Published in final edited form as:

J Food Prot. 2013 November ; 76(11): 1948–1957. doi:10.4315/0362-028X.JFP-13-153.

Customizable PCR-microplate array for differential identification of multiple pathogens

Abdela Woubit, Teshome Yehualaeshet, Sherrelle Roberts, Martha Graham, Moonil Kim, and Temesgen Samuel*

Department of Pathobiology, College of Veterinary Medicine, Nursing and Allied Health, Tuskegee University, AL 36088

Abstract

Customizable PCR-microplate arrays were developed for the rapid identification of *Francisella tularensis* subsp. *tularensis*, *Salmonella* Typhi, *Shigella dysenteriae*, *Yersinia pestis*, *Vibrio cholerae* *Escherichia coli* O157:H7, *Salmonella* Typhimurium, *Salmonella* Saintpaul, *Francisella tularensis* subsp. *novicida*, *Vibrio parahaemolyticus*, and *Yersinia pseudotuberculosis*. Previously, we identified highly specific primers targeting each of the pathogens above. Here, we report the development of customizable PCR-microplate arrays for simultaneous identification of the pathogens using the primers. A mixed aliquot of genomic DNA from 38 different strains was used to validate three PCR-microplate array formats. Identical PCR conditions were used to run all the samples on the three formats. Results show specific amplifications on all the three custom plates. In a preliminary test to evaluate the sensitivity of these assays in laboratory-inoculated samples, detection limits as low as 9 cfu/g/ml *S. Typhimurium* were obtained from beef hot dog, and 78 cfu/ml from milk. Such microplate arrays could serve as valuable tools for initial identification or secondary confirmation of these pathogens.

Keywords

foodborne pathogen; biothreat agents; PCR-microplate; molecular pathogen detection

1. Introduction

Food-related illnesses account for the majority of the infectious disease problems in the US and other developed countries. According to recent reports 48 million Americans suffer from domestically acquired foodborne illness associated with 31 identified pathogens and a broad category of unspecified agents (19–21). The potential impact of deliberate or accidental adulteration of food by organisms such as *Francisella tularensis*, *Yersinia pestis*, *Bacillus anthracis*, etc. is difficult to estimate. Many documented examples of unintentional foodborne outbreaks that have sickened thousands of people and killed hundreds provide a grim basis for estimating the impact of deliberate food adulteration (2, 22).

* Author for correspondence. Tel: (334) 724 4547; Fax: (334) 724 4110; tsamuel@mytu.tuskegee.edu.

Outbreak by *Salmonella* alone has been reported as 1.3 billion annual cases worldwide, and approximately 3 million patients die from the disease each year (15). The reported incidence of *Shigella* infections was 2,848 cases per 100,000 populations in 2007 (3). Among *Shigella* species, *S. sonnei* accounts for approximately 78% of all isolates in recent surveys from the CDC. As compared to 2006 to 2008, pathogen-specific incidence in 2010 was lower for shiga-toxin producing *E. coli* (STEC) O157 and *Shigella* infections; but a rather higher incidence for non-O157 infections mostly O26 (37%), O103 (24%), and O111 (17%) was observed. Out of a total of 186 (96%) *Vibrio* isolates with species information, *V. parahaemolyticus* (57%) and *V. vulnificus* (13%) were the most prevalent (3).

Fast and accurate identification of microbial pathogens from food samples by Public Health agencies and diagnostic laboratories ensure not only a better quality of products but also the possibility to adopt timely detection and intervention measures in case of an outbreak. Real-time PCR is one of the principal methodologies for rapid diagnosis of foodborne outbreaks (6). Multiplexed simultaneous detection of pathogens is one of the recent approaches to identify pathogens involved in an outbreak. However, the number of pathogens detectable by multiplex methodologies has been limited by the cost to run such assays and technical or instrumentation capacity limits. A customizable multiplex assay platform would be beneficial to limit the number of tests to be run based on a presumptive diagnosis or the likelihood of infection or contamination by certain pathogens. For example, a multiplex detection could be customized to perform the simultaneous screening for selected foodborne pathogens including enteric and toxin-producing bacteria, or to selectively detect pathogens of biothreat potential.

In this study, we have developed customizable 96-well PCR-microplate arrays for simultaneous detection of 12 pathogens, which were selected based on their economic significance as foodborne pathogens and their potential as biothreat food contaminants. These microplate arrays can be adopted as specific tools for monitoring foodborne outbreaks.

2. Materials and Methods

2.1. Bacterial species, DNA sources, and primers

Most of the bacterial strains used in this study, their growth conditions, and DNA sources or preparations are described elsewhere (Woubit, et al., 2012). In addition to the 23 strains used in the previous study, the following strains were obtained from the ATCC; *Escherichia coli* 35334, *S. Typhimurium* ATCC®51812TM, *S. Typhimurium* Strain ATCC®700730TM, *S. Typhimurium* Strain MZ1589 ATCC®BAA-1836TM, *S. enterica* subsp. *enterica* ATCC®11511TM, *S. Typhi* ATCC®39926TM, *S. Typhi* ATCC®6539TM, *S. Enteritidis* var. Danysz ATCC®49216TM, *Vibrio cholerae* Serogroup O1, serotype Ogawa, biogroup El Tor (ATCC®BAA-1508TM), *Y. pestis* Yokohama (ATCC®BAA-1508TM), *Y. pestis* K25; D21 (ATCC®BAA-1511TM), *Y. pseudotuberculosis* ATCC® 11960, *Y. pseudotuberculosis* ATCC® 908TM, *Y. enterocolitica* subsp. *enterocolitica* ATCC®700823, *Y. kristensenii* ATCC®35669TM. The quality of all DNA specimens was assessed spectrophotometrically using the Nanodrop ND-1000 (Nanodrop Technologies, Inc., Wilmington, DE) and by agarose gel electrophoresis.

Detailed descriptions of the genome data mining, text mining, comparative genome analysis, that preceded localization of specific target regions for the eventual design of primers, including *in silico* validation and in vitro testing of selected primers are described elsewhere (Woubit, et al., 2012). The validation also included comprehensive analyses using V-NTI (Invitrogen, Carlsbad, CA) motif search tool for non-specific binding to a different location within the same genome sequence. Primers were ordered from Integrated DNA Technologies (IDT, Coralville, IA) (27).

2.2. Real-time PCR for primer validation

For the final validation and verification steps, real-time PCR assay was performed using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA). A reaction volume of 20 µl containing 500 nM of forward and reverse primers, 10 µl of 2X Brilliant III Ultra-Fast SYBR Green master mix, 0.3 µl ROX reference dye, 1 µl (2–3.8 ng/µl) of DNA template and nuclease free H₂O added to the final volume was used. Cycling conditions consisted of one cycle of segment 1, 2 min at 95 °C; followed by 27 cycles of segment 2, 10 seconds at 95 °C, 30 seconds at 60 °C; and a final one melting curve cycle of 1 minute at 95 °C, 30 seconds at 65 °C, and 30 seconds at 95 °C.

2.3. Custom microplate PCR array design

Three microplate arrays (96-well, 63-well, or 48-well) were designed using a 96-well plate format. Validated specific primers described above were aliquoted at 0.25nmole concentration per well, and custom-manufactured at the Integrated DNA technologies (IDT, Coralville, IA). Primers were spotted in duplicates for the 96-well customized plate designed to detect both food threat and foodborne pathogens. For example *E. coli* O157:H7 primer pair EC1 was spotted in duplicates in wells A1 & B1; the second specific primer pair EC2 was spotted in duplicates in wells C1 & D1, etc. (see Figure 1). Additionally, for all the pathogens, rows E and F were spotted with specific primers 1 and 2 respectively, and are reserved for use as positive controls. The 48-well PCR microplate array was designed for specific identification of food biothreat agents including *Escherichia coli* O157:H7 (EC), *S. Typhi* (ST), *Shigella dysenteriae* (ShD), *Vibrio cholera* (VC) and threat agents *Francisella tularensis* (FT) and *Y. pestis* (YP). The 63-well PCR-array was designed for selective identification of six major foodborne pathogens including *Escherichia coli* O157:H7 (EC), *S. Typhimurium* (STm), *S. Saintpaul* (SS), *Shigella sonnei* (ShS), *Vibrio parahaemolyticus* (VP), *Y. pseudotuberculosis* (YPs) and *Francisella novicida* (FN). All the three PCR-microplate arrays contain genus-specific primers and assay negative control (no-template) wells.

2.4. Custom plate Real-time PCR

A mixed aliquots of genomic DNA from all the 38 strains at 1.5 ng was used on the three plates as a test sample. Real-time PCR assay was performed using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA). A reaction volume of 20 µl containing 500 nM of forward and reverse primers, 10 µl of 2X Brilliant III Ultra-Fast SYBR Green master mix, 0.3 µl ROX reference dye, 1 µl (2–3.8 ng/µl) of DNA template and nuclease free H₂O added to the final volume was used. Cycling conditions

consisted of one cycle of segment 1, 2 min at 95 °C; followed by 27 cycles of segment 2, 10 seconds at 95 °C, 30 seconds at 60 °C; and a final one melting curve cycle of 1 minute at 95 °C, 30 seconds at 65 °C, and 30 seconds at 95°C.

2.5. Food matrix sensitivity assay

S Typhimurium was grown overnight at 37°C in TSB medium, which in 24 hours reached approximately 3.9×10^9 CFU/ml. The culture was 10-fold diluted up to 10^{-8} in TSB medium. A 100 µl of suspension from each dilution tube was used to inoculate 1ml of skim milk and 1% fat milk. For solid food matrix 300 µl aliquot of each dilution was used to inoculate 1 g of minced beef hotdog that was originally mixed with 2.7 ml of TSB. A plate colony count was conducted simultaneously to enumerate the number of organisms inoculated. After complete homogenization of each of the inoculated food matrix, total DNA was extracted using QiaAmp DNA (Qiagen, Valencia, CA). In the case of meat inoculate; the matrix was vortexed vigorously for complete homogenization. The entire suspension was collected for DNA extraction and 1µl of the extract was used for PCR analysis.

3. Results and Discussion

In the present study we have developed customizable microplate arrays for simultaneous detection of 12 pathogens comprising major foodborne and Biothreat agents. A total of 38 strains of bacteria representing six genera (*Escherichia*, *Francisella*, *Salmonella*, *Shigella*, *Vibrio*, and *Yersinia*) were tested by PCR- microplate in 96-, 48- and 63-well array formats. The primers used in this study were identified and partially validated in our previous work; here we developed a detection platform and validated the primers using 15 additional organisms. To design the array platform, primers were spotted across the rows of the 96- (Figure 1a), 48- (Figure 2a) and 63-well (Figure 3a) PCR-microplates.

Specific amplification plots were obtained only in those wells where primers and the corresponding target DNA intersect (Figures 1b, 2b and 3b). The 96-well PCR-microplate contained two targets for each organism, with the exception of *Shigella sonnei*, *S. Saintpaul* and *Francisella novicida*, which had only one pair each. The two sets of primers per organism spotted in the 96-well microplate provided a reliable identification of the pathogens in question. The genus identification PCR in row G of the 96-well PCR-microplate array also confirmed the species detected in rows A-D. For the detection of an unknown sample, all wells in rows A1 to D12 received test sample, mixed DNA aliquots from 38 strains. Rows E and F received known DNA from the designated species as positive controls. For example, E1 and F1 received purified *E. coli* O157:H7 DNA while E12 and F12 received DNA from *Y. pseudotuberculosis*.

The 96-well microplate array enabled the detection of all 12 pathogens listed above. However, it may be desirable and economical to perform a targeted detection of either biothreat agents (CDC category A or B organisms) or foodborne pathogens. Therefore, two subsets of microplate arrays in 48-well (Figure 2a) and 63-well (Figure 3a) formats were designed for selective identification of biothreat and foodborne pathogens, respectively. In these formats, sample DNA was aliquoted along the columns enabling simultaneous testing of 6 to 7 samples per plate. As shown by the results (Figures 2b and 3b), specific

amplification of the organism under question was obtained diagonally at the intersection of the primer row and the pathogen column. Both the 48 and 63 well plates were also tested using a mixture of DNA aliquots from 38 organisms and spotted along the genus column to identify which of the 6 or 7 bacteria would be detected at the genus level. The results demonstrated specific amplification of the different genera in question.

In this study, DNA extracted from beef hotdog artificially inoculated with *S. Typhimurium* yielded a detection limit of 9 cfu/ml at 10^{-7} dilution (Ct values of 29) using STm1. Total DNA extracted from both the 1% and skimmed milk provided detection limits of 78 cfu/ml at Ct values of 28. Using the primer STm2, 86 cfu/ml were detected at 10^{-6} dilution in beef hotdog, 1% milk and skim milk with amplifications being detected at Ct values of 26, 30 and 32, respectively.

The dissociation curve analysis of each PCR reaction in these arrays provided a single, sharp peak from each of the 12 pathogens and their corresponding primers used in this assay (Figure 4). This suggests that specific PCR products were generated with the set of primers used to develop these PCR-microplate arrays. The results also show dissociation curves (Tm values) ranging from 78.3°C generated by PCR products from primers SS2 to 90.3°C by products from primers ShS1 targeting *S. Saintpaul* & *Shigella sonnei* respectively.

PCR-based simultaneous detection methods developed elsewhere (9, 13, 23, 25) are based on both fewer organisms and genomic targets than studied in this work. Compared to those multiplexed PCR assays, this study covers a broader range of strains, uses multiple targets as well as two-tier species and genus level identification of food threat and foodborne pathogens. We have employed 27 primer pairs targeting species of major public health importance and their closely related organisms.

The 48-well array could be deployed when there is a suspected case of any of these six major food threat agents thereby minimizing the cost of testing in a 96-well format. Further or alternatively, this array could also be used as a confirmatory tool for the 96-well PCR assay above. Unlike the 96-well PCR-microplate array, these two PCR-microplate arrays do not use redundant wells for each primer, nor multiple primer sets for each target. Only one primer pair was spotted per row. Sample DNA will be equally aliquoted into all the wells of a given column. For example, if a sample contained a mixture of *E. coli* O157:H7 and *S. dysenteriae* and aliquoted into the wells of column 1, then rows E and F will show positive real-time PCR results, while no DNA amplification will be observed in rows A-D. In this manner, the PCR-microplate array can receive up to six different test samples (each of which may contain DNA from any of the 6 organisms) simultaneously.

Wang and colleagues (2007) detected 10^3 CFU/g of *Salmonella* and 10^4 CFU/g for *Shigella* from artificially contaminated ground beef with a combination of Immunomagnetic separation (IMS) and real-time PCR system for the detection. In our assay, we have shown a capacity of detecting as low as 33 CFU/ml of *Shigella* in milk (Woubit, et al., 2012) and as low as 9 CFU/g *Salmonella* in beef hot dog (current study).

Francisella novicida included in the array has been recently considered a subspecies of *F. tularensis* on the basis of DNA similarity (16). Given the genetic similarity of 98.1%

between *F. novicida* and *F. tularensis* (5), and its endemic presence in North America (8), this organism has been an attractive model for the study of the virulence mechanisms of *F. tularensis* (14). Recently it has been associated with a serious illness in a patient with an underlying case of immunosuppression (1). Because of the unreliable results of phenotypic identification methods, unfamiliarity of the bacterium and close genetic relatedness among *Francisella* subsp., its inclusion in this microplate array was appropriate to avoid misidentification of this species. *F. tularensis* is one of the most infectious pathogenic bacteria known, and the organism survives outside of a mammalian host for weeks, making *F. tularensis* a high impact biological weapon (12, 26) and a food threat agent.

Y. pestis, the bubonic plague agent, is a highly virulent pathogen and is considered a likely threat agent for committing an act of agro-bioterrorism (18). Although gastrointestinal plague is extremely rare, the disease has been caused by the consumption of contaminated meat (18). *Y. pseudotuberculosis* has a high degree of genome similarity with *Y. pestis* (4), as a result of which this organism has long been considered a surrogate of *Y. pestis* (24). Recently reported foodborne outbreaks associated with *Y. pseudotuberculosis* (7, 10, 11, 17) stress its significance, thus providing its necessity of inclusion in our assay.

Overall, we have developed a customizable array platform for simultaneous detection of high-impact food threat and foodborne pathogens with high degrees of specificity and sensitivity. The genus specific primers included in these arrays add the benefit of identifying the pathogenic genus, even if the species or strain was not arrayed on the plates. If necessary, additional focused tests could then be performed to identify the specific strain or species involved in the particular food terrorism or foodborne illness. These assay formats could be used to provide initial information to public health authorities about the causative agent involved in a foodborne outbreak, allowing for a more accurate, and timely response.

Acknowledgement

The U.S. Department of Homeland Security through NCFPD Award Number 2007-ST-061-000003 had supported this work. We want to recognize Dr. Frank Busta, Emeritus of the NCFPD Minnesota for his constructive suggestions and review of the manuscript. We thank Dr. Karl Klose, who provided us the *Francisella* DNA and BEI Resources for *Francisella* and *Yersinia* DNA. Tuskegee University shared instrument facility is supported by NIH/NCRR/RCMI G12MD007585. Research in part supported by NIH grants # SC2CA138178 233 and U54CA118623. The views and conclusions contained in this document are those of the authors and should not be interpreted as necessarily representing the official policies, either expressed or implied, of the U.S. Department of Homeland Security.

REFERENCES

1. Amornrut L, Samaporn Y, Sunee L, Thitiya Y, Pattarachai K. Emergence of *Francisella novicida* Bacteremia, Thailand. *Emerging Infectious diseases*. 2008; 14:1935–1937. [PubMed: 19046526]
2. Busta, FF.; Kennedy, SP. Defending the Safety of the Global Food System from Intentional Contamination in a Changing Market. In: Hefnawy, M., editor. *Advances in Food Protection*. Netherlands: Springer; 2011. p. 119-135.
3. CDC. Vital signs: incidence and trends of infection with pathogens transmitted commonly through food-foodborne diseases active surveillance network, 10 U.S. sites, 1996–2010. *Morbidity and Mortality Weekly Report*. 2011; 60:749–755. [PubMed: 21659984]
4. Eppinger M, Rosovitz MJ, Fricke WF, Rasko DA, Kokorina G, Fayolle C, Lindler LE, Carniel E, Ravel J. The complete genome sequence of *Yersinia pseudotuberculosis* IP31758, the causative agent of Far East scarlet-like fever. *PLoS Genetics*. 2007; 3:e142. [PubMed: 17784789]

5. Forsman M, Sandstrom G, Sjostedt A. Analysis of 16S ribosomal DNA sequences of *Francisella* strains and utilization for determination of the phylogeny of the genus and for identification of strains by PCR. *Int J Syst Bacteriol.* 1994; 44:38–46. [PubMed: 8123561]
6. Fukushima H, Kawase J, Etoh Y, Sugama K, Yashiro S, Iida N, Yamaguchi K. Simultaneous Screening of 24 Target Genes of Foodborne Pathogens in 35 Foodborne Outbreaks Using Multiplex Real-Time SYBR Green PCR Analysis. *Int J Microbiol.* 2010; 2010:1–18.
7. Hannu T, Mattila L, Nuorti JP, Ruutu P, Mikkola J, Siitonen A, Leirisalo-Repo M. Reactive arthritis after an outbreak of *Yersinia pseudotuberculosis* serotype O:3 infection. *Annals of the rheumatic diseases.* 2003; 62:866–869. [PubMed: 12922960]
8. Hollis, G D, Weaver RE, Steigerwalt AG, Wenger JD, Moss CW, Brenner DJ. *Francisella philomiragia* comb. nov. (formerly *Yersinia philomiragia*) and *Francisella tularensis* biogroup novicida (formerly *Francisella novicida*) associated with human disease. *J Clin Microbiol.* 1989; 27:1601–1608. [PubMed: 2671019]
9. Hyeon YJ, Park C, Choi IS, Holt PS, Seo KH. Development of multiplex real-time PCR with Internal amplification control for simultaneous detection of *Salmonella* and *Cronobacter* in powdered infant formula. *Int J Food Microbiol.* 2010; 144:177–181. [PubMed: 20961642]
10. Jalava K, Hallanvuo S, Nakari UM, Ruutu P, Kela E, Heinasmaki T, Siitonen A, Nuorti JP. Multiple outbreaks of *Yersinia pseudotuberculosis* infections in Finland. *Journal of clinical microbiology.* 2004; 42:2789–2791. [PubMed: 15184472]
11. Kangas S, Takkinen J, Hakkinen M, Nakari UM, Johansson T, Henttonen H, Virtaluoto L, Siitonen A, Ollgren J, Kuusi M. *Yersinia pseudotuberculosis* O:1 traced to raw carrots, Finland. *Emerging Infectious diseases.* 2008; 14:1959–1961. [PubMed: 19046537]
12. Kaufmann, F A, Meltzer MI, Schmid GP. The economic impact of a bioterrorist attack: are prevention and postattack intervention programs justifiable? *Emerg Infect Dis.* 1997; 3:83–94. [PubMed: 9204289]
13. Kawasaki S, Fratamico PM, Horikoshi N, Okada Y, Takeshita K, Sameshima T, Kawamoto S. Multiplex real-time polymerase chain reaction assay for simultaneous detection and quantification of *Salmonella* species, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 in ground pork samples. *Foodborne Pathog Dis.* 2010; 7:549–554. [PubMed: 20132032]
14. Lai, H X, Shirley RL, Crosa L, Kanistanon D, Tempel R, Ernst RK, Gallagher LA, Manoil C, Heffron F. Mutations of *Francisella novicida* that alter the mechanism of its phagocytosis by murine macrophages. *PloS one.* 2010; 5:e11857. [PubMed: 20686600]
15. Lee, H S, Jung BY, Rayamahji N, Lee HS, Jeon WJ, Choi KS, Kweon CH, Yoo HS. A multiplex real-time PCR for differential detection and quantification of *Salmonella* spp., *Salmonella enterica* serovar Typhimurium and Enteritidis in meats. *J Vet Sci.* 2009; 10:43–51. [PubMed: 19255523]
16. McLendon, K M, Apicella MA, Allen LA. *Francisella tularensis*: taxonomy, genetics, and Immunopathogenesis of a potential agent of biowarfare. *Annu Rev Microbiol.* 2006; 60:167–185. [PubMed: 16704343]
17. Nuorti, P J, Niskanen T, Hallanvuo S, Mikkola J, Kela E, Hatakka M, Fredriksson- Ahomaa M, Lyytikainen O, Siitonen A, Korkeala H, Ruutu P. A widespread outbreak of *Yersinia pseudotuberculosis* O:3 infection from iceberg lettuce. *The Journal of infectious diseases.* 2004; 189:766–774. [PubMed: 14976592]
18. Porto Fett, C A, Juneja VK, Luchansky JB, Tamplin M. Validation of cooking times and temperatures for thermal inactivation of *Yersinia pestis* strains KIM5 and CDC-A1112 in ground beef. *Journal of Food Protection.* 2009; 72(3):564–571. [PubMed: 19343945]
19. Scallan E, Griffin PM, Angulo FJ, Tauxe RV, Hoekstra RM. Foodborne illness acquired in the United States--unspecified agents. *Emerging Infectious diseases.* 2011; 17:16–22. [PubMed: 21192849]
20. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM. Foodborne illness acquired in the United States--major pathogens. *Emerging Infectious diseases.* 2011; 17:7–15. [PubMed: 21192848]
21. Scharff RL. Economic burden from health losses due to foodborne illness in the United States. *Journal of Food Protection.* 2012; 75:123–131. [PubMed: 22221364]

22. Sobel, J. Food and beverage sabotage. In: Pilch RF, ZR., editor. Encyclopedia of Bioterrorism Defense. New York: Wiley-Liss, Inc.; 2005.
23. Suo B, He Y, Tu SI, Shi X. A multiplex real-time polymerase chain reaction for simultaneous detection of *Salmonella* spp., *Escherichia coli* O157, and *Listeria monocytogenes* in meat products. *Foodborne Pathog Dis*. 2010; 7:619–628. [PubMed: 20113204]
24. Tomioka K, Peredelchuk M, Zhu X, Arena R, Volokhov D, Selvapandiyan A, Stabler K, Mellquist-Riemenschneider J, Chizhikov V, Kaplan G, Nakhasi H, Duncan R. A multiplex polymerase chain reaction microarray assay to detect bioterror pathogens in blood. *J Mol Diagn*. 2005; 7:486–494. [PubMed: 16237218]
25. Wang J, Yang Y, Zhou L, Jiang Y, Hu K, Sun X, Hou Y, Zhu Z, Guo Z, Ding Y, Yang R. Simultaneous detection of five biothreat agents in powder samples by a multiplexed suspension array. *Immunopharmacol Immunotoxicol*. 2009; 31:417–427. [PubMed: 19555207]
26. World Health Organization. Geneva, Switzerland: World Health Organization; 2007. Epidemic and Pandemic Alert and Response. WHO guidelines on tularaemia.
27. Woubit A, Yehualaeshet T, Habtemariam T, Samuel T. Novel genomic tools for specific and real-time detection of biothreat and frequently encountered foodborne pathogens. *Journal of Food Protection*. 2012; 75:660–670. [PubMed: 22488053]

Figure 1.

a. 96-well PCR-microplate array layout where primers are spotted across the rows (A–H); rows A and B contain the first specific primer set for all the organisms and rows C and D contain the second specific primer set for all the pathogens except for *Salmonella* Saintpaul, *Shigella sonnei*, *Francisella novicida*, where the first set of primers were spotted along the four wells. Rows E, F, G and H contain controls. Genus detection Primers in row G are indicated by letters: E= *Escherichia*, S= *Salmonella*, Sh= *Shigella*, F= *Francisella*, V= *Vibrio*, Y= *Yersinia*. **b.** Result of the 96-well PCR-microplate array simultaneously amplifying 12 bacterial pathogens. Rows E and F don't show results because they are reserved for control DNA. EC= *E. coli* O157:H7, ST= *Salmonella* Typhi, SS= *Salmonella* Saintpaul, STm= *Salmonella* Typhimurium, ShD= *Shigella dysenteriae*, ShS= *Shigella sonnei*, FT= *Francisella tularensis*, FN= *Francisella novicida*, VC= *Vibrio cholerae*, VP= *V. parahaemolyticus*, YP= *Yersinia pestis*, YPs= *Y. pseudotuberculosis*, P₁P₂= primer1 & primer2.

Figure 2.

a. 48-well PCR-microplate array layout; primers were spotted across the rows A-F. EC2 amplifying *E. coli* O157:H7 spotted in wells A1-A6, FT1 amplifying *Francisella tularensis* spotted in wells B1-B6, ShD1 amplifying *Shigella dysenteriae* spotted in wells C1-C6, ST1 amplifying *Salmonella* Typhi spotted from D1-D6, VC1 amplifying *Vibrio cholerae* spotted in wells E1-E6, and YP1 amplifying *Yersinia pestis* spotted in wells F1-F6. Column 7 contains genus specific primers in wells A7-F7 amplifying genus *Escherichia*, *Francisella*, *Shigella*, *Salmonella*, *Vibrio*, *Yersinia*. Column 8 is a no-template control spotted with species-specific primers in the respective row. **b.** A test result from the 48-well PCR-microplate array, showing specific amplification of biothreat agent at the intersection where the primer rows meet the DNA columns, if all the organisms were amplified simultaneously one would see “V” shaped result line on the raw data tab of real time PCR assay.

Figure 3.

a. 63-well PCR-microplate array layout; primers were spotted across the rows A-G. EC2 amplifying *E. coli* O157:H7 spotted in wells A1-A7, FN1 amplifying *Francisella novicida* spotted in wells B1-B7, ShS1 amplifying *Shigella sonnei* spotted in wells C1-C7, STm1 amplifying *Salmonella Typhimurium* spotted in wells D1-D7, SS1 amplifying *Salmonella Saintpaul* spotted in wells E1-E7, VP1 amplifying *V. parahaemolyticus* spotted in wells F1-F7, and YPs2 amplifying *Yersinia pseudotuberculosis* spotted in wells G1-G7. Column 8 contains genus-specific primers in wells A8-G8 amplifying genus *Escherichia*, *Francisella*, *Shigella*, *Salmonella*, *Salmonella*, *Vibrio*, and *Yersinia*. Column 9 is a no-template control spotted with the species-specific primers in the corresponding row. **b.** Result from the 63-well PCR-microplate array showing specific amplification of the pathogens under question at the intersection where the primers rows met the DNA columns, if all the organisms were amplified simultaneously, one will see a “V” shaped result line from the raw data tab of real time PCR assay.

Figure 4.

Dissociation curves of PCR products after real time PCR amplification of *E. coli* O157:H7 using EC2 primers (a), *Francisella tularensis* using FT1 primers (b), *Francisella novicida* using FN1 primers (c), *Shigella dysenteriae* using ShD1 primers (d), *Shigella sonnei* using ShS1 primers (e), *Salmonella* Typhi using ST1 primers (f), *Salmonella* Saintpaul using SS1 primers (g), *Salmonella* Typhimurium using STm1 primers (h), *Vibrio cholerae* using VC1 primers (i), *Vibrio parahaemolyticus* using VP1 primers (j), *Yersinia pseudotuberculosis* using YPs2 primers (k), and *Yersinia pestis* using YP1 primers (l). Dissociation curves were

calculated for those primers used to design the 48-and 63-well PCR-microplate arrays. Melting temperature (T_m) ranged between 78.3°C and 90.3°C for *S. Saintpaul* and *S. sonnei* PCR products, respectively.