Application of DNA Chips for Rapid and Comprehensive Diagnosis of Fish Pathogens

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Outbreak of infectious diseases caused by viruses, bacteria and parasites could cause severe economic losses in aquaculture. The precise diagnosis and appropriate treatment in the early stage of the outbreak is therefore important in order to prevent further spread of the aquatic disease. This article is based on a study conducted by researchers of the Aquatic Animal Health Division of the National Research Institute of Aquaculture, Fisheries Research Agency, Japan, which aims to develop DNA chips for the immediate diagnosis of pathogens in aquaculture.

It has been reported that in Southeast Asia, the occurrence of viral diseases in many cultured shrimps and fishes have caused devastating losses in aquaculture production. White spot syndrome (WSS) of the black tiger shrimp (*Penaeus monodon*) and viral nervous necrosis (VNN) of marine fishes are among the examples of such viral diseases that occurred in the Southeast Asian region. A number of researches have been conducted to establish and standardize diagnostic techniques for such diseases by using the polymerase chain reaction (PCR) method, which is applicable and most practical for the region (Nagasawa, 2005).

Specifically, for the detection of specific region of the genome in targeted pathogen, the PCR method has been considered as the most popular and rapid diagnostic method. Moreover, the multiplex PCR method would allow the detection of several targets at a time. However, although PCR-based diagnostic method performs high-sensitivity, it could sometimes make non-specific reaction. The use of DNA-DNA hybridization has been found to detect a target DNA fragment from a pool of DNA fragments with high accuracy and sensitivity. Thus, it could overcome a drawback of the PCR-based method. The DNA-DNA hybridization based diagnostic DNA chips had been developed for multiple species of fish pathogens. The DNA chips consisted of an array of pathogen specific DNA probes, and are able to discriminate particular DNA fragments from DNA samples which are prepared from the specimens. The principles of the DNA-DNA hybridization are shown in **Fig. 1**.

DNA chip for detection of multiple bacterial pathogens (16S chip)

For the multi-bacterial 16S DNA chip construction (**Fig. 2**), fifty bases of oligonucleotide[1] probes specific to the 16S rRNA[2] gene in individual species of fish bacterial pathogens were spotted on a nylon membrane. For diagnosis, 16S rRNA gene was amplified by PCR using universal 16S rRNA primers from the bacterial specimens and Digoxigenin-11-

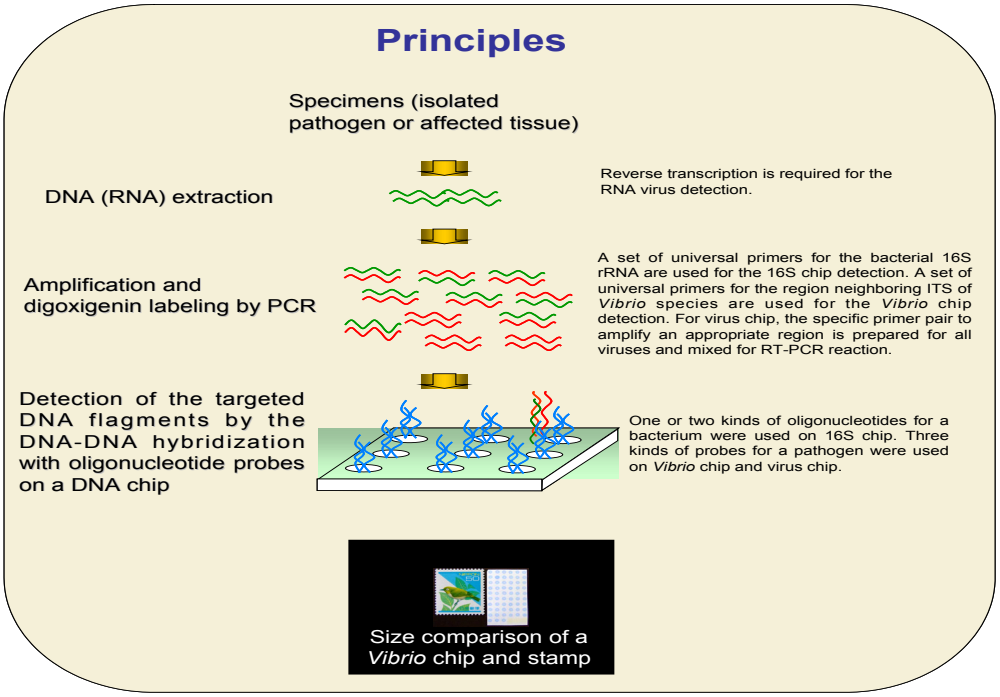


Fig. 1. DNA-DNA hybridization process

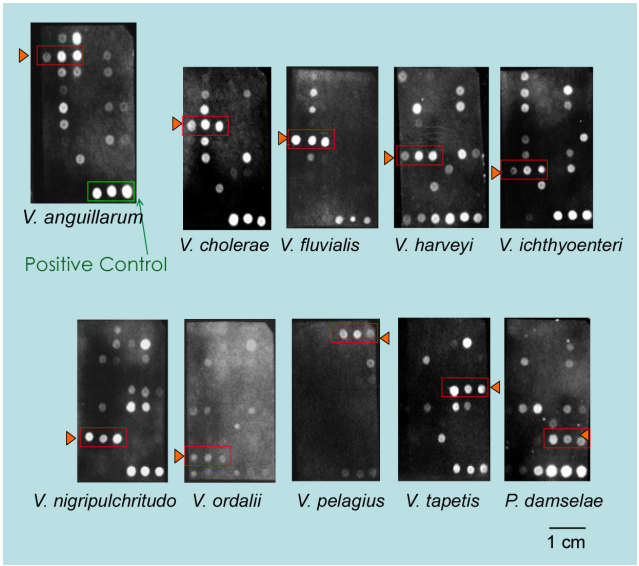
dUTP was incorporated to the PCR products. The amplified PCR product was subjected to hybridization on the DNA chip to determine the species of the bacterial specimen. Alkaline phosphatase-conjugated anti-DIG monoclonal antibody was used to detect the signals.

DNA chip for detection of *Vibrio* species (*Vibrio* chip)

PCR-amplified intergenic transcribed spacer (ITS) between 16S and 23S rRNA gene of individual *Vibrio* species were spotted on the *Vibrio* chip (**Fig. 3**). The ITS region from the specimens was amplified with universal primers, then subjected to hybridization following the same procedure as with the 16S chip and was used for detection of the *Vibrio* species.

Detectable species

1. *V. alginolyticus*
2. *V. anguillarum*
3. *V. carcariae*
4. *V. cholerae*
5. *V. fluvialis*
6. *V. harveyi*
7. *V. ichthyoenteri*
8. *V. nigripulchritudo*
9. *V. ordalii*
10. *V. parahaemolyticus*
11. *V. pelagius*
12. *V. penaeicida*
13. *V. salmonicida*
14. *V. splendidus*
15. *V. tapetis*
16. *V. tubiashii*
17. *V. vulnificus*
18. *P. damsela*
19. *Vibrio* sp. from striped jack
20. positive control



There may be cross-hybridization, and the pattern is reproducible and also useful information

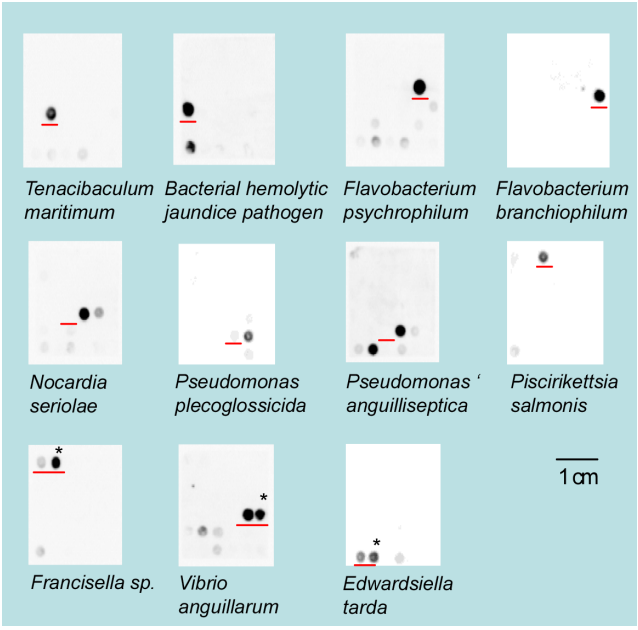
Fig. 2. Images of hybridization on the 16S chip

DNA chip for detection of viral pathogens (virus chip)

For the virus chip, 50 bases of fish virus specific oligonucleotide probes were spotted. To detect both RNA and DNA viruses by the virus chip, DIG-labeled[3] RT-PCR[4] product was amplified by RT-PCR using a mixture of PCR primer sets for 15 fish viral species.

Detectable bacterial species

1. *Francisella* sp. From fishes
2. *Francisella* sp. From abalones
3. *Piscirikettsia salmonis*
4. *Renibacterium salmoninarum*
5. *Streptococcus dysgalactiae*
6. *Lactococcus garvieae*
7. *Streptococcus iniae*
8. *Streptococcus parauberis*
9. *Streptococcus agalactiae*
10. *Flavobacterium columnare*-1
11. *Flavobacterium columnare*-2
12. *Flavobacterium columnare*-3
13. *Flavobacterium psychrophilum*
14. *Flavobacterium branchiophilum*
15. Bacterial hemolytic jaundice pathogen
16. *Tenacibaculum maritimum*
17. *Mycobacterium marinum*
18. *Nocardia seriolae*
19. *Vibrio anguillarum*
20. *Photobacterium damselae*
21. *Vibrio* sp. from striped jack
22. *Pseudomonas anguilliseptica*
23. *Pseudomonas plecoglossicida*
24. Abalone withering syndrome pathogen
25. *Edwardsiella tarda*
26. *Yersinia ruckeri*
27. *Aeromonas hydrophila*
28. *Aeromonas salmonicida*



*Cross-hybridization was observed between related species

Fig. 3. Images of hybridization on the Vibrio chip

[1] Oligonucleotide is a short nucleic acid polymer, composed of DNA that is used in PCR analysis

[2] rRNA - ribosomal ribonucleic acid is the RNA component of the ribosome, the protein manufacturing machinery of all living cells

[3] Digoxigenin-labeled

[4] RT-PCR - Reverse Transcription PCR is a variant of PCR used to generate many copies of a DNA sequence

Detectable viral species

1. Iridovirus
   - RSIV (red sea bream iridovirus)
   - ISKNV (infectious spleen and kidney necrosis virus)
   - TRBIV (turbot reddish body iridovirus)
2. GIV (grouper iridovirus)
3. LCDV (lymphocystis disease virus)
4. FHV (flounder herpes virus)
5. HGRV (hosigarei rhabdovirus or spotted halibut rhabdovirus)
6. HRRV (hirame rhabdovirus)
7. VNNV (viral nervous necrosis virus)
8. Birnavirus
   - YTAV (yellowtail ascites virus)
   - IPNV (infectious pancreatic necrosis virus)
9. VHSV (viral hemorrhagic septicemia virus)
10. OMV (*Oncorhynchus masou* virus)
11. EHNV (epizootic hematopoietic necrosis virus)
12. KHV (koi herpes virus)
13. IHNV (infectious hematopoietic necrosis virus)
14. SVCV (spring viremia of carp virus)
15. PFRV (pike fry rhabdovirus)

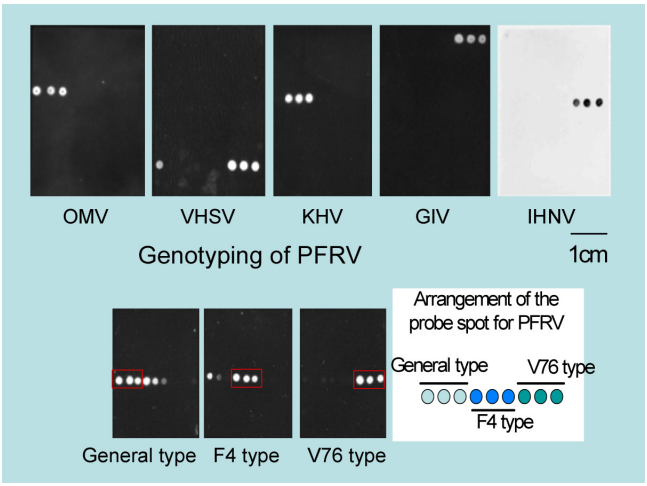


Fig. 4. Images of hybridization on the virus chip.

Discussion

Twenty-eight (28) bacterial species were detected by the DNA chip targeting 16S rDNA. Seventeen (17) *Vibrio* species and two *Photobacterium* species were discriminated by *Vibrio* chip targeting the ITS region, while fifteen (15) viral species can be discriminated by virus chip. These results indicate that DNA chip is useful for the comprehensive detection of fish pathogens. Simple equipments, such as a thermal cycler, a shaker and a 42 m^2 incubator are required for this diagnosis. The cost of an assay is also cheap, approximately USD1.00 per diagnosis. Thus, this technique may be easily introduced to the field of aquaculture.

Taken together, DNA chip which was developed through this project is comprehensive, highly sensitive, and easy, and is a low-cost diagnostic tool for fish pathogens. Further studies on the comparative genomics among fish pathogens will yield more species-specific regions, and these may contribute to detect a broader range of pathogens and more sensitive diagnostic DNA chips. Currently, the project is evaluating the comparative genomics for fish pathogens using the next-generation DNA sequencer. Hence, the information from this upcoming study is expected to improve and modify the diagnostic DNA chips.

Acknowledgements

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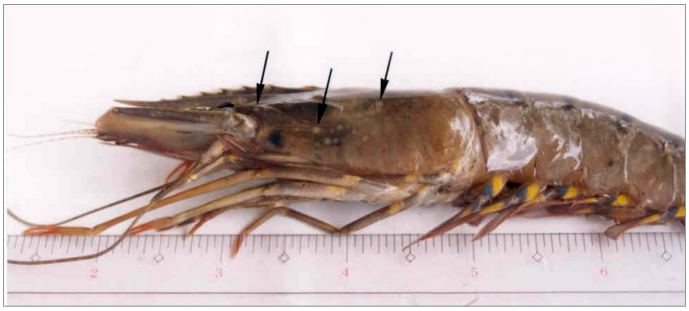

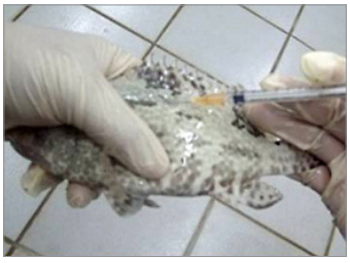

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