Design of a Chimaeric Antigen And Its Use In The Detection of IgG Antibodies Against Rubella Virus

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Research Article

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Abstract

**Background:** Rubella virus (RV) is the causative agent of rubella or German measles. Although most infections cause only mild self-limited measles-like illness, the infection in pregnant women can cause severe foetal malformation or even miscarriage, especially in the first 3 months of pregnancy. Therefore, it is of great practical significance to establish a simple and sensitive RV detection method.

**Methods:** The partial epitopes of the E1 and E2 proteins from Rubella Virus were selected as the target sites, the sequence of the selected antigenic sites of the E1 and E2 were linked by a linker. The expression plasmid P6T was constructed by inserting the gene into PET-32A+ with a His tag. The P6 protein was induced and expressed in *Escherichia coli* L21 DE3 and purified by nickel column affinity. The protein P6 antigen was identified by Western blotting, and an anti-P6 antibody ELISA was established to test known serum samples to evaluate the capability of this method.

**Results:** After purification, the concentration and purity of the protein P6 were 0.283 mg/mL and more than 80%, respectively. Western blotting showed that the protein P6 could react with rubella virus positive serum. By ELISA, 36 negative sera and 58 positive sera were detected. The coincidence rate, specificity and sensitivity of the ELISA were 88.89%, 84.48% and 84.48%, respectively. The P6 ELISA with a *kappa* coefficient of 0.709, *P*<0.05, indicated excellent consistency.

**Conclusions:** The P6 protein with excellent antigenicity obtained from prokaryotic expression followed by chromatography purification could prove useful for early diagnosis of RV infection.

**Background**

Rubella virus (RV) is the causative agent of rubella or German measles [1]. Although most infections cause only mild self-limited measles-like illness, the infection in pregnant women can cause severe foetal malformation or even miscarriage, especially in the first 3 months of pregnancy [2, 3]. Therefore, it is of great practical significance to establish a simple and sensitive RV detection method. After Rubella virus infection, IgM appears earlier. However, it is maintained for a short period of time and decreases in expression after 1 month of pregnancy until it turns negative. IgG antibody appears after IgM, and the level of IgG antibody shows a rising trend. The peak IgG expression appears 1 ~ 2 months after infection. Its level decreases very slowly. IgG can remain in the body for long periods of time, even up to a few decades [4–6]. Therefore, IgM can be used as a marker for early pathogen infection. IgG antibody can indicate a current infection and a previous infection history of the pathogen.

RV has three structural proteins: one capsid protein (C) and two envelope proteins (E2 and E1). C is rich in arginine and proline residues, which make it positively charged and facilitate its reaction with genomic RNA during nucleocapsid formation [7]. As type I membrane proteins, E1 and E2 are heterodimerized to form complexes on the surface of the virus [8]. The main function of the spinous process complex is to collect host cell receptors and to mediate the fusion between the virus and host cell membrane [9]. For RV IgG antibodies, E1 has the most epitopes compared to E2 and C protein[10]. It has been found that a protein expressed by serially linking different epitopes of the virus can be used for the detection of RV-specific IgG with good diagnostic efficacy [11]. In this paper, the immunodominant regions of two RV structural proteins were expressed in tandem on the same protein, and the recombinant protein was applied to an indirect ELISA for detecting RV-IgG.

**Methods**

**Materials and reagents**

*Escherichia coli* (E. coli) DH5a and the BL21 DE3 were obtained from our own laboratory. T4 DNA ligase and all restriction enzymes were purchased from NEB. HRP-labelled goat anti-human IgG was purchased from Santa Cruz (USA). Samples from 94 umbilical cord serum samples collected in 2014 from HuNan Province were collected and provided by the Chinese Center for Disease Control and Prevention. The samples origin is from individuals with history of 2B genotype infection. All of them were confirmed by a RV ELISA Kit/Germany/VirionSerion, of which 58 were positive and 36 were negative.

**Construction of the expression plasmid P6T**

Based on references [12-14], sequences with antigenic sites of E1 (199-286) and E2 (1-115) were selected, they were linked by a linker (GGGGSGGGSGGGGS). After codon optimization, restriction endonuclease sites for BamHI and HindIII were added at both ends. The construct was then composed by the Invitrogen company (named P6T). P6T/18ng/uL was digested by BamHI and HindIII. P6T was purified and ligated to the pGEM-T expression vector. Then, the plasmid pGEM-T-P6T was transformed into E. coli DH5a. The positive clones were further identified with the restriction endonuclease digestion of BamHI and HindIII. The sequence was identified by SinoGenoMax Company.

**Preparation of the protein P6**

The plasmid P6T was purified and ligated to the pET-32a (+) expression vector. The plasmid pET-32a (+)-P6T that was extracted from a positive transformant was transformed into the BL21(DE3) with spectinomycin resistance. The clones were picked and shaken at 37 °C overnight in Luria-Bertani (LB) broth containing 50 μg/mL spectinomycin. Then, the expressed bacteria were collected by centrifugation (4000 r/min, 8 min, 10 °C) and stored frozen. Freeze-stored bacteria were added to lysis buffer 1 (20 mM Tris, 0.5 M NaCl, 2 mM EDTA, 5% glycerine, 0.5% Triton X-100, pH 8.0) and resuspended evenly. The bacterial solution was lysed by ultrasound. Supernatant 1 and precipitate 1 were separated by centrifugation. Supernatant 1 was preserved. Precipitate 1 was washed with lysis buffer 2 (0.5 M NaCl, 5% glycerine, 0.5% Triton X-100, pH 8.0). Supernatant 2 and precipitate 2 were separated by centrifugation. Supernatant 2 was preserved. Precipitate 2 was dissolved in Binding Buffer (20 mM Tris, 0.5 M NaCl, 10 mM imidazole, pH 8.0). Supernatant 3 and precipitate 3 were separated by centrifugation. Precipitate 3 was preserved. Supernatant 3 was filtered with a 0.45 μm filter.
The sample was purified by a nickel column. The nickel column was treated with 60 mmol/L and then 300 mmol/L imidazole. The content and distribution of the target protein were observed and analysed by SDS-PAGE. Diluted with 5% skimmed milk solution. The eluant with high protein content was refolded by dialysis and stored at 4 °C.

**Western blotting analysis of the protein P 6**

SDS-PAGE was performed by adding 5 μL of P6 solution. Then, the protein was transferred to a PVDF membrane (PVDF membranes for different samples were cut to the appropriate size). At 25 °C, the membrane was treated with a blocking solution for 1 hour. Anti-RV serum (1:200) diluted with a blocking solution (5% skimmed milk solution) was added. It was incubated at 4 °C overnight and then washed. The secondary antibody was goat anti-human IgG-HRP enzyme (AmericaSanta Cruz Inc (1:5 000)), which was added and incubated at room temperature for 1 hour and then washed. Use DAB to make it color.

**The protein P 6 was detected by indirect enzyme-linked immunosorbent assay**

After diluting the P6 sample with buffer solution, 100 μl of sample was added into each well, and the sample was coated at 4 °C for 12 hours. Then, the plate was washed four times with PBST buffer. Then, 200 μl of blocking solution was added to each well, and the plate was stored at 37 °C for 2 hours. The plate was washed with PBST buffer four times. Then, 100 μl of serum diluted with a blocking solution was added. After being stored at 37 °C for 1 hour, the plate was washed four times with PBST. Then, 100 μL of goat anti-human antibody (AmericaSanta Cruz Inc) diluted with blocking solution was added and incubated at 37 °C for 30 min. The plate was washed as described above. Then, 100 μL of TMB (AmericaKPL) was added to each well. The plate was stored in the dark for 5 ~ 15 min at room temperature. The optical density (OD) value at 450 nm (A450) was measured by an enzyme-labelled instrument, and the OD value at 630 nm (A630) was used as a reference. When the ratio of positive serum to negative serum A450s (P/N) was the best, the coated antigen concentration was the best [15, 16].

**Data analysis**

An ROC curve was drawn according to the OD value measured by the enzyme labelling method. The optimum critical value was determined by the Yoden index (Yoden index=sensitivity + specificity-1). The sensitivity and specificity of the new detection method were calculated according to the critical value. SPSS18 software was used for statistical analysis of the experimental data.

**Results**

**P6 was obtained by prokaryotic expression**

The recombinant plasmid with multiple epitopes (named P6T) was constructed by selecting the immunoactive sites on the two structural proteins of RV. The P6T plasmid was approximately 666 bp (Fig. 1). The plasmid was successfully constructed and identified by sequencing and double restriction enzyme digestion (Fig. 2). P6T was expressed in E. coli, and the product was named P6. SDS-PAGE showed that the molecular weight of the protein P6 was approximately 44 kDa (Fig. 3). The expected fusion protein expressed encoded by just the pET-32a(+) vector alone would be around 20.4 kDa. In addition, the expected fusion protein expressed encoded by the P6T plasmid would be around 23.29 kDa (GSGLQPRADMAAPPAPPQPPRAHGGHYHGGQLLLLQFGLMGHSGHGTQRVQHHRNASDVLKPGW7LGWLYGCYNLSWDHQTHVCHTKHMDFWVCVHEHRPPATPTF).

The results showed that the protein P6 could be expressed in E. coli. The protein was expressed as inclusion body at 0.283 mg/mL (Table 1).

| Table 1 | Concentration results of the protein P6 |
|---------|----------------------------------------|
|         | Sample number | 1 | 2 | 3 | Mean value |
| absorbance value (A562) | 0.250 | 0.246 | 0.247 | 0.248 |
| concentration (mg/mL) | 0.286 | 0.281 | 0.282 | 0.283 |

Imidazole elution (60 mM, 150 mM, 300mM) was used for the purification of the target protein. The results of SDS-PAGE showed that P6 protein was the purest with 150 mM imidazole concentration. It could be more than 80% pure (Fig. 4).

**The protein P6 reacts with anti-RV serum**

The antigenicity of the protein P6 was identified by Western blotting. The results showed that RV-positive serum as a first antibody and goat anti-human IgG-HRP as a second antibody showed a significant band at approximately 44 kDa. However, RV-negative serum as a primary antibody showed no band (Fig. 5A). No protein P6 was added, and no significant bands were observed with either RV-positive or RV-negative serum (Fig. 5B). The results showed that the protein P6 could react specifically with RV-positive serum.

**P6 indirect enzyme-linked immunosorbent assay for identification of blood samples**

Using the Protein P6 as the first antibody, an ELISA method was established. Ninety-four samples of umbilical cord blood (VirionSerion ELISA method) were selected to verify the accuracy of this method (Table 2). An ROC chart was drawn according to the OD value, and the Yoden index was used to determine the best cut off (cut off = 0.317). According to the critical value (Fig. 6), the coincidence rate, specificity and sensitivity of the ELISA were 88.89%, 84.48% and 84.48%, respectively.
### Table 2

|     | RV+E1E2 ELISA method | VirionSerion ELISA method |
|-----|----------------------|---------------------------|
|     | Positive             | Negative                  | Total   |
| Positive | 49                   | 4                        | 53      |
| Negative | 9                    | 32                       | 41      |
| Total   | 58                   | 36                       | 94      |

Regarding the Chi-square test results, kappa = 0.709 and P < 0.05. The results showed that there was no significant difference between the P6 indirect ELISA and the known results.

### Discussion

There are two types of rubella virus ELISA kits available on the market for testing IgM and IgG antibodies. When pregnant women are infected with rubella virus but not infected in utero, the detection time of IgM resistance in pregnant women is short. When pregnant women are infected with rubella virus in utero, Rubella virus IgM antibody can be continuously detected until before delivery [23]. Therefore, rubella virus IgM test is suitable for early infection and termination of pregnancy detection. The detection of rubella virus IgG is suitable for the seroepidemiological investigation of rubella virus. The monitoring of rubella virus in China began in 1999. At present, the ELISA kit method is mainly used to investigate the evaluation of individual protective antibodies against rubella virus and the investigation of antibody levels in the population. Meanwhile, the neutralization test is used for auxiliary verification. Because the neutralization test process is cumbersome and involves the operation of live poison. At present, the quality of rubella IgG kits produced in China is uneven. Imported kits are expensive and the purchase of such kits is subject to certain conditions [24–25]. Therefore, an indirect ELISA method for serological detecting rubella virus IgG antibody was established in this study.

In this study, recombinant antigen was prepared for serological detection. At present, most of the antigens contained in the kits can be divided into two categories: whole virus antigens and recombinant antigens. The former generally refers to the inactivated virus particle as the encapsulated antigen, the antigen has the complete structure of the virus. Therefore, the results of kit detection of whole viral antigens are usually of good sensitivity. However, compared with the recombinant antigen, the preparation and purification process of the whole virus antigen is complex, the requirements for operators are higher. In addition, the purity of the virus is not ideal, the price is relatively higher. Recombinant antigen is prepared through the expression system by the method of gene recombination. The recombinant antigen has the immunological site of the whole virus antigen, so it has strong specificity. The purification method of recombinant antigen is simple, which can save a lot of cost.

In this study, Escherichia coli BL21(DE3) was selected as the expression system. Prokaryotic expression system is easy to survive, convenient to purchase and low cost. Its genomic information is simple and clear. The method is simple and efficient for the transformation of recombinant plasmid. When used to express foreign protein, the expression amount is high, and the expressed product is stable. Eukaryotic expression system has a good ability to modify the expression protein. Compared with prokaryotic expression system, it has high cost, complex operation and low yield.

In this paper, the critical value of RV-specific IgG was determined by the ROC curve method. At present, there are many methods to determine the critical value of an ELISA, such as the standard curve method, mean plus minus standard deviation method, ROC curve method, response surface optimization method, and uniform design [17–19]. The ROC curve method is a widely used method to determine the critical value considering specificity and sensitivity [20–22]. The practicality of the proposed method was tested by qualitative analysis of serum data, so the ROC curve method was used to determine the critical value of the proposed method.

There may be some deficiencies in the establishment of the ELISA in the experiment. First, the optimization of ELISA experimental processes involves a number of conditions. Not all the conditions have been tried. Second, only the effective antigen sites of rubella virus E1 and E2 were expressed in this study. Within contrast to the whole virus, the other antigen sites were not expressed. Therefore, there was the possibility of missed detection. Finally, this experiment selected a limited number of serum samples. For a more rigorous evaluation of the efficacy of the established method, a larger sample size is needed for validation.

### Conclusions

In conclusion, the recombinant protein P6 was expressed by a prokaryotic expression system. An indirect ELISA method for the detection of IgG antibody in serum was established by using P6. Although this method had some shortcomings, it had strong specificity. After optimization, it would be expected to provide a laboratory basis for the development of an RV detection kit with high specificity and sensitivity. The results provide a practical and effective plan for epidemiological surveys of RV in China.

### Abbreviations

- RV, Rubella virus
- E. coli, Escherichia coli
- LB, Luria-Bertani
OD, optical density

Declarations

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Availability of data and materials

All relevant information is provided in this current manuscript.

Author's contribution:

Writing original draft preparation, Wenyue Xing and Li Li; Writing review and editing, Hong Yuan and Yanhua Ma; supervision, Yanhua Ma and Hong Yuan; project administration, Ruiping Hu; funding acquisition, Ruiping Hu and Li Li, methodology, Wenyue Xing, Xin Xue, Jingnan Zhang, Hong Yuan, Shumei Ye, Chunli Ma. All authors have read and agreed to the published version of the manuscript.

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Figures

P6T → pGEM-T-P6T → DH5α → P6T

Figure 1

Construction of the expression plasmid P6T
Figure 2
Preparation of the protein P6

Figure 3
Results of PCR identification of the plasmid P6T 1: DNA marker, DL2000; 2: the plasmid P6T

Figure 4
Identification of the plasmid P6T by double restriction enzyme digestion
1: DNA marker, DL5000; 2: double restriction enzyme (BamHI, HindII) digestion product of plasmid P6T
3: double restriction enzyme (BamHI, HindII) digestion product of pET-32a(+) 

Figure 5
SDS-PAGE analysis of the Protein P6
1: marker; 2. control (no IPTG induction); 3. expression product (IPTG induction: 2.5h , 1mM IPTG); 4, 6. supernatant of expressed product; 5: inclusion body of expressed product

Figure 6
Purification of the protein P6
1. Marker; 2-7. P6 eluted with 150 mM imidazole; 8-9. P6 eluted with 300 mM imidazole

Figure 7
Western blot analysis of the protein P6
A: 1: marker; 2: control; 3, 4: positive serum +P6; 5: negative serum+P6
B: 1: marker; 2: control; 3, 4: positive serum; 5: negative serum
Figure 8

Detection of the ROC in serum by P6 indirect ELISA