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Identification of antigenic epitopes of the SapA protein of *Campylobacter fetus* using a phage display peptide library

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In this study, we immunized mice with prokaryotically expressed recombinant surface layer protein, SapA, of *Campylobacter fetus*, generated hybridomas secreting mouse monoclonal antibodies (mAb) targeting SapA, and purified the mAb A2D5 from mouse ascites using saturated ammonium sulfate solution. The mAb A2D5, coated onto ELISA plates, was used to screen the phage random 12-peptide library through three rounds of panning. Following panning, 15 phage clones were randomly chosen and tested for reactivity with mAb A2D5 by indirect ELISA. Single-stranded DNA from positive clones was sequenced and compared with the sequence of SapA to predict the key epitope. ELISA and/or Western blot analyses further validated that synthetic peptides and recombinant peptide mimotopes all interact with mAb A2D5. Nine of ten positive phage clones identified by screening were sequenced successfully. Seven clones shared the same sequence HYDRHNYHWWHT; one had the sequence LSKNLPLTALGN; and the final one had the sequence SGMKEPELRSYS. These three sequences shared high homology with SapA J05577 in the region GNEKDFVTKIYSIALGNTSDVDGINYW, in which the underlined amino acids may serve as key residues in the epitope. ELISA and/or Western blot analyses showed that mAb A2D5 not only interacted with the four synthetic peptide mimotopes, but also with 14 prokaryotically expressed recombinant peptide mimotopes. The mimotopes identified in this study will aid future studies into the pathological processes and immune mechanisms of the SapA protein of *C. fetus*.

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1. Introduction

Campylobacter fetus is a gram-negative, microaerophilic bacterium that can cause miscarriage in cattle and sheep and poses a serious threat to human health (Skirrow, 1994). *C. fetus* comprises two subspecies, *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*, highly related at the genomic level (Taylor et al., 1985; Brooks et al., 2004; Kienesberger et al., 2007). Both subspecies can cause abortion in animals (Lander, 1990; Thomas et al., 2003). *C. fetus* subsp. *venerealis* can infect humans causing miscarriage, premature delivery, sepsis and symptoms similar to brucellosis (Rettig, 1979; Rennie et al., 1994).

High-molecular-weight surface layer proteins (SLPs, SapA), which cover the bacterial surface, are the major virulence factors of *C. fetus* and play an important role in miscarriage in cattle and sheep (Dubreuil et al., 1988; Dworkin et al., 1995). Wang et al. (1993) reported that the multiple antigenic epitopes of *C. fetus,* in particular the conserved N termini of SLPs from type A strains, were important for various functions, including prevention of the binding of complement C3b to the cell surface, excretion, self-assembly and formation of the crystal structure. Dubreuil et al. (1990) isolated SLPs from different strains of *C. fetus* and analyzed the N-terminal amino acid sequence, showing that many strains have conserved epitopes such as MLNKTDVSML, KESIDEAGLN and YITIMGMASE.

In previous studies, we expressed two segments of SLPs that are encoded by SapA, rSapA-N (1398 bp) and rSapA-C (1422 bp), in *Escherichia coli* and used rSapA-N recombinant protein to establish an indirect enzyme-linked immunosorbent assay (ELISA) for screening antibodies against *C. fetus* (Zhao et al., 2010). In addition, we immunized mice with rSapA-N recombinant protein and obtained two stable hybridoma cell lines, A2D5 and C1B6, secreting mouse monoclonal antibodies (mAbs) against rSapA-N (Zhao et al., 2009). Based on these results, we screened mimotopes that were recognized by mAb A2D5 in the current study using a phage display peptide library (Parmley and Smith, 1988) and verified the immunogenicity of synthetic peptides and recombinant polypeptides by ELISA and/or Western blot analysis. We also mapped the epitope sequence for mAb A2D5. This study paves the way for future studies on the structure and function of *C. fetus* SLPs.

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2. Materials and methods

2.1. Preparation of mAbs

SapA-N gene amplification, prokaryotic expression vector construction, rSapA-N protein expression and purification, and hybridoma generation were performed as previously described (Zhao et al., 2009, 2010). For generating mAbs, briefly, rSapA-N recombinant protein was used both as the immunogen and the screening antigen. Hybridomas were screened by indirect ELISA. Once established, hybridoma cells were passaged several times in vitro, and cell culture medium was collected for titration. The isotypes of mAbs were determined using the Mouse MonoAb ID Kit (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) according to the manufacturer's instructions. The positive hybridoma cells from screening were expanded, and injected into the abdominal cavity of mice to produce ascites. Finally, immunoglobulin G (IgG) was purified from the ascitic fluid by protein A–agarose affinity chromatography (Horenstein et al., 2003).

2.2. Peptide library screening and biopanning

IgG purified from mouse ascites (100 μg/ml, 150 μl per well) was coated onto 96-well plates (Costar, USA) and incubated at 4 °C overnight. Then, 300 μL of blocking buffer [0.1 M $NaHCO_3$ (pH 8.6), 5 mg/mL BSA, 0.02% NaN_3] was added to each coated well or control well and incubated at room temperature for 1 h. Plates were washed six times with TBST (50 mM Tris–HCl, 150 mM NaCl pH 7.5, 0.1% Tween20). Then, 4×10^{11} plaque forming units (pfu) (Ph.D.-12 Phage Display Peptide Library Kit, New England Biolabs Inc, USA) were diluted in 100 μL of TBST and incubated in antibody-coated wells at room temperature for 1 h with gentle shaking, followed by 10 washes with TBST. Phage binding was disrupted nonspecifically using 100 μL of glycine–HCl [0.2 M (pH 2.2), 1.0 mg/ml BSA] with gentle shaking for more than 10 min. Eluates were transferred to a microcentrifuge tube (Axygen Scientific Inc., USA) and neutralized with 15 μL of 1 M Tris–HCl (pH 9.1). The phage titer was determined using 1 μL of the eluate. The remainder of the eluate was added to 20 ml of *E. coli* ER2738 cells ($OD_{600} \sim 0.5$) provided with the Ph.D.-12 Phage Display Peptide Library Kit, for phage propagation with shaking at 37 °C for 4.5–5 h. Phage were recovered from the culture supernatant using PEG/NaCl (20%, w/v polyethylene glycol-8000, 2.5 M NaCl) precipitation. The phage titer after amplification was determined using LB/IPTG/X-gal (50 μg/ml IPTG, 40 μg/ml X-gal) plates. Then, 2×10^{11} plaque forming units of amplified phage were used in the next round of panning. Panning and amplification were repeated twice as described above. For each round of panning, the amount of phage added (Input) and the amount of phage in the eluate (Output) were recorded to calculate the yield (Yield = Output/Input × 100%). The yields of three rounds of panning were compared to analyze the phage-enrichment efficiency.

2.3. Indirect ELISA

Each amplified single colony phage culture was diluted to 10^{10} pfu/mL and added (100 μL per well) to six wells of 96-well ELISA plates (Costar). After overnight incubation at 4 °C, samples were first blocked using blocking buffer at 37 °C for 1 h, then incubated with mAb60CobaltA2D5 (dilution, 1:1000; all antibodies were diluted with PBST containing 0.05% Tween 20) at 37 °C for 1 h, and finally incubated with HRP-conjugated goat anti-mouse secondary antibody (dilution, 1:5000; Sigma–Aldrich, USA) at 37 °C for 1 h. Color development using tetramethylbenzidine (TMB) substrate (Watson Biotechnologies, Inc., China) was terminated 10 min later by the addition of 2 M H_2SO_4. The optical density at 450 nm ($OD_{450\ nm}$) was measured by the microplate reader EL×800 (BioTek Instruments, USA) using BSA as a negative control .Statistical analysis of the data was executed by SAS software (SAS 8.1, SAS Institute Inc.).

2.4. Sequence analysis of phage single-stranded DNA

Phage single-stranded DNA (ssDNA) was extracted according to the manufacturer's instructions that accompanied the Ph.D.-12 Phage Display Peptide Library Kit (New England Biolabs). Sequencing was performed using the -96 gIII sequencing primer (5′-CCCTCATAGTTAGCGTAACG-3′) on an automated sequencer (Invitrogen, USA). Potential epitope sequences were predicted from similarity of the DNA sequence and the corresponding amino acid sequence from positively-selected phages with the sequences from SapA (DNASTAR.Lasergene.v7.1, DNASTAR Inc., USA).

2.5. ELISA analysis of synthetic peptides

Four peptides (Pep1: HYDRHNYHWWHT, Pep2: HYDRHNYH, Pep3: DRHNYHWW and Pep4: HNYHWWHT) were identified in the sequence analysis screening at high frequency, and were synthesized with a purity of 98% (Invitrogen). Synthetic peptides were dissolved in sterile deionized water, diluted in carbonate buffer (pH 9.6) to 10 μg/ml, added to six wells of 96-well plates (50 μL per well), incubated at room temperature overnight, examined by ELISA as described in Section 2.3, SP2/0 cell culture supernatant was used as a negative control.

2.6. Cloning of mimotopes

The mimotopes (Pep1, Pep5–17) identified in this study were amplified by PCR. Briefly, PCR was performed in a 10 μL reaction mixture containing 4 pmol/μL of primers (Table 1) on a thermocycler (Takara, China). *Bam*HI (gatcc) and *Eco*RI (aattc) restriction sites were added to the 5 and 3 ends of the product, respectively by PCR amplification. The PCR products were cloned into the pET32 vector (Merck–Novagen, Germany) as a *Bam*HI/*Eco*RI fragments, amplified using BL21 competent cells and confirmed by sequencing.

2.7. Prokaryotic expression and recombinant protein purification

In order to locate key amino acids which play a major role in antigen–antibody interaction, prokaryotic expression of recombinant proteins (rPep1, rPep5–17), purification and SDS–PAGE analysis were performed as previously described (Zhao et al., 2010).

2.8. Analysis of purified recombinant proteins

2.8.1. ELISA of purified proteins

The concentration of the recombinant protein preparations was determined using a spectrophotometer (Eppendorf China Limited, China). Proteins (5 μg/mL, 50 μL per well) were coated onto four wells of ELISA plates overnight. The primary mAb A2D5 was used at a dilution of 1:80,000 and HRP-conjugated goat anti-mouse secondary antibody (Sigma–Aldrich) was used at a dilution of 1:10,000. ELISAS performed as above (2.3) or something similar.

2.8.2. Western blot analysis of purified proteins

Recombinant proteins (5 μg) were analyzed by 12% SDS–PAGE and Western blotting as previously described (Sambrook and Russell, 2001). Briefly, proteins were electrotransferred (Semi-Dry Transfer Cell, Bio-Rad, USA) onto nitrocellulose membranes (pore size: 0.45 μm) for Western blotting. PBS buffer (pH 7.4) containing

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