Supporting Information

Fluorescence polarization-based nucleic acid testing for rapid and cost-effective diagnosis of infectious disease

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Experimental section

Materials. DNA oligonucleotides were synthesized by Integrated DNA Technologies. The list of DNA sequences are summarized in Table S1. Based on the transcriptome analysis of *S.* Typhi and *S.* Paratyphi A from the blood of infected humans, six different genes that were highly expressed *in vivo* were selected.[1, 2] Ultra pure DNase/RNase-free distilled water was purchased from Life Technologies. Chloroform and ethyl alcohol were purchased from Sigma-Aldrich. All other chemicals were of analytical grade and used without further purification.

Bacteria culture and RNA extraction. *Salmonella enterica* serotypes (*S.* Typhimurium *str. LT2*, *S.* Typhi *str. CT18*, and *S.* Paratyphi A *str. ATCC 9150*) were provided by the *Salmonella* Genetic Stock Centre (SGSC), and other bacteria (*K. pneumoniae* (#43816)*, E. aerogenes* (#13048)*, C. freundii* (#6879)*, and P. aeruginosa* (#142)) were purchased from the American Type Culture Collection (ATCC). Bacterial cultures (5 mL) were grown to mid-log phase in suitable media: *S.* Typhimurium*, S.* Typhi*,* and *S.* Paratyphi A in Luria-Bertani (LB) media (BD Biosciences); *K. pneumoniae* and *P. aeruginosa* in tryptic soy broth (BD Biosciences); *E. aerogenes* and *C. freundii* in nutrient broth (BD Biosciences). For RNA extraction, bacteria were first centrifuged at 6000 g for 10 min and pellets were treated with Trizol (Life Technologies). After phase separation, the total RNA in the aqueous phase was transferred to a Qiagen RNeasy mini-spin column and purified. The final RNA was quantified by measuring the absorbance at 260 nm with Nanodrop 1000 (Thermo Scientific). To detect bacteria spiked into human blood (1 mL), human blood was purchased from Innovative Research and spiked with the cultured bacteria. For RNA extraction, Trizol LS (Life Technologies) that is more concentrated than Trizol was used, and the same RNA extraction procedure was applied as in pure bacterial cultures.

Reverse transcription-asymmetric PCR. Single-stranded cDNA was synthesized from bacterial RNA using the reverse transcription system (Promega) with thermal cycling conditions of 25 °C for 10 min, 42 °C for 60 min, and 95 °C for 5 min. The asymmetric PCR amplification was carried out on MasterCycler (Eppendorf) in a total reaction volume of 25 μL containing 2.5 μL of cDNA, 0.8 μM of excess primer and 0.08 μM of limiting primer (Table S1), 1× PCR reaction buffer (20 mM Tris-HCl, 20 mM KCl, 5 mM $(NH_4)_2SO_4$, and 3 mM $MgCl_2$), 0.2 mM of each dNTP, and 2.5 U Maxima Hot Start Taq DNA polymerase (Thermo Scientific). The reaction tubes were heated to 94 °C for 2 min, followed by 40 or 50 cycles of 5 s at 94 °C, 15 s at 55 °C, and 15 s at 72 °C, and then a final 10 min extension step at 72 °C. The asymmetric PCR products were analyzed by polyacrylamide gel electrophoresis (Figure S1).

DNA aptamer-based fluorescence polarization assay. In “one-pot” experiment, 20 μL of the PCR solution was mixed with all-in-one master-mix composed of DNA aptamer (187.5 nM) and universal reporter probe that was pre-formed with template (75 nM), primer (75 nM), and FAM-labeled DNA (62.5 nM) at room temperature for 15 min, making a total volume of 40 μL in 20 mM Tris-HCl (pH 8.3), 20 mM KCl, 5 mM $(NH_4)_2SO_4$, and 4 mM $MgCl_2$. In “sequential” experiment, DNA aptamer was first incubated with 20 μL of PCR reaction solution at room temperature for 10 min and the universal reporter probe was added subsequently. The fluorescence polarization values were then measured by a plate reader (Tecan Sapphire 2) on 384-well Greiner Bio-One microplates (ref: 781900). The excitation and emission filters were for 470 nm and 525 nm, respectively. The measured fluorescence polarization (*FP*) was calculated by the instrument software as follows: $FP = 1000\times(G\bullet I_{par} - I_{perp})/(G\bullet I_{par} + I_{perp})$, where I_{par} and I_{perp} are the emission intensities when the emission polarizer is in the parallel and perpendicular position, respectively in relation to the excitation polarizer. The G-factor was determined according to the manufacturer’s instructions. The limit of detection (LOD) was estimated by the conventional definition: 3 × standard deviation (s.d) of background signal. The background signals were from samples without target DNA (Figure 2A) and without bacteria (Figure 2B). We prepared samples with different bacterial concentrations through serial dilution, and the bacterial number was estimated from the dilution factor.

Electrophoretic band shift experiment. Solutions (total volume of 15 μL) containing 100 nM DNA aptamer and 7.5 μL of PCR solution, 15 U Taq DNA polymerase (New England Biolabs) in 20 mM Tris-HCl (pH 8.3), 20 mM KCl, 5 mM $(NH_4)_2SO_4$, and 4 mM $MgCl_2$ were incubated at room temperature for 15 min. The solutions were mixed with 6× loading buffer and subjected to electrophoresis analysis on a 20 % polyacrylamide gel (Life Technologies). The analysis was carried out in 1× TBE (100 mM Tris, 90 mM Borate, 1 mM EDTA) at 120 V for 120 min. After GelRed (Biotium) staining, gels were scanned using a UV transilluminator. DNA polymerase was neither fluorescent itself nor stained by the dye.[3]

SYBR green-based quantitative PCR. The quantitative real-time PCR was carried out on 7500 Fast Real-Time PCR system (Life Technologies) in a total reaction volume of 20 μL containing 2.5 μL of cDNA, 0.4 μM of each primer, 1× SYBR Select Master Mix (Life Technologies). The 96-well reaction plate was heated to 95 °C for 2 min, followed by 40 cycles of 3 s at 95 °C, 15 s at 55 °C, and 30 s at 72 °C. The threshold cycle (Ct) values were obtained using the 7500 Fast software. The instrument automatically calculates the Ct value, which represents the first PCR cycle at which the fluorescence signal exceeds the signal of a given uniform threshold suggested by the instrument software.[4] Negative control (NC) remained undetected, not crossing the established threshold for 40 cycles, which was arbitrarily given a Ct value of 41. The ΔCt was generated by subtracting the Ct value of the specimen from the Ct value of NC.[5] A post-amplification melting curve analysis was performed with the initial denaturation for 15 s at 95 °C, followed by incubation for 1 min at 60 °C. The resulting fluorescence signal was then measured as the temperature was increased to 95 °C and the first derivative plot was used to determine melting temperature (Tm).

Table S1. DNA sequences used in the study.

Salmonella enterica species-specific

Selected genes	Strand name	DNA sequence (5' - 3')
STY3007 (sipC): Pathogenicity island 1 effector protein	Target DNA	ACATGAATCAGGATTTGAATGCCCTGGCAAATAATGTCACGACTAAAGCGAAT
	Detection probe	CAATGTACAGTATTGATTCGCTTTAGTCGTGACATTATTTGCCAG
	Limiting primer	ATTCGCTTTAGTCGTGACAT
	Excess primer	ACATGAATCAGGATTTGAATG
STY1121 (sigD): Cell invasion protein	Target DNA	GCAGCTTCTTCTTGTTTTGTTGCTGGCCGGTCCGCTTTAACTTTGGCTAACTCCATTTG
	Detection probe	CAATGTACAGTATTGCAAATGGAGTTAGCCAAAGTTAAAGCGGAC
	Limiting primer	CAAATGGAGTTAGCCAAAGT
	Excess primer	GCAGCTTCTTCTTGTTTTGT

Salmonella Typhi-specific

Selected genes	Strand name	DNA sequence (5' - 3')
STY0201 (staG): Putative fimbrial protein	Target DNA	GAAGGAAGGTCATCTCAATGGTGAAATAAAAACCGGGAATTGAGGTTTTATACAGCGGAT
	Detection probe	CAATGTACAGTATTGATCCGCTGTATAAAACCTCAATTCCCGGTT
	Limiting primer	ATCCGCTGTATAAAACCTCA
	Excess primer	GAAGGAAGGTCATCTCAATG
STY0207 (staA): Fimbrial protein	Target DNA	AAGAGTGCCGGTAGTGTTAGAGAATGACGAATCCATTACCAGAGAAGCCTTAGTGG
	Detection probe	CAATGTACAGTATTGCCACTAAGGCTTCTCTGGTAATGGATTCGT
	Limiting primer	CCACTAAGGCTTCTCTGGTA
	Excess primer	AAGAGTGCCGGTAGTGTTAG

Table S1 (continued). DNA sequences used in the study.

Salmonella Paratyphi A-specific

Selected genes	Strand name	DNA sequence (5' - 3')
SPA2472: Hypothetical protein	Target DNA	CATCTGTCCCCTCACTAAATACTATGGAGTTTGAAAGTGGATAACC ATCACCTG
	Detection probe	CAATGTACAGTATTGCAGGTGATGGTTATCCACTTTCAAACTCCA
	Limiting primer	CAGGTGATGGTTATCCACTT
	Excess primer	CATCTGTCCCCTCACTAAAT
SPA4291 (hsdR): Subunit R of type I restriction-modification system	Target DNA	ATTTGTTCTTCGATTTCAGGAGTCCAAAGTAATTCTTCTGGAGTAG CATTTGACCAAAG
	Detection probe	CAATGTACAGTATTGCTTTGGTCAAATGCTACTCCAGAAGAATTA
	Limiting primer	CTTTGGTCAAATGCTACTCC
	Excess primer	ATTTGTTCTTCGATTTCAGG

Universal detection probe

Template	CATGAATCTACTCGACGATATTGCTCAACTGTCATAAACTTCTGAGGA
Primer	TCCTCAGAAGTTT
FAM-labeled DNA	(FAM)-TATCGTCGAGTAGATTCATG

Table S2. Summary of *Salmonella* bacteria targeted in this study.[6-8]

Salmonella strains	Description	Selected genes
S. Typhimurium	• A leading cause of human gastroenteritis • Host generalists that infect humans and many other mammalian species • The incidence of non-typhoid salmonellosis is increasing, causing millions of human cases and more than a hundred thousand deaths worldwide	STY3007 STY1121
S. Typhi	• The etiological agent of typhoid fever, a serious and invasive bacterial disease of humans • Host specialists that infect only humans • An annual estimated global burden of approximately 21.7 million cases, leading to 217,000 deaths	STY3007 STY1121 STY0201 STY0207
S. Paratyphi A	• A leading cause of paratyphoid fever • Host specialists that infect only humans • About 5.4 million cases occur a year	STY3007 STY1121 SPA2472 SPA4291

Table S3. Post-amplification melting curve analysis in SYBR green-based qPCR.

	Healthy control	Bacterial mixture	*S.* Typhimurium	*S.* Typhi	*S.* Paratyphi A
STY3007	68.94 (0)	68.30 (0.22)	72.7 (0)	73.6 (0)	73.7 (0.12)
STY1121	—	—	76.7 (0.12)	77.51 (0)	77.6 (0.12)
STY0201	—	—	—	72.5 (0.12)	—
STY0207	—	—	—	74.9 (0)	—
SPA2472	—	—	—	—	72.5 (0.12)
SPA4291	72.2 (0)	72.1 (0.12)	71.8 (0.12)	72.2 (0)	72.6 (0.12)

— : Undetermined
mean ± (s.d)

Table S4. Comparison of the FP assay with other nucleic acid tests.

		FP assay	Gel electrophoresis	Fluorescence-based homogenous assay	
				SYBR green	Taqman
Sensitivity		Single bacterium	Variable[#]	Variable[a]	Single bacterium
Specificity		High	Medium[#]	Medium[a]	High
Assay time (without sample preparation)		< 1.5 hr	< 1.5 hr	< 1.5 hr	~ 1 hr
Cost	Single target	$1 Polymerase, DNA primers, aptamers, universal reporter	$1 Polymerase,DNA primers, agarose, EtBr	$1 Polymerase, DNA primers, SYBR green	$1 Polymerase, DNA primers, Taqman probe ($0.3)
	100 targets	$1	$1	$1	$1 + $30 = $31

[a] Depends on template quality and primer design

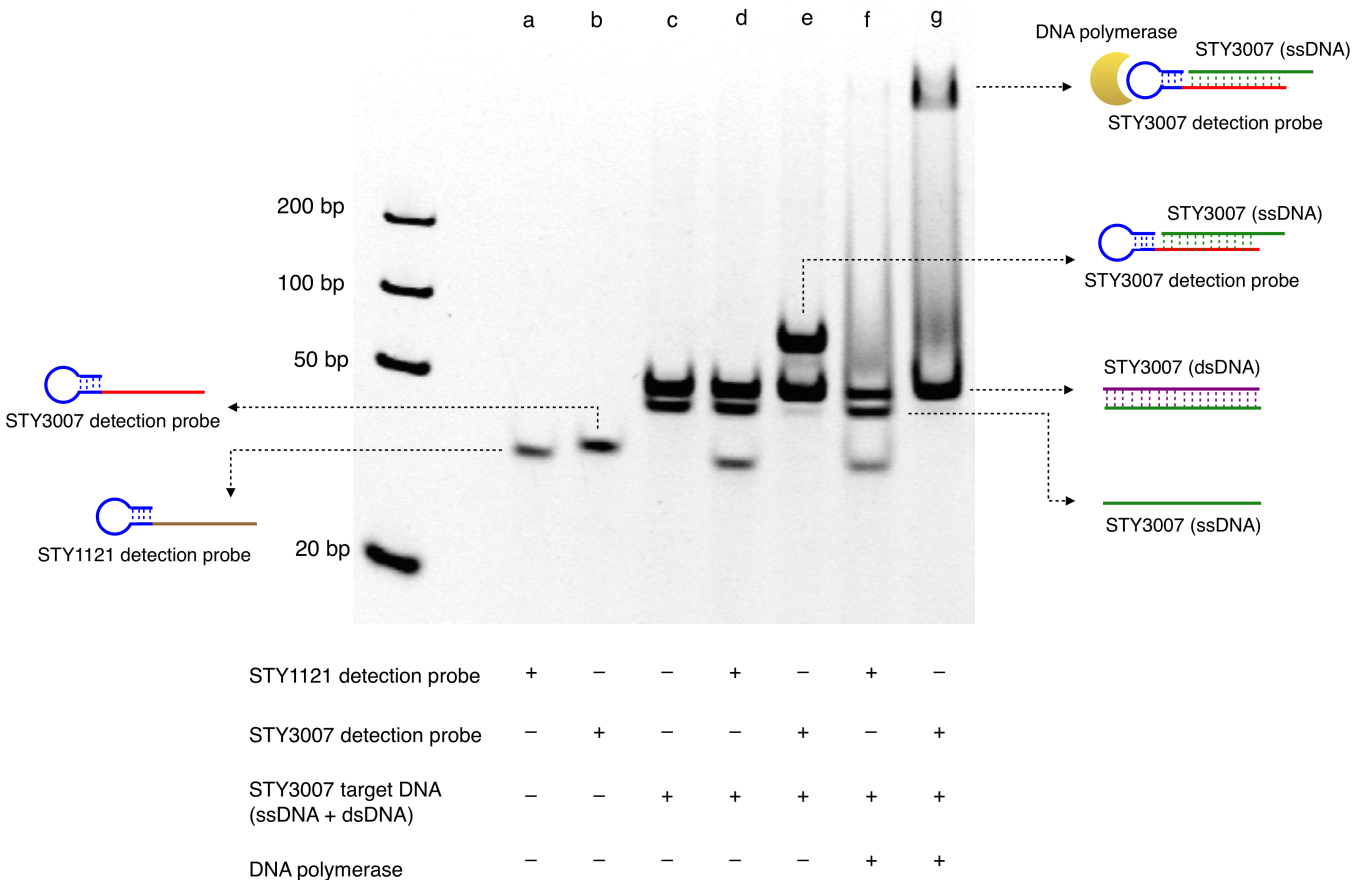

Figure S1. Electrophoretic band shift assay. **(a)** STY1121 detection probe. **(b)** STY3007 detection probe. **(c)** Target amplicon (STY3007) produced by asymmetric RT-PCR. Note that ssDNA appears below dsDNA. **(d)** Mixture of STY3007 amplicon and STY1121 detection probe. The electrophoretic mobility of the amplified products unchanged, indicating no binding. **(e)** Mixture of STY3007 amplicon and STY3007 detection probe. The hybridization between STY3007 detection probe and the ssDNA produced a new band. **(f)** Adding DNA polymerase (M.W., 94 KDa) to (d) caused no changes in gel bands for STY1121 detection probe and STY3007 amplicons. **(g)** Upon addition of DNA polymerase to (e), the band for detection probe and ssDNA hybrid was further shifted to an upper position. The result confirmed the binding of detection probe to DNA polymerase in the presence of complementary amplicon.

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