Asian Fisheries Science **22** (2009): 331-336
ISSN: 0116-6514
E-ISSN: 2073-3720
https://doi.org/10.33997/j.afs.2009.22.1.031

Asian Fisheries Society, Manila, Philippines

Study on rapid detection of *Vibrio parahaemolyticus* with sea food

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Abstract

Indirect enzyme-linked immunosorbent assay (ELISA) was developed for the rapid detection of *V. parahaemolyticus* . Specific polyclonal antibody that produced in New Zealand white rabbits were used in the indirect competitive ELISA.The best reaction concentration of antigen and antibody is 10^7 CFU/ml and 1: 4,000, respectively, and ethe most effective concentration of the secondary HRP-labeled antibody is 1: 1,000. This standardized system was established for detecting *V. parahaemolyticus* in artificially infected seafood and actual seafood respectively. The detection limit is 10^4 CFU/ml, detection time is 8hours; however, the accuracy could be improved to 10^3 CFU/ml after pre-enrichment for 8hours. This system could be of practical application since it was close to the detection result by traditional detection methods.

Introduction

Presently, the methods used for the detection of *V. parahaemolyticus* in food described in the national standard generally include conventional culturing and biochemical identification, which is labor-intensive and time-consuming. These methods commonly will take about 5-7days, before final detection result, which thus could not meet the requirement of the food safety system (Jiao Hong et al., 2004). In recent years, with the development of immune technology, the Enzyme Linked Immunosorbent Assaya (ELISA) has been gradually applied to detect the pathogens in foods. However, in China no such method was developed to detect *V. parahaemolyticus* in seafood. The purpose of this research is to develop a rapid, accurate, sensitive method for detecting *V. parahaemolyticus* in seafood by ELISA.

Materials and methods

Bacterial isolotes

The bacterial isolates used in this study viz., *Vibrio parahaemolyticus*1.2164 and *Salmonella* 50041 were procured from the Institute of Microbiology, Chinese Academy of Science (CAS), whereas *Vibrio auguillaram* was preserved in our laboratory.

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Experimental rabbits

New Zealand big white rabbits purchased from Department of Laboratory Animal Science of Medical College of Fudan University, was used to prepare polyclonal sera against *V. parahaemolyticus*. Polyclonal anti-serum to *V. parahaemolyticus* was produced in 3 male NewZealand big white rabbitsfollowing the method described by WangJun et al., 2001 and preserved in refrigerator at -20°C.

ELISA

The standard difference (SD) and the variation coefficient (CV) of internal-assay of every serum were measured for 3 times. The same serum was tested in this experiment on another 5 plates, and then the ELISA experiment to calculate the variation coefficient among plates was performed (Fan Jing-feng and Liang Yu-bo 2006). ELISA were performed according to the procedure of Zhang Xiao-hua et al. (1997). Different antigen concentration Viz., 10^5, 10^6, 10^7, 10^8 and 10^9 CFU/ml and different antibody dilutions 1:2000 – 1:16000 was used in this study.

Specificity of Anti- V. parahaemolyticus polyclonal antibody measured by indirect competitive

ELISA

Added 10ì L of each test bacterial isolates at the concentration of approximately 10^7 CFU /ml as competitive antigen in each well, and added 90ì L of anti-serum as described in Zhang Xiao-hua et al. (1997). 100ì L of PBST was used as negative control, 100ì L of antiserum without addition of competitive antigen was used (Cheng Fu-sheng et al., 2004).

Preparation of seafood sample artificially infected with V. parahaemolyticus

V. parahaemolyticus on the TCBS plate was washed out with APW to prepare *V. parahaemolyticus* suspension at the concentration of 10^2,10^3,10^4,10^5,10^6 CFU/ml^{-1}. 10g of known *V. parahaemolyticus* -negative sample of big yellow-fin tuna, the shrimp, the river crab, perch was weighed respectively and divided into A and B group. 1ml of *V. parahaemolyticus* suspension at different concentration was added to prepare 5 specimens with infection gradient. The specimens were grinded for complete infection. Two blank controls were set for each group simultaneously. The infected samples were stored at 4°C in the refrigerator for three days and then taken out to room temperature. Added 90ml of APW in group A and B, respectively and then blended. The upper clear liquid of group A after the low speed centrifuge was collected for indirect ELISA detection. Group B was incubated for 8hours at 37°C for the indirect ELISA detection, performed the steps as described before.

Indirect ELISA

One hundred ì L of the upper clear liquid from sample diluted in coating buffer was dried and coated at 60 °C, and then added 100ì L of antibody diluted in 1:4000. Executed this step as described in Cheng Fu-sheng et al., 2004 before for indirect ELISA detection.

Results and Discussion

Indirect ELISA for analyzing the quality of micro plate

The result of Table 1. showed that the micro-plates interior variation coefficient was all below 10% and the variation coefficient among micro-plates (CV=8.943%) also was below 10%. These results indicated the satisfactory quality and good sorption capacity of the micro-plate.

Table 1. The quality analysis of micro plates

Number	OD_{492}								Average	SD	CV(%)
1	0.116	0.123	0.115	0.109	0.113	0.116	0.116	0.117	0.116	0.00395	3.405
2	0.118	0.123	0.116	0.121	0.114	0.115	0.118	0.121	0.118	0.00321	2.720
3	0.128	0.126	0.115	0.106	0.109	0.118	0.118	0.117	0.117	0.00749	6.401
4	0.116	0.126	0.111	0.122	0.113	0.117	0.117	0.114	0.117	0.00489	4.179
5	0.122	0.121	0.117	0.1253	0.118	0.121	0.114	0.116	0.119	0.00363	3.050
Average of microplates interior									0.1174		
SD among microplates										0.0010	
Average CV among microplates											8.943

The specificity of the anti- V. parahaemolyticus polyclonal antibody

Fig. 1 was the result of the anti-*V. parahaemolyticus* polyclonal antibody specificity experiment, which indicated that there was no cross-reaction between *V. parahaemolyticus* and *Staphylococcus aureus*, *Shigella*, *Salmonella* and *Vibrio auguillaram* ä several leading food pathogenic bacteria. All showed that the polyclonal antibody had high specificity. Therefore, the antibody was qualified for the ELISA.

The effects of coating antigen concentration on the sensitivity of indirect ELISA

Fig.2 showed the highest detection sensitivity occurred when the coating antigen concentration is 10^7 CFU /ml, which was the most effective concentration of the single factor analysis.

The effects of antibody dilution on the sensitivity of indirect ELISA

Fig.3 showed the highest detection sensitivity occurred when the antibody dilution was 1:4000, which was the most effective dilution of the single factor analysis.

The effects of IgG-HRP dilution on the sensitivity of indirect ELISA

Fig.4. showed the highest detection sensitivity occurred when the dilution of IgG-HRP was 1:1000 , which was the best IgG-HRP dilution of the single factor analysis.

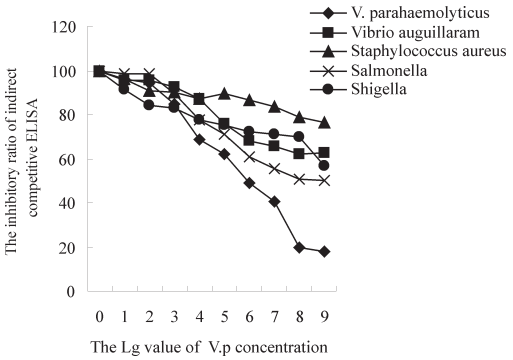

Figure 1. Determination of the specificity of the polyclonal antibody with indirect competitive ELISA

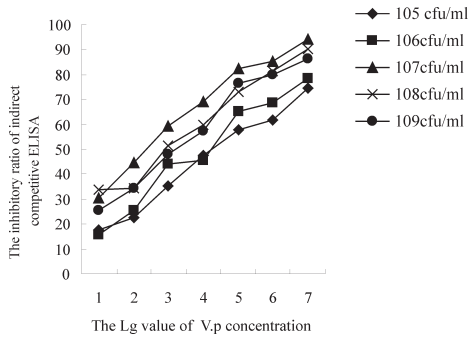

Figure 2. The effects of antigen concentration on indirect competitive ELISA curve

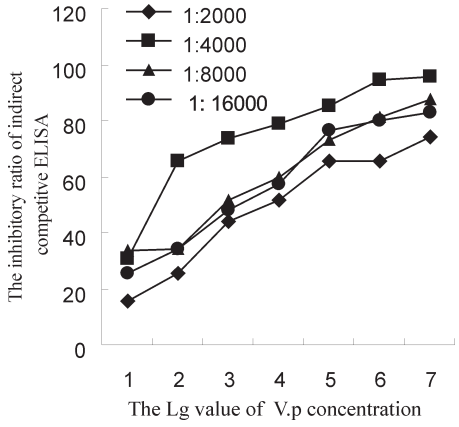

Figure 3. The effects of antibody dilution on indirect competitive ELISA curve

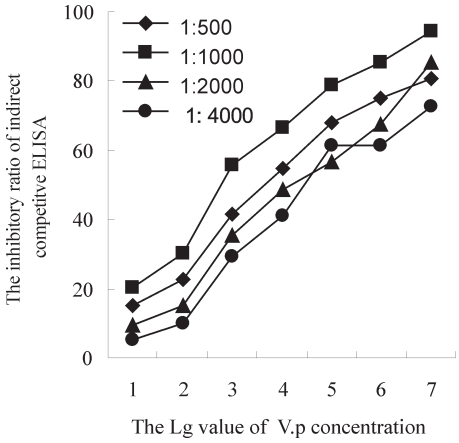

Figure 4. The effects of IgG-HRP dilution on indirect competitive ELISA curve

The detecting result of seafood infected bacteria by simulation

The results of Fig.5-8 showed that the lowest detecting concentration for *V. parahaemolyticus* was 10^4 CFU /ml with indirect ELISA before the four seafood samples were pre-enriched. However, the accuracy for *V. parahaemolyticus* was improved to 10^3 CFU /ml after the samples were pre-enriched for 8hours.

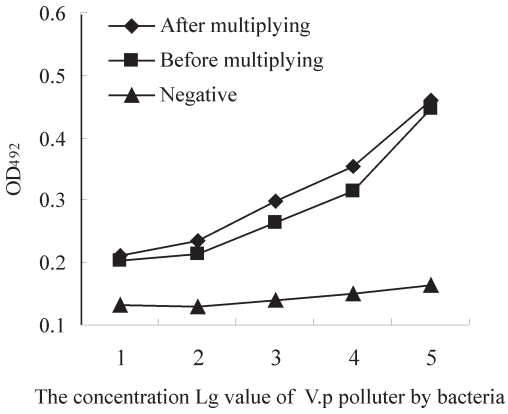

Figure 5. The result of yellow croaker polluted by bacteria artificially by simulation

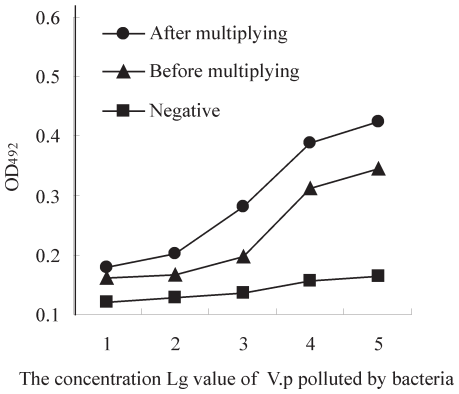

Figure 6. The result of shrimps polluted by bacteria artificially by simulation

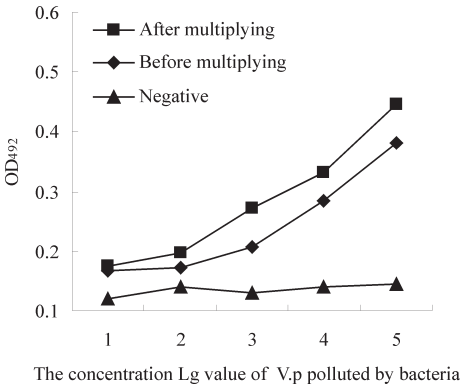

Figure 7. The result of weever polluted by bacteria artificially in simulation experiment

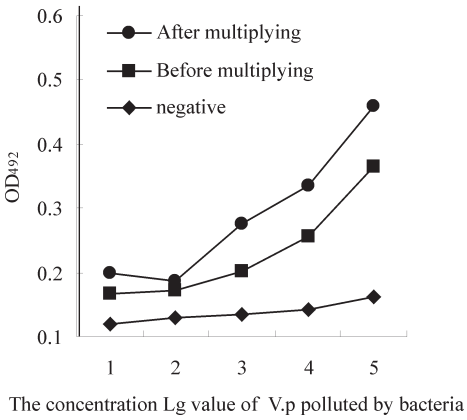

Figure 8. The result of mitten crabs polluted by bacteria artificially by simulation

In this experiment, we developed polyclonal antibody of *V.parahaemolyticus* which had high titer and specificity, and also without cross-reaction with other strains. So this work was of practical significance for detecting *V. parahaemolyticus*. The indirect ELISA method and the experiment parameters were: antigen was dried at 60!; the concentration of antigen was 10^7 CFU /ml; Blocking time was 2.5h; the work dilution of first antibody was 1:4000; the incubation time of antigen reacting with first antibody was 75min; the dilution of IgG-HRP was 1:1000; the incubation time of first antibody reacting with IgG-HRP was 60min; the reaction temperature of enzyme and0substrate was 30 ^{o}C, time was 15min; the detection limit of bacterium suspension was 10^4 CFU / ml, the incubation temperature was 37^{o}C, The minimum detection concentration of sample was 10^4 CFU /ml; the minimum detection concentration was 10^3 CFU /ml after enrichment; the detection time was 6h, however, if adding the time of enrichment, the detection time is 14hours; The advantage of this method was fairly rapid , accurate and of remarkably practical value.

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Received: 07 December 2007; Accepted: 07 November 2008