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# Identification of a conformational neutralizing epitope on the VP1 protein of type A foot-and-mouth disease virus

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ABSTRACT

Foot-and-mouth disease (FMD) caused by foot-and-mouth disease virus (FMDV), is a highly contagious infectious disease that affects domestic and wild cloven-hoofed animals worldwide. In recent years, outbreaks of serotype A FMD have occurred in many countries. High-affinity neutralizing antibodies against a conserved epitope could provide protective immunity against diverse subtypes of FMDV serotype A and protect against future pandemics. In this study, we generated a serotype A FMDV-specific potent neutralizing monoclonal antibody (MAb), 6C9, which recognizes a conformation-dependent epitope. MAb 6C9 potently neutralized FMDV A/XJBC/CHA/2010 with a 50% neutralization titer ($NT_{50}$) of 4096. Screening of a phage-displayed random 12-mer peptide library revealed that MAb 6C9 bound to phages displaying the consensus motif YxxPxGDLG, which is highly homologous to the $^{135}$YxxPxxxxxGDLG$^{147}$ motif found in the serotype A FMDV virus-encoded structural protein VP1. To further verify the authentic epitope recognized by MAb 6C9, two FMDV A/XJBC/CHA/2010 mutant viruses, P138A and G144A, were generated using a reverse genetic system. Subsequent micro-neutralization assays and double-antibody sandwich (DAS) ELISA analyses revealed that the Pro$^{138}$ and Gly$^{144}$ residues of the conformational epitope that are recognized by 6C9 are important for MAb 6C9 binding. Importantly, the epitope $^{135}$YxxPxxxxxGDLG$^{147}$ was highly conserved among different topotypes of serotype A FMDV strains in a sequence alignment analysis. Thus, the results of this study could have potential applications in the development of novel epitope-based vaccines and suitable a MAb-based diagnostic method for the detection of serotype A FMDV and the quantitation of antibodies against this serotype.

## 1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious and economically devastating viral disease of cloven-hoofed animals. FMD has one of the higher levels of infectivity among all animal diseases and causes adverse socioeconomic effects in affected countries. The disease is caused by the foot-and-mouth disease virus (FMDV), which belongs to the genus *Aphthovirus* within the *Picornaviridae* family (Grubman and Baxt, 2004). The virus consists of a non-enveloped icosahedral capsid with a single-stranded positive-sense RNA genome of approximately 8.5 kb. The capsid is composed of 60 copies of four virus-encoded structural proteins, VP4, VP2, VP3 and VP1 (Wang et al., 2011).

FMDV exists as seven distinct serotypes (O, A, C, Asia1, SAT1, SAT2 and SAT3) as well as multiple subtypes that have been generated during the evolution of the virus (Bachrach, 1968). Type A FMDV is considered highly antigenically divergent, which causes these divergent subtypes to often have no cross-protection between them, and therefore FMDV type A infections is one of the most prevalent in the world. In China, the serotype AFMDV strain A/HuBWH/CHA/2009 was first reported in Wuhan in 2009 and was subsequently found in nine other areas of the Chinese mainland (Zheng et al., 2013). Furthermore, two years after outbreaks of the new serotype A strain A/GDMM/CHA/2013 was first reported in Maoming, Guangdong province in 2013. This new strain was subsequently identified in the inland China provinces, the Russian Federation, Kazakhstan, Mongolia, South Korea and Taiwan. These outbreaks led to severe economic losses, and these strains are still widely prevalent in the geographic regions listed above (Zheng et al., 2015).

Due to the error-prone nature of RNA-dependent RNA polymerase, FMDV exhibits a high potential for genetic and antigenic variation, and

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multiple genotypes exist within each serotype (Domingo et al., 2003; Sobrino et al., 2001). Antigenic diversity is one of the major obstacles to preventing FMD. In the case of serotype A FMDV, several studies have been performed to identify antigenic sites present on capsid proteins (Ludi et al., 2014; Saiz et al., 1991). Antigenic sites have been reported for three exposed capsid proteins of serotype A FMDV strains. The βG-βH loop and the carboxy terminus of the viral capsid protein VP1 contribute to neutralizing antigenic site 1, with critical residues at positions 144, 148, 154 and 208. Amino acid residues of VP2 at positions 70–73, 75, 77 and 131 contribute to antigenic site 2. Amino acid residues at positions 58 and 60 of VP3 have been reported to be critical for antigenic site 4, whereas site 5 is characterized by a VP1 amino acid at position 149 and is likely formed by an interaction between the VP1 G-H loop region and other amino acids of the capsid (Mahapatra et al., 2011).

It is generally accepted by FMD researchers thatVP1 is the most accessible protein to the host immune system as it contains a binding site (RGD motif) that attaches to the integrin receptor one pithelial cells and is located on the highly mobile G-H loop of the antigenic site 1. The prominent G-H loop of the VP1 capsid protein that spans residues 134–158 has been identified as a major immunogenic site for eliciting neutralizing antibodies (Bittle et al., 1982; Verdaguer et al., 1995). Guinea pigs and cattle immunized with peptides corresponding to parts of VP1 (amino acids 141–160 or 141–158 and 200–213) acquired neutralizing antibodies and protection against FMDV (Bittle et al., 1982; DiMarchi et al., 1986; Pfaff et al., 1982). Most antibodies that demonstrate neutralization of cell binding or infection are directed against conformational epitope (Mateu, 1995). However, generation of antibodies recognizing conformational epitope is a challenging task, as such epitopes are difficult to mimic, and protein subunit vaccines are difficult to generate cross-responses. Thus, the identification of a highly conserved conformational neutralizing epitope would be a significant milestone in the development of structure-based design of novel vaccine and diagnosis against serotype A FMDV.

In contrast to linear epitopes, conformational or discontinuous epitopes are formed when remote amino acids in the primary sequence of a protein are brought into close proximity in the folded protein (Gustafsson et al., 2009). Thus, discontinuous epitopes are relatively difficult to map using synthetic peptides or the selection of escape mutants (Wang et al., 2011). However, phage-displayed random peptide libraries have proven to be a powerful technique to study protein-protein interactions, to isolate peptide mimics for a variety of biological molecules and to map antibody binding sites or epitopes (Zhang et al., 2006).For the identification of antigenic epitopes, random peptide libraries displayed on the phage surface can be screened by affinity selection or by panning using immobilized antibody molecules (Cui et al., 2003).

In this study, we describe the generation and characterization of a potent neutralizing monoclonal antibody (MAb), named 6C9, against the type A FMDV strain A/XJBC/CHA/2010. Importantly, using a combined approach of phage display and site-directed mutagenesis based on a reverse genetic system, we precisely mapped the conformational epitope recognized by MAb 6C9 on the structural proteinVP1 of serotype A FMDV. Since this conformational epitope is highly conserved among type A FMDV strains, these findings may aid in the design of structure-based novel epitope vaccines and detection techniques for serotype A FMDV antigens and their antibodies.

## 2. Materials and methods

### 2.1. Ethics statement

Mice were acclimatized for one week prior to the beginning of the experiment, were bred in clean and spacious animal facilities and were handled humanely according to the rules described by the Animal Ethics Procedures and Guidelines of the People's Republic of China and the Animal Ethics Committee of HVRI, the Chinese Academy of Agricultural Sciences [SYXK (Hei) 2012-2067]. The mice were euthanized by exsanguination under deep anesthesia (intramuscular injection of chlorpromazine at 2–6 mg/kg) at the end of the experiment.

### 2.2. Viruses and cells

Myeloma cell line Sp2/0 and baby hamster kidney 21 (BHK-21) cells were maintained in Dulbecco's Modified Eagle's Medium (Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum (Gibco-BRL, USA). Serotype A FMDV A/JLYS/CHA/2014 (Sea-97/G2 genotype of the Asia topotype) and A/XJBC/CHA/2010 (Sea-97/G1 genotype of the Asia topotype) were prepared from infected BHK-21 cells; these strains share high amino acid identity (99%) with A/GDMM/CHA/2013 (GenBank accession number: KF450794) (Zheng et al., 2015)and A/HuBWH/CHA/2009 (GenBank accession number: JF792355) (Zheng et al., 2013)for the VP1 protein. The FMDV strains A/JLYS/CHA/2014, A/XJBC/CHA/2010, A FMDV A/KT/58 (AJ131665), Asia1 FMDV Asia1/YS/CHA/05 (GU931682), O FMDV O/Tibet/CHA/99 (AJ539138), O/YS/CHA/05 (HM008917), O/GD/86 (AJ131468) and Bovine enterovirus (BEV) strain BHM26 used in this study have been described previously (Chang et al., 2013; Liang et al., 2016). The FMDV serotype-independent MAb 5D12 against NSP 3B and a FMDV serotype-independent MAb 4B2 against the FMDV structural protein VP2 were previously described by our laboratory (Li et al., 2016; Yu et al., 2011).

### 2.3. Preparation of monoclonal antibodies

The FMDV strain A/XJBC/CHA/2010 was prepared from infected BHK-21 cells and purified using a 5–30% sucrose density gradient after inactivation of the virus with bromo-ethyleneimine (Brown and Cartwright, 1963). Six-week-old female BALB/c mice were injected subcutaneously with 100 μg of the inactivated purified FMDV antigen and an equal volume of adjuvant (ISA206, SEP-PIC, France) three times at 2-week intervals. Hybridomas were generated as published previously. The selected cell clones were cultured in the peritoneal cavities of pristane-primed BALB/c mice to obtain ascitic fluid. The MAbs were purified using the NAb™ Protein G Spin Purification Kit (PIERCE), and their subtypes were identified using the SBA Clonotyping™ System/HRP (Southern Biotechnology Associates Inc., USA).

### 2.4. Indirect immunofluorescence assay

BHK-21 cells in 96-well plates were infected with FMDV strains (A/JLYS/CHA/2014, A/XJBC/CHA/2010, A/KT/58, Asia1/YS/CHA/05, O/Tibet/CHA/99, O/YS/CHA/05 and O/GD/86) at a multiplicity of infection (MOI) of 0.1 for 8 h before being rinsed with PBS and fixed with ice-cold anhydrous ethanol for 15 min at 4 °C. After air drying, 50 μL of hybridoma supernatant was added and incubated for 1 h at 37 °C. After washing with PBS, 50 μL of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma) at a dilution of 1:200 was added and incubated for 1 h at 37 °C. The plates were washed three times with PBS and were observed under an Olympus microscope connected to a Leica DFC 490 digital color camera.

### 2.5. Biopanning

The M13 phage display library displaying 12-mer random peptides (Ph.D-12 Phage Display Peptide Library Kit, New England Biolabs) was used. Affinity selection of the phage clones from the random peptide library was conducted following the manufacturer's recommendations with minor modifications. In the first round of biopanning, a 96-well plate was coated with 10 μg of MAb 6C9 in 0.1 M $NaHCO_3$ buffer (pH 9.6) at 4 °C for 12 h. The coated wells were washed off Tris-buffered saline (50 mM Tris-Cl [pH 7.5] and 150 mM NaCl) containing 0.1% v/v Tween 20 (TBST) followed by blocking with 1 mg/mL bovine