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Diagnostic microarray for 14 water and foodborne pathogens using a flatbed scanner

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ABSTRACT

Parallel detection approaches are of interest to many researchers interested in identifying multiple water and foodborne pathogens simultaneously. Availability and cost-effectiveness are two key factors determining the usefulness of such approaches for laboratories with limited resources. In this study, we developed and validated a high-density microarray for simultaneous screening of 14 bacterial pathogens using an approach that employs gold labeling with silver enhancement (GLS) protocol. In total, 8887 probes (50-mer) were designed using an in-house database of virulence and marker genes (VMGs), and synthesized in quadruplicate on glass slides using an *in-situ* synthesis technology. Target VMG amplicons were obtained using multiplex polymerase chain reaction (PCR), labeled with biotin, and hybridized to the microarray. The signals generated after gold deposition and silver enhancement, were quantified using a flatbed scanner having 2-μm resolution. Data analysis indicated that reliable presence/absence calls could be made, if: i) over four probes were used per gene, ii) the signal-to-noise ratio (SNR) cutoff was greater than or equal to two, and iii) the positive fraction (PF), *i.e.*, number of probes with SNR ≥ 2 for a given VMG was greater than 0.75. Hybridization of the array with blind samples resulted in 100% correct calls, and no false positive. Because amplicons were obtained by multiplex PCR, sensitivity of this method is similar to PCR. This assay is an inexpensive and reliable technique for high throughput screening of multiple pathogens.

1. Introduction

Hybridization-based screening or confirmation of multiple bacterial or viral pathogens often involves fluorescent labeling of the polymerase chain reaction (PCR)-based amplicons or extracted sample DNA, hybridization on a microarray with probes designed for the target genes, and signal quantification using a laser scanner (Cao et al., 2015; Miller et al., 2008; Stedtfeld et al., 2007). High cost of scanning equipment is a bottleneck for use in limited resource settings. Alternative strategies for labeling and signal quantification, *e.g.*, gold nanoparticles labeling with silver enhancement (GLS) followed by quantification with flatbed scanners can address this limitation (Grinev et al., 2017). Several studies have demonstrated its potential focusing on bacterial pathogens (Qi et al., 2010), swine viruses (Wang et al., 2013), and HIV-1 and Hep C (Tang et al., 2011), albeit using less than a dozen probes on the whole array (Table 1). Recently a microarray-based GLS approach demonstrated that it can also be used for detection of mutation in the epidermal growth factor receptor (Xue et al., 2014). Examples of antibody-based lateral flow assays using GLS are numerous (Ngom et al., 2010) but differ significantly in their performance compared to DNA-DNA hybridization.

From the studies conducted using GLS with DNA-DNA hybridization (Alexandre et al., 2001; Liang et al., 2004; Liu et al., 2006; Storhoff et al., 2004; Taton et al., 2000), it is evident that the range of signal intensity produced by the GLS approach is smaller than the range of signals obtained by fluorescent dyes and laser scanners (Wilson et al., 2002). For GLS using flatbed scanners, it is between 0 and 255 on the gray scale (with 0 being black and 255 being white) while for fluorescent dyes with laser scanners is between 0 and 65,000 arbitrary units. As signal-to-noise ratio (SNR), this may translate into 0 to 100 for

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Table 1
Microarray-based studies using gold and silver labeling protocols with visual or flatbed-scanner-based signal quantification.

Target organisms	No. of probes	Scanning device	Max grey value	Reference
Six bacterial pathogens[a]	6	Flatbed scanner	66 ± 6	Qi et al., 2010
Seven swine viruses	9	Flatbed scanner	~100	Wang et al., 2013
HIV-1 and Hep-C	9	Visual	NA	Tang et al., 2011
EGFR gene mutations[b]	16	Visual	~4000	Xue et al., 2014

[a] Yersinia, Shigella, Salmonella, Brucella, *E. coli* O157:H7, and Cholera O139, [b] epidermal growth factor receptor.

GLS and 0 to 1000 for fluorescent dyes. Previous studies have used gray scale (Liang et al., 2004; Taton et al., 2000) or SNR (Wan et al., 2005) when using GLS. Because microarrays designed to screen for multiple pathogens generally use multiplexed PCR amplification step prior to hybridization to enhance detection limit (Call, 2005; Call et al., 2003; Loy and Bodrossy, 2006), and do not provide quantitative information about the detected target, the numerical value of the SNR is not critical. However, to make reliable presence/absence calls, the SNR for target probes must be completely separated from the SNR for non-target probes. Thus, when a large number of bacterial targets must be screened using the GLS approach, it is critical to experimentally establish the cut-off for positive fraction (PF) defined as the number of probes having a SNR greater than a given value.

The objective of this study was to develop and validate a GLS-based approach for simultaneous detection of 14 bacterial pathogens using an *in situ* synthesized microarray and a flatbed scanner. Probes for an additional pathogens were present on the microarray but they were not validated with target VMGs and served only as a control set to evaluate non-specific hybridization. Reliable presence/absence calls were made based on a set of three filters related to the minimum number of probes per probe set, threshold SNR for positive signals, and a threshold for PF. Performance of the GLS approach was validated using blind samples containing mixtures of virulence and marker genes (VMGs) not known to the person hybridizing the array and performing data analysis. Because of the low-cost technologies used for scanning, the method represents a cost-effective approach (Table 2) to screen for multiple pathogens and provides a set of probes for many other pathogens that could be used after further validation. The bacterial pathogens chosen are common contaminants with a potential to be present in water and food globally (Logue et al., 2017).

Table 2
Cost and time comparison between GLS method and fluorescent labeling for microarray based detection.

	Fluorescent labeling	GLS method
1. Scanning equipment cost	$30,000–$60,000	$100–$400 (Flatbed Scanner)
2. Labeling cost per slide	$50–$100	$10–$15
3. Time-to-result		
PCR amplification	1–2 h	1–2 h
Fluorescent labeling	2–3 h	–
Biotin labeling	–	1–2 h
Hybridization	2–10 h	2–10 h
Silver enhancement	–	1 h
Scanning	10–20 min	10–20 min
Total	5–15 h	5–15 h

2. Materials and methods

2.1. Probe design and synthesis of VMG microarray

An in-house database containing 36 genera, 107 pathogens, 539 genes, and 3183 VMG sequences obtained from GenBank was used to design 8887 fifty-mer probes (Table S1-S3) using CommOligo (Li et al., 2005). The probe design criteria listed in Table S1 was similar to that reported in a previous study (He et al., 2005). These probes were synthesized *in situ* on a glass slide in quadruplicate (replicated four times) using a flexible *in situ* microarray technology described previously (Gao et al., 2004, 2001; LeProust et al., 2000). A 32-mer sequence (5′-CCTATAGTGAGTCGTATTAAGCAGCGCAGC-3′) was also synthesized *in situ* and replicated 1214 times over the entire slide to serve as a control. A total of 551 probes targeting 40 VMGs associated with 14 pathogens served as the target probes either during initial development or with blind mixtures. Non-targeted probes were not validated further as part of this study and therefore only served for evaluated the specificity of the microarray.

2.2. Design of sample with known VMG mixture for method development

During method development, 22 VMGs targeted by 228 probes associated with 10 pathogens were used (Table 3; Known VMG mixture). Hybridization experiments to develop the protocol and data analysis scheme were carried out in triplicate. The 22 VMGs selected for method development were amplified from genomic DNA of the targeted pathogens by PCR. Target VMGs were amplified in five multiplexed reactions and the amplicons were labeled with biotin. It is worth noting that the overall VMG mixture also contained the amplicons for six additional VMGs that were filtered at the time of data analysis because only 1–2 probes per VMG were present on the array. A biotin labeled sequence complementary to the 32-mer *in situ* synthesized sequence was synthesized by Integrated DNA Technologies (Coralville, IA) and spiked in the hybridization mixture. This biotin labeled sequence served as a positive control for hybridization to the 32-mer *in situ* sequence mentioned above. The complete target mixture was hybridized to the microarray. Hybridized biotinylated targets were incubated with gold nano-particles using streptavidin-gold conjugate solution followed by silver enhancement to further amplify the generated signals. Thereafter, the VMG microarray was scanned using a conventional flatbed scanner and the data was analyzed using Genepix 5.0 software (Axon Instruments, Union City, CA).

2.3. Strategy for blind samples with unknown VMGs for method validation

Two separate blind samples were prepared using amplicons generated by monoplex PCR of multiple VMGs including four additional pathogens that were not hybridized during development (Table 3: Blind mixtures 1 and 2). All PCR amplicons were cleaned using a QIAGEN PCR clean-up kit (QIAGEN, Valencia, CA), and quantified using the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Approximately 2.5×10^{-14} mol of each amplicon was added to the blind sample mixture, and 250 ng of the final mixture was used for biotin labeling using Bioprime DNA labeling kit (Invitrogen, Carlsbad, CA). The blind samples targeted some VMGs that were included in the known VMG (Table 3, Blind VMG mixtures 1 and 2 marked as + for Spiked). The identity of the prepared samples in terms of pathogens targeted or VMGs mixture used was not revealed to the person carrying out the hybridization and data analysis for the blind samples until the after presence/absence calls were made.

2.4. DNA extraction from bacterial cultures

Target pathogens from which DNA was extracted for method development and validation included: *Aeromonas hydrophila* (ATCC