



# Generation and characterization of a monoclonal antibody against pseudorabies virus glycoprotein C

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## Abstract

Pseudorabies Virus (PRV) glycoprotein C (gC) expressed by baculovirus expression system was used to immunize BALB/c mice. Anti-PRV gC monoclonal antibody (mAb) 1F2 was achieved and presented with different test procedures. Indirect Enzyme-Linked Immune absorbent Assay (ELISA) and western blot (WB) results showed that mAb 1F2 had good reactivity and specificity to PRV gC protein. IFA results indicated it could react with PRV vaccine, classical and current epidemic strain. The mAb 1F2 can also be applied to IPMA and IHC to recognize the antigen, which suggested it is a useful reagent to study the biological properties of PRV gC protein.

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## Introduction

Pseudorabies Virus (PRV), also named Aujeszky's disease virus or Suid herpesvirus type 1, belongs to the genus *Varicellovirus* and family *Herpesviridae*, which causes pseudorabies (PR) in livestock and wild animals especially pigs [1]. PRV was prevalent in US and some Europe in 1980s and has been eradicated from above countries through gene-deleted modified-live vaccines such as Bartha-K61, which was also widely used in China. Nevertheless, PR outbreaks in China on Bartha-K61 vaccinated pig herds in late 2011, and characterized by nervous system disorders, respiratory disease, reproductive failure, fever, itching and even high mortality [2]. Since then, PRV has nationally spread with many prevalent PRV variants and caused huge losses to the pig industry. So far, the commercial PRV vaccines in China are mainly based on foreign virus strain such as Bartha-K61 and Bucharest strains, the experimental vaccines based on local PRV variants are under development [3].

Three major glycol proteins (including gB, gC and gD) of PRV play a key role in inducing protective immune responses against PRV infection, and are considered as the targets of the host immune system [4-8]. In addition, some researchers found that glycoprotein gC (i.e. glycoprotein III or g III) of PRV and other herpesvirus (for instance, herpes simplex virus) could induce cell-mediated immunity [9-11].

In order to study the biological functions of glycoprotein C (gC), we firstly prepared a mouse monoclonal antibody (mAb) against PRV gC that effectively reacted with different PRV strains (including vaccine strain, classical virulent strain and the current emerging variant strain), as described in detail in the article.

## Materials and methods

### Cells and viruses

SP2/0 myelomas were grown in modified RPMI-1640 medium (HyClone) supplemented with 17% fetal bovine serum (FBS, Hyclone) with 5% CO<sub>2</sub> in air at 37°C. Vero cells were purchased from cell resource center of Shanghai Institutes for Biological Sciences (Chinese Academy of Sciences) and then were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10% fetal bovine serum (Gibco) with 5% CO<sub>2</sub> in air at 37°C. PRV strain HN1201 was isolated, identified and stored in our laboratory [3]. PRV strains Bartha-K61 (or Bartha), and MA were purchased from the National Research Center for China Veterinary Medicine. Glycoproteins (gB, gD and gE) were respectively produced by Bac-to-Bac baculovirus expression system in Sf9 cells, purified and then identified in our laboratory [12-13].

### Preparation of PRVgC

The preparation method of PRVgC was performed as previously described [12]. Nucleic acid was extracted from PRV strain HN1201, and the truncated PRVgC gene was amplified by PCR. By using the Bac-to-Bac baculovirus expression system (Invitrogen), the recombinant PRVgC protein was expressed and purified by size-exclusion chromatography. The final protein product was identified by 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE).

### Mice immunization and anti-PRVgC mAbs preparation

BALB/c mice were from Beijing Vital River Laboratory Animal Technology Co., Ltd. (China). Complete and incomplete Freund's adjuvant were obtained from Sigma-Aldrich (St. Louis, MO, USA). For the first immunization at different sites on the

back of four 6-week-old female BLAB/c mice, purified recombinant PRVgC (80µg/mouse) were emulsified with equal volume of complete Freund's adjuvant. Another two boosted immunizations were then kept up with the same incomplete Freund's adjuvant at intervals of 14 days.

The level of specific anti-PRVgC antibody was detected by indirect ELISA after three times of immunization, and the highest mouse was chosen for the last immunization without adjuvant. Followed three days later, the splenic cells were fused with SP2/0 myeloma cells with traditional hybridoma techniques. Positive hybridoma clones were simultaneously selected both indirect Enzyme Linked Immunosorbent Assay (ELISA) and Immune Fluorescence Assay (IFA) and subcloned by limited dilution at least three times. At last, a set of positive hybridoma clones were produced, and one of mAbs (named 1F2) was inoculated intraperitoneally into pristinely-primed BALB/c mice for achieving ascites. After purification as described previously [14], the concentration was quantified with Enhanced BCA Protein Assay Kit (Beyotime Institute of Biotechnology, China) and the subtype was determined using Pierce Rapid ELISA Mouse MAb subtyping Kit (Thermo Scientific) according to the manufacturer's instructions. The comprehensive evaluations of mAb 1F2 were then assessed by the different methods as follows.

### Indirect ELISA

The antibody level was all evaluated by indirect ELISA as described previously with some modifications [15]. 100µl of purified PRV gC (18ng/well in 0.1M carbonate buffer pH9.6) were coated onto the 96-well plates at 4°C overnight. After washing with phosphate buffer solution (PBS, pH7.4)-0.05% Tween (PBST), the plates were blocked with 5% skimmed milk for 2h at 37°C. After washing, mAb 1F2 was diluted with PBS (from 1:1000 to 1:8192000), and pipetted into the 96-well plates with negative control for 1h at 37°C. After another washing, Goat anti Mouse IgG-HRP (Pierce) as secondary antibody was added to the wells, and the plates were incubated for 1h at 37°C. After last washing, the substrate (100µl/well, 0.2mg/ml TMB and 0.2% H<sub>2</sub>O<sub>2</sub> in 0.05mol/l citrate buffer, pH4.6) was carried and reacted for 15min at 37°C. The chromogenic reaction was stopped by adding 2MH<sub>2</sub>SO<sub>4</sub>, and then the OD450nm value were obtained by microplate spectrophotometer. In order to test the specificity of mAb1F2, PRV gB, gD and gE were used as coating antigen and then established similar indirect ELISA as mentioned above.

In addition, the specificity reactivity of mAb 1F2 was analyzed by western blot (WB) assay. The PRVgC was separated on SDS-PAGE and then transferred to nitrocellulose membrane. After blocking with 5% skim milk in PBS for 2h at room temperature, the membrane was incubated with mAb1F2 (1:200 diluted in PBS) for 1.5h at room temperature. After washing with PBS, the membrane was incubated with Goat anti Mouse IgG-HRP (1:2000 diluted in PBS) for 1h at room temperature. After washing, the refine reaction result was colored by using DAB as the substrate DAB.

### IFA test

Before washing with PBS, the vero cells individually infected with PRV different strains (HN1201, MA or Bartha) were fixed with 80% cold acetone, and then followed by incubation mAb 1F2 dilution (from 1:100 to 1:51200) for 1h at 37°C. After washing, anti-mouse IgG (whole molecule)-FITC antibody produced in rabbit (1:400, Sigma) was applied and incubated for 45min at

37°C. The cells were observed with a fluorescence microscopy (Olympus) after washing.

### Immunoperoxidase monolayer assay (IPMA)

IPMA was applied to detect the presence of against PRV gC antibodies, as described previously with some modifications [16]. Briefly, PRV-infected cells were placed onto 36-well glass slides and then fixed with 80% cold acetone for 10min at 4°C. After washing in PBS, a series of mAb dilution (from 1:100 to 1:51200) was applied onto the slides and reacted with the immobilized antigen for 1h at 37°C. After washing, Goat anti Mouse IgG-HRP (Pierce) as secondary antibody (1:500 in PBS) was added and incubated for 30 min at 37°C. After washing, the wells were protect from light and incubated in diaminobenzidine substrate solution for 5min at room temperature. Cell staining was determined using hematoxylin eosin stain, and then investigated by an inverted light microscope.

### Immunohistochemistry (IHC) assay

In order to detect clinical tissue samples, IHC assay was established in our laboratory as previously described [17]. After cutting, fixing and blocking tonsillar tissue samples, mAb 1F2 was diluted 1:1000 in PBS, and added for incubating with the PRV antigen of samples for 30min at 37°C and then 4°C over night. After rinsing with PBS, the slides were added by Goat anti Mouse IgG-HRP (Pierce) as secondary antibody, and incubated for 1h at 37°C. After rinsing, the slides were dyed with AEC with 5min in dark for viewing. After another rinsing, the slides were used hematoxylin eosin stain, and then observed by an inverted light microscope.

## Results

### Identification of recombinant PRVgC protein

The recombinant PRVgC protein was expressed with Bac-to-Bac expression system, and the final soluble protein with about 40kD was identified by SDS-PAGE after purification (Figure 1).

### Production of against PRV gCmAb

After multiple immunizations, the hybridoma cells of two mice with highest ELISA titers were prepared and screened by ELISA and IFA methods, and 32 positive hybridoma clones (data not shown) were subcloned and identified. To further investigate their reactivity, 1 hybridoma (named 1F2) was chosen and then prepared ascites. After purification, mAb 1F2 was IgG antibody and quantitated with 3.8mg/ml, as shown in Table 1.

### Specificity and reactivity of mAb 1F2 by indirect ELISA and WB

Antibody titers of mAb 1F2 was measured by ELISA with PRV gC as coating antigen, and the results shown that the mAb had high titers of 1:2048000 (Table1). In addition, there was no cross-reactivity with PRV gB, gD and gE (data not shown). As shown in Figure 2, the WB assay results also showed that mAb 1F2 had higher specificity to PRVgC protein.

### Application of mAb 1F2 in IFA

Three reaction plates with PRV different strain (HN1201, MA or Bartha) were respectively prepared, and used for determining the antibody titer by means of IFA. As mentioned in Table 1 and Figure 3, mAb 1F2 could recognized three strains of PRV, and had the same titers of 1: 6400.

### Application of mAb 1F2 in IPMA

The result of IPMA test indicated that mAb 1F2 could react with PRV variant HN1201. In particular, we found that the high antibody titer of mAb 1F2 was 1:25600 (displayed in Table 1 and Figure 4).

### Application of mAb 1F2 in IHC

IHC was performed with mAb 1F2 as first antibody. The results of IHC experiment revealed that mAb 1F2 could detect the virus gC protein in tonsillar tissue samples infected with PRV variant HN1201 (Figure 5). As shown by Figure 5, the result remained to be good positive when the dilution of mAb 1F2 was 1:1000.

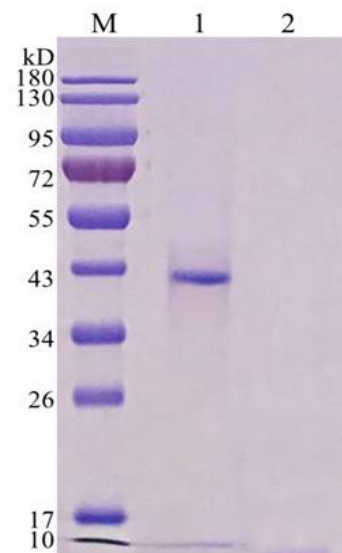
## Discussion

There were few reports on anti-PRV gCmAb. As reported in 1995, Nauwynck and Pensaert found that both mAb 8P19 and polyclonal sheep antiserum against PRV gC protein had no effect on the cell-associated spread of PRV early strain in monolayers of different cell types [18], which was first report on PRV gCmAb. However, since PRV variants outbreak in 2011, no reports about anti-PRV gCmAb were indexed.

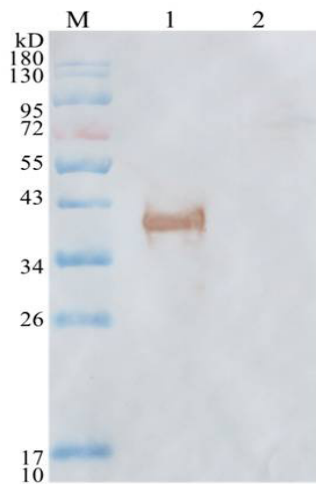
In our study, anti-PRV gCmAb 1F2 was screened and had broad response with PRV different types of representative strains (including PRV vaccine strain Bartha, PRV classic strain MA and PRV variant HN1201). In addition, mAb 1F2 could identify recombinant PRV gC protein by indirect ELISA and WB, and could recognize PRV through different methods, for instance, IFA, IPMA and IHC.

In conclusion, anti-PRVgC mAb 1F2 was generated and characterized by a variety of methods (consist of indirect ELISA, WB, IFA, IPMA and IHC) in our study. As a result, mAb 1F2 can apply as a valuable tool for further biological analysis of PRV.

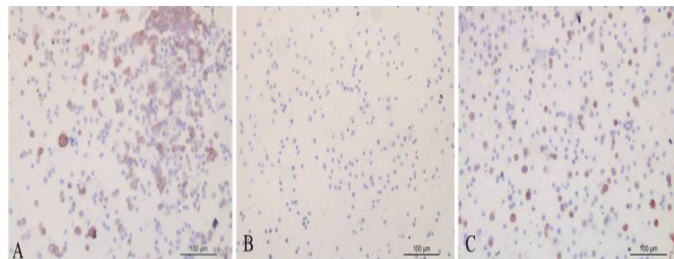
## Figures



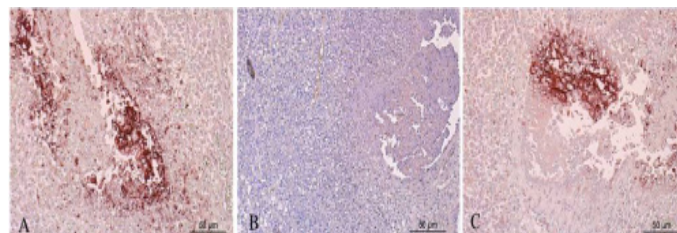
**Figure 1:** Expression and purification of PRVgC and analysis by SDS-PAGE. M, protein marker; lane 1, purified PRVgC using immunoaffinity chromatography; lane 2, sf9 cells transfected with the empty vector.



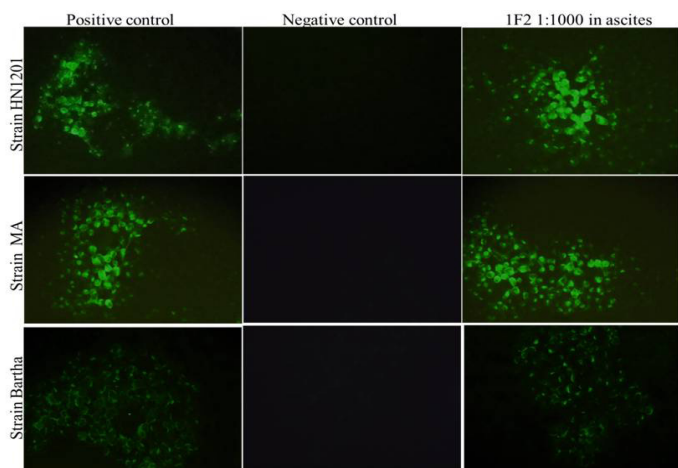
**Figure 2: Specificity of mAb1F2 against PRVgC analyzed by WB.** M: protein marker; lane 1, mAb 1F2 1:1000 in ascites reacts with PRVgC; lane 2, mAb 1F2 1:1000 in ascites does not react with supernatant of sf9 cells.



**Figure 4: Reactivity of mAb 1F2 with the vero cells infected with PRV variant HN1201 by IPMA.** A: Positive control (mice serum); B: Negative control; C: 1F2 1:1000 in ascites



**Figure 5: Reactivity of mAb 1F2 with the tonsillar tissue samples with PRV variant HN1201 attacked by IHC.** A: Positive control (mice serum); B: Negative control; C: 1F2 1:1000 in ascites



**Figure 3: Reactivity of mAb 1F2 with the vero cells infected with PRV different strains by IFA.** Mice serum was used as positive control (Microscopic magnification 200x)

**Tables**

**Table 1: Results of different evaluation methods with anti-PRV gCmAb 1F2 in ascites**

Anti-PRVgCmAb	Antibody-subtyping (Heavy/light chain)	Concentration (mg/ml)	Results of different evaluation methods					
			ELISA titer	IFA titer			IPMA titer	IHC
				Strain HN1201	Strain MA	Strain Bartha		
1F2	IgG2a/Kappa	3.8	1:2048000	1:6400	1:6400	1:6400	1:25600	Positive



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