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Research Article

Development of an IMB-ELISA Method for Analyzing *Pseudomonas fluorescens*

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Abstract: To develop an immunemagnetic-bead-based enzyme-linked immunesorbent assay (IMB-ELISA), antisera were prepared12 from New Zealand white rabbits immunized with inactivated *Pseudomonas fluorescens* (*P. fluorescens*) and conjugated with magnetic-bead to get immunomagnetic-beads. IMB-ELISA would be developed through IMB collecting *P. fluorescens*. The results showed that the limit of detection ELISA and IMB-ELISA are, *respectively* $10^{1.1}$ cfu/mL and $10^{1.0}$ cfu/mL. T*he IMB-ELISA is* more sensitive and higher efficient than IC-ELISA by calculating the recovery rate and the coefficient of variation, because of *IMB* quickly enriching the target bacteria. The IMB-ELISA is a quick and accurate method for the detection of *P. fluorescens.*

Keywords: ELISA, IMB, *Pseudomonas fluorescens*

INTRODUCTION

Psychrophile bacteria is a general term for a class of bacteria, which is generally suitable for the growth between 4~20°C. In the moderndairy processing production, low-temperature storage and transport of raw milk have become indispensable to maintain its freshness, which could lead to psychrophile bacteria to become one of the important risk factors which affect the quality of dairy products (Murata *et al*., 2006).

Currently some of the psychrophile bacteria could be obtained from the raw milk, such as the *Pseudomonas*, *Alcaligenes*, *Achromobacter*, *Flavobacterium* and *Klebsiella*, *Micrococcus* (G^+), etc. The majority of them can produce thermal stability degradation of extracellular enzymes, mainly including proteases, lipases and alkaline phosphatase (Chai, 2002; Yu *et al*., 2006). The most common psychrophile bacteria found in milk is Gram-negative *Pseudomonas*, in which *Pseudomonas fluorescens* (*P. fluorescens*) is one of the major bacteria to cause the spoilage of milk at low temperature condition through producing enzymes (Gao *et al*., 2008; Ercolini *et al*., 2009; Jin *et al*., 2008; Xu, 2003).

There are several methods used for detecting *P. fluorescens* in domestic and international standards. For example, in the International Dairy Federation (IDF) (1991a, 1991b) standards, the detection of *P. fluorescens* mainly includes two colony count methods, respectively for the inoculation period of 10 days at the 4~6°C condition and 24±1 h at the 21±5°C condition. In view that the above methods are time-consuming, it is difficult for rapid detection of *P. fluorescens*. In the past decades, some new detecting methods had been developed for *P. fluorescens*, including the direct fluorescent filtration; electrical impedance method and polymerase chain amplification and so on (Lu *et al*., 2009).

Currently, Enzyme-Linked Immunosorbent Assays (ELISA) are widely used as screening method for detecting chemical residue and microorganism in foods and environmental samples under the restricted levels (Wanatabe *et al*., 2001). ELISA has several advantages of rapidness with short incubation time and simplicity without complicated clean-up procedures in analysis.

Immunomagnetic separation (immunomagentic bead-based separation, IMS), is one of the separation technology methods based on the specific antigen-antibody reaction. When the sample is mixed with the immunomagentic beads (IMB), the sample can be wrapped in the beads because of the specifically reaction between the sample and antibody which is conjugated to the Magentic Beads (MB). The compounds can be rapidly separated by NdFeB magnet (Fitzmaurice *et al*., 2004; Li and Wu, 2008). Therefore, this technology not only has the advantages of solidphase immunological reactions, such as high specificity, but can also reduce the detection time and improve the detection sensitivity (Wang *et al*., 2014). With these advantages, IMS is widely used in biomedical and food safety, such as the cell separation and the pathogen detection.

In the experiment, magnetic-bead would be conjugated with special antibody to get

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immunomagnetic-beads and the new enzyme-linked immunosorbent assay (IMB-ELISA) would be developed through IMB collecting *P. fluorescens* in the milk.

MATERIALS AND METHODS

Animal preparation: Two New Zealand white rabbits were obtained from the experimental Animal Research Institute of Hangzhou Normal University. Animal welfare and experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (The Ministry of Science and Technology of the People's Republic of China, 2006) and were approved by the animal ethics committee of Hangzhou Normal University, Zhejiang, China.

Bacterial strains: Strains of *P. fluorescens*, *Escherichia coli* O157 (*E. coli*) and *Enterococcus faecalis* were preserved in the laboratory.

Reagents and instrument: Freund's Complete Adjuvant (FCA) and Freund's Incomplete Adjuvant (FIA) were purchased from Sigma (USA); Goat Anti-rabbit IgG/HRP was purchased from Sangon Biotech (Shanghai, China); TMB-component color liquid was purchased from Aladdin (Shanghai, China); magentic beads were purchased from Huier Biotech (Luoyang, China); other reagents were routine laboratory reagents(analytical grade) and were purchased from Mike Chemical Instrument Corporation Limited (Hangzhou, China).

Main buffers and solutions: The following buffers were used in ELISA. MES buffer (0.05M, pH 6.0): Dissolve 9.76 g of MES (2-(N-morpholino) ethanesulphonic acid) in 1L deionised water, adjust pH to 6.0 with 1 M NaOH; phosphate-buffered saline (PBS, pH 7.4) containing 138 mM NaCl,1.5 mM KH2PO4, 8 mM Na2HPO4 and 2.7 mM KCl; washing buffer (PBST): PBS that contained 0.05% (v/v) Tween20; coating solution: carbonate-buffered saline (CBS, pH9.6) containing 1.59 g Na2CO3, 2.93 g NaHCO3 in 1 L water; blocking buffer: 0.01 M PBS that contained 5% (v/v) skim milk; substrate solution: TMB-component color liquid; stopping solution: 2 M H2SO4.

Immunization for rabbits: Two New Zealand white rabbits were immunized with subcutaneous and intramuscular injections of the inactivated bacteria of *P. fluorescens*. Seven days before the first immunization, the rabbits were bled from the ear vein. The initial immunization was performed by injecting 1×10^9 cfu/mL of the bacteria emulsified with 1 mL FCA. The rabbits were boosted six times at two-week intervals by injecting 1 mL solution (containing 1×10^8 cfu/mL of the bacteria emulsified with 1 mL FIA). After the third booster, each rabbit was bled from the ear vein on the 7^{th} day after each immunization. The serum titers were determined by ELISA to monitor the quality of the antisera from the immunized rabbits. The last booster was administered by injecting 1 mL stroke-physiological saline solution which contained 1×10^8 cfu/mL bacteria in it, when the titer reached a certain level. Seven days after last booster, the blood was collected from the carotid artery of each rabbit.

Separation of serum: Each blood sample was incubated in the centrifuge tube at 37°C for an hour and then placed at 4°C until the serum precipitated. The blood was centrifuged at 4°C and 5000 rpm for 10 min to separate the serum. Antisera were stored at -20°C after they were purified by bitterness-ammonium sulfate precipitation.

Activation of MB and its combination with the antibody: After the 200 μL MB were treated by using the ultrasound device (200 w, 10 min) and dispersed through shocking 30 min on the vortex concussion instrument, then the supernatant was removed after MB was completely absorbed by NdFeB magnet. The MB was washed by the 1 mL MES buffer for two times, after that it was resuspended by 100 μL MES and 100 μL EDC (10 mg/mL), the sample was mixed on the vortex concussion instrument for 30 min at room temperature, so we can get the activated MB. After MB was washed by the 1 mL MES buffer for two times, adding 500 μL antibody in the MB, the mixture had a reaction overnight at room temperature, Immunomagentic beads (IMB) would be achieved by NdFeB magnet. After IMB was washed with 1 mL MES buffer for 2 times and resuspended by 1 mL of PBS which was contained 0.1% Bovine Serum Albumin (BSA). The mixture was stored at 4°C.

Enzyme linked immunesorbent assay: For the ELISA, each well of a plate was coated with 100 μL coating antigen at the optimal dilution. After an incubation period of one hour at 37°C, the plate was washed three times with PBST for 3 min and blocked with 200 μL of blocking solution per well, followed by an additional incubation at 37°C for 1 h. After the microtitreplate was washed three times, the antiserum was diluted from $1:10^3$ to $1:128\times10^3$ and 100 μL was added to each well; the mixed solution was then incubated at 37°C for 1 h. The microtitreplate was washed three times with PBST for 3 min and 50 μL of horseradish peroxidase-labeled goat antirabbit IgG (Goat Anti-rabbit IgG/HRP) solution that had been diluted 1000 times was added to each well to serve as the secondary antibody. The microtitreplate was incubated at 37°C for 30 min. After the microtitreplate was washed three times with PBST, 100 μL of TMB

reagent was added to each well. Finally, the plate was incubated at 37°C for 10-15 min in the dark before 100 μL stopping solution was added to stop the reaction. The result was read at 450 nm using an ELISA plate reader. PBS solution and negative pre-immune rabbit serum were read as blank and negative controls.

Determination of antisera titer: Titer is the maximum dilution of serum which can produce positive reaction. When the absorbance values of negative sera absorbance value is less than 0.2 and the positive sera absorbance is 2.1 times greater than the negative control, serum dilution at this time is the titer of the antisera (Wang *et al*., 2006). The titer was generally determined by the indirect ELISA.

Determination of the optimal working concentration: The optimal working concentration was determined by the phalanx titration with the antiserum of highest titer. The antibody and antigen-coated were diluted with PBS and CBS, respectively. According to *ELISA procedures*, the antibody and antigen dilutions were selected as the best working concentration when the OD450≈1.

Establishment of the standard curve and the sensitivity determination: According to the ELISA methods, the difference is to choose the best working concentration of antigen and antibody (Antiserum or IMB) dilution in this step. This means ELISA plates were coated with 100 μL 10^5 CFU/mL colonies in each well, adding the 90 μL antibody which should be mixed with 10 μL different dilution times colonies so that the final concentration of antigen were 0, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8 cfu/mL. The specificity was determined with the IC-ELISA and the half maximal inhibitory concentration (IC50) and the detectable limitation (IC10) were calculated.

In the result, the competitive inhibition (%) = B/B_0 ×100%, where, B is the absorbance of the well with the competitor and B_0 is the absorbance of the well without the competitor.

Specific detection: These established ELISA methods were used to detect *E. coli* and *Enterococcus faecalis* to test the cross reaction of *P. fluorescens* antiserum with other bacteria. The assay specificity was evaluated by testing the Cross-Reactivity (CR) of the antibody with other pathogenic microorganisms. The CR values was calculated according to the following formula:

Cross-reactivity (%) = (IC50 of *P. fluorescens*) / (IC50 of other bacteria) ×100%

Repeatability detection:

Repeatability tests with the same plate: According to the established ELISA methods, the antisera and IMB was respectively used as the antibody to detect the concentration of *P. fluorescens* in samples. In this step, the concentration of *P. fluorescens* was known as 10^5, 10^6 and 10^7 cfu/mL, respectively in samples, repeated each concentration in 10 different wells. Blanks were included in the detection, in which the antibody was omitted and replaced by PBS. Coefficient of variation of the same plate was calculated under the different operating methods and different concentration of the *P. fluorescens*.

Repeatability tests between different plates: According to the established ELISA methods, the antisera and IMB were respectively used as the antibody to determine the *P. fluorescens* in samples. In this step, the same concentrations of *P. fluorescens* was determined in three microtiter plates, respectively, repeated each concentration in 10 different wells in one plate. Blanks were included in all experiments, in which the antibody was omitted and replaced by PBS. Coefficient of variation of the same plate was calculated under the different operating methods and different plates.

Recovery study in samples: The different concentrations of *P. fluorescens* were used as samples in the established two detection methods. In the IC-ELISA, each 0.1 mL sample was coupled with 0.9 mL antiserum; in the IMB-ELISA, each 0.1 mL sample was coupled with 0.9 mL IMB and the mixture was shocked on the vortex concussion instrument for 45 min at room temperature and magnetic separated for 3 min in order to capture the *P. fluorescens* in samples. The rate of recovery was determined for IC-ELISA and IMB-ELISA, respectively.

RESULTS AND DISCUSSION

Determination of antisera titers: During this experiment, the New Zealand White rabbits remained in good health during immunization, which indicated that the immunization dose, immune intervals and immune method were appropriate. The results demonstrated that the absorbance values of positive serum and negative was 0.436 and 0.214, respectively, the P/N = 2.06 when the dilution of the antiserum was 128000 of the first rabbit; similar result was obtained with the second rabbit, the dilution of the antiserum was 128000 while the absorbance values of positive serum and negative was 0.495 and 0.227, respectively, the P/N = 2.18. In this step, the concentration of *P. fluorescens* was 10^6 cfu/mL. The antiserum of the first rabbit will be used in the following experiment in order to ensure the accuracy of the results.

Determination of the optimal working concentrations: According to the ELISA procedures, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9 and 10^{10} cfu/mL of *P. fluorescens* were used as the coated antigen to do the

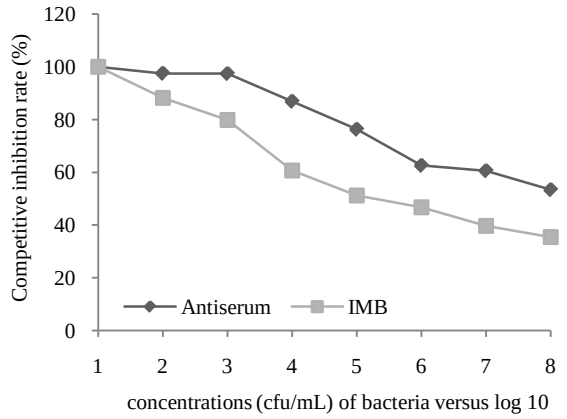

Fig. 1: ELISA standard curves for two different ELISA methods

Table 1: Result of the repeatability test in one plate

Concentrations of bacteria (cfu/mL)	Coefficient of Variation[1] (%)	Coefficient of Variation[2] (%)
10^5	8.09	7.25
10^6	4.24	0.69
10^7	2.34	2.88

*[1]: IC-ELISA; [2]: IMB-ELISA

Table 2: Result of the repeatability test in three plates

	OD450 of first plate	OD450 of second plate	OD450 of third plate	CV (%)
IC-ELISA	0.519	0.514	0.492	2.82
IMB-ELISA	0.579	0.595	0.601	1.96

Table 3: Recovery rate of *P. fluorescens* in samples

Bacteria concentration (cfu/mL)	Recovery rate[1] (%)	Recovery rate [2] (%)
10^7	88.7	97.3
10^6	95.8	114.2
10^5	93.4	107.6
10^4	101.3	98.5
10^3	93.1	100.4
10^2	88.8	95.7
10^1	84.5	91.0

*[1]: IC-ELISA; [2]: IMB-ELISA

phalanx titration. When the $OD_{450}\approx1$, the concentration of antigen was 10^5 cfu/mL and the antiserum dilution was 1:800, which was the optimal working concentration in the IC-ELISA method.

According to the above step, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9 and 10^{10} cfu/mL of *P. fluorescens* were used as the coated antigen. The different was the antibody replaced by *IMB* in the following operation steps. The optimal working concentrations as follows: the concentration of antigen was 10^5 cfu/mL and the dilution of IMB was 1:800. As can be seen from the results, these two detective methods had the same optimal working concentration.

Establishment of the standard curve and the sensitivity determination: Under optimum conditions, the 90 μL antibody with the optimum dilution was mixed with 10 μL different dilution times colonies so that the final concentration of antigens were 0, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8 cfu/mL to construct standard curves. As is shown in Fig. 1, the calculated curve was $y = -7.448x+112.8$, $R^2 = 0.949$ in the IC-ELISA and $y = -9.5655x+105.76$, $R^2 = 0.9606$ in the *IMB-ELISA*. The IC50 (concentration causing 50% inhibition of binding), which is a key criterion for evaluating the sensitivity of these methods were $10^{8.3}$ cfu/mL and $10^{5.1}$ cfu/mL, respectively. The results indicated the established *IMB-ELISA* was more sensitive than IC-ELISA for monitoring *P. fluorescens* in food.

Specific detection: The specificity was determined with the IC-ELISA and *IMB-ELISA*, respectively, to detect *E. coli* and *Enterococcus faecalis* in samples. The results demonstrated that the CR for the above-mentioned pathogenic microorganisms were all less than 0.1%, which suggested the antibody possessed a high specificity for detecting the *P. fluorescens* in samples.

Repeatability detection:

Detection of the repeatability with the same plate: Under the IC-ELISA, the coefficient of variation were 8.09, 4.24 and 2.34% when determining the *P. fluorescens* in samples of known concentration (10^5, 10^6, 10^7 cfu/mL), respectively in the same plate, the mean was 4.89%; Meanwhile, when using the *IMB-ELISA*, the coefficient of variation were 7.25, 0.69 and 2.88% in samples of known concentration (10^5, 10^6, 10^7 cfu/mL), the mean was 3.54%. The coefficient of variation were all less than 10%, it indicated these two detection methods have a good repeat accuracy and compared with IC-ELISA, the *IMB-ELISA* has a better repeatability (Table 1).

Detection of the repeatability between different plates: The 10^5 cfu/mL of *P. fluorescens* was determined in three microtiter plates, respectively. Under the IC-ELISA, the coefficient of variation was 2.82% between three different plates; Meanwhile while using the *IMB-ELISA*, the coefficient of variation was 1.96%. The coefficient of variation were all less than 10%, it indicated these two detection methods have a good repeatability and compared with traditional IC-ELISA, the *IMB-ELISA* has a better repeatability (Table 2).

Recovery rate in samples: To these established detection methods, IC-ELISA and *IMB-ELISA*, the recovery rates were shown in Table 3. When the concentration of *P. fluorescens* was more than 10^1 cfu/mL, the mean recovery rate was 92.2% in IC-ELISA and 100.6% in the *IMB-ELISA*, respectively. They can basically meet the needs of the actual

application; however, *IMB-ELISA* had a better recovery rate than the former.

CONCLUSION

Aiming at the detection of *P. fluorescens* in milk, two different methods were established, IC-ELISA and *IMB-ELISA*. Compared with some traditional enrichment culture assays, the two detection methods are more time-saving and have a better detection result and they can basically meet the actual needs. The limit of detection (the concentration causing 90% inhibition of binding) are $10^{1.1}$ cfu/mL and $10^{1.0}$ cfu/mL, respectively. In contrast to the IC-ELISA, the *IMB-ELISA* is more sensitive and higher efficient by calculating the recovery rate and the coefficient of variation because of *IMB* quickly enriching the target bacteria. It can be speculated that *IMB-ELISA* would have a good prospect in detecting *P. fluorescens.*

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REFERENCES

Chai, T.T., 2002. Test on endospore, heat-resistant endospore, psychrophile and antibiotic residues in raw milk. China Dairy, 3: 28-30.

Ercolini, D., F. Russo, I. Ferrocino and F. Villani, 2009. Molecular identification of mesophilic and psychrotrophic bacteria from raw cow's milk [J]. Food Microbiol., 26(2): 228-231.

Fitzmaurice, J., G. Duffy, B. Kilbride, J.J. Sheridan, C. Carroll and M. Maher, 2004. Comparison of a membrane surface adhesion recovery method with an IMS method for use in a polymerase chain reaction method to detect Escherichia coli O157:H7 in minced beef. J. Microbiol. Meth., 59: 243-252.

Gao, Z.P., T.L. Yue, Y.H. Yuan and F. Qin, 2008. Separation and identification of hypothermal spoilage organisms from dairy products. J. Chinese Inst. Food Sci. Technol., 8(1): 67-71.

International Dairy Federation, 1991a. Enumeration of Psychrotrophic Microorganisms Colony Count Technique at 6.5 ºC. FIL-IDF Standard No. 101A, Brussels, Belgium.

International Dairy Federation, 1991b. Estimation of Numbers of Psychrotrophic Microorganisms: (Rapid Colony Count Technique, 25 Hours at 21 ℃). Fed Int Laiterie-Int Dairy Fed Standard No. 132A. Brussels, Belgium.

Jin, L., G.S. Fan and J.P. Lv, 2008. The research on heat resistant lipase and its effect on UHT milk quality. Food Res. Dev., 29(4): 173-177.

Li, Y.J. and S.Q. Wu, 2008. Application of immunomagnetic separation technique in detection of food-borne diseases. Sci. Technol. Food Ind., 2: 248-251.

Lu, Y., Y.M. Yin, J.N. Tang and D.H. Liu and X.Q. Ye, 2009. Research progress on rapid methods for detecting psychrophilic bacteria in raw milk. Food Sci., 30(3): 274-280.

Murata, Y., T. Homma, E. Kitagawa, Y. Momose, M.S. Sato *et al*., 2006. Genome-wide expression analysis of yeast response during exposure to 4°C. Extremophiles, 10(2):117-128.

The Ministry of Science and Technology of the People's Republic of China, 2006. Guidance Suggestions for the Care and Use of Laboratory Animals. Sep. 30.

Wanatabe, S., S. Ito, Y. Kamata, N. Omoda, T. Yamazaki *et al*., 2001. Development of competitive enzyme-linked immunosorbent assays (ELISAs) based on monoclonal antibodies for chloronicotinoid insecticides imidacloprid and acetamiprid. Anal. Chim. Acta, 427(2): 211-219.

Wang, X.H., K. Xu, Y. Wang, R.K. Wang, X.M. Shi *et al*., 2006. An indirect competitive ELISA method for quantitative analysis of Staphylococcal enterotoxin B. Food Sci., 27(8): 227-231.

Wang, Y.K., Y.C. Wang, H.A. Wang, W.H. Ji, J.H. Sun *et al*., 2014. An immunomagnetic-bead-based enzyme-linked immunosorbent assay for sensitive quantification of fumonisin B1. Food Control, 40: 41-45.

Xu, B.Q., 2003. Cold-addicted germ in dairy products. China Food Ind., 3: 50.

Yu, W.J., R.Q. Wang and Q.J. Zhao, 2006. Study of the factor which will influence the quality of the UHT milk and related measurement. China Dairy Ind., 34(6): 55-57.