PCR detection of *gyrA* markers of reduced susceptibility to fluoroquinolones in DNA extracted from *Salmonella* Typhi antigen RDTs.

wellcome trust

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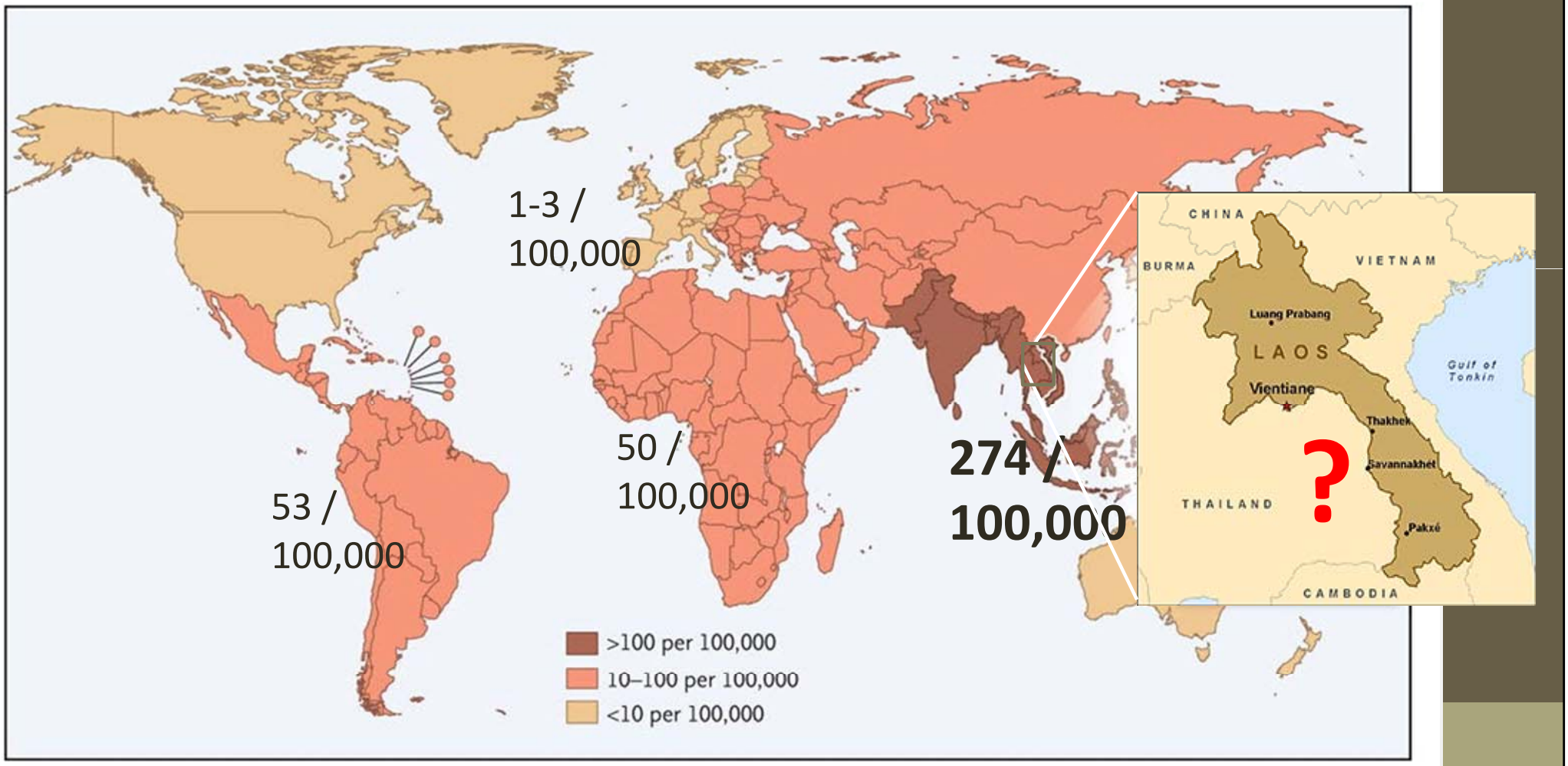
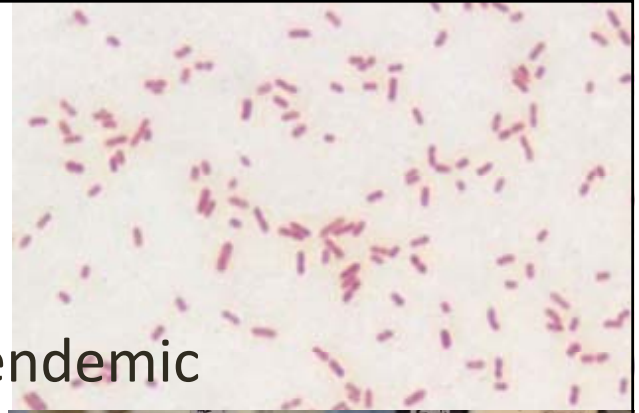
Global Burden of Typhoid[1,2]



1. DeRoeck et al. NEJM 2007; 357: 1069-1071
2. Crump et al. Bull WHO; 82(5): 346-353

Diagnosis & Treatment



- Undifferentiated febrile illness mimicking other endemic infections

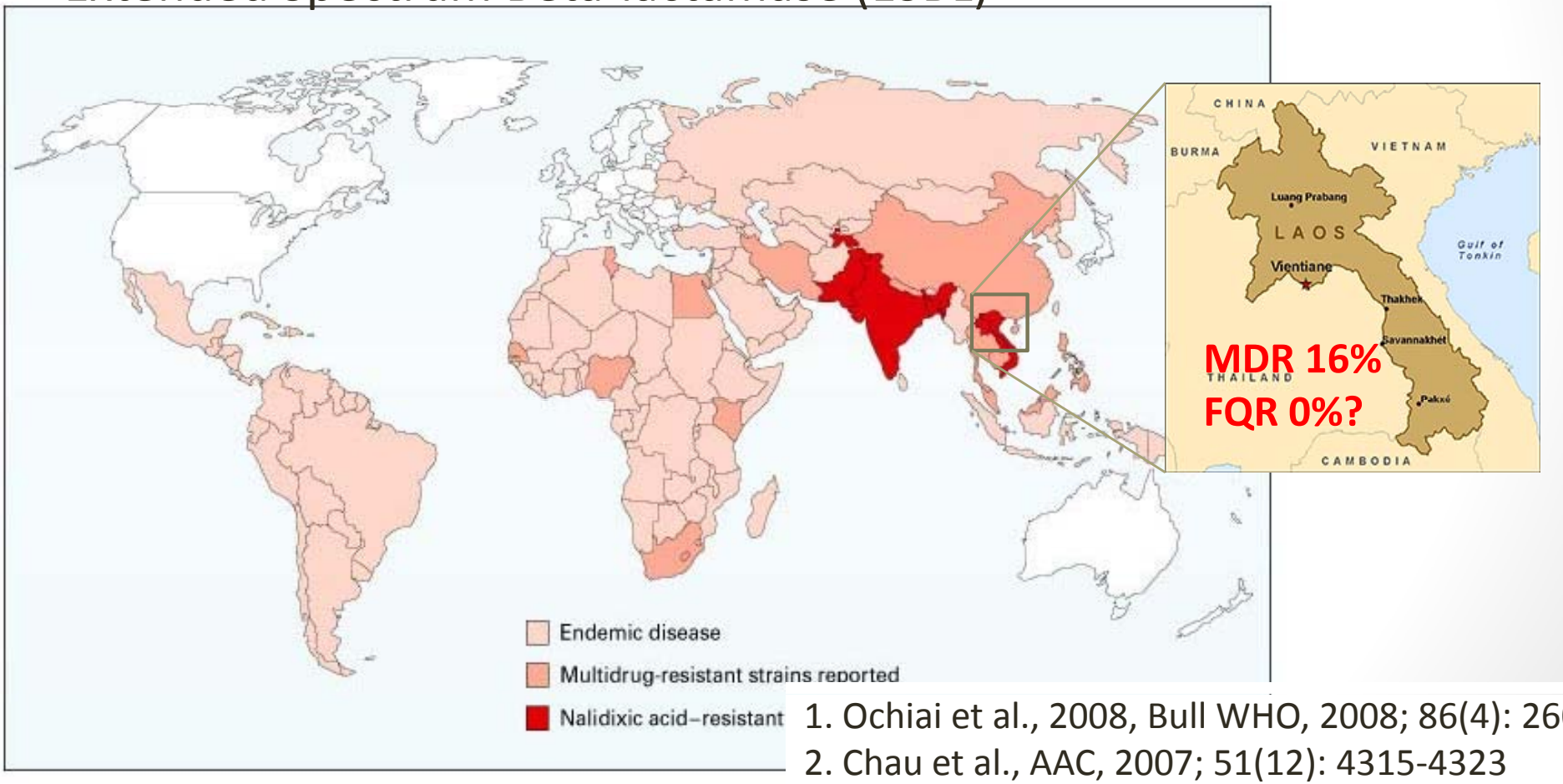
- Blood culture : 40-70% sensitive
- Widal test unreliable (especially in endemic areas)
- Antibody / Antigen detection kits in development

- Treatment must be based on *local susceptibility* data

- Complications in 10-15%, life-threatening in 3%

Antimicrobial Resistance SE Asia

- Multidrug resistance (Amoxicillin, Chloramphenicol, Co-Trimoxazole) up to 65%[1,2]
- Fluoroquinolone *reduced susceptibility* (FQRS): 40-60% [1,2]
- Extended Spectrum Beta-lactamase (ESBL)



1. Ochiai et al., 2008, Bull WHO, 2008; 86(4): 260–268
2. Chau et al., AAC, 2007; 51(12): 4315-4323
3. Parry et al., N Engl J Med 2002; 347(22):1770-82

FQ resistance mechanisms

- Point mutations in genes encoding DNA gyrase*:*
  gyrA (92%) Ser83→Phe, *gyrB* (1-11%) [1]
- Point mutations in genes encoding topoisomerase IV: *parC, parE*
- Plasmid-mediated: *qnr, aac*

- ***FQRS shown to correlate with adverse clinical outcomes even with high dose FQ treatment [2,3]:***

  - Delayed fever clearance

  - Clinical failure

  - Increased mortality

1. Humphries et al., CID, 2012; 15: 1107
2. Crump et al., AAC, 2008; 52: 1278-1284
3. Parry et al., PLoS NTD, 2011; 5(6): e1163

FQ resistance detection (CLSI)

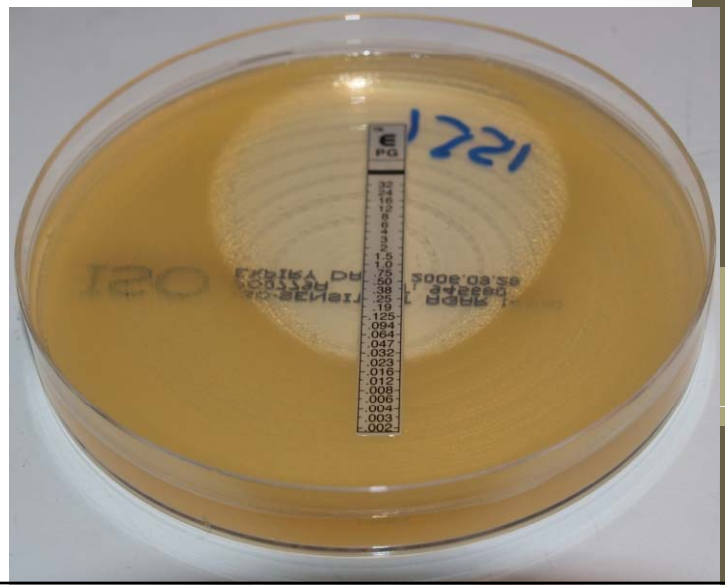
Disk diffusion:

- Nalidixic acid zone diameter ≤ 13mm = R
- Cipro zone diameter < 31 = reduced susceptibility

MIC E-test Cipro (revised 2012)

- 0.008 - 0.06 µg/ml = S
- 0.12 - 2 µg/ml = reduced susceptibility
- ≥ 4 µg/ml = R

Methods for typhoid diagnosis and susceptibility testing is challenging endemic areas



S. Typhi RDT

- Lateral flow RDT detecting O9 antigen
- Stored at room temp
- Cost $2
- Developed for detection of *S.* Typhi from stool
- Effective in detection of *S.* Typhi directly from positive blood cultures in 10-20 minutes

	No. of patients (n = 221) with diagnosis			
RDT result	*S.* Typhi	Non-*S.* Typhi	*Salmonella* group D	Non-*Salmonella* group D
Positive	29	4	31	2
Negative	1	187	3	185
Sensitivity (%) (95% CI)	96.7 (82.7–99.4)		91.2 (76.3–98.0)	
Specificity (%) (95% CI)	97.9 (94.7–99.4)		98.9 (96.2–99.8)	
Negative predictive value (%) (95% CI)	99.5 (97.1–99.9)		98.4 (95.4 –99.7)	
Positive predictive value (%) (95% CI)	87.9 (71.8 –96.5)		93.9 (79.7–99.1)	

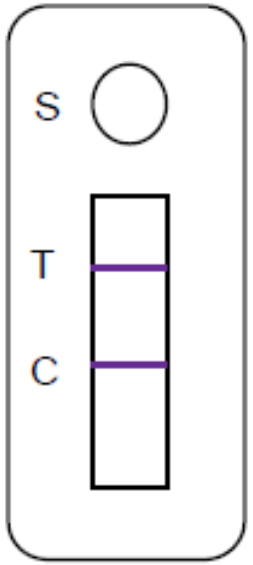
Castonguay-Vanier et al., J. Clin. Micro., 2012

Could the RDT platform be used to obtain DNA for molecular detection of FQRS?

What is the optimal extraction method?

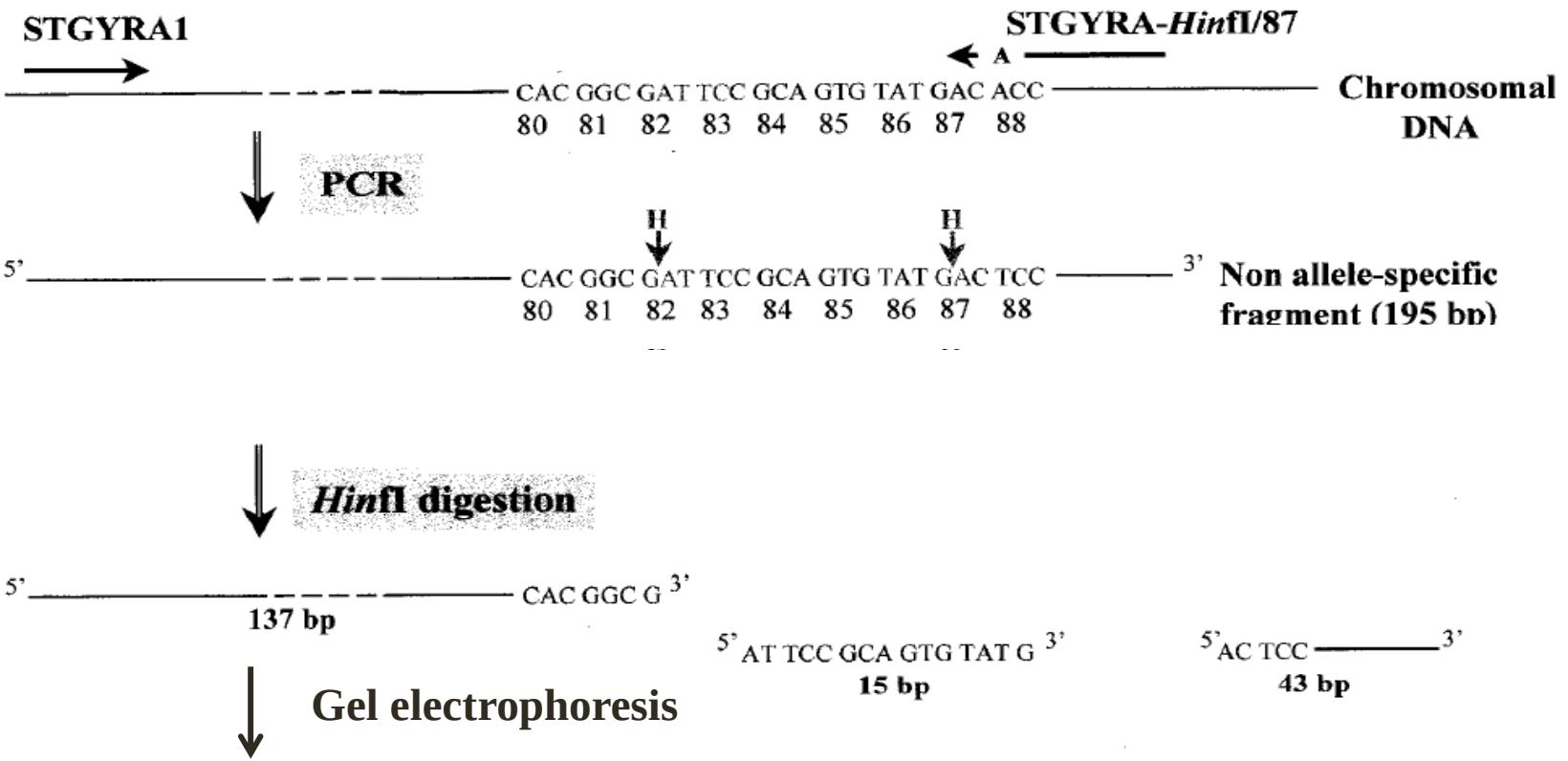
Which section of the RDT yields most DNA?

Can inhibitors from blood / culture broth be overcome?



Positive

Method – *gyrA* PCR & RFLP

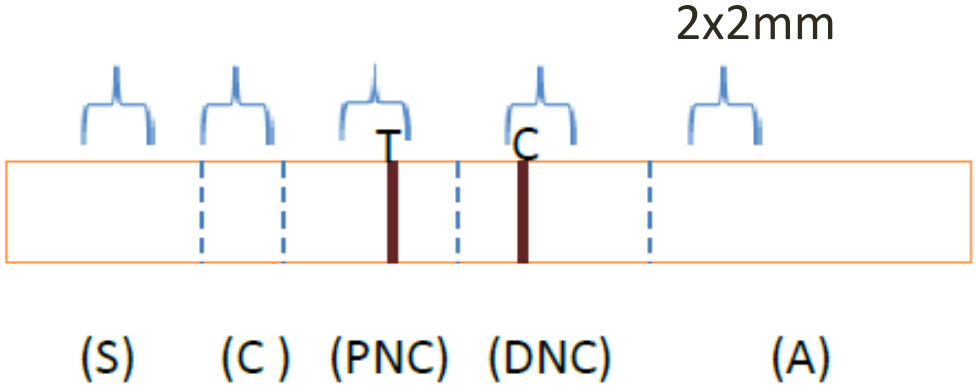


83WT	**87WT**	**=**	**137bp**	**+**	**15bp**	**+**	**43bp**
83M	**87WT**	**=**		**152bp**		**+**	**43bp**
83WT	**87M**	**=**	**137bp**	**+**		**58bp**	
83M	**87M**	**=**	**195bp (uncut)**				

Adapted from Giraud et al., AAC, 1999; 43(9): 2131-2137

Extraction from RDTs (seeded)

- 10 Day 7 negative blood cultures were inoculated with dilute (10^{-6}) suspensions of NCTC *S.* Typhi & incubated
- Once turbid, 2 drops of blood/broth mix used to perform 10 RDTs
- Positive RDTs stored in ziplock bags at 4°C until PCR performed
- RDTs cut into 5 sections; 2 x 2mm strips taken from each section



- 5 RDTs underwent boiling extraction (95°C 10 mins)
- 5 RDTs underwent Qiagen extraction

gyrA PCR & RFLP on RDT DNA extracts

- Method (Giraud et al., 1999)
- Combinations of dilutions and addition of bovine serum albumin were used to overcome inhibitors
- Intensity of bands produced by gel electrophoresis (for serial dilutions) used to semi-quantify amount of PCR product
- Comparison of 2 extraction methods
- Comparison of RDT sections
- Optimised protocol devised for use in prospective study

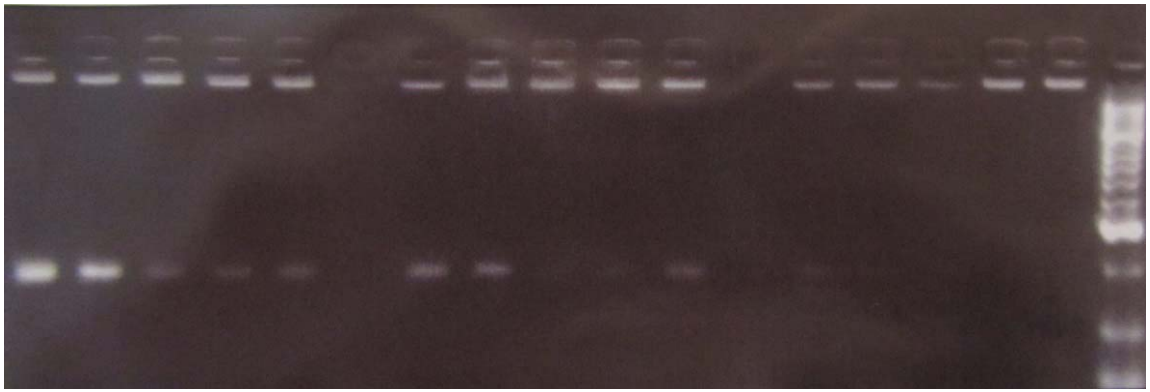
Prospective study: RDT, PCR & RFLP on *S.* Typhi blood cultures

- Mahosot Hospital, Vientiane, Lao PDR
- Angkor Hospital for Children, Siem Reap, Cambodia
- May – Oct 2013
- *S.* Typhi positive blood cultures
- Identification of *S.* Typhi : (RDT), biochemical, API, and serological methods
- Antibiotic susceptibility : DD (incl. Nalidixic acid, Ciprofloxacin)
  E-test (Ciprofloxacin, Ofloxacin, Azithromycin)

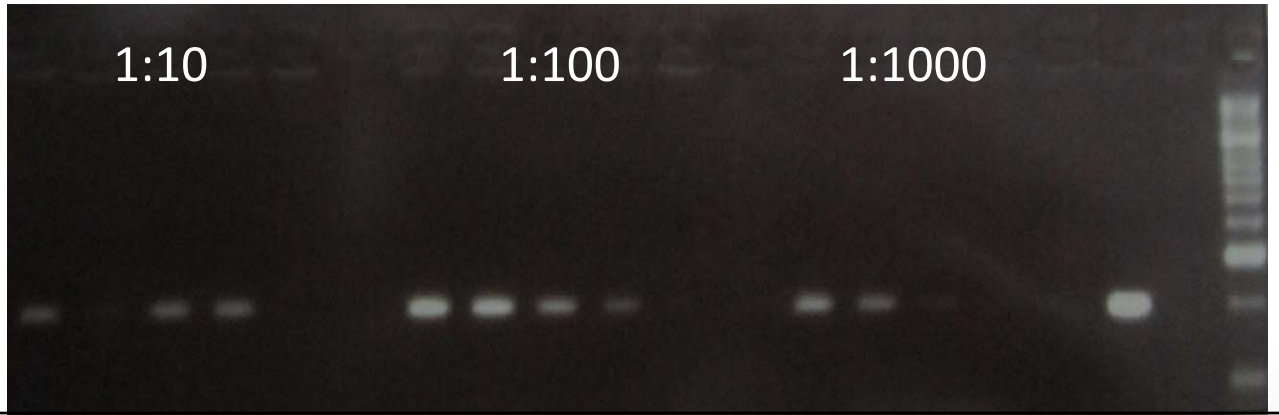
- Positive RDTs stored in ziplock bags at 4°C until PCR and RFLP according to optimised protocol

Results – RDT Extraction

- Boiling method yielded more DNA
- Sample and conjugate pads yielded most DNA:
  - Sample optimal for Qiagen extraction (4/5)
  - Conjugate optimal for boiling extraction (3/5)



S C P D A S C P D A S C P D A



Prospective study

Optimised protocol:

RDT: 2mm strip from each of sample and conjugate pads

Extraction: Boiling for 10 mins

DNA: 4 µl of 1:10 dilution

PCR: 2 µl BSA added, ↑MgCl2

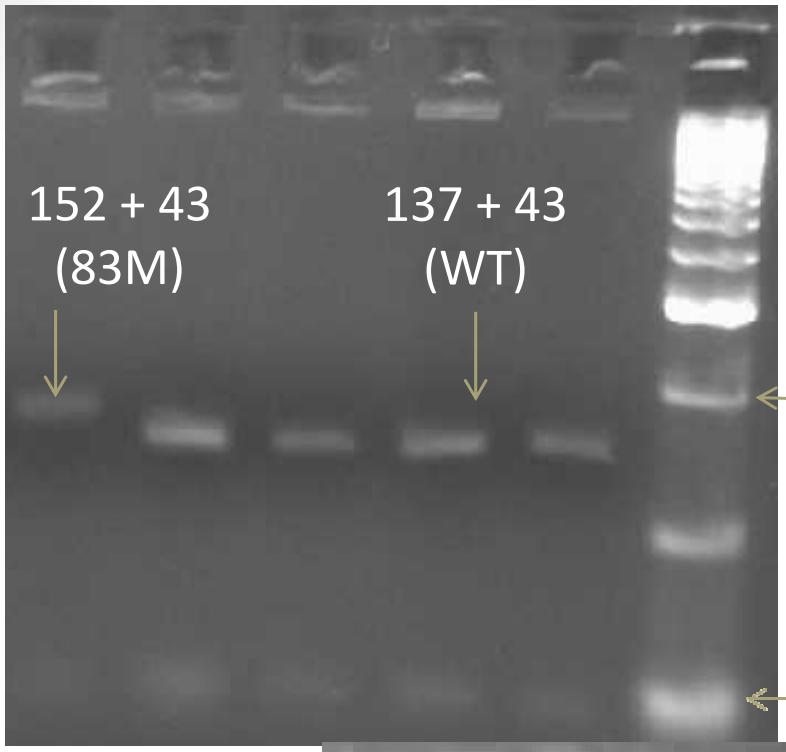
Samples:

26 *S*. Typhi positive blood cultures (Laos 19, Cambodia 7)

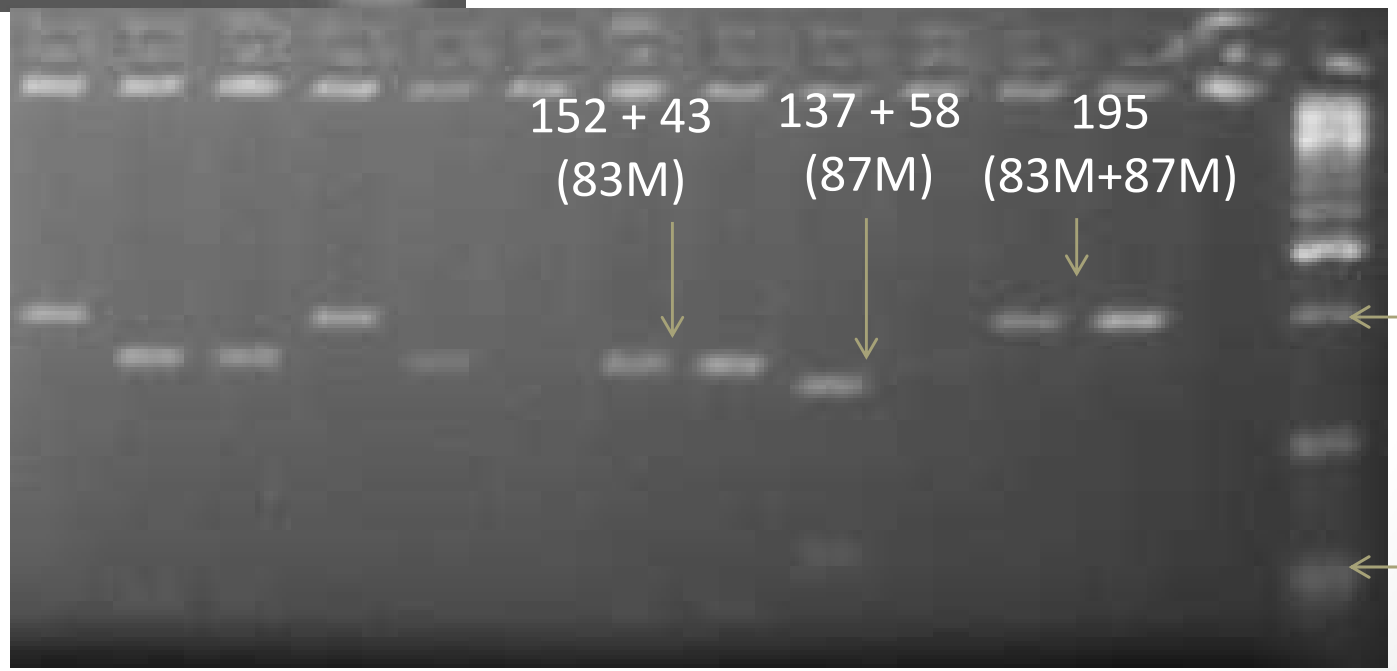
Antibiotic Susceptibility:

8 Nalidixic Acid R, Cipro I (Laos 1, Cambodia 7)

gyrA PCR & RFLP



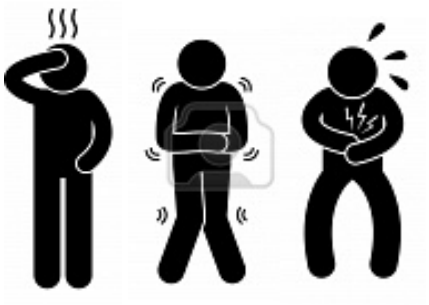
- Laos: 1/1 FQRS (83M)
- Cambodia: 6/7 FQRS
  (4 x 83M, 2 x 83 + 87M)
- 1 no PCR product
- 96.2% sens, 100% specific



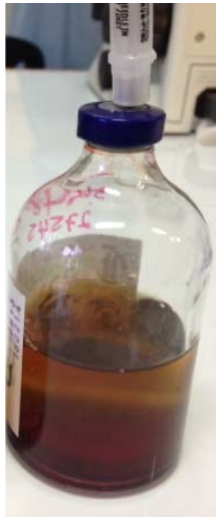
Conclusions

- *S*. Typhi RDT can be used as a source of DNA for molecular susceptibility testing
- Simple 10 mins boiling for optimal extraction
- Sample and conjugate pads yield most DNA
- Dilution and addition of BSA can overcome inhibition
- Could be used for “Same-day” susceptibility or surveillance of resistance purposes
- FQRS a significant problem in Cambodia

Potential application?

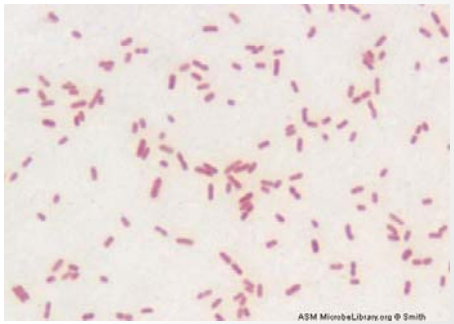


OUTBREAK

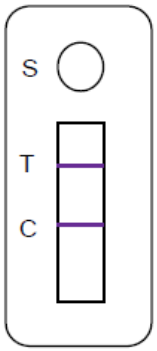


Incubate at ambient temp

Gram's stain



RDT



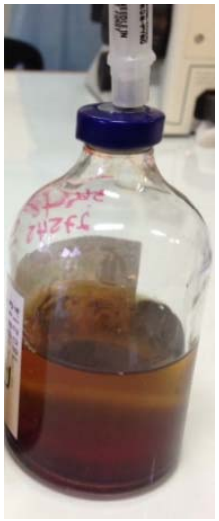
Positive

Rapid diagnosis and empiric treatment

Send to central lab for *gyrA* PCR to determine *optimal treatment*

Potential application?

INDIVIDUAL CASES



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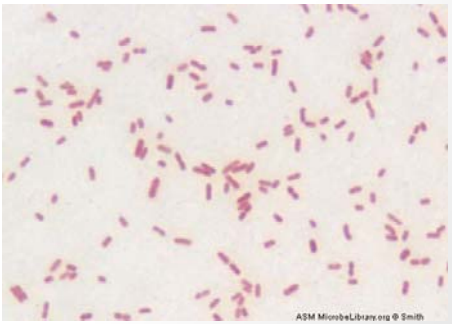
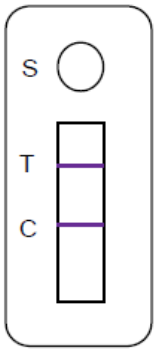


RDT



Positive

Rapid diagnosis and empiric treatment

Send batches for *gyrA* PCR for *surveillance* of resistance

Limitations

- Reliability on infrastructure
  - Transport of RDTs to the laboratory
  - Communication of results
- RDTs may still be infectious material, therefore, require safe handling during transport
- Conventional PCR and RFLP more labour intensive than real-time PCR

Thankyou

Prof. Paul Newton, Dr David Dance, Dr Rattanaphone Phetsouvanh, laboratory and medical staff, Mahosot Hospital, Government of Lao PDR, Wellcome Trust, and Health Protection Agency / Public Health England