Evaluation of Method of Visual Gene Chip Detection for Four Pathogenic Bacteria in Aquatic Products

Lin Lu
National Institute of Metrology, China, Beijing 100029, China

Keywords: Aquatic Products, Pathogenic Bacteria, Visual Detection, Gene Chip, Evaluation.

Abstract: In this manuscript, the author developed a gene chip detection method that combined tyramine signal amplification technology and nanogold-labeled silver staining technology. Four pathogenic bacteria (Listeria monocytogenes, Vibrio parahaemolyticus, V. cholera, and Staphylococcus aureus) in aquatic products were used as targets to evaluate the sensitivity, specificity and repeatability of this method. The detection sensitivity of this method can reach 103CFU/mL, and it is no different from the specificity of fluorescence detection. The coefficient of variation CV value of the hybridization repeatability results of different points on the same chip and different batches of chips were also less than 15%. The sensitivity, specificity, and reproducibility of the four visual gene chip detection methods for pathogenic bacteria in aquatic products all show good performance, which has practical promotion significance in the detection of pathogenic bacteria in aquatic products.

1 INTRODUCTION

Food safety has become a widespread and far-reaching social issue. With the development of society and people's concerns regarding health, the safety and health of aquatic products is receiving increasing attention. Consumers and government authorities are paying more and more attention to the quality and safety of aquatic products, and the safety and quality management of aquatic products has become an important factor in ensuring food quality and promoting social stability (Gao et al., 2007). China is one of the largest producers of aquatic products in the world. Over the past 20 years, total aquatic production has continuously grown, and China's aquatic production reached about 49 million tonnes in 2011, accounting for 35% of the world's aquatic production, ranking it first in the world. In particular, aquaculture production accounted for more than 70% of the world total (Weng et al., 20211, Shuai et al., 2011). However, the detection rate of Listeria monocytogenes in salmon in China was 5–7%, of Vibrio parahaemolyticus in elephant mussels was 9–10%, and of V. parahaemolyticus in jumbo crab was 7–8%. Vibrio cholerae, a national Class I controlled infectious disease, had a detection rate of about 3%, and pathogenic Escherichia coli and Salmonella were also detected at high rates (Wang and Tao, 2009, Wang et al., 2008, Jin et al., 2008).

Internationally, the European Union, the United States, Japan, and others have strengthened their health and safety testing measures for imported aquatic products or increased their detection programs. For example, the European Union stipulates that the four pathogenic Vibrio species with the highest risk − V. parahaemolyticus, V. alginolyticus, V. cholerae and V. vulnificus – should not be detected in shrimp and fish products, and other aquatic products also have similar requirements for corresponding Vibrio species. Because the quality of China's exports of aquatic products often varies and can exceed the required acceptable pathogen levels of importing countries, products are often rejected, creating a series of "green barriers". Such measures result in greater challenges to China's aquatic products export trade (Bobrow et al., 1989).

Because chilled and fresh aquatic products are not easy to preserve, a rapid and sensitive detection method is urgently needed to protect the interests of businesses and the health of consumers. The existing methods of detecting pathogenic bacteria in aquatic products in China, whether for import or export industry standards, or national standards, take a long time. Negative results need at least 3–4 days, and positive results need 5–10 days. Existing detection

Lu, L.
Evaluation of Method of Visual Gene Chip Detection for Four Pathogenic Bacteria in Aquatic Products.
DOI: 10.5220/0011595700003430
In *Proceedings of the 8th International Conference on Agricultural and Biological Sciences (ABS 2022)*, pages 50-55
ISBN: 978-989-758-607-1; ISSN: 2795-5893

methods such as immunochromatography, ELISA, and PCR can only detect one kind of foodborne pathogenic bacteria at a time. Many experiments are needed to identify or investigate a suspicious sample, and the operation is cumbersome and time-consuming, and cannot meet the needs of multiple detection (Meany et al., 2011, Deng et al., 2011, Qi et al., 2010). To address this need, the author has combined tyramine signal amplification technology and nanogold-labeled silver staining technology. A visualized gene chip detection method was established for simultaneous detection of four pathogenic bacteria in aquatic products: L. monocytogenes, V. parahaemolyticus, V. cholerae, and Staphylococcus aureus.

2 MATERIALS & METHODS

Strains: The standard strains used in this experiment were selected by the Academy of Military Medical Sciences: L. monocytogenes, V. parahaemolyticus, V. cholerae, and S. aureus. The strain names and standard strain numbers are shown in Table 1.

Table 1: Experimental standard strains and their numbers.

Strain	Bacteria number
Listeria monocytogenes	54003, 54005, 54006, 54007, 7644
Vibrio parahaemolyticus	20502, 20506, 20507, 20511
Vibrio cholerae O139	M045
Staphylococcus aureus	26001, 26111, 26113, 13565, 27661

Clinical samples: The 10 positive reference strains used in our laboratory were positive strains of imported aquatic products. They were identified as pathogenic bacteria of L. monocytogenes, V. parahaemolyticus, V. cholerae and S. aureus by conventional culture methods. The samples were cultured in enrichment solution for 3 h before use.

Instruments and reagents: The instruments used included an iCycler PCR instrument (Bio-Rad Co.), GenePix 4000B scanner (Axon Co.), PixSys 5000 spotter (Cartesian Co.), and Model 8909 DNA synthesizer (ABI Co.). The PCR-related reagents were purchased from Bao Biological (Dalian) Co. Ltd., gene chip substrate from CEL Associates Co., Cy3 fluorescent dye from Amersham Co., tyramine signal amplification-Cy3 reagent from PE Co., and streptavidin–HRP and coupling buffer from Sigma Co.

Evaluation of gene biochip specificity: The standard strains of the target pathogenic bacteria and the standard strains of related proximate and distant genera were selected and amplified by multiplex PCR with universal primers. They were then hybridized with a variety of pathogenic detection probes obtained by screening, and the specificity of each probe on the gene chip was systematically evaluated.

Evaluation of gene chip sensitivity: The sensitivity standards of various pathogenic bacteria were provided by the China Institute of Drug and Biological Product Identification and the Institute of Microbiology and Epidemiology of the Academy of Military Medical Sciences. The standards were diluted with saline in a 10-fold gradient, and the DNA processed with reference to the genomic DNA extraction procedure.

Evaluation of gene chip repeatability: Three batches of L. monocytogenes were randomly selected from different batches of biochips for repeatability tests. Then three chips were randomly selected from three different batches of chips, and the detection results obtained by multiplex PCR amplification and gene chip hybridization were statistically analyzed to evaluate the repeatability of the chips.

Preparation of fresh shrimp liquid contaminated with L. monocytogenes: Fresh shrimp detected by conventional culture methods in our laboratory were pulped by apparatus, and 1 mL of serially diluted L. monocytogenes DNA diluent (10^1–10^6 CFU/mL) was added to each 0.1 g of fresh shrimp pulp to make a final concentration of 10^1–10^6 CFU/mL/0.1 g suspension of fresh shrimp pulp. Then 9 mL of 75% ethanol was added, mixed, and centrifuged at 100×g for 1 min to remove large particles. The supernatant was transferred to a new centrifuge tube and centrifuged at 10,000×g for 10 min. Then the supernatant was carefully aspirated, sterilized saline was added for one washing, and DNA extract added to the final precipitate, boiled for 10 min, and centrifuged. Of the supernatant, 8 μL was used for PCR detection. Preparation of sensitivity simulation samples of V. parahaemolyticus, V. cholerae, and S. aureus: V. parahaemolyticus, V. cholerae, S. aureus sensitivity simulation samples were prepared referring to the previous section.

3 RESULTS

3.1 Specificity of Gene Biochip Detection

To examine the specificity of the method for the detection of the four pathogenic bacteria, the positive and negative references provided in Table 1 and from the Academy of Military Medical Sciences were compared. The results of gene biochip hybridization showed that the probes for the four pathogenic bacteria were highly specific under the above optimized reaction system (Figure 1). For the other non-target pathogenic bacteria, no fluorescent signal appeared at any probe position except for the positive signal of the internal control probe, while no fluorescent signal appeared for the negative probe or the blank control (Azizipour et al., 2020).

3.2 Determination of Cutoff Value

Cutoff value is the criterion to determine whether the fluorescence signal value of a gene chip is positive or not. In this experiment, the cutoff value of the probes was calculated and determined based on the specificity evaluation. For each probe, a positive strain, a negative strain, and a blank were selected for gene chip hybridization. The hybridization fluorescence signal value of the positive strain was much higher than the background value of the negative strain and the blank control hybridization (Figure 1). Through repeated experiments, we determined the background statistical average of the negative strain and the blank control + 2 standard deviations as the cutoff value of each probe (Table 2).

Table 2: Cutoff values of four aquatic product pathogenic bacteria probes.

Bacterial name	Fluorescence mean of negative bacteria and blank control	Standard deviation	Cutoff value
Listeria monocytogenes	485.25	24.89	535.03
Vibrio parahaemolyticus	586.75	34.13	655.01
Staphylococcus aureus	696.27	52.60	801.47
Vibrio cholerae O139	467.6	36.15	539.9
Vibrio cholerae non-O139	278.625	30.28	339.185
Internal control	221.16	22.90	266.96

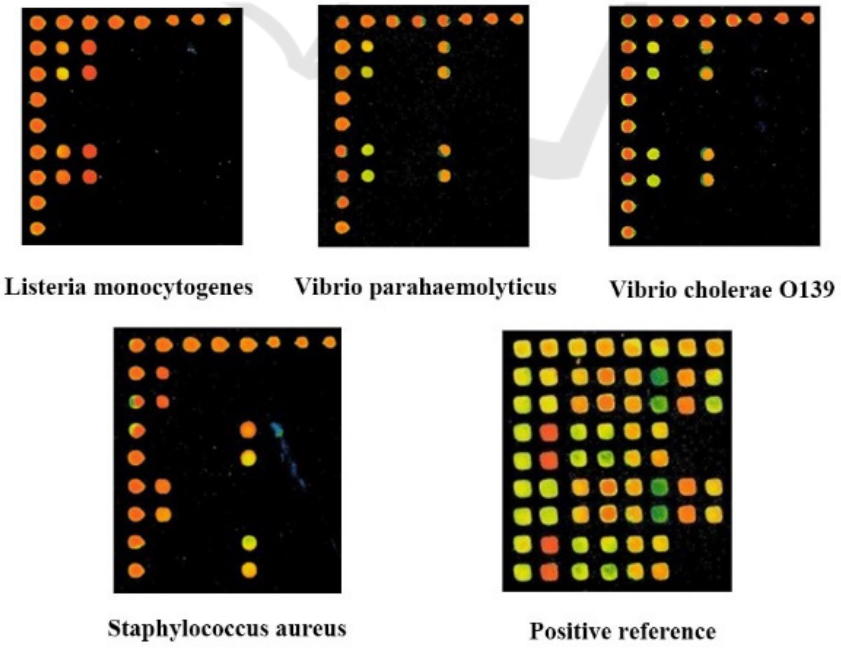

Figure 1: Specificity detection results.

3.3 Sensitivity of Pure Culture Pathogenic Bacteria

In this experiment, the sensitivity of pure cultures of L. monocytogenes, V. parahaemolyticus, V. cholerae, and S. aureus were evaluated separately. The sensitivity of the detection was determined according to the cutoff values of the probes (Table 2) after the initial concentrations of the above target bacteria were diluted with saline in a 10-fold gradient, directly boiled and lyse, PCR amplified, and hybridized. The results are shown in Figures. 2–5 (Park et al., 2018, Bunin et al., 2020).

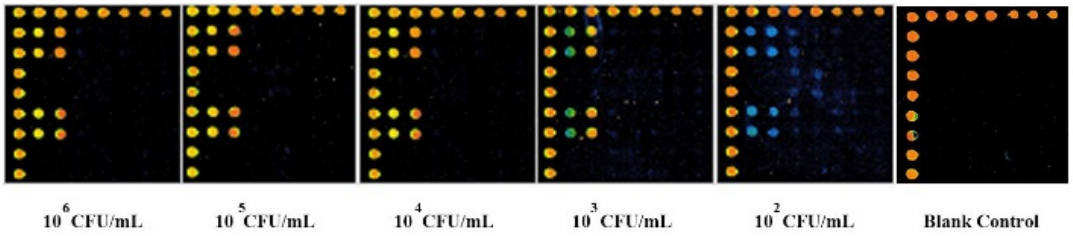

Figure 2: Sensitivity of detection of Listeria monocytogenes.

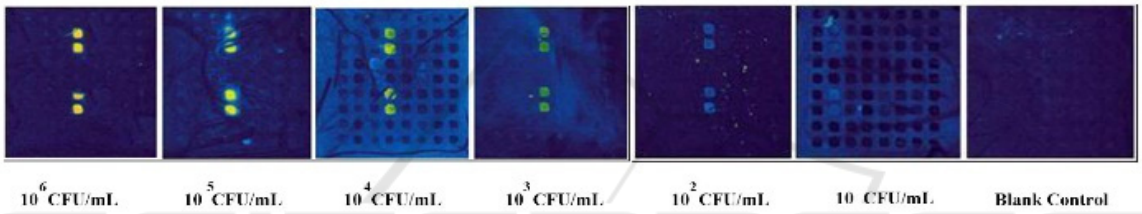

Figure 3: Sensitivity of detection of Vibrio parahaemolyticus.

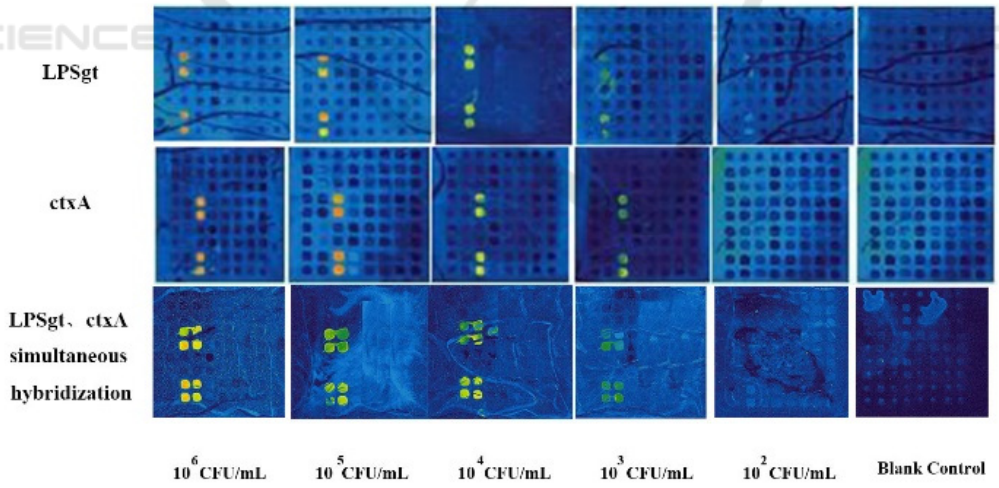

Figure 4: Sensitivity of detection of Vibrio cholera.

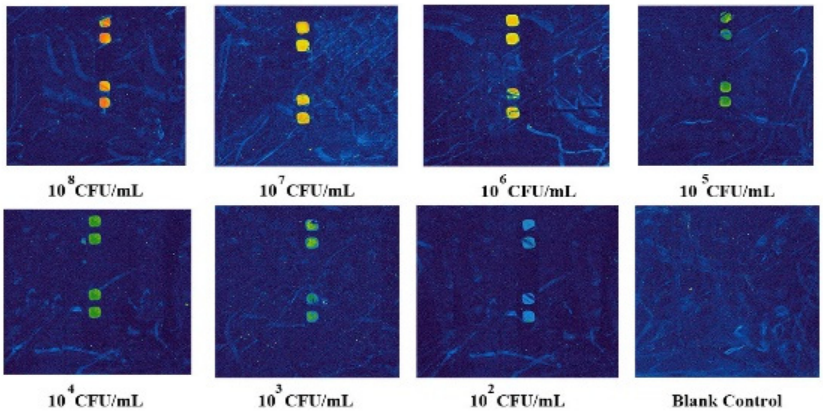

Figure 5: Sensitivity of detection of Staphylococcus aureus.

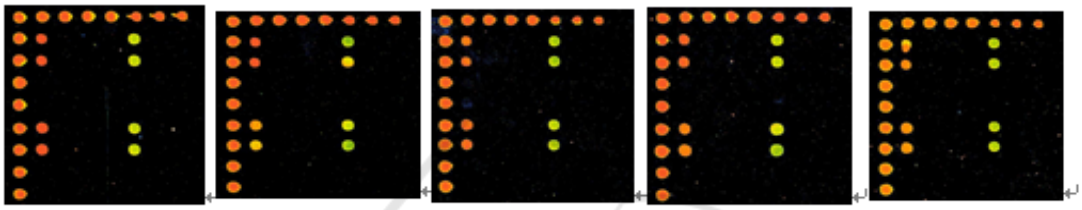

Figure 6: Gene biochip repeatability evaluation.

The sensitivity of all four pathogenic bacteria reached 10^3CFU/mL (Figures 2–5), while L. monocytogenes and S. aureus corresponding to 10^2 CFU/mL showed fluorescent signals in the hybridization plot, but the signal value was lower than the cutoff value of the probe of the bacteria, so it could not be taken as a positive result. Meanwhile, we observed from Figure 4 of hybridization of LPSgt and ctxA genes of V. cholerae O139 that when hybridizing one gene alone, the fluorescence signal value of each gradient was higher than the value of simultaneous hybridization. Apparently two genes hybridized at the same time affected each other, probably because the annealed PCR products hybridized with each other, reducing the amount of hybridization with the corresponding probe, but the statistical analysis of simultaneous hybridization showed that sensitivity reached 10^3 CFU/mL (Dou et al., 2019).

3.4 Repeatability of Gene Biochip Detection

Listeria monocytogenes was selected to evaluate the repeatability of the gene biochip. The PCR amplification and gene biochip hybridization were performed with 10^6 CFU/mL of the pathogenic bacteria, and a negative control without a template was set up. The experiment was repeated five times and intra- and inter-slice repeatability were calculated, thus assessing the repeatability of this method of detection. The results of the experiments are shown in Figure 6, and the statistical analysis results are shown in Table 3. For the single-amplified L. monocytogenes probe, the coefficients of variation (CV) of the repeatability of different spots on the same chip were less than 15%, and the CVs of the hybridization repeatability results of different batches of spot systems of the chip were also less than 15%, indicating that repeatability of detection was good (Park et al., 2018).

Table 3: Statistical results of gene biochip repeatability evaluation.

Repeatability	Number of experiments	Internal control probe 1	*Listeria monocyt ogenes* 1
Intra-Slice repeatability V (%)	1	8.31	6.38
	2	7.23	9.46
	3	5.29	8.33
	4	9.33	8.74
	5	7.45	9.12
Inter-slice repeatability CV (%)		7.82	8.65

4 DISCUSSION

Cutoff value is a criterion to determine whether a hybridization fluorescence signal is positive or not. In this experiment, the background values of positive fluorescence signal value, negative fluorescence signal value, and blank control were statistically analyzed through multiple repetitions, and the corresponding cutoff value determined for each probe (Table 2). The cutoff value is not only used to judge the hybridization results, but also provides a reliable basis for the evaluation of sensitivity. The sensitivity of this experiment was respectively evaluated for L. monocytogenes, V. parahaemolyticus, V. cholerae, and S. aureus, and the sensitivity reached 10^3 CFU/mL. This experiment evaluated the reproducibility with L. monocytogenes and showed that the CV of inter- and intra-slice reproducibility of this gene chip was less than 15%, indicating that the method was accurate, reliable, and repeatable.

Therefore, the results of this method for the evaluation of gene chip detection for the visualization of the four aquatic pathogenic bacteria showed the high specificity of each probe, the intra- and inter-slice CV was less than 15%, the repeatability was good, and the sensitivity of the four pathogenic bacteria reached 10^3 CFU/mL. Therefore, the detection method can be used for sensitive and high-throughput detection of four kinds of aquatic products pathogens, which provides fast and accurate detection technology for improving the pathogenic bacteria of imported and exported aquatic products, and is of great significance for ensuring the quality and safety of imported and exported aquatic products.

REFERENCES

Azizipour, N., Avazpour, R., Rosenzweig, D. H., et al (2020). Evolution of biochip technology: A review from lab-on-a-chip to organ-on-a-chip. *Micromachines*, 11(6): 599.

Bobrow, M. N., Harris, T. D., Shaughnessy, K. J., et al (1989). A novel method of signal amplification. *Journal of Immunology Methods*, 125(2):279–85.

Bunin, E., Khatisashvili, G., Varazi, T., et al (2020). Study of arsenic-contaminated soil bacterial community using biochip technology. *Water, Air, & Soil Pollution*, 231(5): 1-12.

Deng, Z., Ge, Y., Cao, Q., et al (2011). The detection of a transgenic soybean biochip using gold label silver stain technology. *Bioorganic & Medicinal Chemistry Letters*, 21(22):6905–8.

Dou, M., Macias, N., Shen, F., et al (2019). Rapid and accurate diagnosis of the respiratory disease pertussis on a point-of-care biochip. *EClinicalMedicine*, 8: 72-77.

Gao, S., Xie, M. and Jin, D., et al (2007). Study on the establishment of a method for the detection of common pathogenic microorganisms in aquatic foods using gene chip technology. *Biotechnology Communication*, 18(1):72–6.

Jin, D., Qi, H., Chen, S., et al (2008). Simultaneous detection of six human diarrheal pathogens by using DNA biochip combined with tyramide signal amplification. *Journal of Microbiology Methods*, 75(2):365–8

Meany, D. L., Hackler, L. Jr., Zhang, H., et al (2011). Tyramide signal amplification for antibody-overlay lectin biochip: a strategy to improve the sensitivity of targeted glycan profiling. *Journal of Proteome Research*, 10(3):1425–31.

Park, M., Kang, B. H., Jeong, K. H. (2018). Based biochip assays and recent developments: A review. *BioChip Journal*, 12(1): 1-10.

Qi, H. J., Chen, S. H., Zhang, M. L., et al (2010). DNA biochips for visual detection of human pathogenic microorganisms based on tyramine signal amplification coupled with gold label silver stain. *Analytical And Bioanalytical Chemistry*, 398(6):2745–50.

Shuai, J., Fu, L., Zhang X., et al (2011). Rapid detection of major pathogenic Vibrio spp. in aquatic products by dual-channel fluorescent PCR. *Chinese Journal of Food*, 11(5):151–7.

Wang, N. and Tao, Y. (2009). Establishment of a multiplex PCR method for the detection of three pathogenic bacteria in aquatic products. Journal of Food Biotechnology, 28(3): 397–402.

Wang, Y., Long, L., Cai, X., et al (2008). Isolation and serological identification of Salmonella in aquatic products. *Inspection and Quarantine Science*, 18:42–44.

Weng, S., Zhu, J., and Li, J. (2011). Establishment and evaluation of a multiplex PCR method for the detection of four common pathogenic bacteria in aquatic products. *Journal of Aquaculture*, 35(2):305–14.