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Construction of an Indirect ELISA Method for Detection of Antibody of West Nile Virus E Protein

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West Nile fever is an emerging disease caused by West Nile virus (WNV) and gives rise to fever, rashes, including encephalitis or meningitis and fulminate hepatitis, which can be fatal. It was described as a serious disease with a strong influence on public health. In this study, WNV E protein was cloned and expressed and used as an immunogen for detection polyclonal antibodies against West Nile virus by indirect ELISA Method. The ELISA was conducted using an optimal concentration of antigen at 2 μg/mL and serum was diluted to 1:2 000, and the HRP secondary antibody was diluted to 1:8 000. The antigen coating was incubated at 37°C for 1 h, then at 4°C overnight. It was blocked by adding 10% fetal calf serum and the antigen with serum response time was 90 min. For second anti-serum, the enzymatic reaction time was 60 min, and the effect of substrate reaction at 37°C was 15 min. Through specific and reproducible tests repeated by indirect ELISA using 25 samples of chicken serum, the test results showed that chicken serum was negative at the West Nile virus and the critical value was 0.077. These results strongly suggest that the coating antigen of the recombinant E protein serve as a good source in the indirect ELISA method for the detection of WNV antibody and implementation methods for rapid diagnosis of WNV.

Key words: West Nile virus, E protein, Antibody, Indirect ELISA.

West Nile virus (WNV) contains a single molecule of linear, single-stranded RNA, having three structural proteins, an envelope glycoprotein E, a core protein C and a membrane protein M[1]. Envelope Glycoprotein (E) is shown to be the major antigen capable of inducing virus neutralizing antibody that could confer protective immunity against infection of WNV. The diagnosis of WNV infection is performed by a number of laboratory techniques. Virus can be detected in clinical specimens by polymerase chain reaction (PCR), after isolation of virus. Serological testing includes WNV-reactive IgM and IgG by Indirect ELISA method to detect WNV-specific antibody present into a single serum sample of an animal[2,3]. We developed an indirect ELISA using a rabbit antibody with the coating antigen and enzyme (horseradish peroxidase [HRP]) conjugated goat anti-mouse IgG.

Currently, no specific treatment or vaccine license is available for the prevention against this pathogenic virus. Therefore, it is urgent to develop an effective prophylactic vaccine to prevent WNV infection. Therefore, it is important to develop antiviral agents for the treatment of flavivirus infections[4].

The objective of this present study was:

1 To clone the gene E of West Nile Virus into the pET-32a and express it in *E. coli* BL21.

2 To purify the proteins for preparing the antigen

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to immunize rabbits.

3 To develop a rapid and simple indirect ELISA method using recombinant E protein. These allow us to detect the antibodies of West Nile Virus from chickens and other animals.

MATERIALS AND METHODS

Materials

The E protein was collected based on the results from the second part.

Animal

New Zealand white rabbits were purchased from Experimental Animal Center of Southern Medical University.

Serum

positive serum JE, dengue-positive serum, kindly provided by Dr. Changwen Ke, the control by the Guangdong Center for Disease Control and Prevention; West Nile virus-positive serum, kindly provided by Dr. Zhiliang Wang, the Ministry of Agriculture Animal Quarantine of Qingdao.

HRP secondary antibody

HRP labeled goat anti-mouse secondary antibody, mouse anti-human HRP labeled secondary antibody is Boster products.

Coating solution (0.05 mol/L pH 9.6 carbonate buffer)

Sodium carbonate 1.5 g, sodium bicarbonate 2.93 g, dissolved in 1 000 mL double distilled water, pH adjusted to 9.6 with HCl.

PBS (0.01 mol/L pH 7.4)

Sodium chloride 8.0 g, potassium chloride 0.2 g, ferric disodium hydrogen phosphate 2.90 g, Potassium dihydrogen phosphate 0.2 g, dissolved in 1 000 mL double distilled water.

Lotion (PBST) pH 7.4

PBS with 0.05% Tween-20, kept at -20°.

Antibody Dilution

Containing 0.1% BSA in PBST, repackaging, -20° preservation.

HRP secondary antibody diluents

Sterile 0.01 mol/L pH 7.4 in PBS.

Stopping solution (2 mol/L sulfuric acid)

22.2 mL of concentrated sulfuric acid drop and 177.8 mL of distilled water was taken and mixed at room temperature for further use.

ELISA plates

Grenier products imported in Germany.

Microplate Reader

Bio-Rad Model 680

Methods

Preparation of E protein antigen

The recombinant protein was expressed and purified adjuvant was used to improve or enhance an immune response to antigens.

Immunization of rabbits

Two rabbits were immunized four times at intervals of 10 days for two consecutive months and each rabbit received 400 µg of the antigen-E protein and an equal volume of Freunds complete adjuvant mixed with emulsified. After immunization, blood was collected and stored at -20° until use.

Indirect ELISA

Procedure for indirect ELISA

ELISA was performed according to the method described by Feinstein (1985) and Martin (2000) with slight modifications.).

RESULTS

The concentration of purified recombinant E glycoprotein was too low to immunize rabbits. Large amount of glycoproteins E were contained in the inclusions. After detecting by SDS-PAGE, fusion protein bands in gel were cut at 73 kDa indicated by the marker. The recombinant protein E may serve as a good antigen in the indirect ELISA for detecting antibodies against WNV.

Determination of serum antibody levels in rabbits

After four times of immunization, the serum antibody levels in rabbits were measured by the indirect ELISA. E protein was coated in the concentration of 2 µg/mL and serum antibody titers were up to 1:300 000. Results were shown in Table 1.

Determination of the optimum reaction conditions of Indirect ELISA method

Uniformity of the reaction plates

The results of the homogeneity between reaction plates were shown in Table 2. After statistical analysis, the average pore $\overline{X}$ at $OD_{450\text{-}630\ nm}$ was 2.490, the SD (standard deviation) is 0.1139049, CV=SD/X=4.6%, less than 10%, which is indicating that the

ELISA plate had good homogeneity.

Determination of the best dilution of serum and the concentration of antigen

Appropriate value of P/N was

determinated by positive and negative sera and was used to determine the optimal concentration of antigen and dilution of serum. The results showed that the best serum dilution was 1:2 000 with the maximum P/N values between 21.031 and 21.896 respectively, from concentrations of 4μg/ mL and 2μg/ mL (Table 3). However, the maximum P/N value indicates that the best dilution of serum

Table 1. Determination of serum antibody levels in rabbits

		Dilution of serum							N	Blank
		5000	10000	20000	40000	80000	160000	320000		
Rabbits	No.1	1.544	1.348	1.139	0.850	0.594	0.393	0.241	0.064	0.070
	No.2	1.624	1.430	1.208	0.978	0.688	0.478	0.308	0.057	0.070

Table 2. Determination of the homogeneity between the reaction plates

	1	2	3	4	5	6	7	8
A	2.545	2.405	2.463	2.335	2.367	2.351	2.214	2.305
B	2.549	2.703	2.573	2.361	2.428	2.480	2.429	2.215
C	2.426	2.517	2.713	2.464	2.423	2.655	2.528	2.368
D	2.559	2.464	2,535	2.435	2.406	2.432	2.455	2.363
E	2.592	2.499	2.527	2.443	2.434	2.610	2.490	2.382
F	2.608	2.527	2.649	2.474	2.394	2.338	2.511	2.503
G	2.565	2.641	2.605	2.696	2.476	2.370	2.401	2.401
H	2.611	2.580	2.631	2.524	2.495	2.418	2.343	2.719

Table 3. Determination of the best dilution of serum and the concentration of antigen

Concentration of antigen (μg/mL)		Dilution of serum					
		1:250	1:500	1:1 000	1:2 000	1:4 000	1:8 000
8	+	1.737	1.505	1.420	1.320	1.072	0.842
	-	0.119	0.096	0.102	0.069	0.064	0.068
	P/N	14.597	15.678	13.921	19.130	16.750	12.382
4	+	1.630	1.496	1.499	1.346	1.136	0.879
	-	0.116	0.096	0.086	0.064	0.071	0.063
	P/N	11.724	15.583	17.430	21.031	16.000	16.903
2	+	1.439	1.482	1.393	1.270	1.092	0.784
	-	0.095	0.081	0.072	0.058	0.080	0.063
	P/N	15.147	18.296	19.348	21.896	13.650	12.444
1	+	1.186	1.334	1.393	1.135	1.011	0.063
	-	0.078	0.073	0.066	0.058	0.075	0.058
	P/N	13.790	18.273	19.060	19.569	13.480	12.914

Table 4. Determination of the best dilution of secondary antibody

	Dilution of secondary antibody						
	1:1 000	1:2 000	1:4 000	1:8 000	1:10 000	1:20 000	1:40 000
+	2.284	3.061	1.775	0.964	0.775	0.334	0.118
-	0.894	0.544	0.259	0.107	0.107	0.049	0.029
P/N	2.555	5.627	6.853	9.009	7.234	6.816	4.069

could be done with a concentration 2 µg/mL of antigen.

Best dilution of secondary antibody

The optimal serum dilution of secondary antibody was determined using the ELISA reaction. The results presented in Table 3.4 indicated the best limit on the ratio P/N to determine the best dilution of secondary antibody was 1:8 000.

Determination of the best conditions for coating of the recombinant protein

The group 2 was considered better with a reaction condition of 1 hour at 37°C and overnight at 4°C, and the best P/N value is 12.120 (Table 5).

Table 5. Determination of the best antigen coating condition

	Coating condition			
	Group 1	Group 2	Group 3	Group 4
+	0.976	1.006	0.979	1.076
-	0.094	0.083	0.084	0.216
P/N	10.383	12.120	11.655	4.981

Table 7. Determination of the best time of serum incubation

	Serum reaction time			
	30 min	60 min	90 min	120 min
+	1.073	1.102	1.473	1.387
-	0.055	0.099	0.052	0.069
P/N	19.509	11.131	28.327	20.101

Table 9. Analysis of chicken negative serum

	1	2	3	4
A	0.064	0.060	0.061	0.068
B	0.066	0.066	0.060	0.059
C	0.064	0.064	0.061	0.061
D	0.076	0.064	0.066	0.061

Determination of the best condition of blocking

The value of each group was calculated by reading the absorbance of positive and negative wells and the means of each positive/negative (P/N) were calculated. The maximum P/N has been obtained at 90 min, with 10% calf serum, corresponding to the value of 17.462 (Table 6).

Best time of serum incubation

The results showed that P/N ratio of

Table 6. Determination of blocking condition

Blocking solution		Blocking time		
		120 min	90 min	60 min
1% BSA	+	1.273	0.982	1.419
	-	0.122	0.162	0.137
	P/N	10.434	6.062	10.358
10% calf	+	1.450	1.135	1.031
serum	-	0.112	0.065	0.067
	P/N	12.955	17.462	15.388
5% milk	+	1.039	1.074	1.155
	-	0.091	0.086	0.105
	P/N	11.418	12.488	11.000

Table 8. Determination the conditions of serum and secondary antibody incubation

	The reaction time of serum and secondary antibody			
	30 min	60 min	90 min	120 min
+	0.724	0.993	1.085	1.303
-	0.035	0.033	0.051	0.074
P/N	20.686	30.091	21.275	17.608

Table 10. Analysis of chicken negative serum

	1	2	3	4
A	0.064	0.060	0.061	0.068
B	0.066	0.066	0.060	0.059
C	0.064	0.064	0.061	0.061
D	0.076	0.064	0.066	0.061

Table 3.11. Specificity test

	JE positive serum	Dengue feverpositive serum	+	-
$OD_{450\text{-}630\ nm}$	0.090	0.086	1.674	0.068

serum increased when the reaction was last from 30 min to 90 min. P/N value was highest at 90min with a value of 28.327, and then the ratio decreased at 120 min (Table 7). However the P/N value at 120 min was higher than those obtained at 30 min and 60min. According to this result, the best reaction time of serum was 90 min.

Determine the conditions of serum and secondary antibody incubation

The antigen was diluted to 2 μg/mL, as well as the serum to 1:2 000 and the HRP secondary antibody was diluted to 1:8 000. The results showed that the value of N was the lowest and the ratio of P/N reached the peak point at 60 min. It can be determined that the optimum reaction time of the serum and the second antibody was 60 min (Table 8).

Substrate reaction time

When substrate was added, the increasing value of serum $OD_{450\ nm}$ and $OD_{630\ nm}$ appeared after 15min, the best substrate reaction time was 15 min at 37°C which was considered as the best reaction condition of enzyme and substrate, and the P/N value was highest (Table 9).

Determination of the critical value of P/N with indirect ELISA method

The critical value to determine the serum of negative and positive of the value of $OD_{450\text{-}630\ nm}$ is 0.077, when the value of the sample $OD_{450\text{-}630\ nm}$ ≥0.077, and the value of P/N is more than 2.1, the determination is positive.

Specificity tests

The positive serum of Japanese Encephalitis (JE) and dengue fever were tested by indirect ELISA (WNV E protein-coated microtiter plates) and the result was negative (Table 11).

DISCUSSION

In this study, the WNV E protein was expressed and a rapid and simple assay was developed by indirect ELISA method for the detection of WNV antibody using the recombinant E protein. The WNV E protein has been expressed in bacteria[5-7]. The *Escherichia coli* expression systems used in this study provided an easy means of producing recombinant proteins. After 4h induction of protein expression by IPTG, the fused proteins can concentrate to 8mg/L. It was also reported that the recombinant E protein has been expressed in bacteria E. coli and often gives rise to inclusion body[8].The most of glycoprotein E was contained in the inclusions when expressed. It can be ascribed to the lack of post-translational modification or accumulation of recombinant proteins in the cytoplasm[8]. These studies have shown that the pre-membrane protein was necessary for immunity, either for the genesis of a protective response directed against the (pre)membrane protein or by stabilizing the E protein. Our data demonstrated that the recombinant E protein affords full protective immunity by itself, and moreover, the E proteins are sufficient for partial immunity. Although WN virus infection in mice was similar with human disease, such as neuroinvasion, it remains to be determined whether E protein vaccination will be effective in other experimental model systems in humans[9, 10]). Furthermore, WN virus isolates in the U.S. have been demonstrated striking genetic similarities, vaccination with recombinant E protein against diverse isolates can be examined[9,10]. Neutralizing antibodies induced by the domain III of flavivirus E proteins have been reported elsewhere as a major factor responsible for the greatest protection in vivo[11, 12], and all types of vaccines recently developed by bioengineering the target E protein[13-20]. ELISA may be useful to quickly test the status of the protective immune response in individuals immunized (i.e., rabbits). The use of recombinant of WNV E to develop diagnostic tests are based on indirect ELISA [21-23]. We found that WNV neutralizing antibodies in the serum of the rabbit did not inhibit binding to the recombinant E protein expressed in *Escherichia coli*, ELISA tests can cross-react with other flaviviruses and showed that the detection of IgG and IgM antibodies against WNV in blood serum using an ELISA test indicating the presence of neutralizing antibodies (SN), a positive ELISA indicates that the subject has been exposed to WNV or flavivirus closely apparent. Finally we considered a case as confirmed when virus isolation was positive[24].

CONCLUSION

The results indicated that recombinant E protein is a suitable source of coating antigen in the indirect ELISA for detection of WNV antibody.

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