

Study of Drug Resistance in Relapsed and New Cases of Leprosy in select rural and urban areas of Maharashtra using PCR and Mouse Footpad Assays

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Abstract- Background: At a stage where concerted effort is being made to control leprosy through multidrug therapy (MDT), reports of bacterial resistance to constituents of MDT demand vigilance.

Aim: To investigate bacterial resistance to Dapsone (DDS), Rifampicin (RIF) and Ofloxacin (OFX) in relapsed and new cases of leprosy in select areas in Maharashtra, India.

Subjects and Methods: Clinical samples from 76 relapsed, and 69 new cases were tested for drug resistance using PCR and nucleotide sequencing for mutations. Among these, 46/76 relapses and 24/69 new cases were also tested using the Mouse footpad (MFP) method. Results of two methods were further compared for their detection sensitivity.

Results: PCR products identified *M.leprae* specific fragments in 60/76 (79%) relapsed and 48/69 (70%) new cases. Mutation in the *rpoB* gene associated with resistance to RIF was not observed in any of the 94 amplicon positive clinical samples. Mutation in *folP1* (Pro55Arg) and *gyrA* (Gly89Cys) genes associated with resistance to DDS and OFX respectively, were observed in 2/53 (4.7%) and 1/48 (2%) of relapsed cases. A further 17.5% of all strains demonstrated several single nucleotide polymorphisms (SNPs) that so far have not been identified with drug resistance. In the MFP assay, 13/46 (28%) relapsed and 13/24 (54%) new cases gave positive yield. Resistance to DDS (0.01 gm%) was observed in 1/11 (9%) relapsed cases, this isolate also had *folP1* mutation and others were sensitive. No resistant phenotype was observed in any of the 13 new cases.

Conclusions: No secondary or primary resistance to RIF was observed, while resistance to DDS and OFX were observed in 4.7% and 2% respectively of relapsed cases. For quick screening PCR direct sequencing method of drug resistance testing is a robust and sensitive assay as compared to MFP. Further studies are required to establish the significance of SNPs observed outside the Drug Resistant Determining Region (DRDR) of the 3 genes in 17.5% of the strains tested.

Index Terms- Leprosy; Relapse; New cases; Drug Resistance; PCR; Mouse Foot-pad; Active screening

I. INTRODUCTION

Multidrug therapy (MDT) for leprosy was introduced by the WHO in 1982, in response to the threat to leprosy control posed by Dapsone resistance.¹ Since then MDT remains the cornerstone of National Leprosy Elimination Programs (NLEP).

Following sporadic reports of bacterial resistance to Rifampicin, being the most important component of current MDT regime, surveillance for drug resistance was deemed important and initiated in seventeen endemic countries, including India.^{2,3,4} Detection of resistance to Rifampicin (RIF), Dapsone (DDS) and Ofloxacin (OFX), using PCR and nucleotide sequencing to identify mutations was developed and commonly used.^{5,6} As a result, number of countries reporting relapsed cases, increased from 49 in 2008 to 122 in 2012. Of the 3427 globally reported relapsed cases in the year 2012, 1709 (50%) were from Brazil and 697 (20%) were from India.⁷ These were considered to underestimate the true situation.^{8,9}

Disease relapse in leprosy could result from re-infection, 'persisters', or poor efficacy of drugs in use. Relapse due to development of bacterial resistance to components of MDT would be detrimental to the control programme.

A multi-centric study was initiated in India under the aegis of and funding from the Indian Council of Medical Research (ICMR) with two main objectives:

(a) To determine the *extent* of relapse and other deleterious events such as reactions and nerve function impairments (NFI) in patients registered, treated and released from WHO-MDT at select primary health centres (PHCs) in Maharashtra; (b) To determine the *level* of drug resistance in the relapsed and new cases in the areas under study.

As a part of the three year study, 577 available and consenting patients, released from treatment (RFT) between April 2005 and March 2010 in 5 PHCs in Panvel block/ Raigad district (rural) viz. Gavan, Apta, Nere, Wavanje and Ajivali and 3 health posts in Mumbai (urban) viz. G-North, G-South and H Municipal Wards in Mumbai, were clinically examined in three annual visits. Those detected with deleterious events including relapse were further investigated.

The clinical profile of the entire cohort and data on deleterious events are part of a separate report.¹⁰ The present article reports the findings on bacterial resistance, using PCR nucleotide sequencing and Mouse Foot pad (MFP) methods, in relapsed and new cases detected in the study area. Also studied was the detection sensitivity of the two assay systems.

Study Subjects:

a) Sixty relapsed cases were detected during active screening of patients, over a period of 3 years (i.e. July 2011 to June 2014), in a cohort of 577 leprosy patients (350 Multibacillary [MB] and 277 Paucibacillary [PB], WHO operational classification), RFT between April 2005 and March 2010 were included in the study.

b) An additional, sixteen relapsed MB cases of Boderline lepromatous (BL) and Lepromatous leprosy (LL) type (Ridley-Jopling classification) detected in the same study area during active survey, although not a part of the above cohort, were also subjected to drug sensitivity testing.

Total number of clinical samples (biopsy or slit-skin smear) tested from relapsed cases were 76, of which 46 were tested using both MFP assay and PCR nucleotide sequencing method, and in 30 cases only PCR nucleotide sequencing was done.

Note: Relapse is defined as recurrence of active disease any time after the release from multidrug treatment.¹¹

2) New cases:

Sixty nine newly detected cases during June 2011 to July 2014, from the same study area were included, of which 24 were tested using both MFP assay and PCR nucleotide sequencing, and in 45 cases only PCR nucleotide sequencing was done.

Parameters studied were; a) Bacteriological index (BI) of clinical samples b) Detection sensitivity of PCR direct sequencing and MFP method in BI positive vs BI negative clinical samples c) Primary and secondary resistance in PCR and MFP methods and 4) Tally between genotypic and phenotypic resistance in the clinical samples.

II. METHODS

Bacteriological index (BI):

The clinical samples were checked for the presence of acid fast bacilli (AFB). Spot slides prepared from tissue homogenate or Slit Skin Smear (SSS) slides, stained by Ziehl- Neelsen (ZN) method, were examined under 100x oil immersion lens. The acid fast bacilli (AFB) were counted in 100 fields or more and BI determined.¹²

PCR and nucleotide sequencing:

PCR amplification of drug resistance determining region (DRDR) of *M. leprae* using *rpoB*, *folP1* and *gyrA* specific primers was performed. The PCR products were subjected to Sanger sequencing for DNA analysis using the same forward and reverse primers as in PCR.

DNA was extracted from the tissue homogenate using standard DNeasy Blood and Tissue kit as per manufacturer's protocol (Qiagen GmbH, Hilden, Germany). Briefly, the skin

biopsy was homogenised, subjected to proteinase K treatment and DNA isolated using a spin column. Polymerase Chain Reaction (PCR) was used to amplify *folP1*, *rpoB* and *gyrA* gene.^{5, 6} The PCR products were electrophoresed on 2% agarose gel and the amplicons were sequenced on automated Genetic Analyser ABI3100 (Life Technologies, Amsterdam). The PCR amplification and nucleotide sequencing primers were as follows:

folP1-Forward primer -5'-CTTGATCCTGACGATGCTGT-3'

folP1-Reverse primer -5'-CCACCAGACACATCGTTGAC-3'

rpoB-Forward primer -5'- GACGCTGATCAATATCCGT -3'

rpoB-Reverse primer -5'- ACGGTGTTTCGATGAACCCG -3'

gyrA -Forward primer -5'-GA TGGTCTCAAACCGGTACATC-3'

gyrA -Reverse primer -5'- TACCCGGCGAACCGAAATTG -3'

Sequencing and analysis: The sequencing of samples were done at SciGenome, Kochin. The sequences obtained were compared with corresponding sequences of the reference *M. leprae* TN strain using NCBI-BLAST tool. Based on the hot-spot codons and their concerned aminoacid changing pattern, mutations were identified. Any nucleotide variations in the samples observed were compared with the known hotspot sequence and subsequently mutation analysis was carried out.

Mouse Foot pad (MFP) assay:

Clinical samples from 46 relapsed cases and 24 new cases were tested by the MFP assay for growth and only BI positive clinical samples were tested for sensitivity to DDS and RIF (phenotypic) in the primary passage, using the standard technique. Briefly, the biopsy was homogenized with a glass homogenizer within 24 hours of collection of the samples. Spot slides were prepared, stained using ZN stain and bacillary count estimated. The bacilli were, adjusted to 1×10^4 AFB / 30 μ l of homogenate and injected into both hind foot-pads of twenty-four adult Swiss white (S/W) mice. They were further grouped into three groups of 8 mice each, to test for sensitivity to DDS (0.01 gm %), RIF (0.03 gm %) and the third group comprised of untreated controls. The drugs were given to the test mice from day zero (continuous method), through feed prepared on a day-to-day basis by wet mixing and blending at room temperature. They were maintained in 12 hr day/night cycle in an air conditioned room. Foot pads were harvested on 6th, 7th and 8th month post inoculation. At 12th month the remaining mice were harvested and bacterial yield/foot pad were calculated using the standard method. One or more per foot pad counts showing $\geq 1 \times 10^5$ *M. leprae* in the harvests carried out at 6 month or later was considered as positive yield.^{13, 14}

Ethical clearance:

The study followed International Ethical Guidelines for Biomedical Research involving human subjects (CIOMS/WHO, 1993). The study was approved and received ethical clearance from the Ethics Committee of Foundation for Medical Research (IEC No. FMR/IEC/LEP/02/2011). Written informed consent was obtained from individual subjects prior to inclusion in the study. Approval from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) for the use of animals in the study was obtained. The Foundation for

Medical Research is registered with CPCSEA (valid registration number is 424/PO/Re/S/01/CPCSEA).

III. STATISTICAL METHODS

Data entry was done in MS Excel. Quantitative analysis was performed using SPSS version 19.

IV. RESULTS

Determination of bacteriological index (BI) followed by PCR direct sequencing was used to test 145 clinical samples comprising 76 relapsed cases and 69 newly detected cases.

Bacteriological index (BI):

Of the 76 cases with histopathology and/or clinical evidence of relapse, 27(35.5%) were BI positive (2+ to 6+), BL to LL cases and 49 (64.5%) were BI negative, Boderline Tuberculoid (BT) to Boderline Boderline (BB) leprosy cases.

Of the 69 new (untreated) cases, 23 (33.3%) were BI positive and 46 (66.7%) were BI negative (Table 1).

PCR analysis:

PCR amplification and nucleotide sequencing of the PCR products identified *M. leprae* specific fragments in 27/27 (100%) BI positive and 33/49 (67%) BI negative clinical samples from relapsed cases. Among the new cases, 21/23 (91%) BI positive and 27/46 (59%) BI negative clinical samples showed *M. leprae* specific fragments (Table 1) (Figs 1, 2, 3). Overall, both relapse and new cases combined, detected *M. leprae* specific amplimers using PCR in 96% of BI positive (48/50) and 63% (60/95) of BI negative clinical samples.

Mutations in *folP1*, *gyrA* and *rpoB* genes:

***folP1* gene mutation associated with DDS resistance, detected at Codon 55 (CCC to CGC) with Pro substituted to Arg (Fig 4)** was observed in 2 relapsed cases (2/42= 4.7%); one of these was a MB case (WHO operational classification), BI negative i.e. no acid fast bacilli seen in the SSS as well as in the biopsy sample and the histopathology confirmed to BT leprosy. The MFP test was not done in this case (as it was a BI negative case). The second was an LL case with BI of 5+. This isolate showed a high degree resistance to DDS (i.e. at conc. of 0.01 gm%) in the MFP assay.

***gyrA* gene mutation at Codon 89 (GGC to TGC) with Gly substituted to Cys (Fig 5)** was observed in one BL relapse case with a BI of 2+ (1/52=2%). This mutation has been shown to be associated with OFX resistance.

***rpoB* gene mutation associated with RIF resistance was not observed** in the 94 amplimer-positive clinical samples, including 53 relapsed and 41 newly detected cases (Table 2).

Further, single nucleotide polymorphism/s (SNPs) at different codon positions were observed in 19/108 (17.5%) clinical samples including 13 relapsed cases and 8 new cases in either *rpoB*, or *gyrA* or *folP1* gene. All except one, were

observed *outside* the DRDR region. Nine of the SNPs were non-synonymous; all were outside the DRDR region. Ten were synonymous of which one was detected within the *rpoB* DRDR region (Table 3).

Mouse Footpad assay:

Of the 46 relapsed cases and 24 new cases tested in the MFP assay, positive yields in the absence of drug (control) were obtained in 13 (28%) relapsed cases and 13 (54%) new cases (Table 4).

Resistance to DDS at the conc. of 0.01 gm% was observed in 1/11 (9%) relapse case in the MFP test. This strain also demonstrated *folP1* mutation at Codon 55. Resistance to RIF was not observed in any of the samples. Resistance to OFX was not tested in the MFP assay. Thirteen new cases tested for DDS and RIF susceptibility proved sensitive in MFP assay as well as in genotypic assay.

Clinical details of patients detected with drug resistance:

Of the two patients detected with DDS mono-resistance, one was a 64 year old male, with nodular lesions and smear BI of 5+ at the time of relapse examination. The medical history revealed that he had received DDS monotherapy for more than 20 years prior to receiving 12 months of MB-MDT, and was not on any treatment for the past 25 years. No SSS test reports of the past were available.

The second case was a 44 year old male, with multiple new lesions and reactivation of old lesions, 42 months after RFT. Histopathology of lesion biopsy on relapse confirmed BT leprosy was BI negative. The third patient detected with *gyrA* gene mutation was a female aged 65 years, who had received 2 courses of WHO-MB-MDT in the year 1990 and 2010. She was a case of BL relapse, with average BI of 4+.

V. DISCUSSION

We studied bacterial resistance to DDS, RIF and OFX using genotypic and phenotypic assays in relapsed and new cases. Study subjects, 76 relapsed cases and 69 new cases are from select parts of western Maharashtra which remain leprosy endemic.¹⁵ Mutation in *folP1* gene at Codon 55 (CCC to CGC) with Pro substituted to Arg was observed in 2 relapsed cases; *gyrA* gene mutation at Codon 89 (GGC to TGC) with Gly substituted to Cys was observed in one BL relapsed case. These mutations are known to be associated with DDS and OFX resistance respectively.^{16,17} Overall, in the PCR assay secondary drug resistance to DDS was observed in (4.7%) and probable primary resistance to OFX in one case (2%). Mutation in the *rpoB* gene associated with RIF resistance was not observed in any, showing that bacterial resistance is not a major concern and is not the underlying cause of high number of relapse cases detected in the study area.

Different studies report varying levels of drug resistance, reflecting differences in local practice of drug usage or the type of clinical samples studied. A retrospective study carried in 941 Colombian patients with and without previous leprosy treatment, demonstrated 5.77% (22/381) of new cases and 3.04% (17/560) of previously treated cases with resistant genotypes. Proportion

showing resistance to RIF was highest 30/941 (3.17%) followed by OFX 11/941 (1.17%) and DDS 4/941 (0.43%).¹⁷

In a study carried out in Vietnam, 423 clinical samples were studied which included 83 new cases, 321 patients 'receiving treatment' and 19 relapsed cases. Mutation in *folP1* gene was detected in 10.2% (19/187) cases, while mutations in *rpoB* and *gyrA* genes were not observed in any. It was further noted that cases of *folP1* mutation was highest in relapsed cases (8/14=57%) as compared to new cases (2/33 = 6.1%), whereas in patients 'receiving treatment' it was 6.4% (9/140). Patients who had received DDS mono-therapy had high mutation rates (78%) as compared to MDT (33%).¹⁸ In a study conducted at The Leprosy Mission (TLM) Hospital in North India, primary resistance to RIF was observed in 7/16 (43%) clinical samples, highest reported so far.¹⁹ In contrast, neither secondary nor primary RIF resistance was observed by us in any of the clinical samples tested from parts of Western Maharashtra in the current study. Likewise the study conducted by a partner institution of the current ICMR study, namely Blue Peter Public Health and Research Centre, Hyderabad, did not observe any primary RIF resistance in clinical samples from two districts, Adilabad and Hyderabad in Andhra Pradesh.²⁰

SNPs outside the DRDR region:

Single nucleotide polymorphisms (SNP's), were observed in 19/108 (17.5%) clinical isolates and all, except one, were seen outside the DRDR region. In terms of frequency, SNPs outside the DRDR region of *rpoB* gene were more common (n=10), followed by *gyrA* (n=5) and *folP1* (n=4). Presence of such SNPs have been reported by several other groups but significance of the same is yet to be established^{17,18,19,21} The SNPs may indicate association with drug resistance or may represent single nucleotide polymorphism of no consequence to drug resistance.

Comparison of detection sensitivity of PCR vs MFP method:

Notably, *M.leprae* specific DNA was obtained from 48/50 (96%) of BI positive and 60/95 (63%) of BI negative relapsed and new cases combined. In contrast, 22/34 (65%) of BI positive and 4/36 (11%) of BI negative cases scored positive in the MFP test. Thus for quick screening, PCR direct sequencing method of drug resistance testing is a robust and sensitive assay as compared to MFP. One limitation of our study is the small number of clinical samples tested in the MFP assay, barring this there was good concordance between genotypic and phenotypic results.

A noteworthy finding in our study is that two of the lepromatous relapsed cases detected with resistant genotypes including one each *folp1* and *gyrA* had received prolonged and irregular anti-leprosy treatment indicating a relationship between high bacterial load and drug misuse. Another important finding is that systematic and active screening of the 577 strong study cohort resulted in the detection of **60 relapsed cases (10.3%) over a period of three years**. Apart from this, 16 lepromatous relapse cases that were not part of the cohort, were detected by chance during the active survey in the same area. In this group, all except four had nodular lesions and the duration between RFT and time of detection of relapse ranged from 9 years to 30 years. Two (2/16=12.5%) demonstrated mono-drug resistant mutant genotypes viz. *folp1* and *gyrA*. Point of concern is that, these

having been undetected for a long time, undoubtedly forming a source of infection in the community. The patients and the health visitors' failure to recognize the symptoms highlight lack of awareness. In Maharashtra, Raigad district, is one of the high endemic areas with several blocks recording high incidence of child cases.^{15,22} Periodic examination of all RFT cases along with contact examination may go a long way in bringing the transmission level down.

VI. CONCLUSIONS

In the areas covered, no secondary or primary resistance to RIF was observed, while resistance to DDS and OFX were observed in 4.7% and 2% respectively of relapsed cases. Thus drug resistance was not a major problem but undetected lepromatous relapse cases are of concern, could well be a major sources of infection in the community. For quick screening PCR direct sequencing method of drug resistance testing is a robust and sensitive assay as compared to MFP. Further studies are required to establish the significance of SNPs observed outside the DRDR region of the 3 genes in 17% of the strains tested.

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Table 1:

Bacteriological Index (BI) +ve and BI –ve clinical samples from relapsed and new cases in PCR and Mouse Foot Pad (MFP) assay

BI group	PCR	MFP
	Amplified / tested (%)	Growth / tested (%)
Relapsed cases		
BI +ve (n=27) (35.5%)	27/27 (100)	11/20 (55)

BI –ve (n=49) (64.5%)	33/49 (67)	2/26 (8)
Total (n= 76)	60/76 (79)	13/46 (28)
New cases		
BI +ve (n=23) (33.3%)	21/23 (91)	11/14 (79)
BI –ve (n=46) (66.7%)	27/46 (59)	2/10 (20)
Total (n= 69)	48/69 (70)	13/24 (54)

Table 2: PCR results of *rpoB*, *gyrA* and *folP1* genes in Relapsed and New cases

Category	<i>rpoB</i> (%)	<i>gyrA</i> (%)	<i>folP1</i> (%)
Relapsed Cases			
Amplified/Tested	53/76 (70)	52/76 (68)	42/76 (55)
Mutation detected /Amplified	0/53	1/52 (2)	2/42 (4.7)
New cases			
Amplified/Tested	41/69 (60)	42/69 (60)	35/69 (49)
Mutation detected /Amplified	0/41	0/42	0/35

Table 3: Single nucleotide Polymorphisms (SNPs) in relapsed and new cases

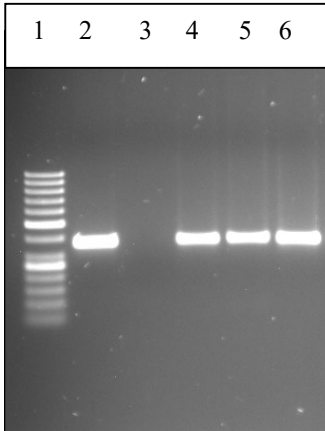
Gene	Codon	SNPs-synonymous (Syn) or Non-synonymous (Non-syn)	Change in Nucleotide	Amino Acid	No. of Relapsed cases (SNP %) n=60	No. of New cases (SNP %) n=48
<i>rpoB</i>						
	511	Non-syn	CTG →CCG	Leu →Pro	4 (6.6)	5 (10.4)
	514	Non-syn	TTC →CTC	Phe → Leu	1 (1.6)	0
	548	Non-syn	CGT →TGT	Arg → Cys	1 (1.6)	0
	507	Non-syn	GGC →AGC	Gly → Ser	0	1 (2)
	509	Syn	AGC →AGT	Ser → Ser	4 (6.6)	5 (10.4)
	516	Syn	GAT →GAC	Asp → Asp	1 (1.6)	0

	531	Syn	TCG→TCC	Ser → Ser	1 (1.6)	0
	533	Syn	CTG→CTC	Leu → Leu	1 (1.6)	0
	534	Syn	GGC→GGT	Gly → Gly	0	1 (2)
	503	Syn	AAG→TAG	Lys → Lys	0	1 (2)
<i>gyrA</i>						
	90	Non-syn	GAC →TAC	Asp → Tyr	1 (1.6)	0
	106	Non-syn	CTG →CGC	Leu → Pro	1 (1.6)	0
	77	Syn	TCA →TCG	Ser → Ser	2 (3.3)	0
	84	Syn	AAT →GAT	Ile → Ile	0	1 (2)
	111	Syn	GTT →GTC	Val →Val	1 (1.6)	0
<i>folP1</i>						
	66	Non-syn	CTC →CCC	Leu → Pro	0	1 (2)
	67	Non-syn	TCT →ACT	Ser → Thr	0	1 (2)
	86	Non-syn	GAT →ATA	Asp →Ile	1 (1.6)	0
	25	Syn	TAC →TAT	Tyr →Tyr	1 (1.6)	0

Table 4: Drug Susceptibility using MFP assay for relapsed and new cases expressed as number showing positive yield/ number tested

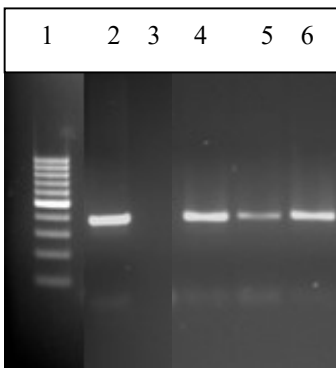
Type	Control group	DDS (0.01gm%)	RFP (0.03gm%)
	Growth /No. tested		
Relapse	13/46 (28%)	1/11 (9%)	0/11
New	13/24 (54%)	0/8	0/8

Fig. 1: PCR amplimers of *rpoB* gene (348bp)



Lane 1: 50bp Ladder, Lane 2: Positive Armadillo control, Lane 3: Negative control, Lane 4-6: Patient samples

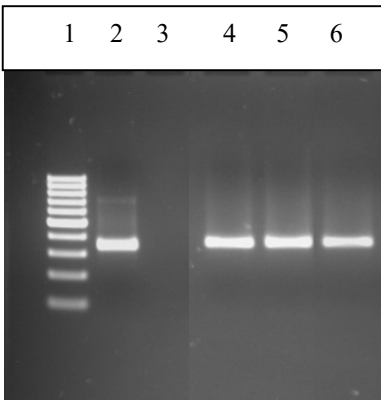
Fig. 2: PCR amplimers of *folP1* gene (388 bp)



Lane 1: 100bp Ladder, Lane 2: Positive Armadillo control, Lane 3: Negative control

Lane 4-6: Patient samples

Fig. 3: PCR amplimers of *gyrA* gene (342 bp)



Lane 1: 100bp Ladder, Lane 2: Positive Armadillo control, Lane 3: Negative control,

Lane 4-6: Patient samples

Fig. 4: Mutation detected in codon 55 with substitution (CCC to CGC) in *folP1* gene

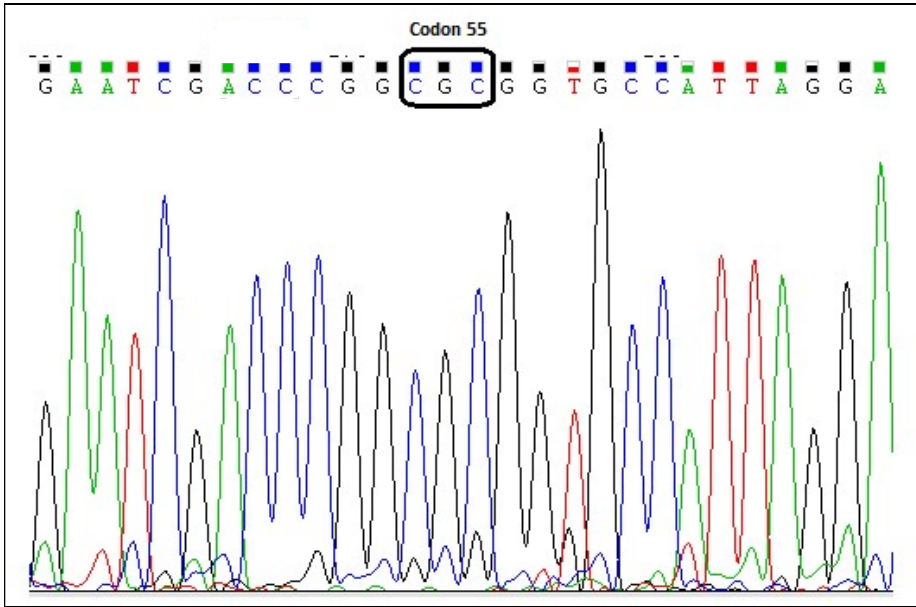


Fig. 5: Mutation detected in codon 89 with substitution (GGC to TGC) in *gyrA* gene.

