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A Subtractively Optimized DNA Microarray Using Non-sequenced Genomic Probes for the Detection of Food-Borne Pathogens

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Abstract In this study, we present the successful detection of food-borne pathogens using randomly selected non-sequenced genomic DNA probes-based DNA microarray chips. Three food-borne pathogens, *Staphylococcus aureus*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium), and *Bacillus cereus*, were subjected for the preparation of the DNA microarray probes. Initially, about 50 DNA probes selected randomly from non-sequenced genomic DNA of each pathogen were prepared by using a set of restriction enzyme pairs. The proto-type of DNA microarray chip for detecting three different pathogens simultaneously was fabricated by using those DNA probes prepared for each pathogen. This proto-type DNA microarray has been tested with three target pathogens and additional seven bacteria, and successfully verified with a few cross-hybridized probes. After this primary verification of the DNA microarray hybridization, this proto-type DNA microarray chip was redesigned and successfully optimized by eliminating a few cross-hybridized probes. The specificity of this redesigned DNA microarray chip to each pathogen was confirmed without any serious cross-hybridizations, and its multiplexing capability in its pathogen detection was found to be possible. This randomly selected non-sequenced genomic DNA probes-based DNA microarray was successfully proved to be the high-throughput simultaneous detection chip for the detection of food-borne pathogens, without knowing the exact sequence information of the target bacteria. This could be the first fabrication of DNA microarray chip for the simultaneous detection of different kinds of food-borne pathogens.

Jin Yong Lee and Byoung Chan Kim contributed equally to this study.

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Keywords Food-borne pathogen · DNA microarray chip · Non-sequenced genomic DNA probes · Cross-hybridization · Multiplexed detection

Introduction

Bacterial pathogens cause a significant threat to human, animal, and environmental health, and the importance of identification of food-borne pathogens among bacterial cells was raised due to its characteristics related to human health care directly. Moreover, global dissemination of some food-borne pathogens is sometimes in pandemic form, mainly due to the increase in international travel and trade, microbial adaptation and changes in the food production system, as well as human demographics and behavior [1].

Therefore, considerable efforts have been devoted to the detection and identification of the bacterial pathogens with rapid, sensitive, and specific assays [2–4]. The conventional culture methods are mostly used for food-borne pathogen detection in food samples or an industrial process. The food product was blended with a selective enrichment medium to induce the population of the target pathogens. These methods commonly take at least 2–3 days and often fail to characterize minor populations or microorganisms in food and environmental samples to detect non-cultivable cells [2, 5]. Various antibody- or nucleic acids-based methods were also developed to detect food-borne pathogens such as ELISA, denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), and terminal restriction fragment length polymorphism (T-RFLP). However, these methods still required to be the culture-independent techniques and could not overcome the low reproducibility [6–11]. Moreover, DNA fragment analysis based on enzyme restriction, such as T–RFLP, tended to overestimate the diversity because of incomplete or non-specific restriction fragments of nucleic acids in complex samples [12]. The known sequence-based real-time PCR and multiplex PCR have emerged as powerful methods for identification of specific bacteria in the contaminated food and environmental samples [13–17]. These methods show rapid and simultaneous detection of specific genes, but still need the sequence information, which leaves this method difficult in application. Especially multiplex PCR assays require the precise design of primers and high cost to synthesize all the primers for each target pathogens, and non-specific products of multiplex PCR may cause a serious problem in the identification and simultaneous detection of the targets [18, 19]. In other words, not only the identification itself but also the simultaneous detection is not possible if no sequence information is available. Even if the numbers of bacteria are increased for which the whole genome is completely sequenced (currently in NCBI Entrez Genome; http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi), the full genomic DNA sequence for all the pathogens are still not available. Therefore, the random genomic DNA probes on DNA microarray platform [20] can be an alternative method without knowing sequence information, but with rapid and multiplexed characteristics for the detection of food-borne pathogens, because this principle has been successfully proven to be simultaneous, high throughput, and specific in the detection of multiple bacteria in complex environments such as activated sludge [21].

The purpose of this study, therefore, is to simultaneously detect specific food-borne pathogens causing public health problems by using the DNA microarray chips containing randomly selected non-sequenced genomic DNA probes of target pathogens (i.e., without sequence information). In this study, more than 50 non-sequenced genomic DNA probes were randomly selected and successfully obtained from the

targets pathogens, and DNA microarray chips containing these genomic DNA probes obtained from three food-borne pathogens [*Staphylococcus aureus*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium), and *Bacillus cereus*] were successfully fabricated. The specificity of this DNA microarray chip was tested and redesigned by eliminating cross-hybridized probes. After the successful fabrication of redesigned DNA microarray chip, highly specific and multiplexed detection of three food-borne pathogens was found to be possible using this redesigned DNA microarray chip.

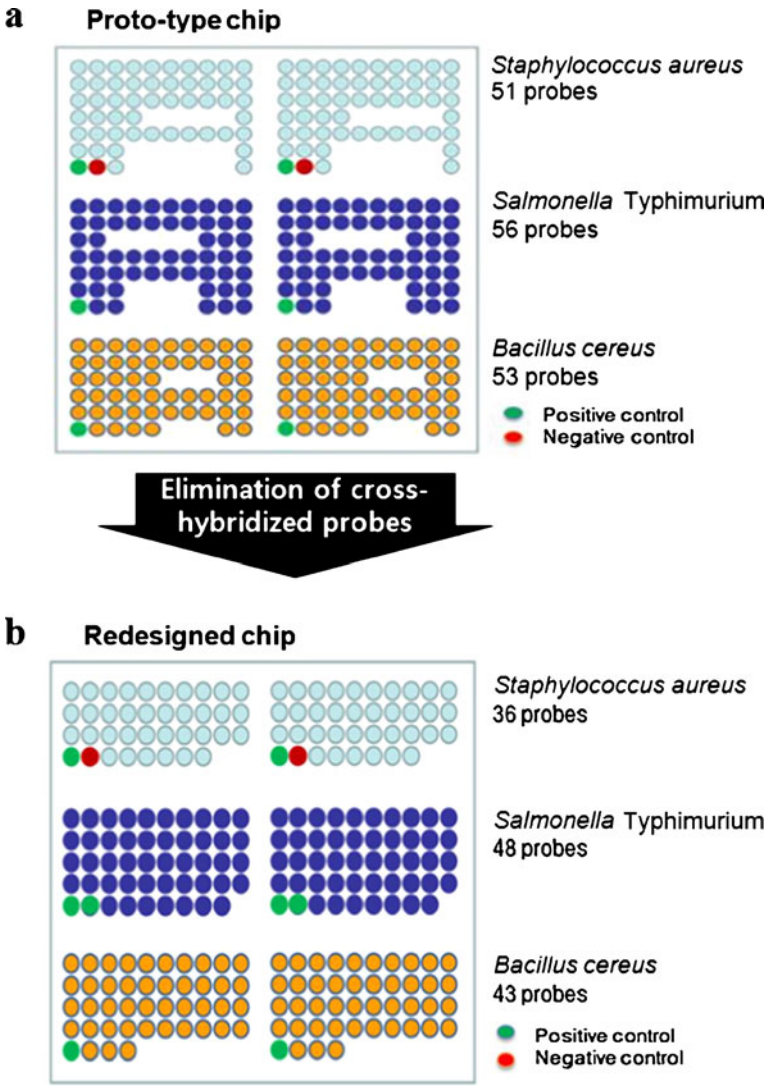


Fig. 1 **a** Schematics of the first proto-type randomly selected non-sequenced genomic DNA probe-based DNA microarray chip for the detection of three different food-borne pathogens. **b** Schematics of the redesigned DNA microarray chip after eliminating the cross-hybridized probes with the seven additional non-target genomic DNAs from the proto-type DNA microarray chip

Fig. 2 Scanned intensities and images of the proto-type DNA microarray chip at 532 nm (Cy3) after individual target genomic DNA hybridization for **a** *S. aureus*, **b** *S.* Typhimurium, and **c** *B. cereus*. The concentration of tested genomic DNA was 50 ng. The *solid black line* indicates the threshold based on the intensity of negative control and blank region, and the *dashed black line* indicates 3SD of average threshold line. The *dashed red line* indicates average intensity signals. The difference of average intensity in each region was compared using *t* test (***$P<0.001$, statistically significant difference between pairs tested)

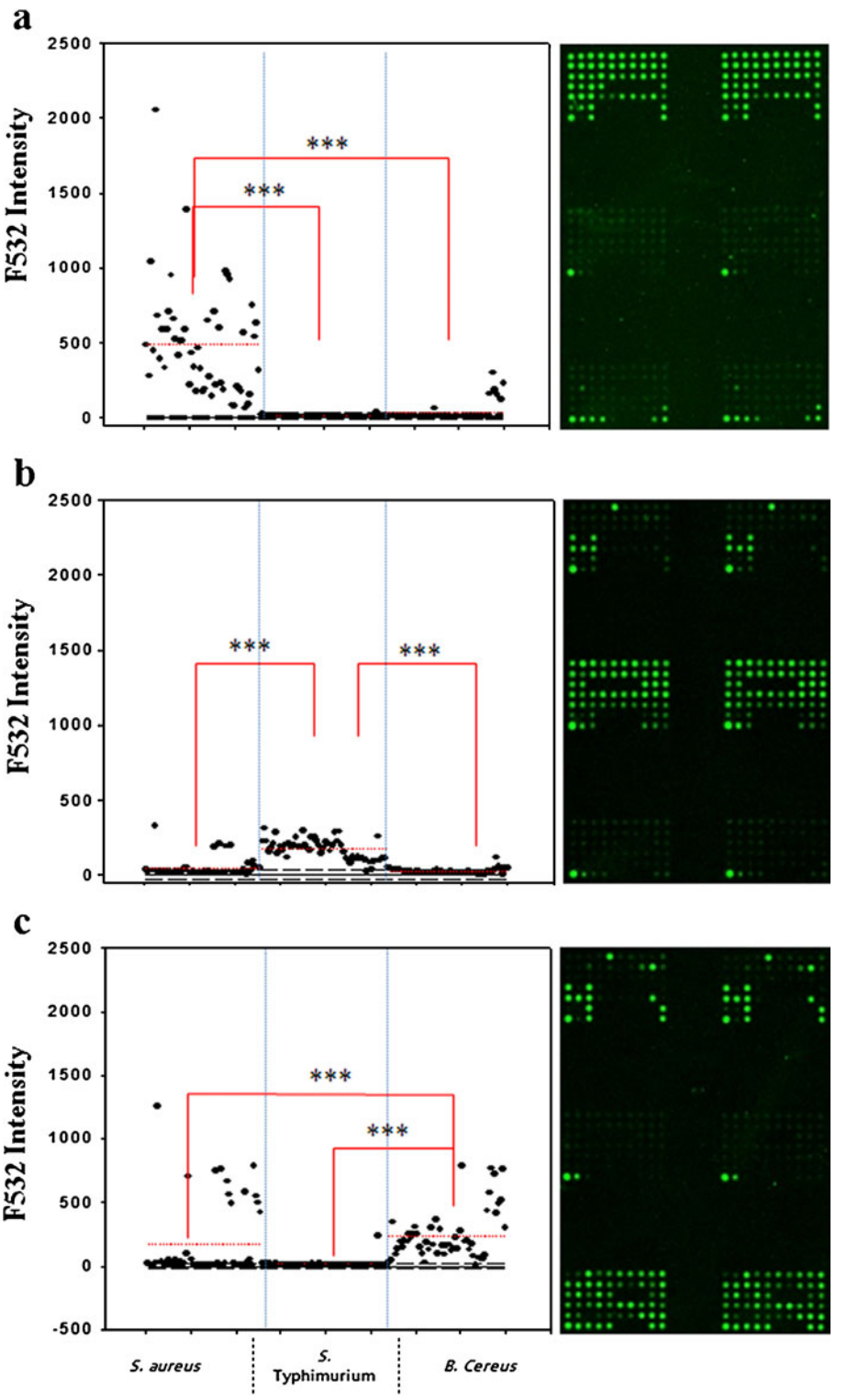
Materials and Methods

Bacterial Strains and Isolation of Genomic DNA

Three microbial food-borne pathogens, *Staphylococcus aureus* (KCTC 1621), *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium) (KCTC 2514), and *Bacillus cereus* (KCTC 1013) were purchased from the Korea Culture Type Collection. Furthermore, the genomic DNAs of seven bacteria, *Alicyclobacillus acidocaldarius* (ATCC 43030), *Alicyclobacillus acidoterrestris* (ATCC 49025), *Alicyclobacillus cycloheptanicus* (ATCC 49029), *Escherichia coli* O157 (ATCC 43895), *Escherichia coli* RFM443 (donated by Dupont Co., USA) [22], *Listeria monocytogenes* (ATCC 19111), and *Yersinia enterocolitica* (ATCC 23715) were used to check the cross-hybridization with fabricated DNA microarray chips. All strains were cultivated by following the culture guidelines without the use of any antibiotics. A stationary-phase culture that had been grown overnight (~24 h) was used as the stock for the extraction of genomic DNA. Bacterial cultures were transferred to a 1.7-mL tube with 15% glycerol and stored at −70 °C until use. Genomic DNAs from all strains except *Alicyclobacillus* species were extracted using DNeasy Blood & Tissue kit (QIAGEN, USA) and the genomic DNAs of *Alicyclobacillus* species were extracted by using Genomic DNA isolation Flexible kit (Nucleogen, Korea). The quality of DNA extracted was checked using Nanodrop (ND-1000; Nanodrop Technologies, Inc., USA) by calculating the ratio of absorbance (260 nm/280 nm) and agarose gel electrophoresis. The DNA was stored at −20 °C until further use.

Random Selection of Genomic DNA Probes and Fabrication of DNA Microarray Chips

To obtain the randomly selected genomic DNA probes, we followed the reported method previously [20, 21]. In brief, the purified genomic DNAs from three different food-borne pathogens were individually fragmented by using three different pairs of restriction enzyme sets (EcoRI/BamHI, HindIII/XhoI, and Hind III/SacII) (NEB, Beverly, MA, USA) at 37 °C. The DNA fragments between 100 bp and 1,500 bp were extracted from electrophorated gel and then purified using QIAquick gel extraction kit (QIAGEN). To construct randomly selected genomic DNA probes of each strain, fragmented DNA pools by restriction enzyme sets were ligated into the pPCR-Script Amp vector (Stratagene, USA) and then transformed into DH5α *E. coli* (RBC, Taiwan). White colonies containing fragmented genomic DNA were randomly selected from the plates and then confirmed with the same restriction enzymes of inserted genomic DNA fragments. Positive clones were amplified with the T3 and T7 primer pairs. After purification, these genomic DNA probes were sent to GenoCheck Co (Gyeonggi, Korea), and these were denatured and then printed on amine-coated glass slide in duplicate in the company. In DNA microarray chip, bacteriophage integrage (MJ 1) gene [23] and peroxisome proliferator-activated receptor (PPAR) γ gene [24] were used as positive and negative control, respectively. The positive control gene was amplified from ΦFC1 DNA with primers 5′-cgggatccatgaaacgtgcagcattg-3′ and 5′-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S. aureus
S. Typhimurium
B. Cereus

Fig. 3 Scanned intensities and images of the redesigned DNA microarray chip at 532 nm (Cy3) after individual target genomic DNA hybridizations for **a** *S. aureus*, **b** *S.* Typhimurium, and **c** *B. cereus*. The concentration of the tested genomic DNA was 50 ng. The *solid black line* indicates threshold based on the intensity of negative control and blank region, and the *dashed black line* indicates 3SD of average threshold line. The *dashed red line* indicates average intensity signals. The difference of average intensity in each region was compared using *t* test (***$P<0.001$, statistically significant difference between pairs tested)

cgggaattcaccgaatgcatgttcgta-3′, and the negative control genes were prepared by RT–PCR from AGS cancer cell RNA with primers 5′-cattctggcccaccaactttgg-3′ and 5′-tggagatg caggctccactttg-3′.

DNA Microarray Experiments and Data Analysis

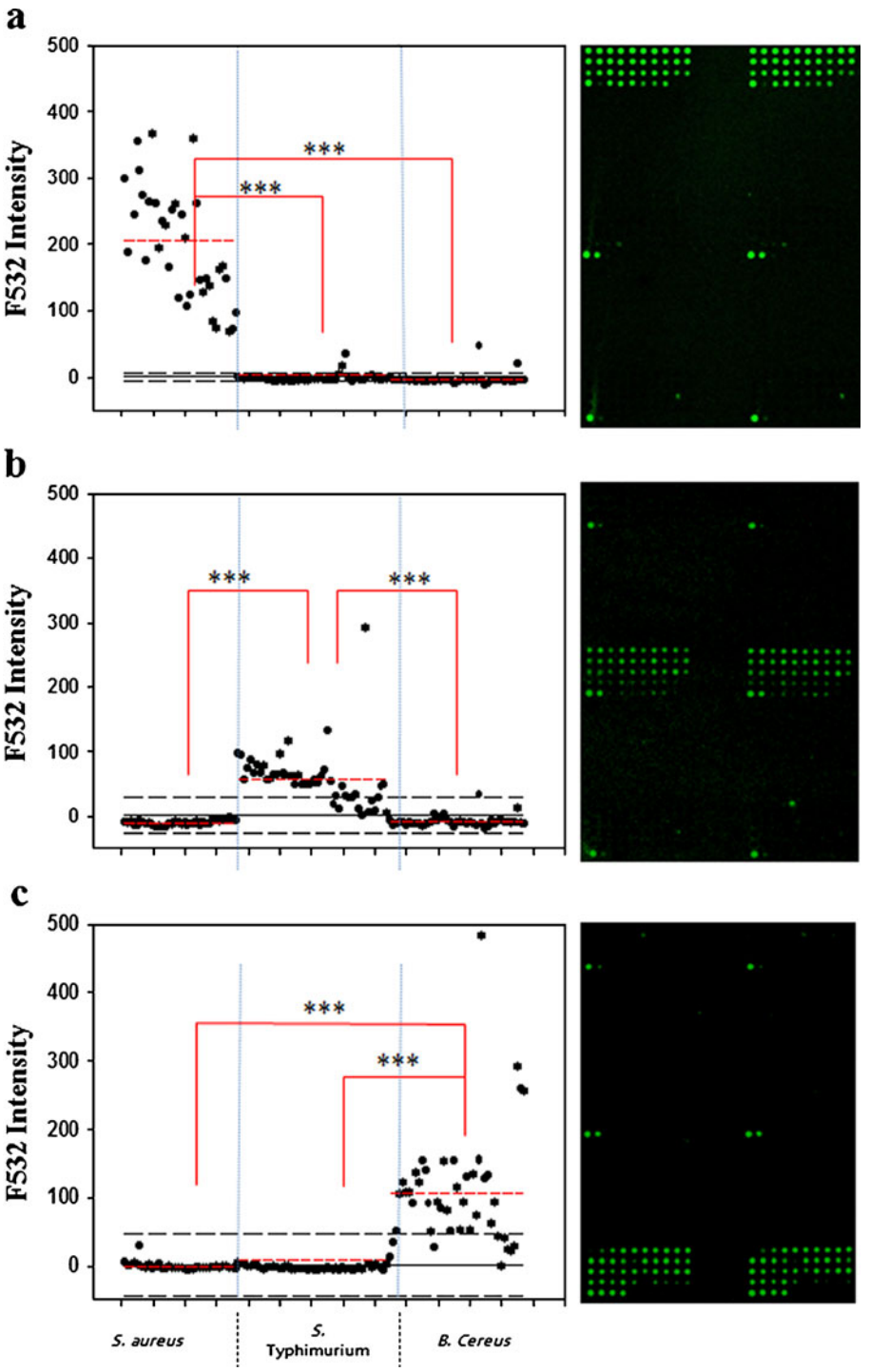
DNA microarray experiments were performed according to the previous reports [20, 21]. The only difference in this analysis compared to previous reports is that we conducted one fluorescent dye analysis using Cy3-dCTP for random priming of target genomic DNA to address the signals of DNA microarray chip. The random priming of target genomic DNA was performed according to the modified protocol using High Prime Labeling kit (Roche, Switzerland) with 10 ng of positive control DNA. After random priming, the reaction pools were purified and eluted using a PCR purification kit (QIAGEN). Further processes such as pre-hybridization, hybridization, washing, and drying in DNA microarray chip were followed by the same protocols with our previous report [20, 21]. The DNA microarray chips were scanned using a Genepix 4000B laser scanner (Axon Instruments, Inc., USA), and fluorescence intensity analysis was performed with GenepixPro 3.0 software (Axon Instruments). All results were taken from two independent samples, from a single culture, conducted simultaneously for error analysis and are shown along with the standard deviation. For the determination of positive binding of target genomic DNAs, the threshold values was set up based on the average intensity of negative control and blank regions (no probes deposited). The presence of target genomic DNA in samples was determined when the average intensity of target region was over the 3SD (standard deviation of average of the average intensity of negative control and blank regions) + threshold intensity. To compare the difference between the intensity of target region and non-target region, the paired *t* test (Mann–Whitney test) was performed.

Results and Discussion

Characteristic Detection of Food-Borne Pathogens Using Primary DNA Microarray Chips Containing Initially Selected Non-sequenced Genomics DNA Probes

To fabricate this microarray chip for the detection and identification of food-borne pathogens, randomly generated genomic DNA probes of each pathogen were obtained. The probes of each strain were 51 of *S. aureus*, 56 of *S.* Typhimurium, and 53 of *B. cereus*. A total of 164 probes obtained and confirmed from the randomly selected colonies after the cloning of randomly selected probes fragmented by restriction enzyme pairs were amplified by PCR and spotted on the glass slide in three different regions including three positive controls and one negative control probe (Fig. 1a). All probes were spotted in duplication.

In order to find the detection limit of this DNA microarray chip for the quality control purpose [25], genomic DNAs of *S. aureus* (0.5, 5, 50, or 100 ng) were used for generating the target signal (Cy3-F532), and the genomic DNAs (each 50 ng) of all three strains were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S. aureus
S. Typhimurium
B. Cereus

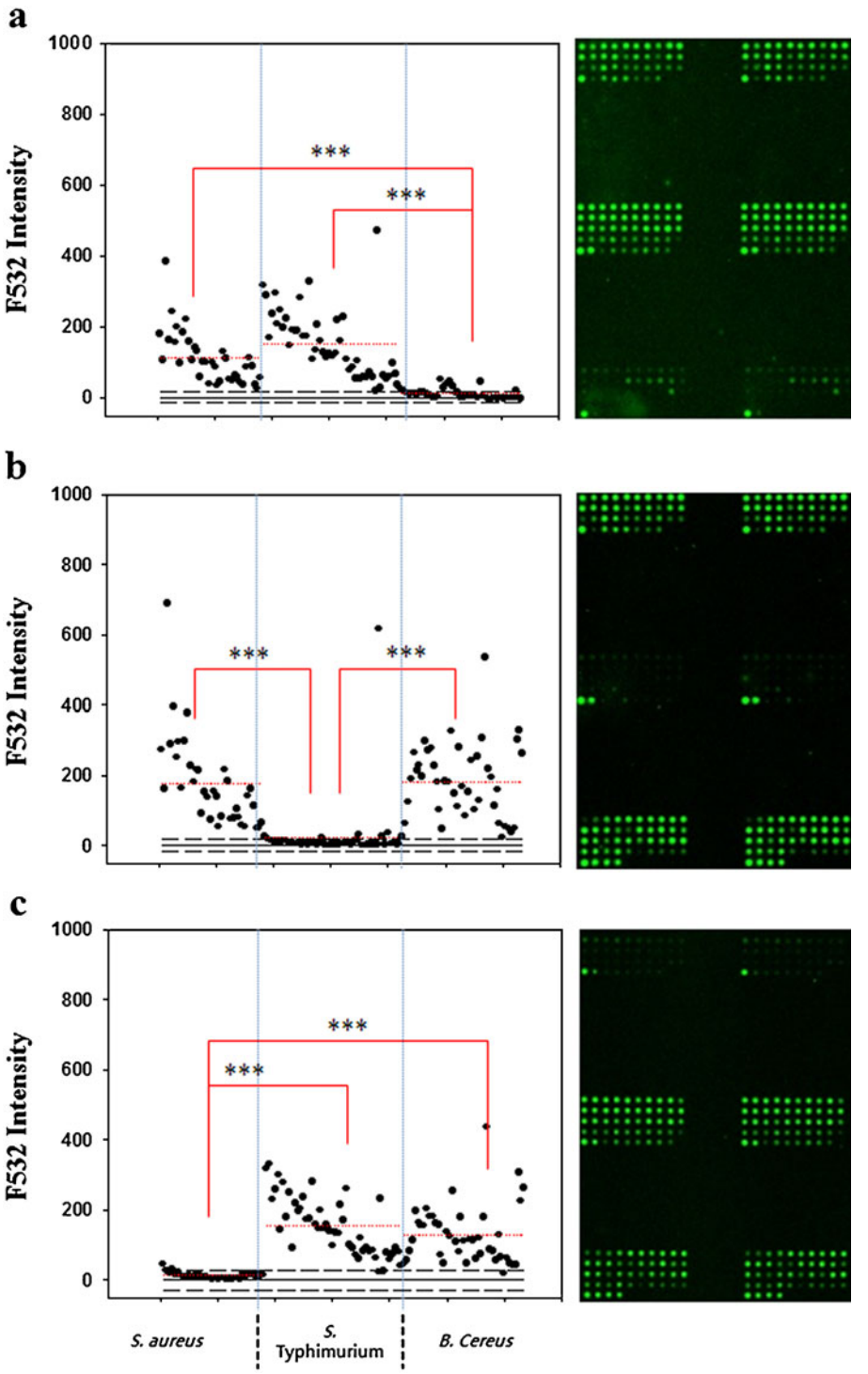
Fig. 4 Scanned intensities and images of the redesigned DNA microarrays at 532 nm (Cy3) after hybridization with the mixture of the bacterial genomics DNAs for **a** *S. aureus* (50 ng) + *S.* Typhimurium (50 ng), **b** *S. aureus* (50 ng) + *B. cereus* (50 ng), and **c** *S.* Typhimurium (50 ng) + *B. cereus* (50 ng). The *solid black line* indicates threshold based on the intensity of negative control and blank region, and the *dashed black line* indicates 3SD of average threshold line. The *dashed red line* indicates average intensity signals. The difference of average intensity in each region was compared using *t* test (***$P<0.001$, statistically significant difference between pairs tested)

used as the reference to check if all the probes are well hybridized with target genomic DNAs (Cy5-F635). From the thorough optimization study, 50 ng of genomic DNA was found to be the optimum for further tests (Supplemental Figure S1a and b). The specificity in the detection was conducted by using a single-dye analysis with Cy3 for 50 ng of target genomic DNA because a single dye was known to be enough to determine if the target genomic DNAs are present in the samples [25]. The meaningful average intensities of Cy3 were observed from the region of the chip where the corresponding probes of the target pathogen were placed, while no significant Cy3 intensities were observed from the other regions where the probes of target pathogens were not spotted (Fig. 2). The average intensity of probes in the target regions was significantly different from the non-target region, and this was confirmed through the paired *t* test (Mann–Whitney test) as well. There were, however, some cross-hybridizations observed in the non-target regions (i.e., some probes in *S. aureus* specific regions showed clear signals with the *B. cereus* DNAs, showing some cross-hybridizations) (Fig. 2c). In that case, the average intensity of non-target regions was over the threshold due to these cross-hybridizations, although the number of hybridized probes is smaller than that in the target region. These results suggested that the randomly selected non-sequenced genomic DNA probes-based analysis for the detection of food-borne pathogens is still valid even if some cross-hybridizations may lead to false-positive interpretation. It should be remembered that we already knew the targets added in the test samples and so the results where the signals would come out for the test samples were easily expected, which means it was not a blind test. Therefore, for more reliable and clear detection for unknown food samples as a blind test, cross-hybridizations with other non-target bacterial genomic DNAs should be minimized as much as possible.

Redesign of DNA Microarray Chip by Eliminating Cross-Hybridized Probes

In order to delete the probes cross-hybridized with other non-target genomic DNAs, seven additional bacterial genomic DNAs (three acidophilic putrefactive bacteria—*A. acidocaldarius*, *A. acidoterrestris*, and *A. cycloheptanicus*; three food-borne pathogens—*E. coli* O157, *L. monocytogenes*, and *Y. enterocolitica*; one non-pathogen—*E. coli* RFM443) were tested with this primary DNA microarray chip, and the results are shown in Supplemental Figure S2. As can be seen in Supplemental Figure S2, all three non-target putrefactive bacterial genomic DNAs were cross-hybridized with the same probes (13 and six probes in *S. aureus* and *B. cereus*, respectively). In the case of *E. coli* RMF443 and *E. coli O157*, six probes were cross-hybridized with the probes from *S. aureus*, which were the same as *Alicyclobacillus* species is applied. Moreover, genomic DNA of *L. monocytogenes* showed a similar cross-hybridization pattern to the cases of three *Alicyclobacillus* species. On the other hand, genomic DNA of *Y. enterocolitica* was found to be mostly cross-hybridized especially with *S.* Typhimurium probes.

The results of these seven DNA microarray experiments suggest that the random selection of highly conserved regions of genomic DNA might generate the unexpected cross-hybridizations and give misleading information about the presence of target bacterial


a
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S. aureus
S. Typhimurium
B. Cereus

genomic DNAs in the samples. Based on these cross-hybridization studies, all the cross-hybridized probes observed were deleted from this primary DNA microarray chip, and the chip was redesigned as shown in Fig. 1b. This redesigned DNA microarray chip resulted in a fabrication with the following number of probes for each strain: 36 of *S. aureus*, 48 of *S.* Typhimurium, and 43 of *B. cereus* probes.

Specific Food-Borne Pathogen Detection Using the Redesigned DNA Microarray Chips

The specificity tests of each target pathogen were performed again with this redesigned DNA microarray chip. As shown in Fig. 3, most cross-hybridizations observed in the previous DNA microarray experiments (see Fig. 2) disappeared and the target was identified clearly well. However, after the redesign of the chips, a total of four probes were found to be newly cross-hybridized. We believe that the signal intensities from these probes could be low enough which should be near the threshold intensity level, and so be considered as unique probes which belong to only a specific bacterium initially designed. These signal intensities from these probes, then, were increased enough to be diagnosed as true hybridizations after a few of the initially spotted probes were eliminated through the chip redesign process. This might be due to some differences of the hybridization efficiencies in DNA microarray experiments. Furthermore, when each of seven additional non-target bacterial genomic DNA was hybridized on the redesigned DNA microarray chips, the reduced number of cross-hybridizations were observed as expected (Supplemental Figure S3). The elimination of cross-hybridized probes can reduce the errors on the identification, and this kind of effort suggests a guide for proper design and selection of probes in the development of highly precise, randomly selected non-sequenced genomic DNA probe-based DNA microarray chip.

Finally, in order to characterize the ability of multiple simultaneous detections, the genomic DNAs of two different target pathogens were mixed and hybridized. Figure 4 shows the results of simultaneous detection of two different target pathogens. The average probe intensities in the target probe regions were significantly higher than those in the non-target regions for all the test cases, which is enough to discriminate the presence of the mixed genomic DNAs of two target pathogens. These results indicated that the randomly selected non-sequenced genomic DNA probes-based DNA microarray can identify the specific target food-borne pathogens without significant cross-hybridizations even in a mixture containing two target food-borne pathogens.

Conclusion

In conclusion, the randomly selected non-sequenced genomic DNA probes were verified to detect specific food-borne pathogens with DNA microarray chip platform. The first proto-type of DNA microarray chip showed good results in discrimination of the presence of target pathogens in samples with a few cross-hybridized probes to other bacterial genomic DNAs. The cross-hybridized probes of this proto-type DNA microarray chip were eliminated, and the chip was successfully redesigned, resulting in a successful reduction in a few cross-hybridizations among targets tested. The redesigned DNA microarray chip showed high specificity to the target food-borne pathogens with little cross-hybridizations. Although the sensitivity of DNA microarray chip in this study might not be enough to detect the small amount of genomic DNA less than pico- or femtogram range in the samples, this sensitivity limitation can be solved by using an appropriate amplification

protocol of genomic DNAs available. This randomly selected non-sequenced genomic DNA probes-based DNA microarray was successfully proved to be the high-throughput simultaneous detection chip for the detection of food-borne pathogen without knowing the exact sequence information of the target bacteria. This type of DNA microarray chips and the probe preparation method suggested here are expected to be applied in the detection of food-borne pathogens in real food samples.

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References

1. Tauxe, R. V. (2002). *International Journal of Food Microbiology, 78*, 31–41.
2. Call, D. R., Borucki, M. K., & Loge, F. J. (2003). *Journal of Microbiological Methods, 53*, 235–243.
3. Lazcka, O., Del Campo, F. J., & Munoz, F. X. (2007). *Biosensors & Bioelectronics, 22*, 1205–1217.
4. Gracias, K. S., & McKillip, J. L. (2004). *Canadian Journal of Microbiology, 50*, 883–890.
5. Hill, W. E., & Payne, W. L. (1984). *Journal of the Association of Official Analytical Chemists, 67*, 801–807.
6. Muyzer, G., Dewaal, E. C., & Uitterlinden, A. G. (1993). *Applied and Environmental Microbiology, 59*, 695–700.
7. Muyzer, G., & Smalla, K. (1998). *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology, 73*, 127–141.
8. Bohaychuk, V. M., Gensler, G. E., King, R. K., Wu, J. T., & McMullen, L. M. (2005). *Journal of Food Protection, 68*, 2637–2647.
9. Anderson, P. N., Hume, M. E., Byrd, J. A., Hernandez, C., Stevens, S. M., Stringfellow, K., et al. (2010). *Poultry Science, 89*, 1293–1300.
10. Powell, S. M., Riddle, M. J., Snape, I., & Stark, J. S. (2005). *Antarctic Science, 17*, 353–360.
11. Sekiguchi, H., Tomioka, N., Nakahara, T., & Uchiyama, H. (2001). *Biotechnology Letters, 23*, 1205–1208.
12. Egert, M., & Friedrich, M. W. (2003). *Applied and Environmental Microbiology, 69*, 2555–2562.
13. Heid, C. A., Stevens, J., Livak, K. J., & Williams, P. M. (1996). *Genome Research, 6*, 986–994.
14. McKillip, J. L., & Drake, M. (2004). *Journal of Food Protection, 67*, 823–832.
15. Rodriguez-Lazaro, D., D'Agostino, M., Herrewegh, A., Pla, M., Cook, N., & Ikonomopoulos, J. (2005). *International Journal of Food Microbiology, 101*, 93–104.
16. Li, X. F., Zhang, S., Zhang, H. W., Zhang, L. H., Tao, H. T., Yu, J., et al. (2009). *International Journal of Food Microbiology, 133*, 252–258.
17. Omiccioli, E., Amagliani, G., Brandi, G., & Magnani, M. (2009). *Food Microbiology, 26*, 615–622.
18. Maynard, C., Berthiaume, F., Lemarchand, K., Harel, J., Payment, P., Bayardelle, P., et al. (2005). *Applied and Environmental Microbiology, 71*, 8548–8557.
19. Volokhov, D., Rasooly, A., Chumakov, K., & Chizhikov, V. (2002). *Journal of Clinical Microbiology, 40*, 4720–4728.
20. Kim, B. C., Park, J. H., & Gu, M. B. (2004). *Environmental Science & Technology, 38*, 6767–6774.
21. Kim, B. C., Park, J. H., & Gu, M. B. (2005). *Analytical Chemistry, 77*, 2311–2317.
22. Vandyk, T. K., Majarian, W. R., Konstantinov, K. B., Young, R. M., Dhurjati, P. S., & Larossa, R. A. (1994). *Applied and Environmental Microbiology, 60*, 1414–1420.
23. Park, M. O., Lim, K. H., Kim, T. H., & Chang, H. H. (2007). *Journal of Microbiology and Biotechnology, 17*, 342–347.
24. Andersen, O., Eijsink, V. G. H., & Thomassen, M. (2000). *Gene, 255*, 411–418.
25. Rasooly, A., & Herold, K. E. (2008). *Foodborne Pathogens and Disease, 5*, 531–550.