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A multiplex single nucleotide polymorphism typing assay for detecting mutations that result in decreased fluoroquinolone susceptibility in *Salmonella enterica* serovars Typhi and Paratyphi A

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Objectives: Decreased susceptibility to fluoroquinolones has become a major problem for the successful therapy of human infections caused by *Salmonella enterica*, especially the life-threatening typhoid and paratyphoid fevers.

Methods: By using Luminex xTAG beads, we developed a rapid, reliable and cost-effective multiplexed genotyping assay for simultaneously detecting 11 mutations in *gyrA*, *gyrB* and *parE* of *S. enterica* serovars Typhi and Paratyphi A that result in nalidixic acid resistance (Nal^R) and/or decreased susceptibility to fluoroquinolones.

Results: This assay yielded unambiguous single nucleotide polymorphism calls on extracted DNA from 292 isolates of *Salmonella* Typhi (Nal^R=223 and Nal^S=69) and 106 isolates of *Salmonella* Paratyphi A (Nal^R=24 and Nal^S=82). All of the 247 Nal^R *Salmonella* Typhi and *Salmonella* Paratyphi A isolates were found to harbour at least one of the target mutations, with GyrA Phe-83 as the most common one (143/223 for *Salmonella* Typhi and 18/24 for *Salmonella* Paratyphi A). We also identified three GyrB mutations in eight Nal^S *Salmonella* Typhi isolates (six for GyrB Phe-464, one for GyrB Leu-465 and one for GyrB Asp-466), and mutations GyrB Phe-464 and GyrB Asp-466 seem to be related to the decreased ciprofloxacin susceptibility phenotype in *Salmonella* Typhi. This assay can also be used directly on boiled single colonies.

Conclusions: The assay presented here would be useful for clinical and reference laboratories to rapidly screen quinolone-resistant isolates of *Salmonella* Typhi and *Salmonella* Paratyphi A, and decipher the underlying genetic changes for epidemiological purposes.

Keywords: genotyping, DNA gyrase, mechanisms of resistance, *Salmonella*

Introduction

Salmonella enterica serovars Typhi and Paratyphi A are invasive, life-threatening human pathogens, causing the systemic diseases typhoid and paratyphoid fever, respectively, which still pose significant threats to public health in many developing countries. In 2000, a population-based study estimated that the global disease burdens associated with typhoid and paratyphoid fever were 21.6 million cases (with 216000 deaths) and 5.4 million cases, respectively.[1] Resistance and decreased susceptibility to fluoroquinolones in *Salmonella*, often accompanied by resistance to nalidixic acid (Nal^R), will

lead to treatment failures for typhoid and paratyphoid fever.[2,3] The most common cause of resistance to nalidixic acid and decreased susceptibility to fluoroquinolones in serovar Typhi is amino acid substitutions in the quinolone resistance-determining region (QRDR) of the DNA gyrase subunit GyrA.[4,5] Mutations in the QRDR of the other subunit of DNA gyrase (GyrB) and both subunits of DNA topoisomerases IV (ParC and ParE) will also result in increased resistance to quinolones.[6,7]

The rapid detection of Nal^R *Salmonella* Typhi and *Salmonella* Paratyphi A is vital in clinical practice to guide antibiotic therapy, and the traditional disc-diffusion protocol is still the most commonly used method for identifying resistant isolates. Microbiologists have attempted to develop molecular assays for rapidly screening Nal^R isolates and for determining underlying genetic changes in resistant isolates as well. These assays include denaturing HPLC (dHPLC),[8] real-time PCR,[9,10] restriction fragment length polymorphism,[11] pyrosequencing,[12] high-resolution melting curve assay[13] and mismatch amplification mutation assay.[14] However, none of these assays is capable of detecting multiple single nucleotide polymorphisms (SNPs) in different genes simultaneously with medium to high throughput.

We have previously described six different mutations in the QRDR of GyrA within *Salmonella* Typhi,[5] and have also identified additional mutations in GyrB and ParE in unpublished work, some of which are associated with decreased susceptibility to fluoroquinolones without concomitant resistance to nalidixic acid (P. Roumagnac and M. Achtman). In order to efficiently test for the presence of these mutations individually and in combination, we designed a rapid and cost-effective multiplex genotyping assay based on Luminex xTAG beads, which can simultaneously screen for 11 mutations in *gyrA*, *gyrB* and *parE* of *Salmonella* Typhi and *Salmonella* Paratyphi A.

Materials and methods

Bacterial isolates

Four sets of isolates including a total of 292 *Salmonella* Typhi and 106 *Salmonella* Paratyphi A were tested in this study. Strains of *Salmonella* Typhi had been isolated between 1991 and 2006 in Asia, Europe and Africa, while *Salmonella* Paratyphi A strains were isolated between 1917 and 2006 across Asia, Europe, Africa and America. Twelve *Salmonella* Typhi isolates with known mutations in *gyrA*, *gyrB* and *parE* were used to develop the assay. These mutations are based on genomic sequences for four isolates [CT18, 150(98)S, E02-2759 and AG3][15,16] and on mutations discovered by dHPLC plus sequencing in eight others.[5] The assay was validated with a second group of 193 *Salmonella* Typhi isolates, for which the corresponding regions in *gyrA*, *gyrB* and *parE* had been tested previously by dHPLC (unpublished for *gyrB* and *parE*, P. Roumagnac and M. Achtman).[5] A third group of 87 isolates of *Salmonella* Typhi that had not been previously tested was also subjected to the Luminex assay, including 80 Nal^R and 7 Nal^S isolates. Lastly, 106 isolates of *Salmonella* Paratyphi A (24 Nal^R and 82 Nal^S) were also included in this study. The MICs for isolates of nalidixic acid (resistant breakpoint 32 mg/L) and ciprofloxacin (resistant breakpoint 4 mg/L) were determined according to the guidelines of the CLSI,[17] and genomic DNAs were extracted using standard protocols.

Mutations and oligonucleotides

We tested 11 mutations in nine polymorphic sites, including two biallelic polymorphisms (Bips) in *gyrA* ($gyrA_{247}$ and $gyrA_{260}$), three Bips in *gyrB* ($gyrB_{1391}$, $gyrB_{1394}$ and $gyrB_{1398}$) and two Bips in *parE* ($parE_{1246}$ and $parE_{1258}$), as well as two triallelic polymorphisms (Trips) in *gyrA* ($gyrA_{248}$ and $gyrA_{259}$) (Figure 1).[5] Primers were designed with PrimerPlex V1.0 (PREMIER Biosoft International, USA) and are shown in Table 1, including 6 primers for amplifying the three gene fragments in one multiplex PCR and 20 allele-specific primer extension (ASPE) primers for detecting all 11 mutations. The 20 ASPE primers target each of the wild-type and mutant alleles by allele-specific nucleotides at the 3′ end, and contain unique TAGs (specific 24-mer oligonucleotide sequences as 'tags' for different ASPE primers) at the 5′ end that are reverse complements to the anti-TAGs on xTAG beads (Luminex Corp., USA). They also contain degenerate nucleotides at internal positions, which will ensure primer extension despite known polymorphisms neighbouring the target SNPs in all isolates from *Salmonella* Typhi as well as *Salmonella* Paratyphi A. All oligonucleotides were synthesized by Invitrogen Corp.

Multiplex PCR

The overall scheme of the assay is shown in Figure 2. The following steps were carried out in 96-well microplates. Each 10 μL multiplex PCR consisted of 5 μL of 2× Multiplex PCR Master Mix (Qiagen, Germany), 1 μL of a mixture of 0.5 μM PCR primers for amplifying *gyrA*, *gyrB* and *parE*, and 4 μL of template DNA (5 ng/μL). One well per microplate contained 4 μL of distilled water (dH_2O) instead of template DNA and served as a blank control. PCR cycling was performed in a four-block DNA Engine Tetrad 2 Thermocycler (Bio-Rad Inc., USA), with an initial denaturation at 95°C for 10 min, followed by 30 cycles at 94°C for 30 s, 58°C for 30 s and 72°C for 30 s, and a final extension step at 72°C for 3 min.

Exo-SAP treatment

To remove the unincorporated PCR primers and dNTPs, 1 μL of Exo-SAP containing 5 U of exonuclease I (Exo) and 0.5 U of shrimp alkaline phosphatase (SAP) (USB Corp., Germany) was added to each multiplex PCR product and mixed. The mixture was then incubated at 37°C for 30 min, followed by 10 min at 80°C to inactivate the enzymes.

Multiplex ASPE

The ASPE used here follows the protocol recommended by Luminex (http://www.luminexcorp.com/support/protocols/xtag_protocols.html). Briefly, each 10 μL multiplex ASPE mixture reaction contained 5 μL of Exo-SAP-treated multiplex PCR product, 0.3 U of *Tsp* DNA polymerase (Invitrogen), 25 nM ASPE primer mixture for all 11 SNPs (Table 1), 5 μM dATP/dTTP/dGTP and biotin-dCTP (Invitrogen), 20 mM Tris-HCl (pH 8.4), 50 mM KCl and 1.25 mM $MgCl_2$. Thermocycling was performed at 95°C for 5 min, followed by 25 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, and a final extension at 72°C for 3 min.

Hybridization

A mixture of 20 distinct Luminex xTAG beads (shown in the last column of Table 1) containing 250 beads/μL for each bead type was prepared in 2× Tm hybridization buffer (0.4 M NaCl/0.2 M Tris/0.16% Triton X-100, pH 8.0). Then, 2 μL of the bead mixture was added per well containing 10 μL of ASPE products, followed by 23 μL of 2× Tm hybridization buffer and 15 μL of dH_2O for a total volume of 50 μL. A bead control consisted of 2 μL of bead mixture, 23 μL of 2× Tm hybridization buffer and 25 μL of dH_2O. The hybridization mixture was then incubated at 95°C for 1 min and 37°C for 30 min.

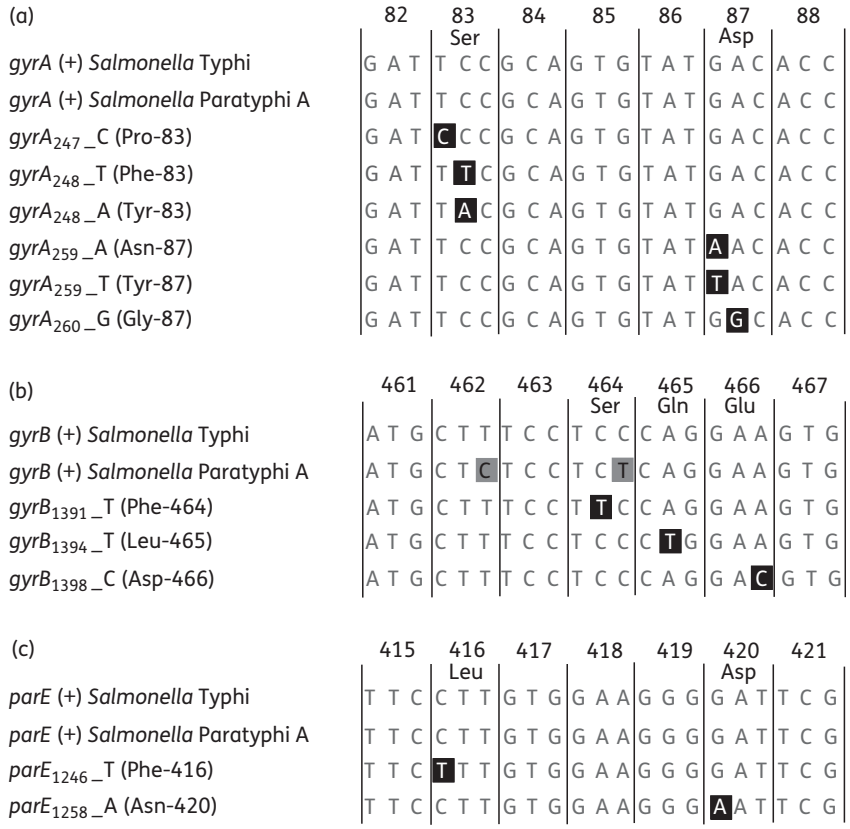

Figure 1. Multiple alignments of partial *gyrA*, *gyrB* and *parE* fragments in *Salmonella* Typhi and *Salmonella* Paratyphi A. Wild-type alleles for each gene are indicated by a plus symbol (+). The mutations tested in this paper are shown in black squares and the wild-type amino acid is indicated on top of the alignments for codons containing target mutations. The amino acids for mutations are shown in brackets after the allele names. (a) Alignment of *gyrA* showing polymorphisms in codons 83 and 87, of which two are triallelic polymorphisms (Trips). (b) Alignment of *gyrB* with three mutations. Two silent mutations in *Salmonella* Paratyphi A are shown in grey squares. (c) Alignment of *parE* with two mutations.

Washing and detecting

The hybridization mixture was centrifuged at 3220 **g** for 3 min and the supernatant was discarded. The xTAG bead pellets were then washed twice by adding 75 μL of 1× Tm hybridization buffer (0.2 M NaCl/0.1 M Tris/0.08% Triton X-100, pH 8.0), centrifuged at 3220 **g** for 3 min and the supernatant discarded. Then, 75 μL of 1× Tm hybridization buffer containing 2 mg/L streptavidin-R-phycoerythrin (Invitrogen, USA) was added to each well and the mixture was incubated at 37°C for 15 min. For detection, 50 μL of the final solution was injected into a Luminex 200 station (Luminex Corp., USA), at a sample plate temperature of 37°C. Fluorescence signals were measured for 100 beads for each of the 20 types of beads.

SNP calling

The Luminex station provides median fluorescence intensity (MFI) values for 100 beads of each bead type for all tested samples. The MFI values for samples were corrected by subtracting the values of the bead control, and the blank control was used to ensure that contamination and unspecific signals were not introduced during the multiplex PCR and ASPE process. SNPs were called with the software MasterPlex (MiraiBio, USA) only when the background-corrected MFI was >300 and the ratio of $MFI_{called\ allele}/(MFI_{wild\ allele}+MFI_{mutant\ allele})$ was >0.9. For the two Trips in *gyrA* (Figure 1 and Table 2), the denominator consisted of the MFI for the wild-type plus both of the mutant alleles.

Reproducibility

DNA from isolate CT18 was used to evaluate the reproducibility of the assay in five independent repeats, following the steps described above.

Direct detection from bacterial colonies

Twelve random isolates of *Salmonella* Paratyphi A (4 Nal^S and 8 Nal^R) were streaked to single colonies on trypticase soy agar plates. For each isolate, a single colony was resuspended in 100 μL of H_2O and incubated at 95°C for 15 min in order to kill the bacteria and release genomic DNA. After centrifugation, the supernatants were transferred to new tubes and used as templates for multiplex PCR.

Results

Development of the Luminex xTAG assay

The Luminex xTAG genotyping assay consists of four steps (Figure 2), as follows.

(i) A multiplex PCR to amplify three gene fragments of *gyrA*, *gyrB* and *parE* containing 11 target SNPs (Table 1), after which unincorporated dNTPs and PCR primers are removed by Exo-SAP treatment.

Table 1. Detailed information on mutations in *gyrA*, *gyrB* and *parE*, and oligonucleotides used in this study

Gene	PCR primer pairs	Amplicon	ASPE primer sequence[a]	Target allele[b]	Amino acid	xTAG beads[c]
gyrA	GCCCTTCAATGCTGATGTCTTC TCTCCTCTGTGTCGCCTCTG	805 bp	*CAATAAACTATACTTCTTCACTAA*ATGGTGTCATACACTGCG**D**A	$gyrA_{247}$_T(+)	GyrA Ser-83	13
			*ATACTTCATTCATTCATCAATTCA*ATGGTGTCATACACTGCG**D**G	$gyrA_{247}$_C	GyrA Pro-83	15
			*CAATTCATTTACCAATTTACCAAT*GATGGTGTCATACACTGCGG	$gyrA_{248}$_C(+)	GyrA Ser-83	7
			*TAATCTTCTATATCAACATCTTAC*GATGGTGTCATACACTGCGA	$gyrA_{248}$_T	GyrA Phe-83	9
			*TACAAATCATCAATCACTTTAATC*GATGGTGTCATACACTGCGT	$gyrA_{248}$_A	GyrA Tyr-83	11
			*TACACTTTATCAAATCTTACAATC*GCCATACGAACGATGGTG**Y**C	$gyrA_{259}$_G(+)	GyrA Asp-87	3
			*TACATTACCAATAATCTTCAAATC*GCCATACGAACGATGGTG**Y**T	$gyrA_{259}$_A	GyrA Asn-87	4
			*CAATTCAAATCACAATAATCAATC*GCCATACGAACGATGGTG**Y**A	$gyrA_{259}$_T	GyrA Tyr-87	5
			*CTTTAATCTCAATCAATACAAATC*CGCCATACGAACGATGGTGT	$gyrA_{260}$_A(+)	GyrA Asp-87	1
			*CTTTATCAATACATACTACAATCA*CGCCATACGAACGATGGTGC	$gyrA_{260}$_G	GyrA Gly-87	2
gyrB	ACTGGCGGATTGTCAGGAAC ATCGGCTTCGGTCAGAGTTG	640 bp	*AATCCTTTCTTTAATCTCAAATCA*GCTTCGACAAGATGCTTTCCTC	$gyrB_{1391}$_C(+)	GyrB Ser-464	21
			*CTTTAATCTACACTTTCTAACAATG*CTTCGACAAGATGCTTTCCTT	$gyrB_{1391}$_T	GyrB Phe-464	81
			*TCAATTACCTTTTCAATACAATACC*GACAAGATGCTTTCCT**YY**CA	$gyrB_{1394}$_A(+)	GyrB Gln-465	24
			*CTTTTCAATTACTTCAAATCTTCA*CGACAAGATGCTTTCCT**YY**CT	$gyrB_{1394}$_T	GyrB Leu-465	25
			*TTACTCAAAATCTACACTTTTTCA*AAGATGCTTTCCT**YY**C**W**GGAA	$gyrB_{1398}$_A(+)	GyrB Glu-466	26
			*CTTTTCAAATCAATACTCAACTTTA*AGATGCTTTCCT**YY**C**W**GGAC	$gyrB_{1398}$_C	GyrB Asp-466	27
parE	ATACGGTATAGCGGCGGTAG CGGAACAACTGGCAGAGATG	493 bp	*TCAATCAATTACTTACTCAAATAC*GCCGAATCCCCTTCCACAAG	$parE_{1246}$_C(+)	ParE Leu-416	19
			*CTATCTATCTAACTATCTATATCA*GCCGAATCCCCTTCCACAAA	$parE_{1246}$_T	ParE Phe-416	78
			*AATCAATCTTCATTCAAATCATCA*GGAACCGCCTGCCGAATC	$parE_{1258}$_G(+)	ParE Asp-420	16
			*CTTTAATCCTTTATCACTTTATCA*GGAACCGCCTGCCGAATT	$parE_{1258}$_A	ParE Asn-420	17

[a]The italic nucleotides for each ASPE primer are the TAG sequences provided by Luminex. The nucleotides in bold are degenerate sites (D, A or G or T; Y, C or T; and W, A or T).

[b]Alleles detected by individual ASPE primers. The subscript numbers indicate the nucleotide positions in *gyrA*, *gyrB* and *parE*, followed by nucleotides at these positions. '(+)' indicates wild-type alleles. Because the primer design was carried out on the CT18 genome and the reading frame of *gyrA* is on the minus strand of the genome, nucleotides in *gyrA* alleles are complementary to the 3′-end nucleotides in corresponding ASPE primers. In total, 11 mutations at nine polymorphic sites are shown, including two triallelic polymorphisms (Trips) in *gyrA* ($gyrA_{248}$ and $gyrA_{259}$), two biallelic polymorphisms (Bips) in *gyrA*, three Bips in *gyrB* and two Bips in *parE*.

[c]Different xTAG beads used to call the alleles in the Luminex assay. The numbers are indexed to the coupled anti-TAG, which are reverse complimentary to the italic TAG sequences appended to the ASPE primers.

(ii) A multiplex primer extension step using 20 ASPE primers, during which biotin-dCTP is incorporated if there is a perfect match at the 3′ end. This step includes oligonucleotide primers specific for each of the wild-type and mutated alleles at each of the Bips and Trips (Figure 1), and which contain allele-specific unique TAG sequences. *Tsp* polymerase is so specific that only one of the alternative primers is extended for each nucleotide position and the other primers for that position serve as internal controls.

(iii) The ASPE products are hybridized to a mixture of 20 types of Luminex xTAG beads, each containing one anti-TAG sequence that is complementary to one of the TAG sequences appended to an ASPE primer. After washing the beads, the fluorescent dye streptavidin-R-phycoerythrin is added to bind to biotin-dCTP in the ASPE products.

(iv) The ASPE products/xTAG beads/streptavidin-R-phycoerythrin complex is quantified with a Luminex station, which calculates the MFI for 100 beads of each type. The entire procedure was conducted in two 96-well plates per test, with one plate for step (i) and the second one for the other three steps.

MFI values were corrected by subtracting the bead control and arbitrarily set to 0.5 if this correction resulted in negative values. Examples of such corrected data for the 12 reference isolates used to develop this assay are shown in Table 2. SNPs were called when corrected MFI was >300 and the ratio of $MFI_{called\ allele}/(MFI_{wild\ allele}+MFI_{mutant\ allele})$ was >0.9. These calls agreed completely with prior assignments based on dHPLC and sequencing.[5] In Table 2, the MFI values of called alleles ranged from >1000 to 5961. In contrast, the MFI of uncalled alleles ranged from 0.5 to 31.5, only slightly greater than the values for the H_2O control (12.0–29.0). These results demonstrate that the Luminex assay provides a tool to unambiguously call all the targeted SNPs.

Reproducibility of the assay was tested by five independent trials with isolate CT18 (Figure 3). CT18 is wild-type for all the nine polymorphic positions in *gyrA*, *gyrB* and *parE*. MFI values for mutant alleles were almost negligible whereas corrected MFI values for wild-type alleles were strong. Positive MFI results showed considerable variation between individual tests, possibly reflecting variability between different runs in PCR and ASPE efficiencies. However, the ratios of $MFI_{called\ allele}/(MFI_{wild\ allele}+MFI_{mutant\ allele})$ were very uniform, with an average ratio of called MFI of >0.99 and a coefficient of variation of <0.5%. The MFI of called alleles was always >50 times higher than that of the uncalled ones. The use of MFI ratios instead of net

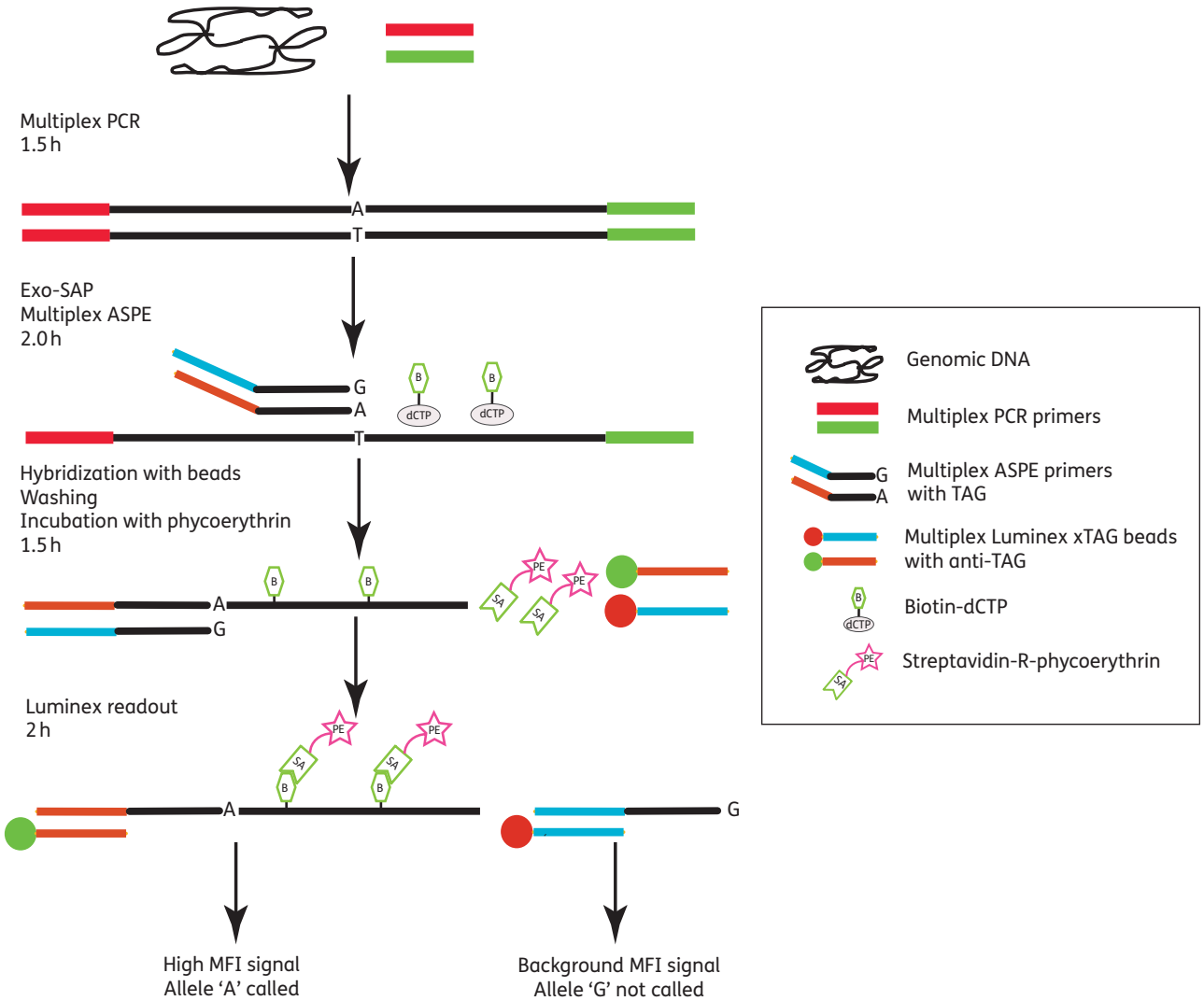

Figure 2. Overview of the Luminex xTAG assay developed in this paper. More details of all steps can be found in the Materials and methods section. The estimated time for each step is shown on the left, based on testing two 96-well plates of samples. Only one fragment with one biallelic polymorphism site (Bip) is shown for illustrative purposes.

MFI values minimizes the variation among different tests and yields unambiguous SNP calling results.

The assays just described were performed with purified DNA as template. We also tested whether boiled single colonies from agar plates could be used as a template with eight Nal^R and four Nal^S isolates of *Salmonella* Paratyphi A. The results were congruent with results obtained with purified DNA from these isolates, namely all four Nal^S isolates contained only wild-type alleles, seven Nal^R isolates were called as GyrA Phe-83 and one Nal^R isolate was called as GyrA Tyr-83. This result indicates that this assay could be used by clinical microbiologists immediately after cultivation of *Salmonella* isolates. Starting with template DNA, testing 188 samples, two H_2O controls and two bead controls in two 96-well microplates took a total of ~7 h (Figure 2), with hands-on time of <1 h. Hence, the assay can be completed within 1 working day, producing 2068 datapoints (188 samples×11 SNPs).

Validation with a larger panel of isolates

A panel of 205 isolates of *Salmonella* Typhi (including the 12 isolates used to develop the assay) was tested to validate the Luminex assay. The mutation profiles for *gyrA*, *gyrB* and *parE* of these isolates had previously been determined, either by whole genome sequencing or dHPLC (Table 3). [5,15,16]

The results with the 62 Nal^S isolates were fully consistent with previous dHPLC data: 54 were wild-type for all the targeted SNPs; six contained GyrB Phe-464; one was GyrB Leu-465; and one was GyrB Asp-466. All of the 143 Nal^R isolates harboured at least one of the target mutations. The mutation profiles of these isolates were: GyrA Phe-83 (77 isolates); GyrA Tyr-83 (18 isolates); GyrA Pro-83 (1 isolate); GyrA Asn-87 (4 isolates); GyrA Tyr-87 (1 isolate); GyrA Gly-87 (10 isolates); GyrA Phe-83+ParE Asn-420 (31 isolates); and GyrA Phe-83+ParE Phe-416 (1 isolate). These results were only 88.8% (127/143) concordant with the prior dHPLC results, because 11 isolates were GyrA

Table 2. Raw data of background-corrected MFI values for the 12 *Salmonella* Typhi isolates used for setting up the assay[a]

		Salmonella Typhi isolates												
Allele	Amino acid	CT18[b]	MP0150	150(98)S[b]	MP0247	MP0248	MP0327	8(04)N[b]	E02-2759[b]	MP0068	ct 42	MP0562	AG3[b]	H_2O
gyrA$_{247}$_C	GyrA Pro-83	27.5	**2331.5**	17.0	26.0	22.0	0.5	16.0	19.0	22.0	25.5	17.0	11.0	15.0
gyrA$_{247}$_T(+)	GyrA Ser-83	**2321.0**	28.0	**1639.0**	**1594.0**	**1497.0**	**1244.0**	**1850.0**	**1373.0**	**2349.0**	**2055.0**	**1864.5**	**1942.0**	16.0
gyrA$_{248}$_T	GyrA Phe-83	25.0	10.0	**2302.0**	24.0	13.0	0.5	5.0	16.5	15.5	13.0	**2784.0**	**2823.0**	12.0
gyrA$_{248}$_A	GyrA Tyr-83	12.0	0.5	12.0	**1610.0**	12.5	0.5	0.5	1.0	13.5	22.5	17.0	19.0	15.0
gyrA$_{248}$_C(+)	GyrA Ser-83	**1453.5**	**1129.5**	7.5	5.0	**1252.0**	**1100.5**	**1675.0**	**1972.0**	**1547.0**	**1845.0**	31.5	27.0	16.5
gyrA$_{259}$_A	GyrA Asn-87	11.0	13.0	19.0	18.0	**1969.0**	1.0	12.0	13.0	19.0	18.5	14.0	15.0	18.0
gyrA$_{259}$_T	GyrA Tyr-87	16.0	25.0	23.0	22.5	17.0	**1251.5**	21.5	21.5	13.5	12.0	18.0	17.0	29.0
gyrA$_{259}$_G(+)	GyrA Asp-87	**1621.5**	**1225.5**	**1226.5**	**1250.5**	21.5	0.5	**1657.5**	**1060.0**	**1673.5**	**2024.5**	**1554.5**	**1618.5**	23.0
gyrA$_{260}$_G	GyrA Gly-87	25.0	24.0	19.0	22.0	19.0	9.0	**3812.0**	22.5	22.5	28.5	25.0	28.0	15.0
gyrA$_{260}$_A(+)	GyrA Asp-87	**4591.5**	**3483.5**	**3562.5**	**3515.0**	**5238.0**	**1679.5**	24.5	**2769.5**	**4425.0**	**3078.5**	**4326.5**	**4733.5**	14.5
gyrB$_{1391}$_T	GyrB Phe-464	15.0	18.0	19.0	26.0	26.0	9.0	12.0	**1140.0**	27.0	19.0	15.0	18.0	20.0
gyrB$_{1391}$_C(+)	GyrB Ser-464	**1971.0**	**1718.0**	**1374.5**	**1478.0**	**1522.0**	**1592.0**	**1338.5**	16.0	**1974.0**	**1755.0**	**1427.0**	**1750.0**	23.0
gyrB$_{1394}$_T	GyrB Leu-465	17.0	21.5	14.5	21.5	10.0	11.5	18.5	17.5	**3005.5**	24.5	18.5	11.5	16.0
gyrB$_{1394}$_A(+)	GyrB Gln-465	**5961.0**	**5578.0**	**4700.0**	**5516.5**	**5372.0**	**2709.0**	**5198.0**	**1721.0**	14.5	**4967.0**	**5478.0**	**5935.0**	15.0
gyrB$_{1398}$_C	GyrB Asp-466	27.5	18.5	22.0	2.5	21.5	10.0	10.5	0.5	16.5	**3984.5**	21.0	22.5	15.0
gyrB$_{1398}$_A(+)	GyrB Glu-466	**4463.0**	**4221.0**	**3633.0**	**3550.0**	**3923.0**	**1634.0**	**3694.5**	**1486.5**	**1140.0**	18.5	**4045.0**	**4349.0**	20.0
parE$_{1246}$_T	ParE Phe-416	20.0	22.5	16.0	20.0	22.0	14.5	17.0	16.0	20.0	21.0	**2722.0**	16.0	17.0
parE$_{1246}$_C(+)	ParE Leu-416	**4119.5**	**3655.0**	**3842.0**	**3692.0**	**3833.0**	**2455.0**	**3866.0**	**3801.5**	**5039.0**	**4362.0**	23.0	**2382.0**	25.0
parE$_{1258}$_A	ParE Asn-420	22.5	11.5	2.0	21.0	18.0	6.5	8.5	8.0	11.5	17.5	21.0	**1918.5**	24.0
parE$_{1258}$_G(+)	ParE Asp-420	**1498.5**	**1388.0**	**1189.0**	**1178.5**	**1462.5**	**1762.0**	**1417.5**	**1158.0**	**1760.0**	**1636.5**	**1532.0**	15.0	29.0

[a]The MFI values were corrected by subtracting the value of the bead control; values of <0 were arbitrarily set to 0.5. For easier visualization, the values of called SNPs are shown in bold. The isolates MP0562 and AG3 contain dual mutations in *gyrA* and *parE*.
[b]Isolates from which whole genome sequences are available.[15,16]

Phe-83 according to dHPLC whereas they were GyrA Phe-83+ ParE Asn-420 based on the Luminex assay, and five isolates were GyrA Tyr-83 in dHPLC and GyrA Phe-83 in the Luminex assay. The 16 discrepancies were resolved by direct sequencing using the primers listed in Table 1. The direct sequencing results confirmed the Luminex SNP calls for all 16 isolates, showing that the Luminex assay made the correct calls, including multiple SNPs, for all *Salmonella* Typhi isolates (both Nal^S and Nal^R). We were surprised at the large number of prior false calls with dHPLC, which was thought to be highly reliable until now. Possibly, these false dHPLC calls reflect the fact that dHPLC was performed with multiplexed DNA templates of 5 or 10 isolates in order to increase throughput and it was not sufficiently sensitive to signals from mixtures of alleles.[5]

Mutation profiles for GyrA, GyrB and ParE of Salmonella Typhi and Salmonella Paratyphi A

We also tested additional isolates of serovars Typhi (87 isolates) and Paratyphi A (106 isolates) for which levels of susceptibility to nalidixic acid were known (Table 3), but which had not been previously evaluated in regard to their DNA gyrase and topoisomerase SNP profiles. All Nal^S isolates of serovars Typhi (7 isolates) and Paratyphi A (82 isolates) were wild-type for all alleles that were tested. In contrast, mutations in GyrA or GyrA + ParE were found in all Nal^R isolates of serovars Typhi (80 isolates) and Paratyphi A (24 isolates). One of the Typhi isolates even contained two mutations in GyrA (Table 3).

Overall, we have tested a total of 292 isolates of *Salmonella* Typhi (Nal^R=223 and Nal^S=69) and 106 isolates of *Salmonella* Paratyphi A (Nal^R=24 and Nal^S=82) with the Luminex assay. All 247 Nal^R *Salmonella* Typhi and *Salmonella* Paratyphi A isolates were found to harbour at least one of the target mutations, which demonstrated 100% sensitivity for identifying Nal^R mutations in this strain collection. The most common mutation in the Nal^R *Salmonella* Typhi isolates was GyrA Phe-83 (143/223=64.1%). Three other less common mutation profiles were GyrA Phe-83+ParE Asn-420 (33/223=14.8%), GyrA Tyr-83 (27/223=12.1%) and GyrA Gly-87 (10/223=4.5%). These four mutation profiles account for 95.5% (213/223) of all Nal^R *Salmonella* Typhi isolates that were tested. The predominating mutation profile of *Salmonella* Paratyphi A Nal^R isolates was also GyrA Phe-83 (18/24=75.0%).

GyrB mutations

The Luminex assay also recognized mutations in GyrB within eight Nal^S *Salmonella* Typhi isolates (Table 4). Six of these isolates are mutated at GyrB Phe-464, and one each at GyrB Leu-465 and GyrB Asp-466. These isolates were designated Nal^S, because their MICs of nalidixic acid are below the breakpoint of 32 mg/L associated with resistance to nalidixic acid.[17] However, as shown in Table 4, all of them, except isolate E98-4364, showed decreased susceptibility to ciprofloxacin, which is of clear significance in clinical practice. Different groups have already reported such decreased ciprofloxacin

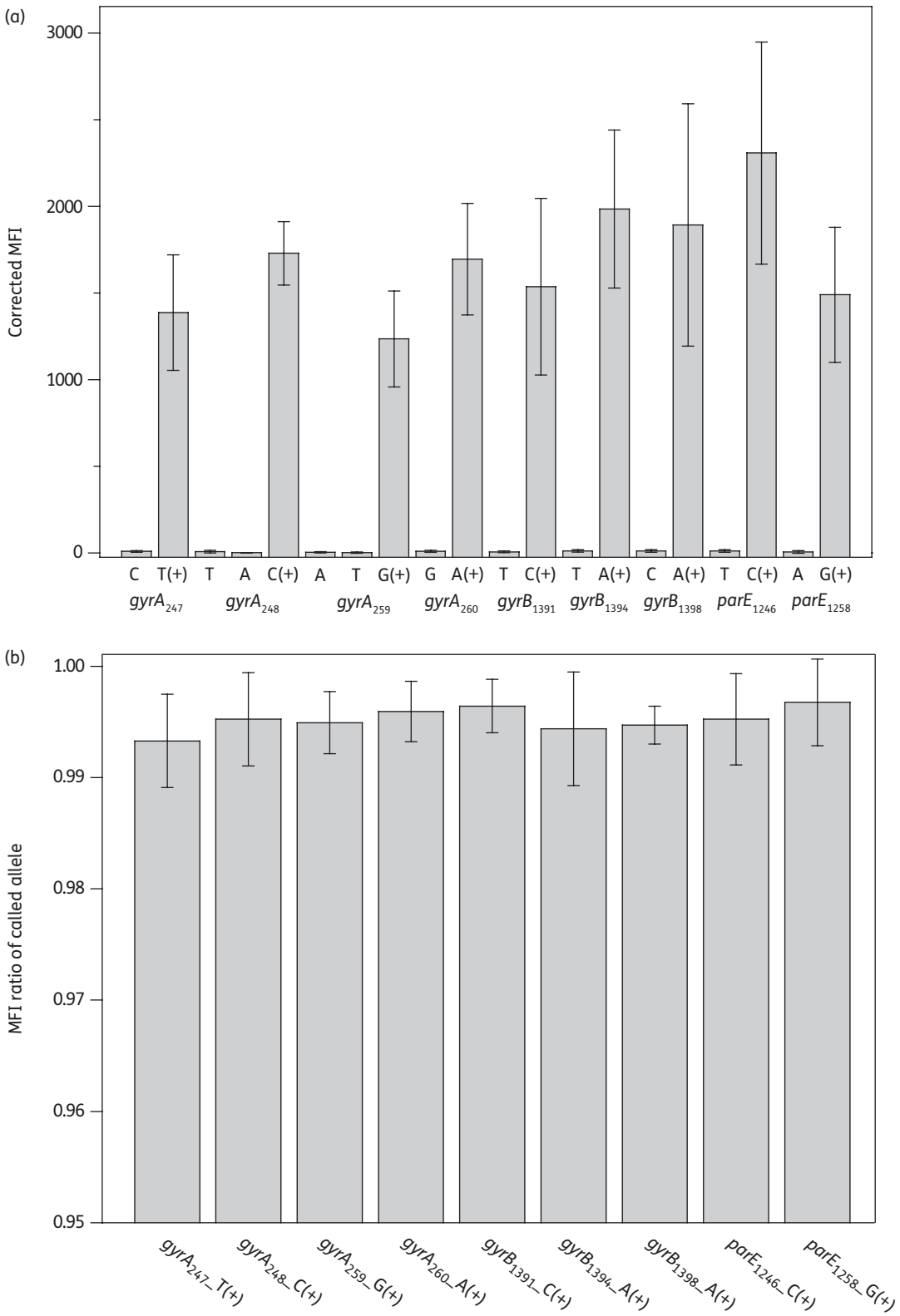

Figure 3. Reproducibility of the Luminex assay in five independent tests with *Salmonella* Typhi CT18. (a) Raw data of average corrected MFI values (MFI minus the MFI of bead control). Error bars indicate the standard deviation. (b) Ratios of $MFI_{called\ allele}/(MFI_{wild\ allele}+MFI_{mutant\ allele})$. The average ratio for the called allele is shown, with error bars indicating the standard deviation.

Table 3. Overview of mutation profiles for *gyrA*, *gyrB* and *parE* of all isolates included in this study

Mutation profile	Amino acid(s)	Validation panel[a]		*Salmonella* Typhi		*Salmonella* Paratyphi A	
		Nal^R ($n=143$)	Nal^S ($n=62$)	Nal^R ($n=80$)	Nal^S ($n=7$)	Nal^R ($n=24$)	Nal^S ($n=82$)
Wild-type	wild-type		54		7		82
$gyrA_{247}$_C	GyrA Pro-83	1					
$gyrA_{248}$_T	GyrA Phe-83	77		66		18	
$gyrA_{248}$_A	GyrA Tyr-83	18		9		3	
$gyrA_{259}$_A	GyrA Asn-87	4		2		2	
$gyrA_{259}$_T	GyrA Tyr-87	1				1	
$gyrA_{260}$_G	GyrA Gly-87	10					
$gyrB_{1391}$_T	GyrB Phe-464		6				
$gyrB_{1394}$_T	GyrB Leu-465		1				
$gyrB_{1398}$_C	GyrB Asp-466		1				
$gyrA_{248}$_T, $parE_{1246}$_T	GyrA Phe-83, ParE Phe-416	1					
$gyrA_{248}$_T, $parE_{1258}$_A	GyrA Phe-83, ParE Asn-420	31		2			
$gyrA_{248}$_T, $gyrA_{259}$_A	GyrA Phe-83, GyrA Asn-87			1			

[a]The validation panel consists of *Salmonella* Typhi isolates for which dHPLC results on *gyrA*, *gyrB* and *parE* are available, including the 12 isolates in Table 2 used for setting up the assay.[5,15]

Table 4. Characteristics of *Salmonella* Typhi isolates with GyrB mutations

Isolate	Year	Country	NAL MIC (mg/L)[a]	CIP MIC (mg/L)[b]	GyrB mutation
AG-22	2004	Vietnam	6.0	0.250	Phe-464
AS-22541	2003	Bangladesh	8.0	0.125	Phe-464
DT-33	2002	Vietnam	8.0	0.500	Phe-464
DT-94	2002	Vietnam	16.0	0.125	Phe-464
dty1-121	1997	Vietnam	8.0	0.190	Phe-464
E02-2759	2002	India	4.0	0.125	Phe-464
ct 42	1994	Vietnam	16.0	0.160	Asp-466
E98-4364	1998	Mexico	2.0	0.030	Leu-465

[a]MIC of nalidixic acid (NAL), which ranges from 0.75 to 4.0 mg/L for 25 isolates without mutations.
[b]MIC of ciprofloxacin (CIP), which ranges from 0.012 to 0.032 mg/L for 25 isolates without mutations.

susceptibility (DCS) isolates in *Salmonella* Typhi, for which the ciprofloxacin MIC is 0.12–1 mg/L.[18–20] Patients infected with DCS *Salmonella* Typhi isolates normally experience longer times to fever clearance and more frequent treatment failure than those without DCS.[18] According to Table 4, mutations GyrB Phe-464 and GyrB Asp-466 seem to be responsible for the DCS phenotype in some isolates, but this needs to be further confirmed by functional assays. It had already been reported that isolates of *Salmonella* Typhimurium with GyrB Phe-464+GyrA Phe-83+GyrA Asn-87 mutations were fully resistant to ciprofloxacin (MIC 16–32 mg/L).[21] We therefore considered it worthwhile to include these SNPs in the Luminex assay. Thus, this assay recognizes two major sources of decreased susceptibility to fluoroquinolones, only one of which is recognized by tests for resistance to nalidixic acid.

Discussion

Luminex bead-based technology is a recently developed platform for the detection of multiplexed signals. This system incorporates 5.6 μm polystyrene microspheres that are internally labelled with two spectrally distinct fluorochromes. Mixing these two fluorochromes in various proportions yields 100 types of distinguishable, commercially available beads, on the basis of characteristic fluorescence signals. In the past, Luminex assays, also known as bead-based suspension arrays, have been used predominantly for quantitative measurements of multiplexed protein–ligand interactions or high-throughput nucleic acid detection through DNA hybridization.[22,23] For example, a Luminex hybridization assay has been introduced to detect the six most common serogroups of *S. enterica* in the USA.[24] Luminex assays are now being increasingly used for multiplexed genotyping on the basis of individual SNPs.[25] For example, Ducey *et al.*[26] and Ward *et al.*[27] have presented Luminex-based assays for genotyping *Listeria monocytogenes*, which can simultaneously detect 51 or 64 SNPs on the basis of calibrated MFI values.

We have developed a somewhat different strategy for the SNP typing of bacterial DNA in this assay. Instead of attempting to calibrate a range of acceptable MFI values that indicate a positive reaction, we designed ASPE primers that are specific for both the mutated and the wild-type alleles at each targeted polymorphic position. For Bips this involves one mutant-specific and one wild-type-specific ASPE primer, while for Trips, such as in *gyrA*, it involves two mutant-specific and one wild-type-specific ASPE primer. The assay described here uses 20 ASPE primers and 20 types of beads to screen three gene fragments for 11 mutations, seven Bips and two Trips, and results in calls for nine alternative alleles in each assay (Figure 3). This approach has the disadvantage that two or three ASPE primers are needed for each polymorphic site, but this disadvantage is accompanied by very high reliability when measured as MFI ratios (Figure 3).

This characteristic removes the necessity for including positive controls for analysing real samples, which improves the overall throughput. In other assays that have been described, such as dHPLC[8,28] and high-resolution melting curve assays,[13] SNPs are called by examining the curves (including shape and melt temperature/retention time), which can be subject to human errors. Positive controls for every genotype should also be included in every assay batch when using these two technologies, to ensure reliable comparisons and SNP callings.

Another underlying advantage of using ASPE specific for both the mutation and wild-type alleles is that this approach provides a limited ability for mutation discovery. Failure to call any SNP for all ASPE primers within one of the three multiplexed gene fragments implies polymorphisms within the PCR primer-binding region, which we have not yet observed. On the other hand, if only certain mutations are not called in one fragment, this indicates the existence of novel sequence diversity within the ASPE primer binding sites. In its original form, when the assay had been developed only for *Salmonella* Typhi, it failed to call SNPs in codons 465 and 466 in *gyrB* for *Salmonella* Paratyphi A isolates. This proved to reflect two silent mutations in the *gyrB* of *Salmonella* Paratyphi A isolates (Figure 1b), which significantly reduced the ASPE efficiency for neighbouring target mutations. After introducing appropriate degenerate nucleotides into the ASPE primers, the assay now also works well for *Salmonella* Paratyphi A. Similarly, the Luminex assay failed to call SNPs in codon 464 of *gyrB* in two *Salmonella* Typhi isolates, which subsequently turned out to harbour a novel mutation in this codon (F.-X. Weill, unpublished data).

Glass slide-based microarray technology can potentially offer similar or even much higher multiplexing capacity in terms of numbers of targeted mutations. For instance, a microarray has been described that simultaneously scans all potential mutations in codons 83 and 87 of *gyrA* in extraintestinal pathogenic *Escherichia coli*, as well as mutations inside the virulence-related gene *fimH*.[29] However, the glass slide-based microarray can only test one sample per slide, with a maximum throughput of dozens of samples per day. The Luminex assay presented here offers higher throughput (11 SNPs for 188 isolates per day). Although the current Luminex protocol is not simple enough yet for routine use in hospitals with limited numbers of samples, it will be ideal for reference laboratories and central clinical laboratories with large sample collections. The total cost of this assay per sample (including oligos, reagents and xTAG beads) was ~€2.40 (US$ 3.50), which means ~€0.20 per SNP. For testing large numbers of samples, it might be possible to reduce costs dramatically by using an oligonucleotide ligation assay with very low numbers of beads.[30]

The Luminex assay identified one or two mutations in each Nal^R isolate that was tested (100% sensitivity), resulting in nine distinct mutation profiles (genotypes) of Nal^R *Salmonella* Typhi and four profiles of Nal^R *Salmonella* Paratyphi A (Table 3). All the single mutations of *gyrA* were located in codons 83 and 87, which are known to represent 'hotspots' of Nal^R mutations in *Salmonella*.[4,6] The three dual-mutation profiles of *Salmonella* Typhi might reflect the accumulation of point mutations in GyrA and ParE under the selection of fluoroquinolone usage for therapy.[31]

Several papers reported mutations of ParC in ciprofloxacin-resistant *Salmonella* Typhi and *Salmonella* Paratyphi A isolates.[32–34] We failed to identify any resistant strain with a ParC mutation in our current *Salmonella* Typhi and *Salmonella* Paratyphi A collections; therefore, we excluded ParC from the assay due to the lack of control isolates for assay validation. We also did not include probes for plasmid-mediated quinolone resistance [*qnrA*, *qnrB*, *qnrC*, *qnrS*, *qepA* and *aac(6′)-Ib-cr*][35,36] or quinolone resistance related to decreased antibiotic uptake (*mar* and *acr* genes),[6] which were only reported in some serovars of *S. enterica* other than Typhi and Paratyphi A. However, the assay presented in this paper can potentially be extended to detect additional quinolone-resistance-related mutations in *Salmonella*. Luminex is currently bringing a hardware upgrade to market that can distinguish 500 analytes (capable of testing 250 Bips) and it would also be possible to combine multiple SNP typing with direct-hybridization tests in the same assays. Probably, the most limiting feature of the Luminex assay is the number of multiplexed PCR products that can simultaneously be amplified. One human genetic study reported successful 15-plex and 40-plex SNP genotyping,[30] and we have succeeded in developing an 18-plex assay for genotyping *Salmonella* Typhi (Y. Song and M. Achtman, unpublished data). For extending this assay to detect SNPs in QRDRs of some other common serovars of *Salmonella* (Typhimurium, Enteritidis, Dublin etc.), multiplex PCR is not likely to be a problem, as it will only need to amplify four genes (*gyrA*, *gyrB*, *parC* and *parE*). Since there are more mutations in QRDRs of other *Salmonella* serovars than what we have tested here,[6] careful alignments of the targeted regions must be performed before designing ASPE primers (Figure 1). Degenerate nucleotides will be necessary to cover the variations inside the upstream regions (~8 bp) of certain targeted mutations; this has been proven as an effective strategy for *gyrA* and *gyrB* in this study (Table 1).

In summary, we describe a flexible, rapid, medium throughput and cost-effective multiplexed Luminex xTAG assay, which can simultaneously detect 11 mutations in *gyrA*, *gyrB* and *parE* of *Salmonella* Typhi and *Salmonella* Paratyphi A. This assay yields unambiguous SNP calls and possesses a limited mutation discovery potential. It can be used on extracted DNA or on single colonies of *Salmonella* Typhi and *Salmonella* Paratyphi A, which makes it an ideal platform for clinical and reference laboratories to rapidly screen quinolone-resistant *Salmonella* Typhi and *Salmonella* Paratyphi A isolates for guiding therapy, and detect the underlying genetic changes for molecular epidemiological analysis. The genotyping assay presented here can also be readily adapted to decipher evolutionary events and detailed population genetics in a variety of other genetically monomorphic bacterial pathogens, such as *Salmonella* Typhi, *Yersinia pestis*, *Bacillus anthracis* and *Mycobacterium tuberculosis*, in which SNPs can serve as reliable genetic markers.[37]

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Transparency declarations

No conflicts of interest to declare.

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