

# A Novel Technique for Detecting Antibiotic-Resistant Typhoid from Rapid Diagnostic Tests

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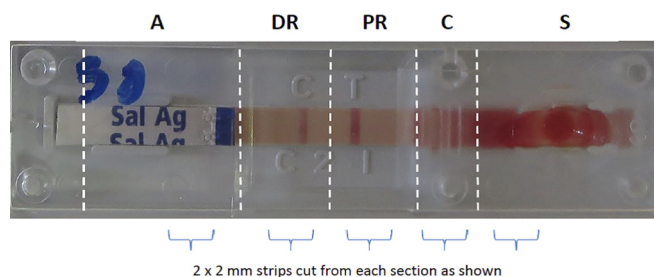
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**Fluoroquinolone-resistant typhoid is increasing. An antigen-detecting rapid diagnostic test (RDT) can rapidly diagnose typhoid from blood cultures. A simple, inexpensive molecular technique performed with DNA from positive RDTs accurately identified *gyrA* mutations consistent with phenotypic susceptibility testing results. Field diagnosis combined with centralized molecular resistance testing could improve typhoid management and surveillance in low-resource settings.**

Antimicrobial resistance is a global problem of increasing concern. Over the past decade, strains of *Salmonella enterica* subsp. *enterica* serovar Typhi with ciprofloxacin MICs in the “intermediate” range have emerged and have been associated with adverse clinical outcomes and increased mortality (1, 2), leading to a lowering of ciprofloxacin breakpoints for *S. Typhi* (3, 4). Fluoroquinolone-resistant (FQR) *S. Typhi* is reported in 40 to 60% of typhoid cases (5, 6), with considerable geographical variation. Prevalence has reached 97% in southern Vietnam (5) and 90% in Cambodia (7), which is of great concern, as fluoroquinolones have become the mainstay of treatment for uncomplicated infection in areas where typhoid is endemic, except where the prevalence of resistance is known to be high, due to their ease of oral administration and low costs. Over 90% of FQR *S. Typhi* isolates are associated with point mutations in the *gyrA* gene, and the most common mutation is Ser83 → Phe (5).

Optimal detection of fluoroquinolone resistance by conventional techniques requires sophisticated laboratories that are able to use expensive consumables, have well-trained staff, and utilize quality assurance measures (8). With such capacities largely lacking in low- or middle-income countries, innovative approaches are needed. We demonstrated the utility of an antigen-detecting *S. Typhi* rapid diagnostic test (RDT) performed with blood culture fluid containing Gram-negative rods (GNRs) in typhoid diagnosis (9). As DNA extracts from RDTs and dried blood spots have been used for molecular surveillance of malaria and HIV drug resistance (10–12), we hypothesized that mutations in the *gyrA* gene could be detected from *S. Typhi*-positive RDTs, facilitating optimized treatment and public health interventions in remote areas. A pilot study was conducted to optimize methodologies, followed by a prospective, multicenter, hospital-based study that evaluated the sensitivity of *gyrA* mutation detection via RDTs and compared these results to those obtained with standard susceptibility testing.

**Pilot study.** Negative blood culture bottles (7 days) were seeded with ~150 cells of *S. Typhi* NCTC 8385, reincubated, and inspected daily. A Gram stain was performed on turbid bottles to confirm the presence of GNRs. Ten bottles containing GNRs were used to inoculate 10 One-Step *Salmonella* Typhi antigen rapid detection kits (Standard Diagnostics, South Korea), which were used to optimize DNA extraction protocols (Fig. 1). Detection of the *gyrA* gene and its mutations was performed using previously



**FIG 1** A *Salmonella* Typhi rapid diagnostic test. Each RDT strip is divided into five sections: sample (S), conjugate (C), proximal result (PR), distal result (DR), and absorption pads (A), which were cut into 2-mm strips to compare DNA extraction by elution or a column-based method.

described primers under slightly modified PCR conditions, followed by restriction fragment length polymorphism (RFLP) analysis (13). DNA from each section and extraction method underwent PCR as neat and diluted (1:10, 1:100, and 1:1,000) samples, plus 40 μg of bovine serum albumin (BSA; New England BioLabs) per reaction mixture to overcome inhibitors (14, 15). Subsequently, the intensities of bands on an agarose gel were compared, and the bands giving the greatest intensities were chosen as indicators for the optimal processing method. Wild-type *S. Typhi* NCTC 8385 and well-characterized strains with known *gyrA* mutations, provided by Oxford University Clinical Research Unit, Ho Chi Minh City, Vietnam (16), were used as controls.

The optimal protocol for DNA extraction was found to be the

Received 25 February 2015 Accepted 3 March 2015

Accepted manuscript posted online 11 March 2015

Citation Nic Fhogartaigh C, Dance DAB, Davong V, Tann P, Phetsouvanh R, Turner P, Dittrich S, Newton PN. 2015. A novel technique for detecting antibiotic-resistant typhoid from rapid diagnostic tests. *J Clin Microbiol* 53:1758–1760. doi:10.1128/JCM.00531-15.

Editor: S. S. Richter

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doi:10.1128/JCM.00531-15

elution method (12), which consistently yielded more DNA than the column-based commercial kit (Qiagen, Germany). Sample or conjugate sections at the final dilution of 1:100 yielded similarly large amounts of DNA (Fig. 1).

**Prospective evaluations.** For the prospective evaluations, blood cultures taken with written and/or verbal informed consent from patients of all ages at Mahosot Hospital, Vientiane, Laos (May to October 2013) and children <15 years old at Angkor Hospital for Children (AHC), Siem Reap, Cambodia (June to October 2013) were included. Ethical clearance was granted by the Oxford Tropical Research Ethics Committee, University of Oxford, United Kingdom, and local ethics committees.

Positive blood culture fluid containing GNRs was used to perform the *S. Typhi* RDT, and positive RDTs were individually stored in ziplock bags at 4°C until extraction and *gyrA* PCR-RFLP. The positive RDT samples from Cambodia were transported to the Mahosot laboratory at ambient temperature (maximum 36 h of travel) (17). Antimicrobial susceptibility testing of confirmed isolates was performed according to published guidelines, including disk-diffusion tests (Oxoid, United Kingdom) for ciprofloxacin (5 µg) and nalidixic acid (30 µg) and MIC testing via Etest (bioMérieux, France) for ciprofloxacin (18, 19). RDTs were performed on GNR-containing blood cultures from 172 patients (Laos, *n* = 136; Cambodia, *n* = 36). RDTs were positive for 38 patients (Laos, 28/136 [20.6%]; Cambodia, 10/36 [27.8%]), including 31 *S. Typhi* and 7 non-*S. Typhi* group D salmonellae. Available RDT samples (from total RDTs, 31/38 [81.6%]; *S. Typhi*, 25/31 [80.6%]; group D salmonellae, 6/7 [85.7%]) were tested under the optimized *gyrA* detection protocol. The median time from RDT to extraction was 42 days (range, 8 to 134 days). All *S. Typhi gyrA* results showed 100% agreement with phenotypic susceptibilities, including 7 FQR cases: 1/19 (5.3%) from Laos had a single mutation at codon 83; 4/6 (66.7%) from Cambodia had a single mutation at codon 83, and 2/6 (33.3%) had double mutations at codons 83 and 87 (Table 1).

The method, albeit with a small sample size, was therefore 100% sensitive and 100% specific in detecting *gyrA* mutations from RDT-derived DNA to predict FQR *S. Typhi*.

Limitations of the study are that the RDT also detects other group D salmonellae that share the O9 antigen, and the current PCR-RFLP only detects *gyrA* mutations. An additional molecular test to confirm *S. Typhi* (20) could be incorporated into a multiplex or nested PCR, which could be further developed to include primers for other resistance genes, should these become more prevalent, with the option of subsequent sequencing to provide more-detailed molecular epidemiology data on resistance and phylogeny.

After RDTs are conducted on *S. Typhi* isolates in the field, the small RDT package can be conveniently transported to a central reference laboratory, eliminating the risk of injuries from sharp medical instruments (needles) and blood-borne infections from transport of blood culture bottles. Although RDTs contain fewer viable organisms than bacterial colonies (21), inactivation techniques, used successfully for other bacterial pathogens (22), could further decrease risks and make our approach more applicable to field conditions. As standard susceptibility testing of blood culture isolates takes two additional days, if RDTs are shipped speedily, the PCR-RFLP can provide accelerated results to guide patient management within the same day. This could be particularly useful during outbreak investigations. However, as rapid transport

TABLE 1 Results of PCR and RFLP for *gyrA* mutations in *S. Typhi* and corresponding ciprofloxacin MIC results<sup>a</sup>

| Lab. ID no. | Country  | CIP MIC (µg/ml) | Interpretation (S/I/R) | PCR-RFLP result (WT or mutated codon[s]) |
|-------------|----------|-----------------|------------------------|--|
| 2956        | Laos     | 0.012           | S                      | WT                                       |
| 2957        | Laos     | 0.008           | S                      | WT                                       |
| 2965        | Laos     | 0.016           | S                      | WT                                       |
| 3009        | Laos     | 0.016           | S                      | WT                                       |
| 27416       | Laos     | 0.023           | S                      | WT                                       |
| 3624        | Laos     | 0.25            | I                      | 83                                       |
| 3770        | Laos     | 0.016           | S                      | WT                                       |
| 2986        | Laos     | 0.016           | S                      | WT                                       |
| 27545       | Laos     | 0.016           | S                      | WT                                       |
| 3823        | Laos     | 0.023           | S                      | WT                                       |
| 27986       | Laos     | 0.012           | S                      | WT                                       |
| 27987       | Laos     | 0.012           | S                      | WT                                       |
| 28247       | Laos     | 0.012           | S                      | WT                                       |
| 28257       | Laos     | 0.012           | S                      | WT                                       |
| 28317       | Laos     | 0.016           | S                      | WT                                       |
| 28373       | Laos     | 0.008           | S                      | WT                                       |
| 28403       | Laos     | 0.032           | S                      | WT                                       |
| 28412       | Laos     | 0.008           | S                      | WT                                       |
| 3862        | Laos     | 0.023           | S                      | WT                                       |
| 3286        | Cambodia | 0.5             | I                      | 83, 87                                   |
| 3472        | Cambodia | 0.5             | I                      | 83                                       |
| 3473        | Cambodia | 0.5             | I                      | 83                                       |
| 3489        | Cambodia | 0.5             | I                      | 83, 87                                   |
| 3543        | Cambodia | 0.5             | I                      | 83                                       |
| 4402        | Cambodia | 0.25            | I                      | 83                                       |

<sup>a</sup> Isolates were identified by using the API 20E test (Laos) or an in-house biochemical test set (Cambodia) and Salmonella Omni-O, O9, Vi, and Hd antisera (Pro-lab Diagnostics, United Kingdom) at both sites. Every PCR-RFLP investigation included the following controls to guide interpretation. S83F (*gyrA* codon 83): nalidixic acid (NA) MIC of 256 µg/ml, ofloxacin (OFX) MIC of 0.38 µg/ml, ciprofloxacin (CIP) MIC of 0.125 µg/ml. D87A (*gyrA* codon 87): NA MIC of 48 µg/ml, OFX MIC of 0.19 µg/ml, CIP MIC of 0.094 µg/ml. S80I (*gyrA* codons 83 and 87; *parC* codon 80): NA MIC of 256 µg/ml, OFX MIC of 16 µg/ml, CIP MIC of 8 µg/ml. S, sensitive; I, intermediate; R, resistant; WT, wild type.

depends on the local infrastructure, larger feasibility studies are needed to investigate the real-life impact of molecular resistance testing on patient or outbreak management. Even if patient management cannot be directly influenced due to transport constraints, batched results will provide valuable data for FQR surveillance to inform public health guidelines and treatment policies.

In conclusion, with the increasing global frequency of drug resistance, use of molecular markers from RDTs represents an innovative, accurate, and potentially cost-effective method for both individual patient diagnosis and public health surveillance in countries without accessible clinical microbiology laboratories.

#### ACKNOWLEDGMENTS

We are very grateful to Stephen Baker and Duy Pham Thanh of the Oxford University Clinical Research Unit, Ho Chi Minh City, Vietnam, for providing the *gyrA* mutant control strains of *S. Typhi* and to the directors and staff of Mahosot Hospital and Angkor Hospital for Children for their assistance. We thank Standard Diagnostics for kindly donating the tests.

The study was funded by the Wellcome Trust of Great Britain and Public Health England.

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