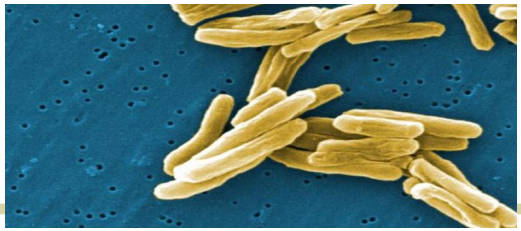
Association of *gyrA* mutation in *Mycobacterium tuberculosis* isolates with phenotypic ofloxacin resistance detected by resazurin microtiter assay



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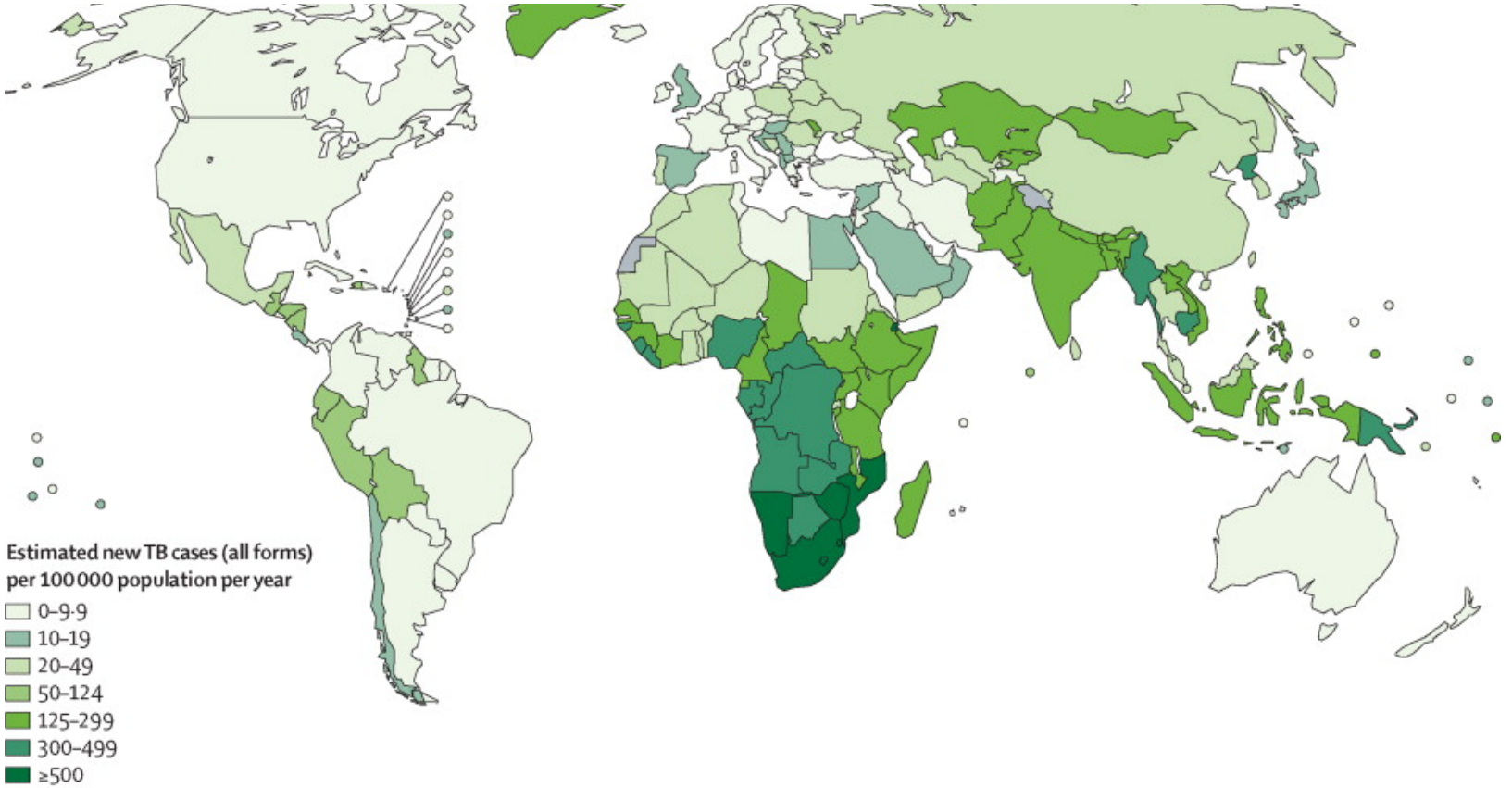
Tuberculosis

- Re-emerging disease of global concern
- Accounts for >3 million deaths every year

Global occurrence of Tuberculosis



(WHO, 2014)

WHO estimates

- >80 % of TB cases occur in the 22 high TB burden countries including India, Bangladesh, China, Russia and Pakistan

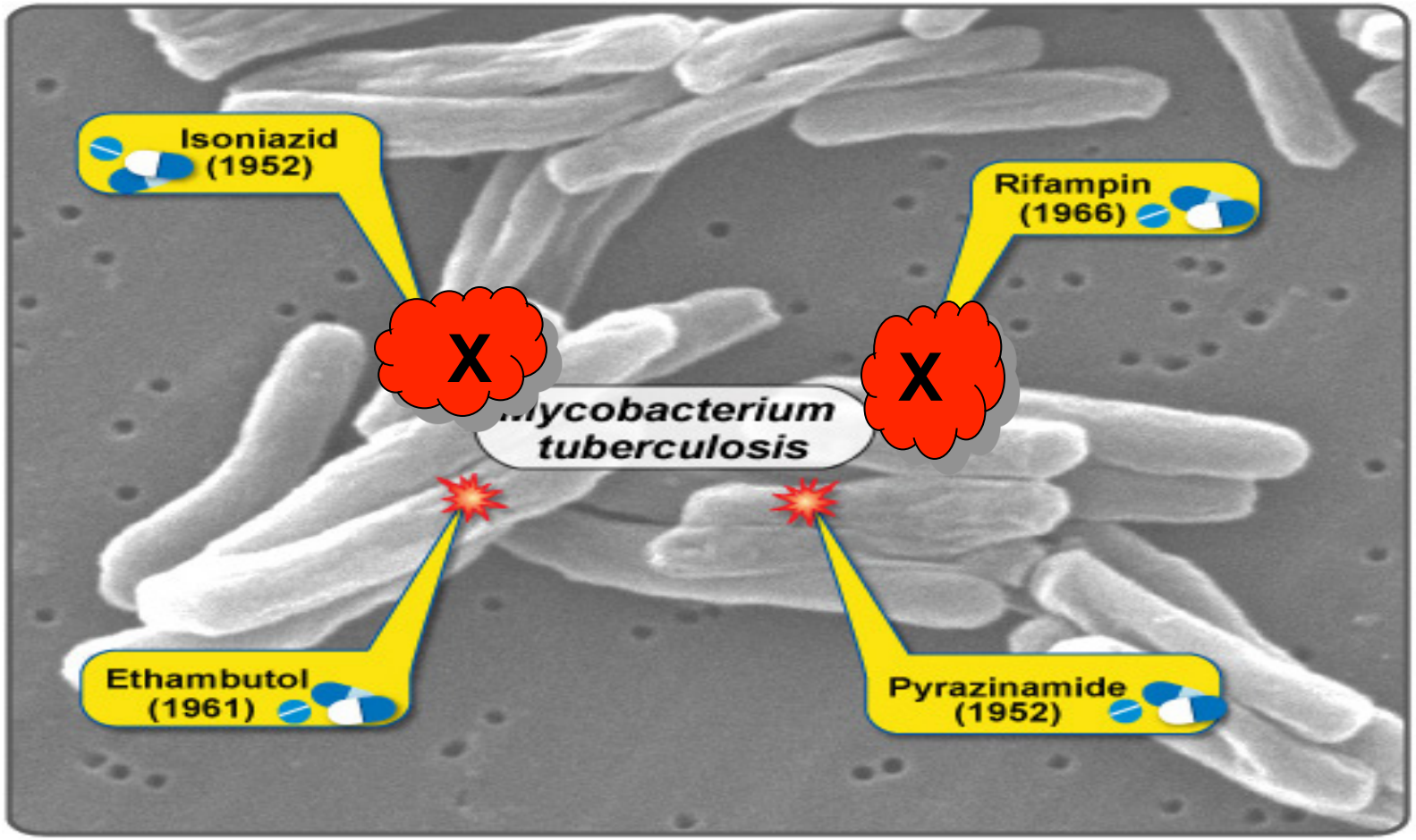
(WHO, 2008)

Tuberculosis

- Effective Therapy
  - Isoniazid
  - Rifampicin
  - Pyrazinamide
  - Ethambutol
  - Streptomycin
- BCG vaccination

- **Drug resistance**
- **Multiple Drug Therapy**

Multi-drug Resistance TB (MDR-TB)



WHO estimates of MDR-TB

- Global occurrence of MDR-TB among new cases is 3.5% and among previously treated TB cases is 20.5%
- 75% of MDR-TB cases occur in Asia
- The success rate for MDR-TB treatment globally is reported to be only 48%.
- 9% of patients with MDR-TB had extensively drug resistant TB (XDR-TB)

(WHO, 2014)

Fluoroquinolone (FQ)- A Key drug for treatment of MDR-TB

- FQs are broad-spectrum antibiotics that were shown to be useful in the treatment of MDR-TB.
- FQ act by inhibiting DNA gyrase, an enzyme encoded by *gyr*A and *gyr*B genes which required for bacterial DNA synthesis.
- MTB resistance to FQ is primarily associated with mutations in DNA gyrase, encoded by *gyr*A and *gyr*B genes.

Resistance in FQs

- 3-35% FQ resistance amongst the MDR-TB strains have been reported from across the globe

(WHO, 2014, Agarwal et al 2009)

Major cause of FQs resistance

- Use of FQs in infections other than TB
- Delayed diagnosis of FQ resistance

Objectives

This study aimed to:

- assess the performance of the ***Resazurin Microtiter assay (REMA)*** method in determining susceptibility to ofloxacin in *M. tuberculosis* clinical isolates.

- identify mutations in *gyrA* and *gyrB* genes of MTB isolates for OFX resistance using sequencing

- compare *gyrA* and *gyrB* gene mutations with MICs of OFX determined by the REMA assay.

METHODS:

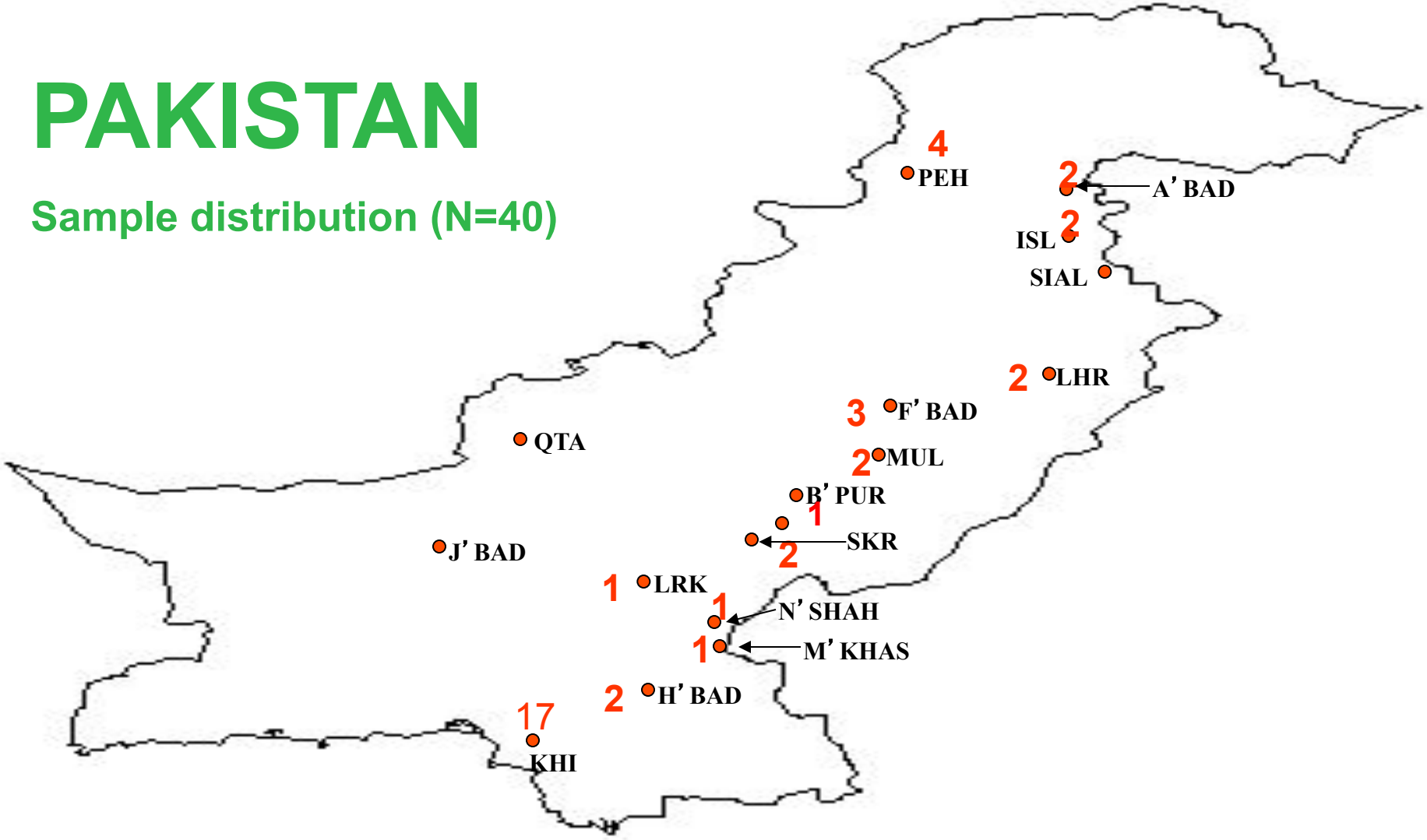
MTB *Strain Selection:*

30 OFX resistant and 10 susceptible MTB strains were selecte from MTB strains bank of Clinical Microbiology Laboratory of the Aga Khan University Hospital, collected during 2006-2009 from across the country.

M. tuberculosis strain selection

PAKISTAN

Sample distribution (N=40)



Overview of methodology

1. ***Culture of Selected MTB strains*** and Control strain H37Rv on 7H10 agar and LJ.

2. ***DNA extraction*** using cetyltrimethylamoniumbromide (CTAB) method

3. ***Polymerase Chain Reaction (PCR)*** of target *gyrA* and *gyrB* genes sequence for Fluoroquinolone (FQ) resistance.

4. ***Sequencing of gyrA and gyrB genes*** for detection of mutation or wild type gene in each of the selected FQ resistance as well as susceptible MTB strains.

5. ***Detection of* Ofloxacin *MIC by using Resazurin Microtiter assay (REMA)***

Resazurin Microtiter Assay (REMA)

1. Inoculum from the Control H37Rv and selected isolates were used to prepare 7H9-S broth adjusted to 1 McFarland standard. This was further diluted to 1:10 with 7H9-S broth.

2. 100µl of 7H9-S broth was dispensed in each well of sterile flat bottom 96-well plate.

3. Serial two fold dilutions of OFL (from 32 µg/ml working solution) drug was prepared directly in the plate

4. 100 µl inoculum was added to each well for positive control strain and negative control

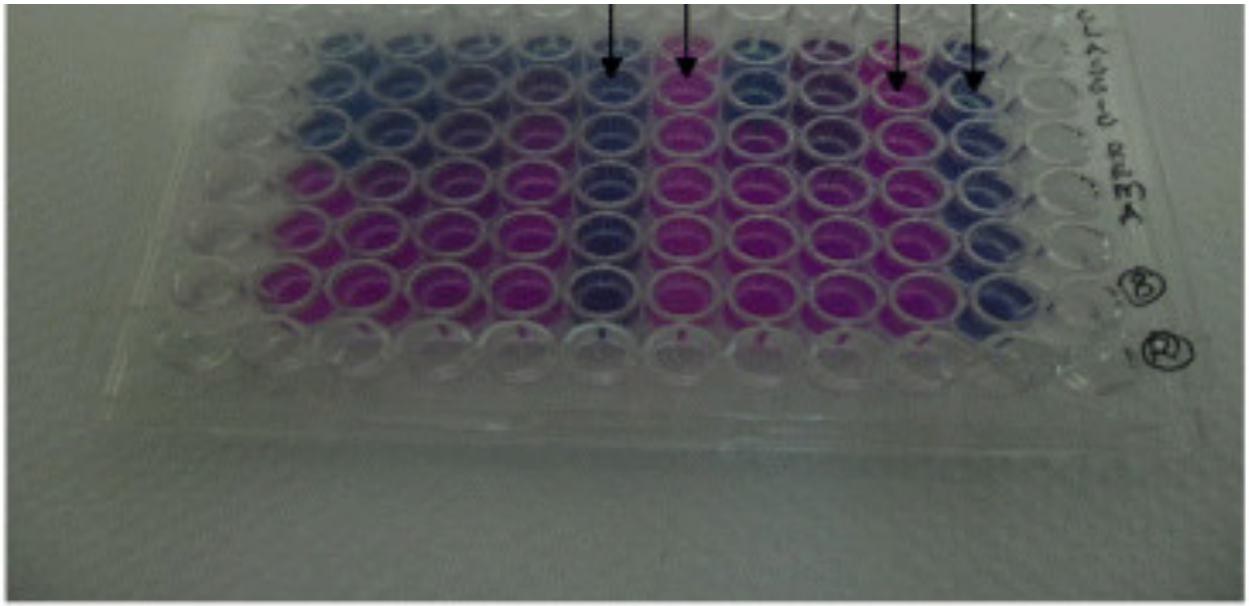
5. Sterile water was added to all perimeter wells to avoid evaporation.

6. Plates were covered and sealed in a plastic bag and were incubated for 7 days.

7. After 7 days of incubation 30 µl of .02% resazurin solution was added and plate was re-incubated overnight.

8. A change in color from blue to pink indicated the growth of bacteria, and the MIC was determined at the lowest concentration of drug that prevented this change in color.

REMA assay results on 96 well-plate



Resazurin indicator is blue when added to specimen wells. Upon oxidation by live organisms, it turns pink, indicating successful growth of microorganisms.

Mutations for OFX resistance

Gene	Sequence change	Amino acid change	No. of isolates	Drug Susceptibility of strain
gyrA	GCG→GTG + AGC→ACC	A90V + S95T	3	XDR (2), MDR+FQ^{r} (1)
	GCG→GTG	A90V	1	XDR
	TCG→CCG + AGC→ACC	S91P + S95T	1	XDR
	GCG→GTG + TCG→CCG + AGC→ACC	A90V + S91P + S95T	1	XDR (1)
	GAC→GGC + AGC→ACC	D94G + S95T	8	XDR (7), MDR+FQ^{r} (1)
	GAC→TAC + AGC→ACC	D94Y + S95T	3	XDR
	GCG→GTG + AGC→ACC + CTG→GGC	A90V + S95T + L96P	1	XDR
	GAC→AAC	D94N	1	XDR
	AGC→ACC	S95T	18^	XDR (7), MDR+FQ^{S} (5), non-MDR+FQ^{s} (6)
		No mutation	2	XDR (1), MDR+FQ^{r} (1)
gyrB		No mutation	39	XDR (25), non-MDR+FQ^{S} (6), MDR+FQ^{s} (5), MDR+FQ^{r} (3)

Prevalent Mutations for OFX resistance

- 64% (18/28) OFX resistant MTB isolates revealed mutations in the *gyrA* gene.
- Four out of 39 (14%) MTB isolates had a mutation at codon 90
- 11/39 (39%) had a mutation at codon 94.
- Two OFX resistant isolates did not any mutation in *gyrA* gene.
- None of the OFX resistant isolates exhibited mutation in *gyrB* gene.
- Mutations were not observed in *gyrA* and *gyrB* gene in all the OFX susceptible MTB isolates.

MIC detected for OFX among MTB isolates by REMA assay

MIC of OFX (µg/mL)	No. of strains	OFX susceptibility
8	13	r (13)
4	8	s(1)*, r (7)
2	0	
1	11	s(5), r (6)
0.5	7	s(6), r (1)^

Strains with Codon 94 mutation showed more high level (4-8µg/mL) OFX resistance as compared to other mutations

*strain found resistant to FQ on re-testing of culture susceptibility test
^strain found susceptible to FQ on re-testing of culture susceptibility test

Conclusion:

- Early detection of OFX resistance in MDR TB is very important for adequate therapy and control. Thus being simple, rapid and cost effective method, REMA method appears to be a good alternative method for use in resource-limited countries

- Although no significant association could not be established between type of mutated codon in *gyrA* gene and level of OFX resistance however isolates with 94 codon mutation were higher in number in high level of OFX resistance.

- Findings also suggest presence of alternate OFX resistance mechanisms among the strains without gyrA mutation such as efflux pump.

Future directions

- **Further investigation of alternate mechanisms of OFX resistance need to be explored.**

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Thank you !


QUESTIONS