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Sandwich Enzyme-linked Immunosorbent Assay for Rapid Detection of *Vibrio Parahaemololyticus* Using IgY and Enzyme Conjugated IgG

Qingping Zhong [1 +], Bin Wang [2], Li Wang [1] and Xiaoqin Liao [1]

[1] College of Food Science, South China Agriculture University, Guangzhou, 510642, China
[2] Guangzhou South China Biological Medicine Co. Ltd, Guangzhou, 510642, China

Abstract. *Vibrio parahaemolyticus* is one of the most important food-borne pathogen which can cause serious infection in human. One kind of sandwich ELISA was developed in this paper to detect *V. parahaemolyticus* rapidly and economically, using anti- *V. parahaemolyticus* IgY as the capture antibody and the specific enzyme conjugated antibody (HRP-IgG) as the detection antibody. The detection limit of the established assay was 2.1×10^4 cfu/ml. The method can sensitively detect *V. parahaemolyticus* in seafood with low intra-assay coefficient of variation (0.90% to 2.36%) and inter-assay CV (0.83% to 3.15%), and has no cross reactions with other bacteria. The IgY coated microtiter plate had high stability. Results suggest that this sandwich ELISA developed in this study is a simple and convenient preparation for the rapid detection of *V. parahaemolyticus* in seafood.

Keywords: *Vibrio parahaemolyticus*, Sandwich ELISA, Detection, IgY, Enzyme conjugated IgG

1. Introduction

Vibrio parahaemolyticus, a gram-negative marine bacterium, halophilic inhabitant of coastal waters, has been isolated from a variety of different seafood. *V. parahaemolyticus* is an enteric pathogen usually responsible for acute gastroenteritis associated with the consumption of contaminated seafood, such as raw or slightly cooked shellfish, particularly oysters [1]. Food poisoning caused by *V. parahaemolyticus* has spread all over the world include Asia, America, Europe and Africa [2]. In Asia, *V. parahaemolyticus* is a common cause of foodborne disease [3].

Although conventional culturing methods are the best choice for *V. parahaemolyticus* identification in food products, they are labor-intensive, time-consuming, and unsuitable for rapid detection. Numerous new techniques for *V. parahaemolyticus* detection are emerging. PCR, real-time PCR and ELC-PCR are being widely used [4]-[6]. Despite of the high sensitivity, the expensive equipments and materials, strict operating procedure restrict them only in laboratories application. Enzyme-linked immunosorbent assay (ELISA) is also applied to *V. parahaemolyticus* detection [7]. The immunoassay is cheaper, simpler and commercial immunoassay-kit for the outdoor and large scale sample detections can be expediently developed.

IgY is the major antibody produced by hens. It is a promising antibody for immunodiagnosis and immunotherapy [8]. Chicken egg yolks have been proved to be better and more cost-effective than antivenom. Apart from the lower cost of feeding and handling, hens can produce large amounts of homogeneous IgY per month. Hens can be immunized through different routes and the production of antibody involves very simple and inexpensive steps. Therefore, the use of hens for the production of antibody can not only refine painful blood collection in animals but also lower the clinical or research costs. The idea of this study is to develop a rapid and sensitive sandwich ELISA for the detection of *V.*

[+] Corresponding author. Tel.: +86 13535121218; fax: +86-20-85280270.
E-mail address: zhongqp@scau.edu.cn.

parahaemolyticus in foods using IgY as capture antibody and enzyme conjugated IgG (HRP-IgG) as detection antibody.

2. Materials and Methods

2.1. Reagents and instruments

All reagents were of analytical grade unless specified otherwise. Horseradish peroxidise (HRP), Sephadex G-25 were obtained from Sigma (USA). DEAE-Sepharose FF was purchased from Pharmacia (USA). TMB were obtained from Amresco (USA). Both the ELISA washer (1575) and reader (Model 680) were from BIO-RAD (USA). The 96-well microtiter plate was purchased from JET Biochemicals (Canada).

2.2. Bacterial strains and culture conditions

V. parahaemolyticus, Shigella dysenteria, Escherichia coli O157:H7, *Salmonella* spp, *Staphylococcus aureus, Bacillus cereus, Alccaligenes faecalis, Bacillus subtilis* and *Pseudomonas* sp were all from CGMCC (China). All strains except *V. parahaemolyticus* were cultured overnight in liquid Luria-Bertani broth with constant shaking (180 r/min) at 37℃, while the *V. parahaemolyticus* was cultured overnight at 28℃ in liquid Luria-Bertani broth containing 2.5% NaCl.

2.3. Preparation of antigen

V. parahaemolyticus was cultivated in medium of alkaline peptone water (APW) containing 3 % NaCl, incubated at 30℃ for 15-18 h. Then it was collected with physiological brine into 1×10^9 CFU/mL. At last, the bacteria liquid was added with 0.2 % formalin and treated at 30℃ for 12h, and was washed three times with sterile saline, stored at 4℃ until use.

2.4. Preparation and purification of the capture antibody and enzyme conjugated antibody against *V. parahaemolyticus*

The specific IgY was isolated by water dilution methods and ammonium sulfate precipitation, and was purified by Sephadex G-25 gel chromatography followed by DEAE-Sehparose FF chromatography. The specific IgG was harvested from rabbit immunized with *V. parahaemolyticus*. The antiserum was treated by ammonium sulfate precipitation, further purified by Sephadex G-100 gel chromatography and DEAE-Sepharose FF chromatography. Purified antibodies were collected and stored at −20 ℃.

HRP-IgG was prepared by oxidation with sodium periodate. Dissolved 3mg/ml HRP in 0.5ml double distilled water, adding 0.5ml prepared 0.06 mol/ L $NaIO_4$ solution, incubated at 4℃ for 30min； removed by adding 0.5ml of 0.16 mol/ L glycol solution, was added with 1ml of 3mg purified antibody solution after 30min at room temperature, mixing and loading dialysis bag in 0.05mol/L pH 9.5 carbonate buffer slowly stirring dialysis for 6h (or overnight) to make it binding, and then added 0.2ml $NaBH_4$ solution (5mg/ml) standing at 4℃ for 2h; added an equal volume of saturated ammonium sulfate to the mixture of the above conjugates, centrifugated at 4℃ for 30 min, then dissolved in 0.02 mol/L pH 7.4 PBS and dialyzed at 4℃ overnight; and then centrifuged to remove insoluble substances the next day, which were HRP conjugate, added equivalent pure glycerin after dissolving in 0.02 mol/L pH 7.4 PBS, stored at -20℃until use.

2.5. Sandwich ELISA

The sandwich ELISA was developed using the anti-Vp IgY as capture antibody and HRP-IgG as detection antibody. The microplate wells were coated with 100 μL of IgY. After blocked with 200 μL of blocking solution at 37 ℃ for 1 h, the samples (bacterial cells) were allowed to react with the antibodies for 2 h at 37℃.This was followed by the addition of HRP-IgG (detection antibody, 100 μg/well) and incubation was allowed for 1 h. The followed procedures were as normal, the absorbance was read at 450 nm using a microplate reader. Orthogonal experiment was applied to the optimization of sandwich ELISA. All measurements were performed in triplicates. The positive and negative were judged by the P/N values, if P/N >2.1, the result was considered to be positive. P and N were calculated as follows: P= OD450s -OD450b, N= OD450c-OD450b. Where OD450s represents the mean value of samples, OD450b is the mean value of blank well and OD450c is the mean value of negative control group.

2.6. Sensitivity of sandwich ELISA

The *V. parahaemolyticus* standard solution was diluted to 5×10^3 cfu/ml, 10^4 cfu/ml, 5×10^4 cfu/ml, 10^5 cfu/ml, 5×10^5 cfu/ml, 10^6 cfu/ml, 5×10^6 cfu/ml, 10^7 cfu/ml and tested by the established sandwich ELISA. The results were expressed by the P/N values.

2.7. Cross-reactivity of sandwich ELISA

V. parahaemolyticus and other bacteria were respectively detected at 10^6 cfu/ml by the sandwich ELISA method to study the cross-reactivity. P/N > 2.1 was considered to be the criterion of cross-reactivity.

2.8. Seafood samples detection

Yellow croaker, pomfret, prawn were minced and spiked with *V. parahaemolyticus* at different concentration of 0.1 cfu/ml, 1 cfu/ml and 10 cfu/ml, respectively. The spiked samples were cultured at 30 ℃ with shaking at the speed of 150 rpm. Detections were performed by the established sandwich ELISA at an interval of 2 h.

2.9. Statistical analysis

The student t-test was used to analyze the significant difference ($p < 0.5$) between the control group and samples. Statistical analysis software (SPSS for windows, SPSS inc., USA) was used to carry out the one-way analysis of variance and Duncan's multiple range tests on the mean value with standard deviation.

3. Results and discussion

3.1. Determination of the concentrations of capture antibody and detection antibody

With the checkerboard procedure, the appropriate concentrations of coating antibody were prepared by serial dilutions from 10 μg/ml to 320 μg/ml of IgY with a dilution factor of 2, and detection antibody was serially diluted with purified enzyme conjugated antibody（10mg/ml HRP-IgG）from 1:100 to 1:3200, antigens were added with dilution of 10^6 CFU/ml. The ELISA results with different concentrations of capture antibody IgY and detection antibody HRP-IgG have a significant effect ($p < 0.05$), for the homogeneity of variance, multiple comparisons can determine the optimal concentration of antibodies. We can see that P/N value is the largest and best when IgY concentration is 160μg/ml (Table 1). There is no significant difference when IgY concentration is 80μg/ml, 320μg/ml and 160μg/ml, and there is also no significant difference among 20μg/ml, 40μg/ml, 80μg/ml and 320μg/ml concentration of IgY. As a result, to develop an economical detecting model, the concentration of the capture antibody IgY is 20-80μg/ml.

Table 1. The multiple comparison of P/N values of different concentrations of capture antibody IgY

IgY（μg/ml）	P/N value		
	Mean	Standard error	
10	4.009	0.159	D
20	4.365	0.222	BC
40	4.313	0.223	BC
80	4.378	0.201	AB
160	4.723	0.203	A
320	4.452	0.156	AB

The table has the same letters in different rows, which mean that there is no significant difference in the 5% level.

As shown in Table 2, P/N value is the largest when the detection antibody is diluted 100 times, and the P/N value is the second when diluted 200 times, which has significantly difference with other dilution. Considering the economy, 10mg/ml enzyme conjugated antibody was diluted 200 times for subsequent use.

Table 2. The multiple comparison of P/N values of different concentrations of enzyme conjugated antibody

HRP-IgG dilution ratio	P/N value		
	Mean	Standard error	
100	7.767	0.208	A
200	5.406	0.191	B
400	4.790	0.146	C
800	3.444	0.182	D
1600	2.664	0.181	E
3200	2.165	0.156	F

The table has the same letters in different rows, which mean that there is no significant difference in the 5% level.

3.2. Determination of coating condition of capture antibody

Plates were coated with the optimal concentration of capture antibody, the coating conditions are at 4℃ overnight, 37℃ for 1h, 2h and microwave for 5min, 10min and 20min, respectively. In accordance with the steps of sandwich ELISA, the results are shown in Fig.1, the OD_{450} and P/N values are the greatest when antibody is coated at 37℃ for 1h. As a result, 37℃ for 1h was chosen as optimal coating condition.

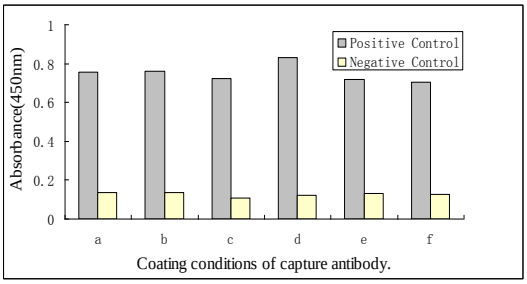

Fig. 1: Determination of optimal coating condition of antibody. a, 4℃ overnight; b, 37℃ for 1h; c, 37℃ for 2h; d, microwave for 5min; e, microwave for 10min; f, microwave for 20min.

3.3. Specificity, sensitivity and reproducibility of the sandwich ELISA

The sandwich ELISA successfully showed a high specificity for *V. parahaemolyticus* detecting, exhibiting no cross-reactivity with any other 8 representative bacteria included in the test. Under the optimized conditions mentioned above, the double-antibody sandwich ELISA procedures were conducted in triple with a set of standard serially diluted concentration of *V. parahaemolyticus*. Then a test curve was obtained, the results show that the limit of detection is 2.1×10^4cfu/ml.

The sandwich ELISA is comprised of capture and detection antibodies with high specificity due to the sandwich reaction of antibodies with antigen. Assays of serially diluted antigen by the established sandwich ELISA method showed that the linear equations of the standard curve was y=0.2992x-1.0708, with the squared correlation coefficient R^2=0.9938 (Fig. 2).

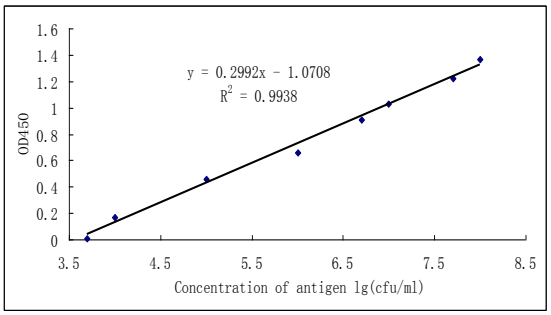

Fig. 2: The standard curve of the established sandwich ELISA

The reproducibility of the assay was determined using the intra-assay coefficient of variation (CV) and inter-assay CV derived from 3 different samples. The assay exhibited low intra-assay variability from 0.90% to 2.36%, and inter-assay variability from 0.83% to 3.15%.

3.4. Analysis of samples with *V. parahaemolyticus*

Several samples (prawn, yellow croaker and pomfret) were tested with sandwich ELISA. These results showed that the developed sandwich ELISA was well suited for *V. parahaemolyticus* analysis in real seafood samples. Results were shown in Fig. 3. With enrichment procedure at 30℃ and 150 rpm, *V. parahaemolyticus* recovers well from inoculated food samples. The results showed positive when the samples (with 0.1～1CFU/g of *V. parahaemolyticus*) are cultured for 10h, and with 1～10CFU/g for 8h.

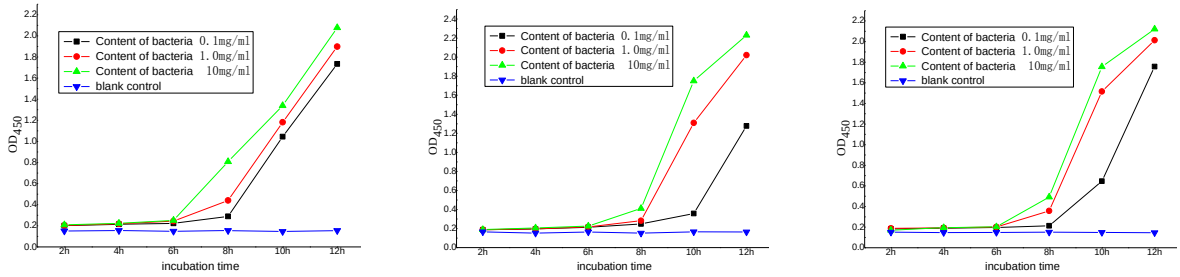

Fig. 3: The detection results of *V. parahaemolyticus* in foods. Left: prawn; Middle: yellow croaker; Right: pomfret.

4. Conclusion

The specific immunoassay developed in this study could be practical to detect *V. parahaemolyticus* in seafood. Antibodies generated in this research are with high titers and good purity, can conjugate with antigens specifically, stably and strongly. We developed an optimal sandwich ELISA with higher specificity and lower detection limit using purified IgY as the capture antibody and HRP-IgG as the detection antibody. The main procedures of the sandwich ELISA were described as following: IgY coating at 37℃ for 1h, the concentration of IgY was 20-80 μg/ml; unblocking; the concentration of HRP-IgG was 50 μg/ml. The assay showed the detection limit of 2.1×10^4CFU/ml, and has no cross reactions with other bacteria. The intra-assay coefficient of variation was from 0.90% to 2.36%. The inter-assay coefficient of variation was from 0.83% to 3.15%. Besides, the developed ELISA takes only 4-5 h which is much less than the time of traditional method. With enrichment procedure, *V. parahaemolyticus* recovers well from inoculated food samples. The results showed positive when the samples (with 0.1～1CFU/g of *V. parahaemolyticus*) are cultured for 10h, and with 1～10CFU/g for 8h. Furthermore, ELISA is a better method with easier operation and simpler procedure, with higher sensitivity and throughput but lower cost. The sandwich ELISA method developed in this study could satisfy the requirement of detection of *V. parahaemolyticus* in several seafoods in China.

5. Acknowledgements

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