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High-resolution melting assay for the detection of *gyrA* mutations causing quinolone resistance in *Salmonella enterica* serovars Typhi and Paratyphi

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Abstract

We developed a novel rapid assay to detect the *gyrA* mutations that cause quinolone resistance in typhoid and paratyphoid fever *Salmonella* spp. using high-resolution melting (Idaho Technology, Salt Lake City, UT) analysis of polymerase chain reaction amplicons. The presence of *gyrA* mutations led to small but consistent changes in amplicon melting temperatures that allowed quinolone-resistant isolates to be differentiated from susceptible ones.

Keywords: High-resolution melting; *Salmonella*; Typhoid fever; *gyrA*; Mutation; Quinolone; Resistance; Fluoroquinolone

Mutations in the *gyrA* gene that lead to quinolone resistance and reduced susceptibility to fluoroquinolones are clinically significant in *Salmonella enterica* serovar Typhi (*S*. serovar Typhi) and *S. enterica* serovar Paratyphi (*S*. serovar Paratyphi) since there is an increased risk of treatment failure and poor response to treatment (Crump et al., 2003; Parry et al., 2002; Slinger et al., 2004; Wain et al, 1997). Detection of these isolates requires 16–18 h for the nalidixic acid (NAL) disk diffusion screening procedure (National Committee for Clinical Laboratory Standards [NCCCLS], 2003a; NCCLS, 2004).

We hypothesized that since common *gyrA* mutations in enteric fever *Salmonella* spp. result in lower GC content and thus lower melting temperatures (T_ms), mutant isolates could be rapidly distinguished from wild-type ones using high-resolution melting analysis. This is a recently described mutation detection method in which a novel intercalating double-stranded DNA dye (LCGreen Plus+, Idaho Technology, Salt Lake City, UT) and a high-resolution melting analysis device (HR-1, Idaho Technology) are used to detect small melting curve differences, such as those produced by single nucleotide mutations (Liew et al., 2004).

Polymerase chain reaction (PCR) primers were designed using the Primer 3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to amplify a *gyrA* fragment that included the critical Ser83 and Asp87 amino acid sites. Before PCR, amplicon T_ms for wild-type and mutant isolates were estimated using the Tm Utility Software v 1.5 program (www.idahotech.com/downloads_up/index.html). Primers amplifying an 89-bp fragment (SA89F GACGTAATCGGTAAATACCATCC and SA89R ATGTAACGCAGCGAGAATG) were selected. Primers were synthesized by Integrated DNA Technologies (Skokie, IL).

Ten microliters of PCR reactions included 500 μg/mL of bovine serum albumin, a 3-mmol/L concentration of magnesium chloride, 200-μmol/L concentrations of each deoxynucleoside triphosphate, a 0.5-μmol/L concentration of each primer, 50–60 pg/μL of DNA, 0.4 U of KlenTaq1 polymerase (Ab Peptides, St. Louis, MO), 88 ng of TaqStart Antibody (ClonTech, Palo Alto, CA), and 1× LCGreen Plus+.

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Table 1
Melting temperatures, nucleotide and amino acid sequences, and antibiotic susceptibilities for NAL-susceptible and NAL-resistant *Salmonella* spp. isolates

NAL susceptibility	*Salmonella* serovar	Nucleotide (position)		Amino acid (codon)		CIP MIC (μg/mL)	NAL MIC (μg/mL)	T_m (°C)[a]	
		135	146	83	87			Mean	Range
S	*S.* serovar Typhi	C	G	Ser	Asp	0.015	2	86.45	86.36–86.53
S	*S.* serovar Typhi	C	G	Ser	Asp	0.015	4	86.45	86.39–86.52
S	*S.* serovar Typhi	C	G	Ser	Asp	0.015	2	86.45	86.43–86.47
R	*S.* serovar Typhi	A	G	Tyr	Asp	0.25	256	86.04	85.92–86.14
R	*S.* serovar Typhi	C	A	Ser	Asn	0.125	128	85.90	85.82–86.04
R	*S.* serovar Typhi	T	G	Phe	Asp	0.25	256	86.13	86.08–86.20
R	*S.* serovar Typhi	T	G	Phe	Asp	0.25	256	86.23	86.17–86.27
R	*S.* serovar Paratyphi	T	G	Phe	Asp	0.5	>512	86.18	86.14–86.23
R	*S.* serovar Paratyphi	T	G	Phe	Asp	0.5	>512	86.15	86.07–86.20
R	*S.* serovar Paratyphi	T	G	Phe	Asp	0.5	>512	86.15	86.07–86.22
R	*S.* serovar Enteritidis[b]	C	T	Ser	Tyr	0.25	>512	85.88	85.79–86.14

CIP = ciprofloxacin.
[a] T_m means and ranges obtained from 3 consecutive runs of isolates.
[b] An *S. enterica* serovar Enteritidis strain was included in the study since its mutation may also be found in enteric fever *Salmonella* spp.

The thermocycler (RapidCycler II, Idaho Technology) protocol consisted of 45 cycles of 54 °C annealing, 72 °C extension, and 94 °C denaturation temperatures with a programmed transition rate of 9.9 °C/s and no holds. After PCR of the *gyrA* gene fragment, samples were transferred to the high-resolution melting instrument (HR-1). A rate of 0.3 °C/s was used when measuring the fluorescence change of the sample in a temperature range of 60–90 °C. The derivative of the measured fluorescence plotted against temperature was displayed using the HR-1 software, and the peak of these curves was measured to obtain the amplicon T_m.

DNA was extracted from isolated bacterial colonies using the PrepMan Ultra reagent (Applied BioSystems, Foster City, CA) according to the manufacturer's protocol.

The assay was fist developed using a previously characterized collection of *Salmonella* spp. containing 4 different *gyrA* mutation types (Slinger et al., 2004). T_ms were used to establish a cutoff value that indicated the presence of a *gyrA* mutation and thus resistance to NAL. The assay was tested on a group of 13 unsequenced *S.* serovar Typhi and *S.* serovar Paratyphi clinical isolates and 2 NAL-susceptible reference strains (ATCC 9150 *S.* serovar Paratyphi and ATCC 13311 *S. enterica* serovar Typhimurium) using the defined cutoff temperature. NAL susceptibility predicted by T_ms was compared to in vitro susceptibility testing results.

Antibiotic susceptibilities were determined using Clinical Laboratory Standards Institute (formerly NCCLS) methods (NCCLS, 2003a; NCCLS, 2003b; NCCLS; 2004).

T_ms for the *Salmonella* spp. of known sequence are shown in Table 1 (measurements obtained from 3 runs). Fig. 1 illustrates the derivative melting curves for 5 isolates that represent the *gyrA* sequences included in our collection (1 wild-type sequence strain and 4 mutation types). T_ms for isolates with *gyrA* mutations were consistently lower than those of wild-type strains (no *gyrA* mutation present). Based on 9 measurements, we calculated the mean wild-type T_m as 86.45 °C with SD of 0.055 °C. We then chose the mean T_m −3 SD (86.29 °C) as a suitable cutoff temperature for calling *gyrA* mutations before testing the unsequenced isolates.

The derivative melting curve for the 2 ATCC strains and the 13 unsequenced clinical isolates is shown in Fig. 2. Of 15, 14 were NAL-susceptible by disk diffusion. The T_m of the single NAL-resistant isolate was 86.12 °C, below the cutoff value, whereas T_m's of all NAL-susceptible isolates were above the cutoff (mean, 86.52 °C; range, 86.42–86.68 °C). Thus, the cutoff T_m chosen seems appropriate as there was 100% (15/15) concordance between NAL susceptibility predicted by amplicon T_ms and disk diffusion results.

The high-resolution melting analysis assay appears to be a rapid and accurate method for detection of clinically significant *gyrA* mutations in *S.* serovar Typhi and *S.* serovar Paratyphi. This method has so far been studied predominantly in the field of human genetics (Liew et al., 2004; Reed

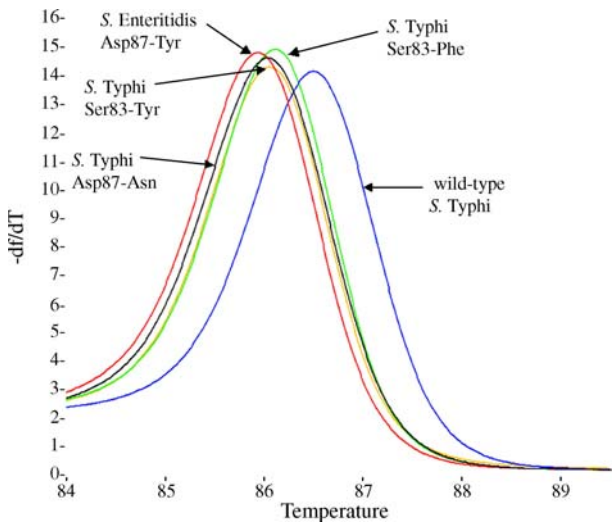

Fig. 1. Derivative melting curves for a wild-type sequence (nalidixic-acid susceptible) *S.* Typhi strain and 4 *Salmonella* spp. that represent the 4 *gyrA* mutation-types studied (all nalidixic-acid resistant strains). Values on the y axis are the first negative derivative of the change in fluorescence (dF) divided by the change in temperature (dT).

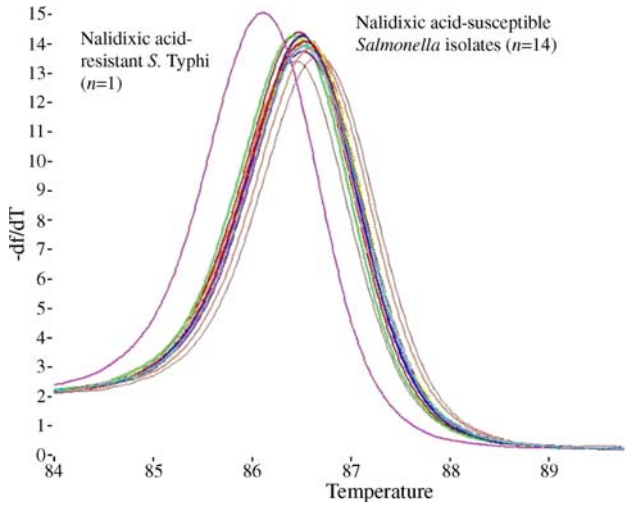

Fig. 2. Derivative melting curves for 14 nalidixic acid-susceptible Salmonella strains (12 enteric fever clinical isolates and ATCC reference 9150 *S*. Paratyphi and 13311 *S*. Typhimurium) and 1 nalidixic acid-resistant isolate, demonstrating a lower Tm for the resistant isolate. Values on the y axis are the first negative derivative of the change in fluorescence (dF) divided by the change in temperature (dT).

and Wittwer, 2004; Wittwer et al., 2003; Zhou et al., 2004). One article has shown that rapidly growing mycobacteria can be differentiated by T_m differences (Odell et al., 2005). However, to the best of our knowledge, we are not aware of other studies applying high-resolution melting analysis to the rapid identification of antimicrobial resistance.

Although the temperature differences observed with our method were small, they appear to be sufficient to allow strains with *gyrA* mutations to be distinguished from wild-type ones. The mutations studied account for the great majority of enteric fever *Salmonella* spp. *gyrA* mutations. The Asp87-Gly mutation (Walker et al., 2001) was not present in our strain collection, but the T_m of isolates with this mutation is also predicted to be lower than wild-type.

Single clinical isolates of *S.* serovar Typhi and *S.* serovar Paratyphi that are fully fluoroquinolone-resistant (ciprofloxacin MIC ≥ 4 μg/mL) have now been reported (Harish et al., 2004; Renuka et al., 2005). The genetic basis for mechanism for the *S.* serovar Paratyphi resistance was not stated. The *S.* serovar Typhi isolate contained both Ser83-Phe and Asp87-Asn *gyrA* mutations. Together, these 2 mutations are predicted by the Tm utility software program to result in a lower T_m than that of single-mutation isolates. Thus, such fluoroquinolone-resistant isolates could theoretically be distinguished from the single-mutation isolates we examined in this study. However, we have not yet assessed such strains, and formal study is still required to determine if this is feasible.

Other rapid *gyrA* mutation detection methods have been studied with non-enteric fever *Salmonella* spp. These include the GAMA (*gyrA* mutation assay) real-time PCR assay (Walker et al., 2001) and denaturing high-performance liquid chromatography (DHPLC) (Eaves et al., 2002). However, DHPLC requires mixing of wild-type and unknown strain PCR products, increasing specimen handling steps and the risks of cross-contamination, and equipment costs are high. GAMA requires individual probes for each of 5 *gyrA* mutations, so that the mutation present can be determined without sequencing (Walker et al., 2001). However, the potential need to run 5 PCR reactions per isolate makes this method more labor-intensive, and knowledge of the mutation present is not important clinically.

Advantages of high-resolution melting assays in the clinical laboratory include simplicity (since only a generic DNA binding dye is needed rather than specific probes) and a lower risk of contamination with the closed-tube method. The assay is also rapid, as the entire procedure, including DNA extraction, can be completed in <1 h, and assay and platform costs are low. Equipment costs for the HR-1 and Rapid Cycler II combination are approximately US$15 000. This compares very favorably to many thermocylers with real-time fluorescence monitoring and melting curve capabilities, which may be priced up to 7 times higher. Since our assay and many other diagnostic PCR assays require only the melting curve analysis results and not product quantitation, the need for real-time monitoring is eliminated. However, for those currently using a real-time device, several have been shown to accurately determine amplicon melting temperatures using LCGreen Plus+ dye, although the HR-1 remains the optimal device (Herrmann et al., 2006). LCGreen Plus+ is itself inexpensive, with a cost per reaction in our assay of approximately US$0.22.

Further study with this method is still needed to determine if rapid detection of *gyrA* mutations is feasible in the clinical laboratory setting and if such testing leads to more timely and appropriate antibiotic treatment for patients with quinolone-resistant *S*. serovar Typhi and *S*. serovar Paratyphi infections.

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