid residues) between the N-terminal ends of the two overlapping peptides. For example, the amino acid sequences TSLNLQKDEPN, NLQKDEPNGRA and KDEPNGASDT overlap by 8 residues and are offset by 3 residues.

4. The peptide stock solution (28.6 µg/ml) can be diluted further down to 1:200, however we found that ELISA sensitivity was reduced. The amount required of each of the diluted peptide depends on how many sera are used in screening. For example, if 20 sera are tested, 40 wells are required (test in duplicate) for each peptide. Therefore, a minimum 4 ml of diluted peptide (0.286 µg/ml) should be prepared.

5. If the plates are not to be used immediately, they should be dried at 37ºC before storing in the dry state at 4ºC. We have tested shelf life stability of peptide coated ELISA plates and found that sensitivity was excellent and, well-to-well and plate-to-plate variation was minimal after 12 months of storage.
6. Note that the diluent of horseradish peroxidase-conjugated antibodies must not contain sodium azide because this would destroy the activity of the peroxidase. In addition, the dilution of the secondary antibody must be determined for each newly purchased batch.

7. A linear B-cell epitope was identified by a universal recognition of sera to the synthetic peptides corresponding to a particular fragment of the target protein. An example of results is shown in Fig. 1. The 51 positive sera screened against the total 50 overlapping peptides recognised four overlapping peptides No. 11, 12, 13 and 14. The amino acid sequences of these peptides are TSLNLQKDEPN, NLQKDEPNGRA, KDEPNGRASDT and PNGRASDTAGQ, respectively. These peptides are equivalent to the N-terminal 20mer-epitope TSLNLQKDEPNGRASDTAGQ (amino acids 62 to 81) of the *R. equi* VapA protein (9) (Fig. 2). The whole/partial region TSLNLQKDEPNGRASDTAGQ is thus identified as a linear B-cell epitope of the antigenic protein.
A total of 50 overlapping peptides was synthesised based upon the amino acid sequences of the antigenic VapA protein of *Rhodococcus equi*, and were screened with 51 positive horse sera. Overlapping peptides No. 11, 12, 13 and 14 were universally recognised by the sera with Peptide No. 12 was the most reactive. These peptides correspond to the region between amino acids 62 to 81 of VapA and this is thus identified as a linear B-cell epitope of the target protein. This figure is reproduced from (2) with the permission of The American Society for Microbiology Inc. who are the publishers.
Fig. 2. Amino acid sequences of the *R. equi* VapA protein (Genbank accession No. D21236) with a linear B-cell epitope is identified as indicated with the underline.

Acknowledgements

The authors would like to thank Stuart Rodda of Mimotopes for his advice and helpful discussion. We are grateful to Glenn Browning from University of Melbourne for kindly providing the sera. We also thank Tuck Weng Kok and staff of Serology Unit of the Institute of Medical and Veterinary Science for the use of equipment and facilities. This work was supported by the Rural Industries Research and Development Corporation (RIRDC) - Horse Programme and Vet Biotechnology Ltd, Adelaide, South Australia.

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