Simultaneous, specific and real-time detection of biothreat and frequently encountered food-borne pathogens

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Abstract

The bacterial genera *Escherichia*, *Salmonella*, *Shigella*, *Vibrio, Yersinia* and *Francisella* include important food safety and biothreat agents causing food-related and other human illnesses worldwide. We aimed to develop rapid methods with the capability to simultaneously and differentially detect all six pathogens in one run. Our initial experiments to use previously reported sets of primers revealed non-specificity of some of the sequences when tested against a broader array of pathogens, or proved not optimal for simultaneous detection parameters. By extensive mining of the whole genome and protein databases of diverse closely and distantly related bacterial species and strains, we have identified unique genome regions, which we utilized to develop a detection platform. Twelve of the specific genomic targets we have identified to design the primers in *F. tularensis* ssp. *tularensis*, *F. tularensis* ssp. *novicida*, *S. dysentriae*, *S. typhimurium*, *V. cholera*, *Y. pestis*, and *Y. pseudotuberculosis* contained either hypothetical or putative proteins, the functions of which have not been clearly defined. Corresponding primer sets were designed from the target regions for use in real-time PCR assays to detect specific biothreat pathogens at species or strain levels. The primer sets were first tested by *in-silico* PCR against whole genome sequences of different species, sub-species, or strains and then by in vitro PCR against genomic DNA preparations from 23 strains representing six biothreat agents (*E.coli* O157:H7 strain EDL 933, *Shigella dysentriae*, *Salmonella typhi*, *Francisella tularensis* ssp. *tularensis*, *Vibrio cholera*, and *Yersinia pestis*) and six foodborne pathogens (*Salmonella typhimurium*, *Salmonella saintpaul*, *Shigella sonnei*, *Francisella novicida*, *Vibrio parahemolytica* and *Yersinia pseudotuberculosis*). Each pathogen was specifically identifiable at the genus and species levels. Sensitivity assays performed using purified DNA showed the lowest detection limit of 640 fg DNA/μl for *F. tularensis*. A preliminary test done to detect *Shigella* organisms in a milk matrix showed that 6–60 colony forming units of the bacterium per milliliter of milk could be detected in about an hour. Therefore, we have developed a platform to simultaneously detect foodborne pathogen and biothreat agents specifically and in real-time. Such a platform could enable rapid detection or confirmation of contamination by these agents.

Keywords

Biothreat agents; PCR; food-borne pathogens

1. Introduction

Food-borne pathogens cause millions of clinical illnesses every year and cost billions of dollars to manage and control. Intentional contamination of food in the form of a biological attack is an even more alarming prospect given that no standards or established routines

exist to test food for these threats (Kennedy, 2008). Intentional contamination could involve among many food products, contamination of water, milk, and other beverages that are distributed from bulk storage or processing sites, not necessarily at the source of the starting product. This has the potential to result in disastrous and far-reaching effects, including direct morbidity and/or mortality, disruption of food distribution, loss of consumer confidence in government and the food supply, business failures, trade restrictions, and ripple effects on the economy (Busta and Shaun, 2011). Therefore, from both food safety and biothreat points of view, it is imperative that food and drink contaminations are detected well before they reach the consumer level.

In the past decades the Polymerase Chain Reaction (PCR) has been transformed into a powerful tool to detect pathogens with extremely high sensitivity and specificity. While the main drawback of PCR still remains to be the inability to distinguish between live and dead organisms, the potential for non-specific detection could also be high if the target sequences are not specific or of contamination of the reactions or rooms occur (He et al., 1994; Lantz et al., 2000; Wright and Wynford-Thomas, 1990). Moreover, biological sample preparation strategies are needed to remove non-specific inhibitors. Multiplexing the PCR for the detection of multiple pathogens or targets is currently employed to enable the testing of samples for multiple pathogens in a single run. Several multiplex or simultaneous PCR systems for the detection of food-borne and other infectious pathogens are also being developed (Fukushima et al., 2010; Jothikumar and Griffiths, 2002; Skottman et al., 2007; Wilson et al., 2005). Despite the advantages, when multiplexing PCR, optimal reaction conditions for the multiple sets of primers have to be identified for all the potential targets to be detected (Markoulatos et al., 2002; McKillip and Drake, 2004).

Pathogenic bacteria of the genera *Escherichia*, *Shigella*, *Francisella*, *Salmonella*, *Vibrio* and *Yersinia* contain species or strains that are considered biothreat agents that, if intentionally introduced into the food supply system, could result in high morbidities, mortalities and severe economic losses. These bacteria therefore constitute organisms of interest in food defense strategies against potential bioterrorism. Molecular diagnostic techniques for the detection of the individual genera or a combination of some of these agents have been developed by various research groups (Pohanka and Skladal, 2009; Song et al., 2005; Versage et al., 2003). However, the progress towards a rapid, fully multiplexed, sensitive and specific real-time PCR platform for the detection of all the six agents is not yet satisfactory.

In an attempt to establish a molecular detection platform amenable to real-time PCR, we first tested several previously reported PCR-primers for their validity for the detection of a wide range of pathogenic and non-pathogenic, as well as related or-unrelated species or strains. While some of the primers or gene targets reported in the literature were further used for our real-time assays, *in silico* validation of many of the primers against a wide array of bacterial genomes revealed cross reactivity or amplicon sizes not compatible with simultaneous real-time PCR detection of multiple pathogens. Therefore, we utilized whole-genome data mining, text-mining, *in silico* target and amplicon analysis, and *in vitro* validation using DNA sequences from representative bacteria. We have identified very specific molecular targets for reliable identification of the six food biothreat agents, and developed a platform with simultaneous detection capability. Interestingly, many of targets we identified have putative functions or code for hypothetical proteins, digressing from the traditional use of previously known gene targets or known pathogenicity-associated molecular detection tools.

2. Materials and Methods

2.1. Bacterial species and DNA preparation for PCR

The bacterial strains used in this study are listed in Table 1. Laboratory level 2 organisms consisted of *Shigella dysentriae, Shigella sonnei*, and *Salmonella enterica* ssp. *enterica* serovar Saintpaul were grown aerobically at 37°C on tryptic soy agar supplemented with 5 % horse blood and tryptic soy broth, the exceptions to this is *Vibrio vulnificus,* which was grown at 30°C. One ml of culture was collected by centrifugation at 10,000-× *g*, and pellet re-suspended in sterile 1XPBS solution. DNA was extracted according to the manufacturer's protocol recommended for bacterial DNA extraction (Wizard genomic DNA purification kit, Promega). Genomic DNAs of strains, *F. tularensis* subspecies *novicida* KM145 and *Y. pestis* ZE 94–2122 were obtained from Biodefense and Emerging Infections Research Resources Repository (BEI Resources). Genomic DNAs of all organisms listed on Table 1, except for *F. tularensis* subspecies *novicida* strain U112 and *F. tularensis* subspecies *tularensis* strain Schu S4, were purchased from ATCC collection (Manassas, VA). The genomic DNA of the two *Francisella* subspecies, *F. tularensis* subspecies *novicida* U112 and *F. tularensis* subspecies *tularensis* Schu S4 were kindly provided by Dr. Karl Klose, University of Texas at San Antonio (UTSA) STCEID.

2.2. Genomic and expressed gene data mining

Completed genome sequence data of all of the select agents, including incomplete genome sequence for *S. enterica* subsp. *enterica* serovar Saintpaul strain SARA 23 were retrieved from the NCBI (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi) microbial genome-sequencing database. Most recently published references of comparative genomics of every select agent were used in target region selection. A BLAST search was used in the selection of specific amino acid sequences of all the organisms used in this study. Specific primers were designed from genes and coding regions specific to the agent of interest. These primers were then analyzed *in silico* for specific binding across the genome sequence of similar species. For more stringency assessment we used V-NTI Advanced-11 (Invitrogen, USA) for the design and modification of primers having a 3` end similarity with the closely related species. All available whole genome sequence and partial sequences from prokaryote genome database of the NCBI were used for the *in silico* validation of the specific primers.

2.3. Comparative genomic analysis

In search for a specific region, a BLAST analysis was performed for all the species containing a coding sequence of > 500 aa. Further COBALT alignment analysis was used to localize more specific regions for species under question. Once the specific amino acid sequence was localized, we used the nucleotide sequence of this sequence for specific primer design.

2.4. Primer design and preliminary analysis

The primers underwent rigorous testing before the experimental validation. This included oligo-dimer, hair-loop formation and successful standardization of all primers to similar melting temperatures, which is one of the requirements for the simultaneous use of these primers. Primers fulfilling the required criteria were further analyzed for possible binding to a different location within the same whole genome sequence using V-NTI motif search analysis.

2.5. In silico PCR

In silico PCR validations were performed using both http://insilico.ehu.es/PCR/ and V-NTI. Only primers giving specific amplifications were selected for further validation using

conventional and real time PCR assay. Primers that showed the most specificity to the target molecule were ordered from Integrated DNA technologies (IDT, IA).

2.6. In vitro PCR and analysis

All PCR reactions were set up in an isolated PCR station (AirClean Systems, NC) that was UV-sanitized daily and after each use. Initial conventional PCR was performed to validate the specificity of the primers for respective organisms. DNA from 23 strains representing major biothreat agents and closely related foodborne pathogens were used for the validation (Table 1). Single target PCR was performed in a 25 μl final volume containing 0.2 μM of forward and reverse primers, 12.5 μl of Pwo Master mix containing 1.25 U of Pwo enzyme, 2 mM MgCl$_2$ and 0.2 mM dNTPs (Roche Diagnostics, Mannheim Germany). The PCR amplification profile for this initial assay consists of 10 min at 95 °C, followed by 30 cycles of 15 seconds at 95 °C, 15 seconds at 60 °C, and 15 seconds at 72 °C using Master cycler pro (Eppendorf, Humburg, Germany). Presences of single band were analyzed using gel electrophoresis.

2.7. Real-time PCR

Real time PCR assay was performed using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies) for final validation & verification. A specific amplification was obtained with all the primers used to amplify the respective organisms. A reaction volume of 20 μl containing 500 nM of forward and reverse primers, 10 μl of 2X Brilliant III Ultra-Fast SYBER Green master mix, 0.3 μl ROX reference dye, 1 μl of DNA template, nuclease free H$_2$O added to the final volume was used for the real-time PCR. 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; completed by one melting curve cycle of 1 min at 95 °C, 30 seconds at 65 °C, and 30 seconds at 95°C.

2.8. Sensitivity assay

Sensitivity of the real-time PCR assay was determined by five-fold serial dilution of the DNA samples (initial DNA concentrations were 2.5 ng/μl for *E. coli* O157:H7 strain EDL933, 2 ng/μl for *Francisella tularensis*, 2.6 ng/μl for *Shigella dysentriae*, 3.7 ng/μl for *Salmonella typhi*, 3 ng/μl *Vibrio cholerae* and 3.8 ng/μl for *Yersina pestis*) in nuclease free double distilled H$_2$O from each of the bacterial species. One microliter of each of the DNA dilutions were used in real time PCR assay mixture. The assay was performed as described above.

2.9. Sensitivity assay in food Matrix

Milk (450 μl) was inoculated with 7.5×10^8 CFU of *Shigella dysentriae* (attenuated strain) culture suspension and diluted serially up to 10^{-8}. After the dilution, bacteria in each aliquot were immediately concentrated by centrifugation at 12,000 RPM ($13,400 \times g$) for 2 min. Whole genomic DNA was extracted from all of the serially diluted tubes according to the PrepMan Ultra Sample Preparation protocol (Applied Biosystems, CA). The DNA was detected using ShD1 primers by real-time PCR. In parallel, bacterial cultures were initiated to determine the CFU of bacteria in those dilutions.

3. Results

3.1. Bacterial Species, Design of primers and in silico validation

The list of strains of bacterial species included in this study to establish the PCR detections is given in Table 1. DNA from these organisms were either purchased or donated through specific agreements. Initially, we aimed at developing a simultaneous foodborne pathogen

detection platform using published primer sequences. Some of the sequences were found to be not highly specific when tested against a wide array of organisms *in silico*. Others were not suitable for simultaneous or multiplex detection of the pathogens in the current study. Therefore, we designed an entirely new set of primers or modified the existing sequences to develop our detection system. To this end, we used text mining, genomic data mining, sequence analysis and comparison tools. Specific primers Typhi-vipR-ST2-F and R were adapted from the work done by Jin et al. (2008) modified by removing G from the 3'end of the forward primer and CT from the 5'end. The list of primers designed or used in this study is shown in Table 2. During the process of selection, the primers were initially validated for unique site recognition and strength of complementarities by using the genomic DNA of each organism as a template.

After the primers were designed, we tested the specificity of the primers by performing virtual (*in silico*) PCR using the publicly accessible tool at http://insilico.ehu.es/PCR/. Examples of pre-validation *in silico* PCR are shown in Fig. 1. Additionally, we further tested the strength of complementarities and the uniqueness of the binding sites for each primer using the Vector NTI software package (Invitrogen, Carlsbad, CA). Reference genomic DNA sequences of each of the organisms to be detected were uploaded to the program and the designed primers were run along the entire genomic sequence. Validated primers with 100% complementary binding at a unique site were selected for each organism. These primers were in turn tested for cross reactivity with other genomic DNA sequences, especially against those phylogenetically closely related bacteria.

3.2. Conventional PCR and gel electrophoresis analysis for the specificity of the primers

Following *in silico* analysis and validation, we tested the primers using DNA isolated from strains of species of the foodborne pathogens. Each PCR experiment was designed so that the primers are tested against their template DNA and DNA from the maximum number of closely related species. In parallel, genus inclusive primers were designed and PCR was performed to verify that the target species as well as other members of the genus could be detected. Genus inclusive primers gave an additional quality control to minimize cross reactivity with other genera or species within the genus. PCR products were resolved on 1.5 % agarose gels; the gels were stained with GelRed (Biotium, Hayward, CA) and photographed using AlphaImager (Cell Biosciences, Santa Clara, CA). As shown in Fig. 2, our species-specific primers gave very specific detection that discriminated each of the target species, whereas the genus-inclusive primers distinctively identified other members of the genus simultaneously with the target. Therefore, these conventional PCR data provided strong evidence that the primers were very specific, and yielded products of the expected size under the experimental conditions employed. Only a minor cross reactivity of the *Escherichia* genus specific primers with *Vibrio cholera* was noticed in this assay. However, the size of the product was larger than expected and also the intensity of the band was weak.

3.3. Validation of the detection of foodborne bacterial pathogens DNA by real-time PCR

The conventional PCR method was employed as described above to visualize the PCR products and determine the specificity of our detection. However, as conventional PCR is time consuming and labor intensive, it is not practical to use it for the detection of pathogens in a high throughput platform. Therefore, we wanted to determine the suitability of our primers and the PCR system in a real-time setup. PCR conditions were selected so that a species-specific primer was used to detect DNA from target and other bacteria arrayed on 96-well plates. The average amount of DNA concentration used per well was 2 ng/μl. On the array, one well was designated for DNA from one bacterial species or strain. While DNA was added to the designated wells, a primer solution was aliquoted to all the 23 wells on the array corresponding the different species listed on Table 1. A positive curve was expected to

be generated only from the wells containing primers and the target DNA in the same well. In a parallel array of 23 wells, genus-inclusive primers were also aliquoted to all the wells containing DNA from the target species as well as other bacteria, including members of the genus. Positive curves were anticipated only from wells designated to the members of the same genus. As shown in Fig. 3, single curves were generated with all of the primers we tested. Since we did not multiplex the PCR, the multiple curves shown in Fig. 3 were generated by merging data from the corresponding single curves originating from parallel simultaneous detection of different strains of the same species.

3.4. Evaluation of the sensitivity of real-time PCR for the detection of DNA from foodborne pathogens

To evaluate the sensitivity of our real-time assay, we serially diluted 1:5 the DNA from each of the major pathogen and performed real-time PCR detection on each of the dilutions. One microliters of each of the dilution was used in a 20 μl total PCR reaction volume and the PCR was run for 27 amplification cycles. At the end of the run, 4 μl aliquots from each of the wells were resolved on a 2 % agarose gel and visualized by GelRed staining (Biotium®). As shown in Fig. 4, the sensitivity of the assay was variable between the organisms used; e.g., starting with 3.8 μg/μl DNA, a 230 fg/μl dilution was detectable for *Y. pestis* DNA while 640 fg/μl of diluted *F. tularensis* DNA was reliably detected for both species at threshold cycles below 27. The overall detection range varied between 234 fg/μl (*Y. pestis*) and 1.18 pg/μl (*S. typhimurium*). The gel electrophoregrams also showed generation of bands of the correct size as well as visible limits of the serial dilution under conditions of the experiment. Therefore, under these conditions, we were able to achieve a high sensitivity of detection combined with the high specificity described above.

3.5. Evaluation of the application of the real-time PCR detection for bacteria in food matrix

Finally, to preliminarily evaluate if our real-time detection approach would be compatible with DNA isolated from bacteria in a food matrix, we spiked commercially available skimmed milk with 7.5×10^8 attenuated *S. dysentriae* bacteria (ATCC) and serially diluted 1:10 the suspension in a total of 500 μl volumes. Immediately after the serial dilution, tubes were centrifuged to concentrate the bacteria and total DNA was isolated as described in materials and methods. Parallel cultures were also initiated from each dilution to count colony-forming units. As shown in Fig. 5, we were able to detect DNA isolated from milk spiked with *Shigella* organisms with an approximate detection limit of 6–60 colony forming units per ml of milk in less than one hour of experiment time.

4. Discussion

We have developed a PCR-based real-time detection platform for simultaneous, rapid, sensitive and differential molecular detection of six primary biothreat agents, namely, *Escherichia, Salmonella, Shigella, Vibrio, Yersinia* and *Francisella*. Through extensive genomic data mining, text mining and multiple layer validation, we have identified 26 new target sequences (Table 2), which enabled us to design the platform with improved specificity. Some of these targets have a well-known functions such as *stx*2A (shiga toxin subunit A from *E. coli* O157:H7), *fus*A (Elongation factor A from *F. tularensis* ssp. *tularensis*), *ipa*H (invasive plasmid antigen from *Shigella* species), *inv*C (invasion protein from *Salmonella* species), *rpo*B (DNA-directed RNA polymerase subunit beta, from *Vibrio* species). However, others have hitherto unknown functions such as in RloF gene from *S. enterica* ssp. *enterica* s. Saintpaul with putative functions, or hypothetical proteins such as *pdp*D (pathogenicity determinant protein D from *F. tularensis* ssp. *tularensis*). Moreover, we preliminarily evaluated the utility of the platform using milk as a food matrix into which *Shigella* organisms were spiked.

One of the known challenges in multiplexed or simultaneous PCR-based molecular detections is the need for optimization of the reaction conditions such as annealing temperatures optimal for all primer sets, avoiding primer dimers, generation of compatible amplicon sizes, and adjustment for different amplification efficiencies (Edwards and Gibbs, 1994). In this study, we have identified PCR conditions that are suitable for the amplification of 105 bp to 371 bp fragments from all the six pathogens under the same reaction conditions. Moreover, the specificities of the primers were tested and validated against a broad array of potential biothreat agents and related species or strains, which do not pose threat. For example, the *Francisella tularensis* ssp *tularensis* detection primers used in this study, designed from a hypothetical protein gene pdpD2, were tested against four other strains, *Francisella* subspecies (*holartica*, *novicida*, and *mediasiatica*) and the closely related *F. philomiragia* ssp *philomiragia*, and showed reactivity only to the *F. tularensis* ssp *tularensis*. On the other hand, *F. tularensis* ssp *novicida* primers based on another gene for hypothetical protein *pdp*D were specific to the subspecies, without detecting any of the other *F. tularensis* subspecies. Despite the degree of specificity we achieved with these primers, our *E. coli* O157:H7 primers EC1 and EC2 still *in-silico* cross detected the non-O157 *E. coli* such as O111 and O103, strains that evolved parallel to the O157 strain (Ogura et al., 2009). However, since such non-O157 strains also possess pathogenic potential (Reid et al., 2000), the ability of our primers to detect non-O157 strains may be a desirable feature in the investigation of *E. coli* outbreaks.

Because of their close evolutionary relationship, differentiation of *Escherichia* from *Shigella* species poses a big challenge (Jin et al., 2002; Pupo et al., 2000). In our study, even challenging was the distinction among Shigella species, especially *Shigella sonnei* from other members of the genus. After extensive comparative genome analysis, we identified the gene for *rhs*A protein in *rhs* element as target for the specific identification of *S. sonnei*. Similarly, *Salmonella enterica* ssp *enterica* s. *typhimurium* was identifiable by targets in the genes for putative inner membrane protein and putative DNA repair ATPase. On the other hand, while *Vibrio parahemolyticus* was identifiable using the hemolysin gene VP1729, *Vibrio cholerae* was identifiable using the gene for phosphotyrosine protein phosphatase VC0916 as a target.

Our primers based on the genes for putative phage-related membrane protein (YPO2127) and the hypothetical protein YpAngola A2197 were able to detect all *Y. pestis* strains except the biovar Microtus strain 91001. Lack of identification of this organism using our present assay would not be a major problem since there has been so far no evidence that human plaque can arise from Microtus strains (Zhou et al., 2004). Furthermore subcutaneous inoculation of strains from serovar Microtus has demonstrated no virulence (Song et al., 2004). The genes for YPTS_2284 and YPTB2194 hypothetical proteins were also specific targets for the identification of *Y. pseudotuberculosis* isolates. These two regions were selected from a set of 67 refined species-specific genes (Mark Eppinger et al., 2007). While the targeting of hypothetical proteins for detection of the bacteria may not directly correlate with the hitherto known pathogenicity or virulence of the organisms, genetic studies may reveal the significance of these gene products in bacterial biology or host interaction, or even pathogenicity. The identification of these genomic regions as specific to the particular species or even sub species is an important step in advancing pathogen detection techniques by providing additional targets.

We were able to verify our *in silico* results by *in vitro* differential identifications under identical PCR conditions. In previous studies other groups had also developed multiplexed PCR assays to simultaneously detect multiple food borne pathogens (Fukushima et al., 2010; Jothikumar and Griffiths, 2002; Skottman et al., 2007; Wilson et al., 2005). While our approach is similar in some aspects, for the first time we have combined into one assay the

detection of primary biothreat agents of bioterrorism potential, and identified highly specific targets capable of discriminating a broad range of pathogens or related bacteria. In this study, we did not multiplex the primers or DNA in a single tube. With added future improvements for even more rapid differential detection and multiplexing capabilities, our assay platform provides a strong foundation to strengthen national and international food defense strategies as a promising component of primary detection or confirmatory platforms.

Through this study, we also have designed highly specific primers that yielded PCR assays with high sensitivity and low detection limits. For example to the detection limit for *F. tularensis* in this study are comparable to or better than some other reports (Sellek et al., 2008; Svensson et al., 2009), although different sources of DNA and procedures of detection may influence the detection limits.

Food matrices provide a critical challenge in amplification-based pathogen detection approaches. Improved pre-analytical sample processing techniques are needed to reduce the time needed to arrive at diagnosis and decision-making (Benoit and Donahue, 2003; Dwivedi and Jaykus, 2011). Previous studies have shown immunomagnetic separation method to provide better concentration of bacteria from food matrices such as chicken meat (Taha et al., 2010). In milk, a combination of the two techniques, i.e. immunomagnetic separation and polymerase chain reaction, provided a detection of 1–10 CFU of salmonellae/ml, after a selective pre-enrichment incubation of 12–16 hrs. However, a decreased sensitivity of 10–100 CFU/ml was observed after 8–10 h of pre-enrichment period (Mercanoglu Taban et al., 2009). In this study we have performed a preliminary test to evaluate the use of real-time PCR for detection of *S. dysentriae* spiked in milk. Using centrifugation to concentrate the bacteria serially diluted in milk, we were able to detect about 6–60 CFU/ml of milk matrix, without an enrichment step. According to CDC, 10–100 organisms of *S. dysentriae* are considered the minimum infectious dose with possible secondary transmission (Sobel et al., 2002).

Caution is needed in correlating the CFU findings with PCR detection limits, because in general, PCR does not discriminate between live and dead organisms. Therefore, any correlation between our DNA detection limit and the observed CFU per dilution may not be direct. For example, if there is a time lapse between sample collection and PCR analysis, the CFU could underestimate the degree of contamination of the food matrix. Alternatively, sterilized products containing non-viable bacteria or their DNA may still yield positive data. Although our studies using food matrix are still preliminary, we were able to detect organisms in a milk matrix with reasonable sensitivity. Further work is needed to improve recovery of pathogen DNA from such matrices and improve the sensitivity. Ultimately the findings of this study will contribute to an effective means of identifying high impact pathogenic agents in human food supply systems before the agents reach the consumer.

Acknowledgments

References

Benoit PW, Donahue DW. Methods for rapid separation and concentration of bacteria in food that bypass time-consuming cultural enrichment. J Food Prot. 2003; 66:1935–1948. [PubMed: 14572237]

Busta, FF.; Shaun, PK. Defending the safety of the global food system from intentional contamination in a changing market. In: Hefnawy, M., editor. Advances in Food Protection [Focus on Food Safety and Food Defense. (Security and Safety against Terrorist Threats and Natural Disasters) NATO/SPS Advanced Research Workshop Proceedings] NATO Science for Peace and Security Series A: Chemistry and Biology. New York: Springer Publishing; 2011. p. 119-135.

Dwivedi HP, Jaykus LA. Detection of pathogens in foods: the current state-of-the-art and future directions. Crit Rev Microbiol. 2011; 37:40–63. [PubMed: 20925593]

Edwards MC, Gibbs RA. Multiplex PCR: advantages, development, and applications. PCR Methods Appl. 1994; 3:S65–S75. [PubMed: 8173510]

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

He Q, Marjamaki M, Soini H, Mertsola J, Viljanen MK. Primers are decisive for sensitivity of PCR. Biotechniques. 1994; 17:82–84. 86–87. [PubMed: 7946322]

Jin Q, Yuan Z, Xu J, Wang Y, Shen Y, Lu W, Wang J, Liu H, Yang J, Yang F, Zhang X, Zhang J, Yang G, Wu H, Qu D, Dong J, Sun L, Xue Y, Zhao A, Gao Y, Zhu J, Kan B, Ding K, Chen S, Cheng H, Yao Z, He B, Chen R, Ma D, Qiang B, Wen Y, Hou Y, Yu J. Genome sequence of Shigella flexneri 2a: insights into pathogenicity through comparison with genomes of Escherichia coli K12 and O157. Nucleic Acids Res. 2002; 30:4432–4441. [PubMed: 12384590]

Jothikumar N, Griffiths MW. Rapid detection of Escherichia coli O157:H7 with multiplex real-time PCR assays. Appl Environ Microbiol. 2002; 68:3169–3171. [PubMed: 12039787]

Kennedy S. Epidemiology. Why can't we test our way to absolute food safety? Science. 2008; 322:1641–1643. [PubMed: 19074334]

Lantz PG, Abu al-Soud W, Knutsson R, Hahn-Hagerdal B, Radstrom P. Biotechnical use of polymerase chain reaction for microbiological analysis of biological samples. Biotechnol Annu Rev. 2000; 5:87–130. [PubMed: 10874998]

Mark Eppinger MJ, Rosovitz, 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; 3:1508–1523.

Markoulatos P, Siafakas N, Moncany M. Multiplex polymerase chain reaction: a practical approach. J Clin Lab Anal. 2002; 16:47–51. [PubMed: 11835531]

McKillip JL, Drake M. Real-time nucleic acid-based detection methods for pathogenic bacteria in food. J Food Prot. 2004; 67:823–832. [PubMed: 15083739]

Mercanoglu Taban B, Ben U, Aytac SA. Rapid detection of Salmonella in milk by combined immunomagnetic separation-polymerase chain reaction assay. J Dairy Sci. 2009; 92:2382–2388. [PubMed: 19447970]

Ogura Y, Ooka T, Iguchi A, Toh H, Asadulghani M, Oshima K, Kodama T, Abe H, Nakayama K, Kurokawa K, Tobe T, Hattori M, Hayashi T. Comparative genomics reveal the mechanism of the parallel evolution of O157 and non-O157 enterohemorrhagic Escherichia coli. Proc Natl Acad Sci U S A. 2009; 106:17939–17944. [PubMed: 19815525]

Pohanka M, Skladal P. Bacillus anthracis, Francisella tularensis and Yersinia pestis. The most important bacterial warfare agents - review. Folia Microbiol (Praha). 2009; 54:263–272. [PubMed: 19826916]

Pupo GM, Lan R, Reeves PR. Multiple independent origins of Shigella clones of Escherichia coli and convergent evolution of many of their characteristics. Proc Natl Acad Sci U S A. 2000; 97:10567–10572. [PubMed: 10954745]

Reid SD, Herbelin CJ, Bumbaugh AC, Selander RK, Whittam TS. Parallel evolution of virulence in pathogenic Escherichia coli. Nature. 2000; 406:64–67. [PubMed: 10894541]

Sellek R, Jimenez O, Aizpurua C, Fernandez-Frutos B, De Leon P, Camacho M, Fernandez-Moreira D, Ybarra C, Carlos Cabria J. Recovery of Francisella tularensis from soil samples by filtration and detection by real-time PCR and cELISA. J Environ Monit. 2008; 10:362–369. [PubMed: 18392279]

Skottman T, Piiparinen H, Hyytiainen H, Myllys V, Skurnik M, Nikkari S. Simultaneous real-time PCR detection of Bacillus anthracis, Francisella tularensis and Yersinia pestis. Eur J Clin Microbiol Infect Dis. 2007; 26:207–211. [PubMed: 17294160]

Sobel J, Khan AS, Swerdlow DL. Threat of a biological terrorist attack on the US food supply: the CDC perspective. Lancet. 2002; 359:874–880. [PubMed: 11897303]

Song L, Ahn S, Walt DR. Detecting biological warfare agents. Emerg Infect Dis. 2005; 11:1629–1632. [PubMed: 16318712]

Song Y, Tong Z, Wang J, Wang L, Guo Z, Han Y, Zhang J, Pei D, Zhou D, Qin H, Pang X, Zhai J, Li M, Cui B, Qi Z, Jin L, Dai R, Chen F, Li S, Ye C, Du Z, Lin W, Yu J, Yang H, Huang P, Yang R. Complete genome sequence of Yersinia pestis strain 91001, an isolate avirulent to humans. DNA Res. 2004; 11:179–197. [PubMed: 15368893]

Svensson K, Back E, Eliasson H, Berglund L, Granberg M, Karlsson L, Larsson P, Forsman M, Johansson A. Landscape epidemiology of tularemia outbreaks in Sweden. Emerg Infect Dis. 2009; 15:1937–1947. [PubMed: 19961673]

Taha EG, Mohammed A, Srivastava KK, Reddy PG. Rapid Detection of Salmonella in chicken meat using Immunomagnetic separation, CHROMagar, ELISA and Real time polymerase chain reaction (RT-PCR). International Journal of Poultry Science. 2010; 9:831–835.

Versage JL, Severin DD, Chu MC, Petersen JM. Development of a multitarget real-time TaqMan PCR assay for enhanced detection of Francisella tularensis in complex specimens. J Clin Microbiol. 2003; 41:5492–5499. [PubMed: 14662930]

Wilson WJ, Erler AM, Nasarabadi SL, Skowronski EW, Imbro PM. A multiplexed PCR-coupled liquid bead array for the simultaneous detection of four biothreat agents. Mol Cell Probes. 2005; 19:137–144. [PubMed: 15680215]

Wright PA, Wynford-Thomas D. The polymerase chain reaction: miracle or mirage? A critical review of its uses and limitations in diagnosis and research. J Pathol. 1990; 162:99–117. [PubMed: 2250198]

Zhou D, Tong Z, Song Y, Han Y, Pei D, Pang X, Zhai J, Li M, Cui B, Qi Z, Jin L, Dai R, Du Z, Wang J, Guo Z, Huang P, Yang R. Genetics of metabolic variations between Yersinia pestis biovars and the proposal of a new biovar, microtus. J Bacteriol. 2004; 186:5147–5152. [PubMed: 15262951]
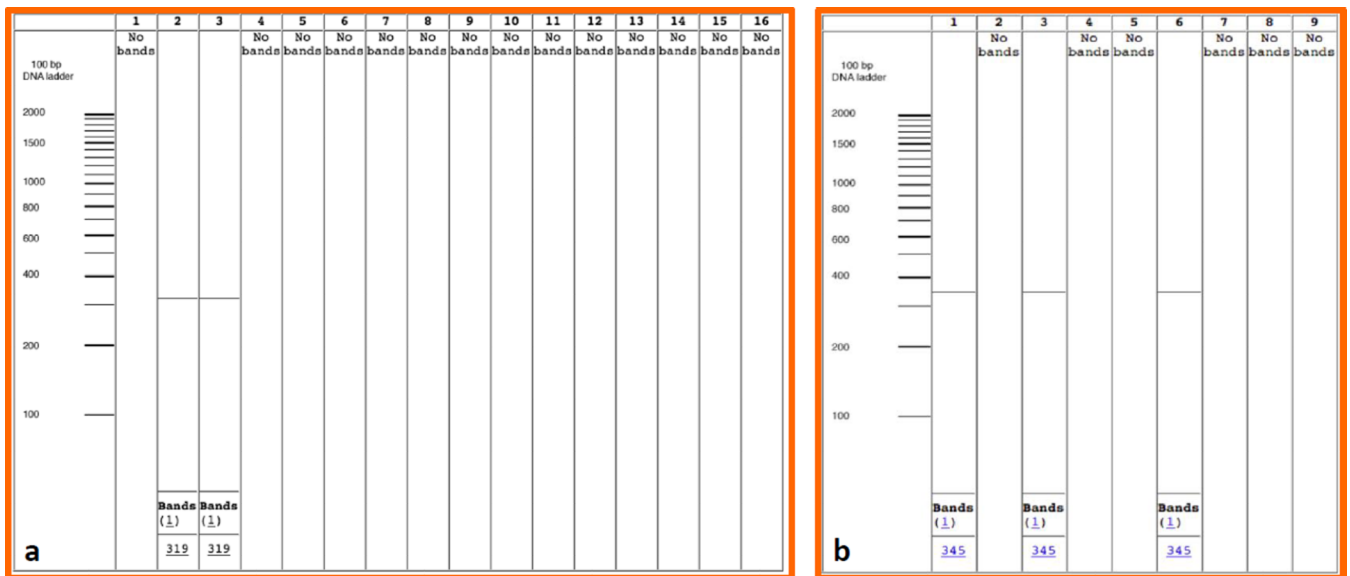
Fig. 1.
Specific *in silico* validation performed using our primers **1a**. Pre-validation of ST1-F-m-2/R-m-2 primers for *S. enterica* ssp. *enterica* s. Typhi 1. *S. enterica* subsp. *enterica* s. Typhimurium LT2, 2. *S. enterica* ssp. *enterica* s. Typhi, 3. *S. enterica* subsp. *enterica* s. Typhi Ty2, 4. *S. enterica* subsp. *enterica* s. Paratyphi A str. ATCC 9150, 5. *S. enterica* subsp. *enterica* s. Choleraesuis str. SC-B67, 6. *S. enterica* subsp. *enterica* s. Paratyphi B str. SPB7, 7. *S. enterica* subsp. *arizonae* s. 62:z4, z23:--8. *S. enterica* subsp. *enterica* s. Newport str. SL254, 9. *S. enterica* subsp. *enterica* s. Heidelberg str. SL476, 10. *S. enterica* subsp. *enterica* s. Schwarzengrund str. CVM19633, 11. *S. enterica* subsp. *enterica* s. Agona str. SL483, 12. *S. enterica* subsp. *enterica* s. Paratyphi A str. AKU_12601, 13. *S. enterica* subsp. *enterica* s. Dublin str. CT_02021853, 14. *S. enterica* subsp. *enterica* s. Gallinarum str. 287/91, 15. *S. enterica* subsp. *enterica* s. Enteritidis str. P125109, 16. *S. enterica* subsp. *enterica* s. Paratyphi C strain RKS4594; **1b**. Pre-validation of FT1-F/R, primers for *F. tularensis* ssp. *tularensis*, 1- *Francisella tularensis* subsp. *tularensis* Schu4, 2 - *Francisella tularensis* subsp. *holarctica*, 3 - *Francisella tularensis* subsp. *tularensis* FSC 198, 4 - *Francisella tularensis* subsp. *holarctica* OSU18, 5 - *Francisella tularensis* subsp. *novicida* U112, 6 - *Francisella tularensis* subsp. *tularensis* WY96-3418, 7 - *Francisella tularensis* subsp. *holarctica* FTNF002-00, 8 - *Francisella philomiragia* subsp. *philomiragia* ATCC 25017, 9 - *Francisella tularensis* subsp. *mediasiatica* FSC147
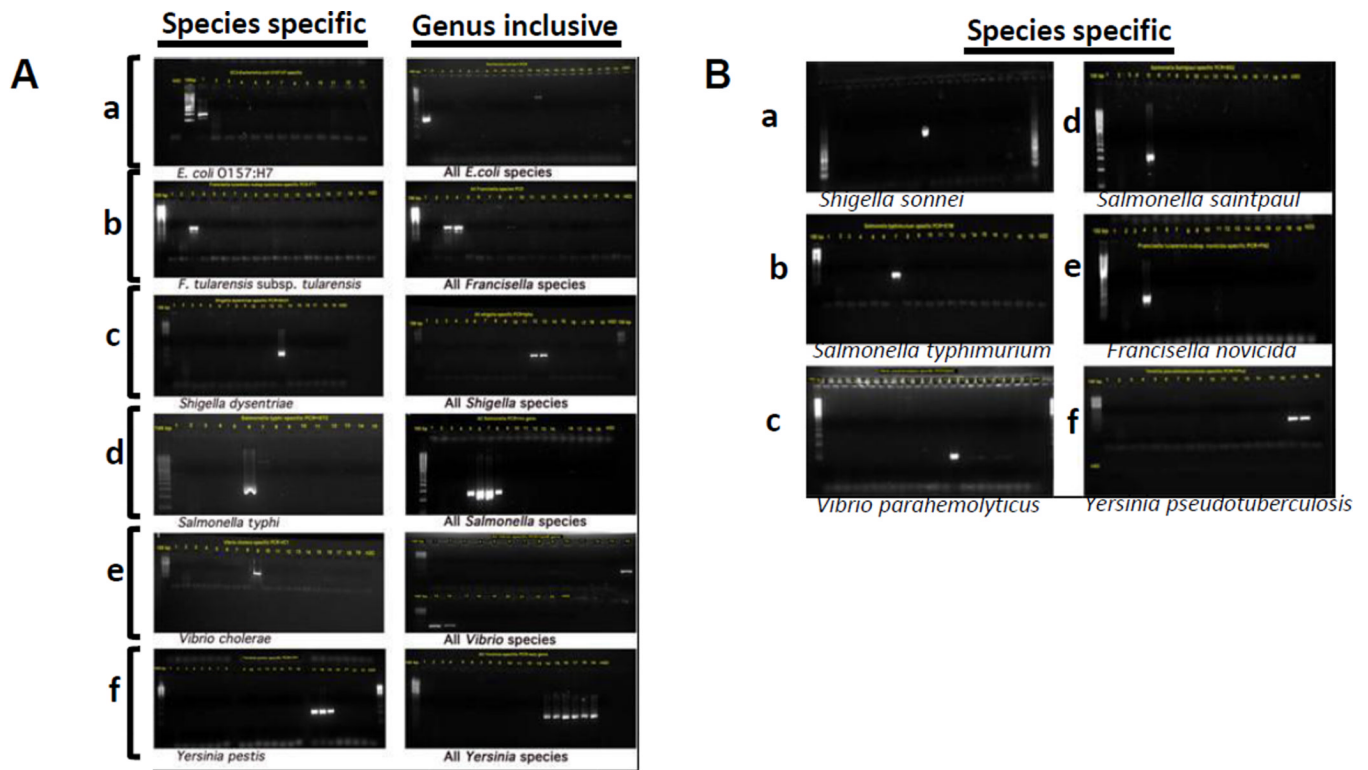
Fig. 2.

(A and B). Validation of the specificity of the primers by conventional PCR and gel electrophoresis to detect biothreat agents (**A**) and major foodborne pathogens (**B**): **A**. **a**. detection of *E. coli* O157:H7 using the EC2 primers and a genus inclusive primer (right panel) **b**. detection Francisella using *F. tularensis ssp tularensis* (FT2, left panel) and genus inclusive primers (*F. tularensis* and *F. novicida* shown in the right panel); **c**. detection of Shigella using *S. dysentriae* primers (ShD1, left panel) and genus inclusive (*S. dysentriae* and *S. sonnei* shown in the right panel) primers **d**. detection of Salmonella using the ST2 primers for *Salmonella typhimurium* (left panel) and genus-inclusive primers (*S. typhi, S. typhimurium*, *S. saintpaul*, and *S. braenderup* shown in the right panel); **e**. detection of *V. cholerae* using VC1 primers (left panel) and *Vibrio* genus inclusive primers (*V. cholerae, V. parahemolyticus*, and *V. vulnificus* (right panel); **f**. detection of *Yersinia* using YP1 primers for *Y. pestis* (left panel) or genus inclusive primers (three different *Y. pestis strains*, two *Y. pseudotuberculosis* strains, and *Y. enterocolitica* (right panel)). **B.** Validation of specificity of primers to major foodborne pathogens: **a**. detection of *Shigella sonnei* using rhs-y-Sonnei-F/R, **b**. detection of *Salmonella enterica* ssp. *enterica* serovar Typhimurium using STM-F-M/R-M, **c**. detection of *Vibrio parahemolyticus* using VpH1-F/R, **d**. *Salmonella enterica* ssp. *enterica* serovar Saintpaul using SS2-F-n/R-n; **e**. *Francisella tularensis* ssp *novicida* using FN2-F-m/R-m; and **f**. *Yersinia pseudotuberculosis* using YPs1-F-M/R.
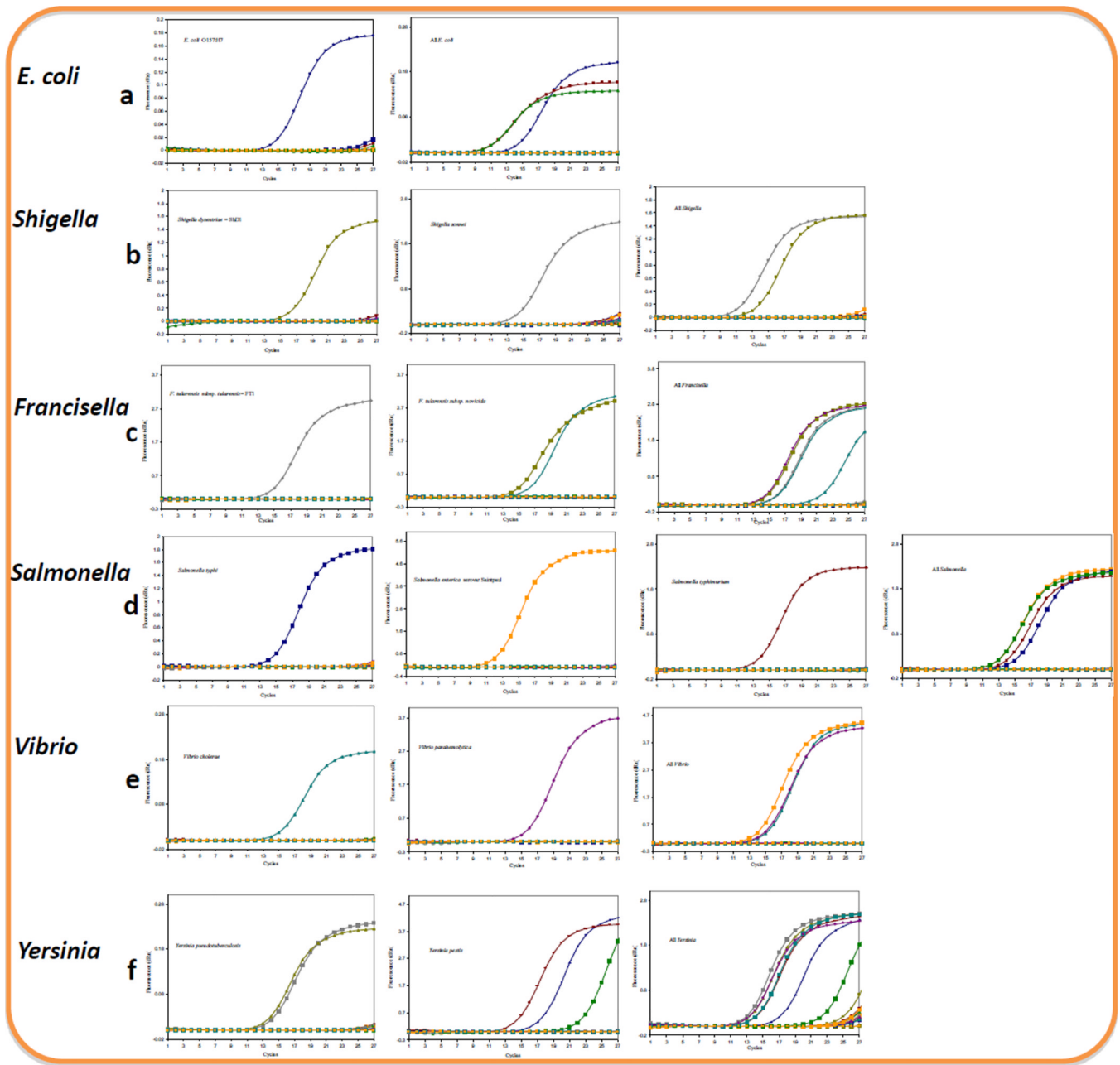
Fig. 3.
Validation of the specificity of the primers against DNA from 23 foodborne pathogens and related bacterial species (listed in table 2) by real-time PCR: Detected were **a**. *E. coli* (left panel 0157:H7, right panel 0157:H7, HB101, and ATCC 1175 strains with genus-inclusive primers) **b**. *Shigella* (left panel *S. dysentriae*, middle panel *S. sonnei*, right panel *S. dysentriae* and *S. sonnei* with genus inclusive primers) **c**. *Francisella* (right panel *F. tularensis ssp tularensis*, middle panel two strains of *F. tularensis ssp. novicida*, and right panel all Francisella with genus inclusive primers) **d**. Salmonella (panels from left to right: 1st *S. typhi*, 2nd *S. enterica* ssp *enterica s.* Saintpaul, 3rd *S. enterica* ssp *enterica s.* Typhimurium, 4th all Salmonella with genus inclusive primers, **e**. Vibrio (left panel *V. choleræ*, middle panel *V. parahemolyticus*, right panel all Vibrios with genus inclusive

primers **f**. Yersinia (left panel *Y. pseudotuberculosis*, middle panel *Y. pestis*, and right panel all Yersinia with genus inclusive primers). PCRs using genus inclusive primers and those on multiple strains of the same species e.g. *Y. pestis* (**f**) were run separately, and the amplification plots were merged. The highest number of cycles used for all the plots is 27.
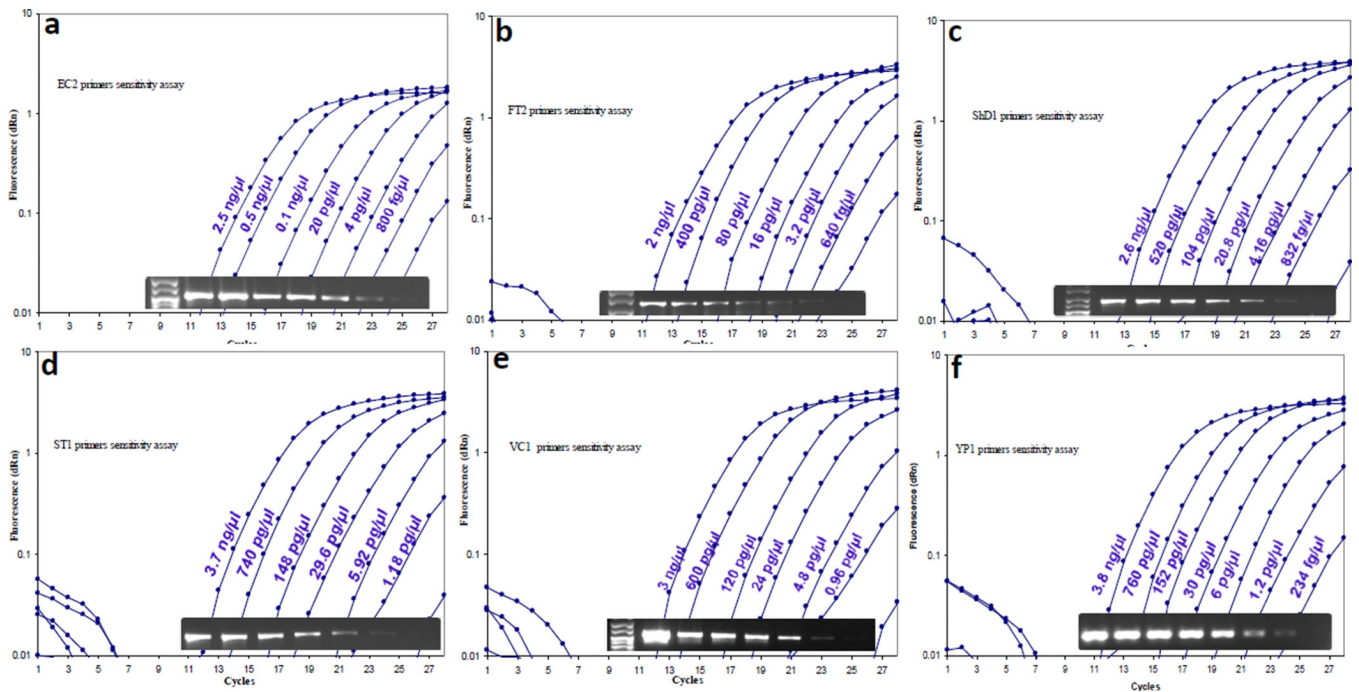
Fig. 4.
Sensitivity of the real-time PCR assay to detect six major foodborne pathogens: Aliquots of DNA from *E. coli* O157:H7 (a), *S. dysentriae* (b), *F. tularensis* ssp. *tularensis* (c), *S. enterica ssp enterica s.* Typhi (d), *V. cholerae* (e), and *Y. pestis* (f), were serially five-fold diluted and analyzed by real-time PCR to determine the lowest detection limit of the assay. At the end of the 27 cycles of PCR, aliquots of the reactions were also resolved by agarose gel electrophoresis to examine the PCR products and intensity from each dilution (lower inset panels).
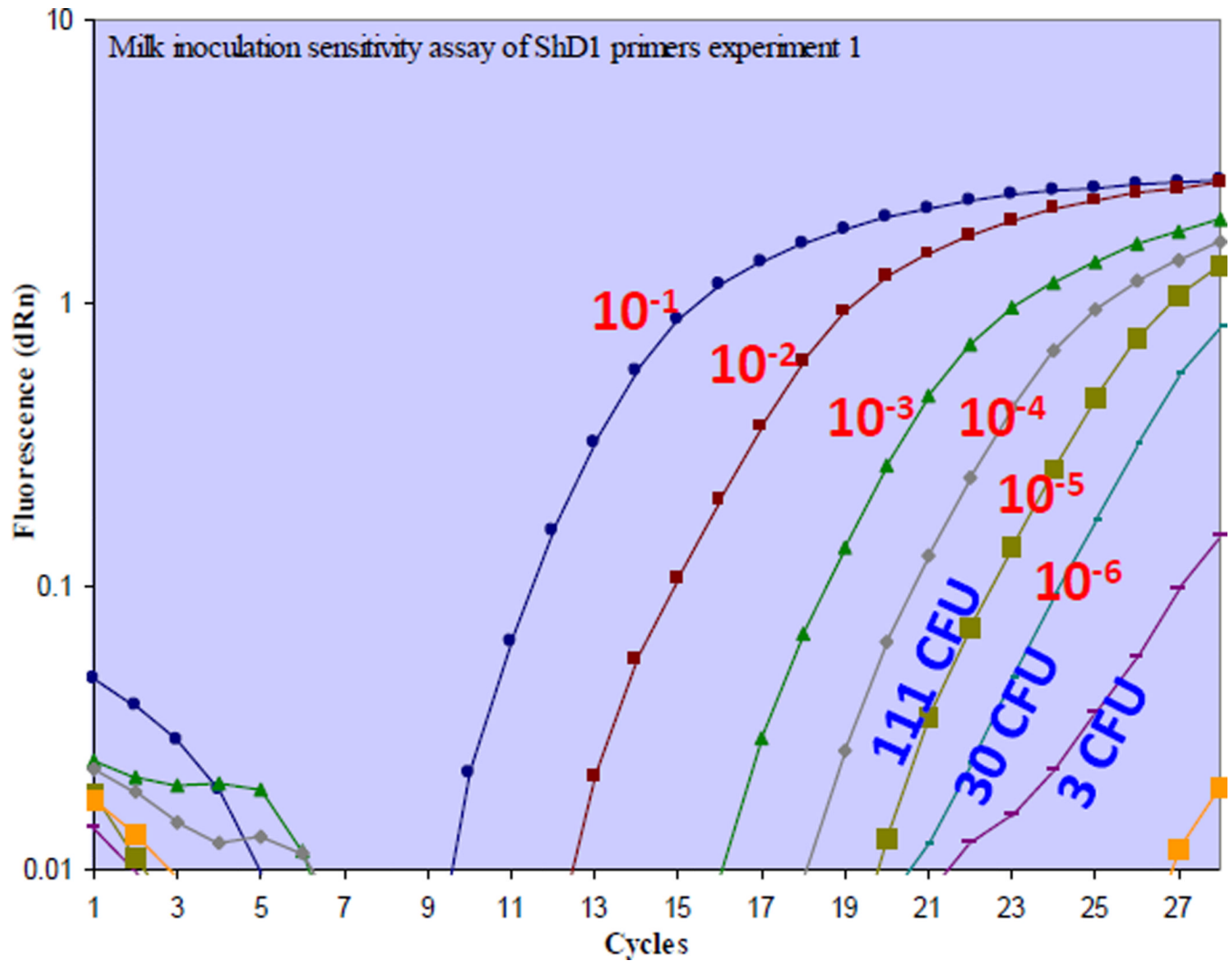
Fig. 5.
Sensitive detection of *Shigella dysentriae* in milk using real-time PCR assay: Attenuated *S. dysentriae* bacteria (7.5×10^8 CFU) were serially 10-fold diluted in milk as described in materials and methods. Bacteria suspended in each of the 500μl serial dilution volumes were concentrated, and genomic DNA was isolated. The DNA was detected using ShD1 primers by real-time PCR. In parallel, bacterial cultures were initiated to determine the CFU of bacteria in those dilutions. The real time PCR assay was able to detect DNA isolated from at least 3–30 CFU/500μl (6–60 CFU/ml) of *S. dysentriae* in milk.

Table 1

List of organisms used in the validation of specific PCR

Species/ Strain	CDC category	Origin
Genus *Escherichia*		
Escherichia coli O157:H7 EDL933	B	ATCC
Escherichia coli 1175		ATCC
Genus *Francisella*		
Francisella tularensis ssp. *tularensis* Schu S4	A	Dr. Karl Klose (UTSA, STCEID
Francisella tularensis ssp. *novicida* U112		Dr. Karl Klose (UTSA, STCEID
Francisella tularensis ssp. *novicida* KM145		
Francisella tularensis ssp. *philomiragia*		ATCC
Genus *Salmonella*		
Salmonella enterica subsp. *enterica* serovar Braenderup ATCC (®BAA-664™)		ATCC
Salmonella enterica serovar *typhimurium* LT2 ATCC® (700720D-5™)	B	ATCC
Salmonella enterica serovar *typhi* Ty2 ATCC (®700931™)	B	ATCC
Salmonella enterica subspecies *enterica* serovar Saintpaul 127 ATCC (®9712™)	B	ATCC
Genus *Shigella*		
Shigella dysentriae ATCC (®11456a™)	B	ATCC
Shigella sonnei ATCC (®11060™)	B	ATCC
Genus *Vibrio*		
Vibrio cholerae ATCC (®39315™)	B	ATCC
Vibrio parahaemolyticus EB 101 ATCC (®17802™)		ATCC
Vibrio vulnificus Type strain Bio-group 1 ATCC (®27562™)		ATCC
Genus *Yersinia*		
Yersinia pestis A1122 BEI (NR-15)	A	
Yersinia pestis KIM10+ BEI (NR-642)	A	BEI
Yersinia pestis ZE 94–2111	A	ATCC
Yersinia pseudotuberculosis P62 ATCC (29910)	B	BEI (NR-804)
Yersinia pseudotuberculosis NCTC 10275 ATCC (29833)	B	ATCC
Yersinia enterocolitica Billups-1803-68 ATCC (23715)		BEI (NR-204)
Yersinia enterocolitica WA ATCC (27729)		ATCC

Table 2

Primers used to establish PCR to detect food threat and major foodborne bacterial pathogens. The primers were used for both conventional and real-time PCR methods established in this study. All the primers except those indicated in the footnote below the table, were newly designed.

Primer Name	Sequence 5´–3´	Bacterial species	Target gene	Gene Accession No.	Amplicon Size
EC2-SLT-R-m EC2-SLT-F-m	CAGACGAAGATGGTCAAAACGCG AGTTTACGATAGACTTTTCGACCC	*Escherichia coli* O157:H7 EDL933	Stx2	NP_286976	201 bp
EC1-Rm EC1-Fm	TCTGGTTGACTCTCTTCATTCACGG TACAGAGAGAATTTCGTCAGGCACTG	*Escherichia coli* O157:H7 str EC4115	Stx2A	YP_002271797	256 bp
lacY-ecoli-F lacY-ecoli-R	GCACTTCAAACTGGCTGGTAATA TGCACCTACGATGTTTTGACC	*Escherichia coli* CFT073	lacY	NP_752393	331 bp
FT1-F FT1-R	GAAGGTCTTCTAGAAAATTCTGCTC TTGCTGGTAATTCGTAGATAATATC	*Francisella tularensis* subsp. *tularensis* Schu4	PdpD2	YP_170620	345 bp
FT2-mR FT2-mF	GGAAGCATAGCTATTAGCATATTCTGG TTGTCTAAAGCAAATATTGAGTGGG	*Francisella tularensis* subsp. *tularensis* Schu4	HP	YP_169554	234 bp
FN2-F-m FN2-R-m	ATGCAAAAGATAAGGCTAACTCTT GAATCAATATTCGTTAGGTCTTCA	*Francisella tularensis* subsp. *novicida* U112	PdpD	YP_898955	214 bp
All F-R-m All F-F-m	GGAACACCGTARTTGTTAGCTTGG ATTGGTATCGTGTCTCACGTTGATG	*Francisella tularensis* subsp. *holarctica* OSU18	fusA	YP_762892	371 bp
ShD1-F ShD1-R	ATGGTGTCGTCGATAATATCGGCC AAGAGCGTATCTGGAGTATTTCACC	*Shigella dysenteriae* Sd197	Z5694-like protein	YP_405526	270 bp
ShD2-F-m ShD2-R-m	GTGATGGTTGTTGTTAGATTCTACCAA ATGCAATTGCCAATAGACAACCA	*Shigella dysenteriae* Sd197	HP	YP_402814	231 bp
rhsA-y-Sonnei-F rhsA-y-Sonnei-R	TATTGCTGCGGTCATACACTGCC CTGATCGAACTTCGATGCCAATCC	*Shigella sonnei* Ss046	rhsA	YP_309648	303 bp
ipaH-F ipaH-R	CACAGTGCCTCTGCGGAGCTTCG GAGAGTTCTGACTTTATCCCG	*Shigella sonnei* Ss046	ipaH	YP_310220	234 bp
Inv-F Inv-R	TCAAGAATAGAGCGAATTTCATCC TGCTTTTTATCGATTCCATGACCC	*Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2	invC	NP_461815	235 bp
ST1-F-m-2 ST1-R-m2	ATGAACCTTTGCAGCTATCGAGTAA AACGAGAGGACGTAATCGCGAA	*Salmonella enterica* subsp. *enterica* serovar Typhi Ty2	cI phage immunity repressor protein	NP_808111	319 bp
*†*Typhi-vipR-ST2-F *†*Typhi-vipR-ST2-R	GGTTTCATCATTTCTGGCCTCC CTGCTCCGTCAAGATCTTTTCACC	*Salmonella enterica* subsp. *enterica* serovar Typhi Ty2	tviA VI	NP_807946	335 bp
STM1-F-M STM1-R-M	CAGATTCATCCATCAAAAAAATGGG GCTAATGCGGGCTCTGAACCTGTG	*Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2	IMP	NP_463357	229 bp
STM2-R-M STM2-F-M	GACATTCTACGTAACCAGCTTGCT TGAGCGTTCACCCATGGCTAACTGTT	*Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2	DNA repair ATPase	NP_463355	189 bp
SS2-F-n SS2-R-n	GGGAGTGGTTAAAGCAACCGTGTCA TCACAGACTCTTCGGTCCATTCCTT	*Salmonella enterica* subsp. *enterica* serovar Saintpaul SARA23	RloF	ZP_03165416	312 bp

Primer Name	Sequence 5´–3´	Bacterial species	Target gene	Gene Accession No.	Amplicon Size
VC1-pho-F VC1-pho-R	AAGGTTTATCAGTATTAGTCGTGTG TTGCTGGACTGGGTTGACCATAGGG	*Vibrio cholerae* M66-2 chromosome I	phosphotyrosine protein phosphatase	YP_002809642	212 bp
VC2-B-tox-R VC2-B-tox-F	CCTCAGGGTATCCTTCATCCTTTC CTTCAGCATATGCACATGGAACACC	*Vibrio cholerae* O1 biovar El Tor str. N16961	Enterotoxin subunit B	NP_231099	239 bp
VpH1-F VpH1-R	GGCGTCGTCTTCTAAATACTGTTC ATGAAACACCATGCACAAACTTCT	*Vibrio parahaemolyticus* RIMD 2210633	thermostable hemolysin delta-VPH	NP_798108	249 bp
VpH2-F VpH2-R	CTTTTTAAGAGCGGCAGATATCA ATGACTGCGACTAACTTATTCGTC	*Vibrio parahaemolyticus* RIMD 2210633	thrA	NP_796873	105 bp
All-Vibrio-rpoBF All-Vibrio-rpoBR	TGGACATTCCATACCTGCTATCG ACCACGGATTTGACATTCTTTA	*Vibrio vulnificus* YJ016	rpoB	NP_935952	203 bp
YP1-F-m2 YP1-R-m2	CCAGCTATTATAGCAAATAGTAAGGG CAGTGTTTGCATTTAATGGCTT	*Yersinia pestis* strain CO92	Putative phage-related membrane protein	YPO2127	206 bp
YP2-R-M3 YP2-F-M2	ATTGCCGTTCGGGTCTTTCC GGCAATCAACAATACAGCCGTT	*Yersinia pestis* strain CO92	HP	YP_002347092	241 bp
YPs1-F-M YPs1-R	AGCAATGTGTCTGAACTTTCTTCA CATATTGCCGTCACCGACTACACC	*Yersinia pseudotuberculosis* IP 32953	HP	YP_070722	348 bp
YPs2-R YPs2-F	CAGGCAACGCTGAGTATTAGGT CTGCTGATGTTGCCATTAGTATGG	*Yersinia pseudotuberculosis* IP 32953	HP	YP_070711	288 bp
wzzR wzzF	TCATCTAAAGCACCAACGAAYACC TATTTGTTGCTCGCAAAGTTGCC	*Yersinia pseudotuberculosis* IP 32953	*rfe*	YP_068715	211 bp

*†*These primers were slightly modified by removing G from the 3'end of the forward primer and CT from the 5'end from published sequences (Jin et al. 2008)

RIoF= putative protein of unknown function

IMP: Inner membrane protein

HP: Hypothetical protein