S6110 RFLP Fingerprinting Analysis of Drug Resistant Mycobacterium Tuberculosis Isolates in Agra Region

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Abstract

Tuberculosis (TB) is a disease with deep social and economical roots. Low-income people with large families, living in dense urban communities with deficient housing conditions, have a high probability of becoming infected, developing active disease, and dying from TB. Also, the risk of becoming infected and ill with TB is higher among people that live in congregated institutions, such as prisons, youth correctional facilities, nursing homes for elderly people, social shelters, day nurseries and schools; the same is valid for elderly people, diabetics and people living with Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome (HIV/AIDS), American Thoracic Society 2000, Castelo-Filho 2004, World Health Organization 2006). The most established typing method, IS*6110*- based restriction fragment length polymorphism (RFLP), exploits the variable presence and copy number (0-.25) of the IS*6110* transposable element among *M. tuberculosis* complex (MTC) strain.**IS*6110* RFLP** (Restriction Fragment Length Polymorphism) is current gold standard method for *M. tuberculosis* typing and is extensively used for epidemiology and population based studies **. RFLP** analysis was used to study the disease dynamics of most MDR-TB outbreaks. In RFLP analysis, where the distribution and number of copies of an insertion sequence, IS*6110*, in a chromosome is monitored, and this event varies among different TB strains.IS*6110*-RFLP banding patterns containing more than five bands which were considered related TB transmission. IS*6110* restriction fragment length polymorphism is a powerful tool to unravel the epidemiology of TB. Therefore, present study was aimed to investigate the initial pattern of transmission of MDR-TB within Agra district by using IS*6110* RFLP fingerprinting.

Keywords

TB, RFLP and Bacterium.

Introduction

TB is an infectious disease caused by the bacterium *Mycobacterium tuberculosis* and transmitted via aerosol droplets. The bacteria usually attack the lungs but can affect any part of the body. One third of the world's population carries the bacterium but does not have the disease (latent TB). Only a small proportion of these people will develop the disease and become infectious. In 2010, some 8.8 million people contracted TB causing 1.45 million deaths, including those among HIV-infected persons1. Progress has been made in tackling the epidemic. Globally, the number of new/ relapsing cases has fallen each year since 2006 and TB death rates have dropped by more than a third since 1990.

In India 2013, there were 8.8 million incident cases of TB and 1.1 million deaths from TB among HIV-negative people and an additional 0.35 million deaths from HIV-associated TB and total case notification rate per 1,00,000 population was 113 which is decreasing since last 5 years**(RNTCP 2014).** Updates to estimates of disease burden follow the completion of a series of consultation with 96 countries between 2009 and 2011, including China, India and 17 African countries in the past years, and much greater availability and use of direct measurement of TB mortality **(WHO 2013).** According to NIH 2015, in 2013 an estimated 9 million people became ill with TB, and 1.5 million people died **(NIH 2015).** In 2014, a total of 9,412 new tuberculosis (TB) cases were reported in the united states, with an incidence rate of 3* cases per 100,000 persons, a decrease of 2.2% from 2013,overall numbers of TB cases and rates continue to declines**.(CDC 2014)**

Tuberculosis is treatable and various anti-tuberculosis drugs that are effective against infection caused by *M. tuberculosis* are available. These include rifampicin, isoniazid, pyrazinamide, ethambutol, streptomycin, fluoroquinolones etc., these drugs are used in DOTS (Directly Observed Treatment short Courses). In recent years the treatment of TB threatened by increasing the number of patient with drug resistant TB especially to rifampicin and isoniazid **(Paramasivan et al 1993 and 1994).** Other drugs that may be useful, but are not on the WHO list of SLDs: Rifabutin, Macrolides: e.g. clarithromycin, linezolid, thioacetazone, thioridazine, arginine, vitamin D, R207910. A new drug has been discovered that may serve as a treatment against multi-drug resistant tuberculosis, a form of the disease that cannot be cured with conventional therapies. Anti-tuberculosis drugs can cure most people of MTB infection, improper use of antibiotics has led to new strains of the bacterium resistant to the two most powerful medications, isoniazid and rifampicin**. (Vasu *et al* 2015)**

World health organization (WHO) estimates that between 220,000 and 400,000 MDR-TB occurred among TB cases notified in the world in 2011. India has the second highest burden of MDR-TB cases following china. RNTCP started a WHO recommended DOTS PLUS programme in a phased manner for the systematic treatment of MDR-TB in 638 districts covering a population of 1089 million (92%) and were rapidly scaled up to include remaining districts by 24th March 2014 **(IJRHS, 2015).** Despite the declaration of tuberculosis as a global emergency by the World Health Organization **(WHO)** twelve years ago, the global problem of Tb has worsened due to increase drug resistance and the HIV. Sub Saharan Africa bears the brunt of HIV fuelled TB epidemic **(WHO 2013).** Globally current health projection show that more than 4 million cases of TB will occurs by 2020, of those cases, nearly 200, 00 will be MDR-TB **(Global health report 2014).** There are major advances in early diagnosis and molecular epidemiology of various diseases including TB. This technology allows for direct detection of PCR product during the exponential phase of the reaction

(Chauhan 2006). For epidemiological study of tuberculosis the discovery of variety of repetitive DNA elements in *M. tuberculosis* genome has led to development of RFLP using IS6110 probe for differentiation of isolates.

The most established typing method, IS*6110*- based restriction fragment length polymorphism (RFLP), exploits the variable presence and copy number (0-.25) of the IS*6110* transposable element among *M. tuberculosis* complex (MTC) strain.**IS*6110* RFLP** (Restriction Fragment Length Polymorphism) is current gold standard method for *M. tuberculosis* typing and is extensively used for epidemiology and population based studies **(Van Embden *et al* 1993). RFLP** analysis was used to study the disease dynamics of most MDR-TB outbreaks. In RFLP analysis, where the distribution and number of copies of an insertion sequence, IS*6110*, in a chromosome is monitored, and this event varies among different TB strains.IS*6110*-RFLP banding patterns containing more than five bands which were considered related TB transmission. **(WHO 2014).** IS*6110* restriction fragment length polymorphism is a powerful tool to unravel the epidemiology of TB. Therefore, present study was aimed to investigate the initial pattern of transmission of MDR-TB within Agra district by using IS*6110* RFLP fingerprinting method.

Materials and Method

The following materials and methods were used in this study-

Table 1:- Equipments used:

Sr. No.	Name of Equipment	Manufacture's Name
1	Autoclave	York Scientific India, Kartos International, NOIDA India.
2	BOD incubator	Widson Scientific works, New Delhi.
3	Bio-Safety Cabinet	Kartos International, NOIDA, India.
4	Electrophoresis apparatus	Banglore Genei, India.
5	Progranmmable thermal cycler	MJ Research, USA applied Biosystem USA.
6	Gel documentation	Bio Rad, USA.
7	Inspissator	Science house, Agra.
8	Oven	Brinkemnn Inatruments Ltd.
9	Deep freezer (-80 and -20C)	Heareus, germany, widson, India.
10	Electronic balance	Sartorius, Germany.
11	Centrifuge	Remi Udyog Ltd.India, sigma, U.S.A.
12	Water bath	Neolab, India.
13	Vacuum oven	Gallenkamp, U.S.A.

Table 2:- Enzymes:

1	Lysozyme	Sigma , U.S.A
2	Proteinase K	Banglore genei, india

4.1 Mycobacterium strains

A total of 22 isolates of *Mycobacterium tuberculosis* recovered from sputum samples of patients suspected for MDR-TB were taken from mycobacterial repository, , National JALMA Institute for Leprosy, Agra, for this study. These isolates were sub-culture further to maintain original isolates in repository. All the isolates were subjected to DNA isolation. Out of 22 isolates, the intact DNA was obtained from 14 isolates. Therefore, IS6110 RFLP was performed for 14 isolates.

Preparation of Reagents

Prepration of reagents used in biochemical tests:

I. Catalase test*:*

3.4 gm Phosphate buffer powder was mixed in 100 ml distilled water & dispensed as aliquots of 1 ml in test tubes & autoclaved.

Reagents: mixture of 1 ml tween 80 + 9ml of distilled water was autoclaved and the 10ml hydrogen peroxide was added.

II. Nitrate reductase test*:*

$NaNO_3$ – 0.085gm

KH_2PO_4 – 0.177gm

Na_2HPO_4 – 0.19gm

The reagents were dissolved in 100 ml of distilled water & dispensed as aliquots of 1ml test tubes & autoclaved.

Reagents

1) 100 ml of HCl & 10 ml distilled water

2) 0.2 gm Sulfanilamide was dissolved in 100ml of Distilled water

3) 0.1 gm N-naphthyl ethylene diamine dihydrochloride was dissolved in 100 ml of distilled water.

Preparation of reagents for DNA isolation

i. Lysozyme:

Concentration: 20 mg lysozyme was dissolved in 1ml distilled water. The solution was stored in small aliquots at -20 ºC for no longer than one year.

Function: it breaks carbohydrate linkage with protein or the peptidoglycan linkage in cell wall and cause lysis of cell envelope.

ii. 10% SDS*:*

Concentration: 10 gm SDS was dissolved in 100 ml of distilled water by heating at 65º C for 20 min.

Function: SDS is found to denature proteins & it gives the protein a negative charge. It breaks cell wall proteins.

iii. Proteinase K*:*

Concentration: 10 mg proteinase K was dissolved in 1 ml of distilled water. The solution was stored in small aliquots at -20º C for no longer than one year.

Function: it digest the highly resistant protein as keratin.

iv. **5M NaCl:**

Concentration: 29.2 gm of NaCl was dissolved in 100 ml distilled water and autoclaved.

Function: It provides the required molarity to precipitate DNA.

v. **10% CTAB (N-acetyl N,N,N trimethyl ammonium bromide)/ 4% NaCl solution:**

Concentration: 4.1gm NaCl was dissolved in 80ml distilled water by stirring. During stirring 10gm CTAB was added. If necessary, solution was heated at 65º C. the volume was raised to 100ml with distilled water.

Function: it removes carbohydrates and polysaccharide from cell.

vi. **Chloroform/isoamyl alcohol (24:1):**

Concentration: chloroform and isoamyl was mixed to prepare solution freshly in 24:1 ratio.

Function: chloroform helps in deproteinization and isoamyl alcohol is used as an antifoaming agent.

vii. **Isopropanol:**

Concentration: 0.784gm/ml isopropanol.

Function: it selectively precipitates DNA.

viii. **70% ethanol:**

Concentration: 70% of absolute alcohol.

Function: ethanol is used to wash the DNA pellet.

ix. **T.E. buffer (pH 7):**

Concentration: 5ml of 1 M tris HCl (pH 7.5) and 2ml of 0.5 M EDTA (pH 8) added & volume was raised up to 500ml with distilled water. It was autoclaved and stored at room temperature. Working conc. = 1x

Function: it is used to dissolve the DNA.

Other Reagents

1) **0.5M EDTA (pH 8.0):**

18.61 gm of EDTA was dissolved in 80ml of distilled water, pH was adjusted to 8.0 and volume was raised up to 100ml. solution was autoclaved and stored at room temperature.

2) **1M tris HCl:**

78.80 gm tris was dissolved in 300 ml of distilled water & pH was adjusted to 7.5. The Volume was made up to 500 ml with distilled water. Solution was autoclaved & stored at room temperature.

Reagents Used for Agarose Gel Electrophoresis:

1) **10 X Tris borate EDTA (TBE) buffer (pH 8.0):**

i) Tris base (0.089M) - 108 gm

ii) Boric acid (0.089M) - 55 gm

iii) 0.5M EDTA - 40 ml

The reagents were dissolved in de-ionized water, pH of the solution adjusted to 8 and the volume was raised up to 1000 ml with de-ionized water. The solution was autoclaved and kept at room temperature.

2) Gel loading dye (6x):

i) Bromophenol blue - 0.25gm
ii) Xylene cyanol - 0.25gm
iii) Ficoll type 400 - 15gm

The reagents were dissolved in de-ionized water and made the volume up to 100 ml with de-ionized water. The dye was kept at 4 ºC.

3) Ethedium bromide (stock solution) :

0.05 gm of ethidium bromide was dissolved in de-ionized water to make up the volume to 5 ml.

4) Agarose gel:

1gm of agarose was weighed and 90 ml of distill water was added in 10 ml of 10x TBE buffer, it was boiled at boiling temperature till the gel becomes transparent. The solution was allowed to cool at 45ºC then 5µl ethidium bromide was added. It was poured into a gel mould fitted with a comb and allowed to solidify so as to form a gel, comb was removed carefully.

Reagents for IS6110 – RFLP Analysis

i) 7µl of DNA
ii) 3µl of 10x restriction buffer.
iii) 2µl of PuyII restriction endonuclease enzyme (10 units / reaction).
iv) Add distilled water to make up the final volume upto 30µl.

Reagents Used in Southern Transfer

(a) Acidic buffer: 12.5 ml of HCl (conc.) was mixed with 487.5ml-distilled water.

(b) Alkaline buffer: 10gm NaOH (0.5M) & 43.87gm NaCl were dissolved in 500ml of distilled water.

(c) Neutralization buffer: 73.05gm NaCl (5M) & 250ml of 1M Tris HCl (0.05M, pH 7.0) were dissolved water & final volume was made up to 500ml.

(d) 20x SSC buffer (Saline Sodium Citrate buffer): 3M NaCl (175.3) & 0.3M Sodium Citrate (88.2gm) were dissolved in 1000ml of distilled water, pH was adjusted to 7.0. The buffer was autoclaved and stored at room temperature for no longer than 1 year.

(e) 6x SSC Buffer: 30ml 0f 20x SSC was mixed with 70 ml of distilled water, prepared freshly.

Reagents Used in Prehybridization

(i) Hybridization fluid:

0.5gm Ficoll 400
0.5gm Bovine serum albumin (BSA)
0.5gm Polyvinyl Pyrrilidone (PVP)
2.5gm Sodium Dodecyl Sulphate (SDS)
125ml 20x SSC
25ml 1M tris HCl (pH 7.5)
1ml 0.5M EDTA
250ml De-ionized formamide

The above reagents were mixed &shaken to dissolve &final volume was made upto 500ml with deionized water. It was prepared fresh.

(ii) tRNA solution: 10mg of tRNA was dissolved in 1ml of autoclaved distilled water.

Reagents used in hybridization

1. hybridization fluid
2. probe

The probe was prepared & labelled by using the reagent present in the kit (Roche diagnostics, Germany). Probe was prepared by PCR ampilification of 245bp of IS6110 fragments of H37Rv (Van Soolingen et al 1991) with primer having sequence:

Primer 1

INS-5'- CGT GAG GGC ATC GAG GTG GC-3'

Primer 2

INS-5'- GCG TAG GCG TCG GTG ACAAA-3'

The DIG random primer DNA labelling kit (Roche diagnostics catalogue no. 1093657) contains all the components need to make random labelled probe with DIG.

Table 3: - reagents present in the DIG random primed DNA labelling kit (Roche diagnostic catalogue no. 1093657):

S. No.	Reagents	Description
1.	Hexanucleotide	290 units1 ml mixture (10x) random hexanucleotides, 500mM $MgCl_2$, 1mMDTE (dithiothreitol), 2mg BSA (pH7.2)
2.	dNTP labelling	1mMdATP, 1mM dCTP mixture, 1mMdGTP,0.65mMdTTP,0.35M DIG-11-dUTP (pH7.5)
3.	Klenow enzyme	2 units/ ml DNA polymerase (Klenow enzyme) labelling grade, from Ecoli.

Reagents Used in Post Hybridization Washing

i) 4x SSC buffer: 20 ml 20x SSC buffer was mixed with 80 ml distilled water &0.1 gm SDS.

ii) 2x SSC buffer: 10ml of 20x SSC buffer was mixed with 90ml distilled water and 0.1gm SDS.

iii) 1x SSC buffer: 5ml 20x SSC buffer was mixed with 95ml distilled water and 0.1gm SDS.

iv) 0.5x SSC buffer: 2.5 ml 20x SSC buffer was mixed with 97.5ml distilled water and 0.1gm SDS.

v) DIG Buffer No. 1: 8.775 gm NaCl (0.15M) and 11.607 gm of maleic acid (0.1M) were dissolved in 1 litre distilled water, pH was adjusted to 7.5 and then autoclaved.

vi) DIG Buffer No. 2(stock conc. 10 %): 10 gm of DIG blocking powder and nucleic acid detection were dissolved in 100 ml of DIG Buffer No.1. Working solution 1%: 10ml stock solution buffer no 2 was mixed with 90ml of DIG Buffer No.1.

vii) DIG Buffer No. 3: 10ml of 1M Tris HCl (pH 9.5), 2 ml of 5M NaCl and 5ml of 1M $MgCl_2$ were mixed with 83 ml of distilled water.

Methods

(a) Preparation of L-J media:

(i) Preparation of salt solution:

600 ml of mineral salt solution was prepared by adding:-

KH_2PO_4 (Monopotassium phosphate)	:	2.4 gm.
$Mg_3(C_6H_5O_7)_2.14H_2O$ (Magnesium citrate)	:	0.60 gm.
$MgSO_4.7H_2O$ (Magnesium Sulphate)	:	0.24 gm.
$C_4H_8N_2O_3.H_2O$ (Asparagine)	:	3.6 gm.
Glycerol + distilled water	:	12 ml

The solution was then autoclaved.

(ii) Preparation of homogenized Eggs:

- 20-25 fresh hens' eggs were properly washed under tap water.
- Sterilized with 70% ethanol for 15 minutes and then were broken.
- Homogenized in a sterilized conical flask with the help of glass beads.
- The homogenized eggs were then filtered into a sterile graduated cylinder.

i) Preparation of 2% malachite green solution:

Malachite green	:	2gm
Distilled water	:	100ml

Mixed well and incubate at 37ºC for 1-2 hours.

ii) Preparation of complete media:

Mineral salt solution	=	600ml
Malachite green solution	=	20ml
Homogenized egg	=	1000ml

➢ To 1000ml of homogenized eggs 600 ml of mineral salt solution and 20 ml of 2% malachite green solution was added and mixed well

➢ 8-10 ml of medium was poured into sterile Mac Cartney bottles and tightly capped.

➢ The slants of L-J media were made by incubating in an inspissator at 80-85ºC for 1 hour a day for three consecutive days.

DNA isolation

Isolation of DNA was done by a method adopted by Van Soolingen et al (1991). The two loopful growth of biochemically identified M.tuberculosis was taken from L-J medium in different eppendrof tubes each containing 400µl of T.E Buffer.

Procedure

➢ **Freezing and thawing treatment:**

Freezing and thawing treatment was given to each eppendrof tube. These tubes were boiled for 5-10 minutes and then snap chilled in ice. This makes the cell wall ruptures.

➢ **Lysozyme addition:**

40µl of lysozyme was added in each tube.

Tubes were incubated at 37ºC for 2 hours in shaking water bath.

Function: lysozyme breaks the 1-4 linkage of glycosides bonds in the cell wall of bacteria.

➢ **SDS and Proteinase K addition:**

After incubation 56µl of 10% SDS and 5µl of proteinase K was added in each tube then tubes were vortexed.

Tubes were incubated at 65ºC for 30 minutes in shaking water bath.

- **NaCl and CTAB-NaCl addition:**

80µl of 5 M NaCl & 64µl of CTAB-NaCl were added in each tube and vortexed and then incubated at 65ºC for 30 minutes.

- **Chloroform and isoamyl alcohol addition:**

Till this step the volume of reaction in each eppendrof is 645µl.

Equal volume of (645µl) of chloroform and isoamyl alcohol mixture (24:1 ratio) was added in each tube.

Tubes were centrifuged at 10,000 rpm for 5 minutes.

After centrifugation two distinct layers were observed.

Proteins and carbohydrate settles down and supernatant of each tube was transferred in another respective tube.

- **Addition of isopropanol:**

0.6 volume of isopropanol i.e., for 300µl (volume of supernatant) 180µl was added in each tube.

Tubes were incubated at -20ºC for 1 hour for DNA precipitation.

Tubes were centrifuged for 15minutes at 10,000 rpm.

After centrifugation supernatant of each tube was discarded & DNA remains in the test tube as pellet.

- **Chilled ethanol addition:**

In each tube 150µl of chilled 70% ethanol was added side by side for washing of DNA.

Tubes were again centrifuged at 10,000 rpm for 5 minutes.

After centrifugation supernatant was discarded and tubes containing DNA were dried.

- **Storage:**

30µl of T.E. Buffer was added side by side in each tube and stored at -20ºC.

IS6110 Based RFLP Analysis (Van Soolingen et al 1991)

i) Preparation of probe:-

Probe was prepared by PCR amplification of 245 bp IS*6110* fragment using INS1 and ISN2 primers. Master Mix (48µl) was prepared by adding 5µl of PCR buffer, 4µl of dNTPs mixture, 0.5ml of primer 1 & 2 each, 0.46µl of Taq Polymerase and rest of distilled water in an eppendrof tube. Then 2µl of DNA template (H37Rv) was added to the reaction mixture to make the final volume upto 50µl. the tube containing DNA & reaction mixture was placed in thermal cycler. The thermal cycler was programmed as follows:

Table 4: - PCR Amplification of IS6110 Probe:

Step	Description	Temperature (ºC)	Time (minutes)
1	Denaturation	96ºC	1
2	Annealing	65ºC	1
3	Extension	72ºC	2

ii) Labelling of probe:

Probe was DIG labelled by random primed labelling method. All reagents required for probe labelling were provide in the DIG labelling kit (Roche Catalogue No. 1093657)

1. Preparation of reaction mixture:

In an eppendrof tube containing 2µl of DNA hexanucleotides mixture, 2µl of dNTP labelling mixture & 1µl of Klenow enzymes was added. The final volume was made to 20µl with distilled water. The mixture was centrifuged for few seconds & then incubated at 37ºC for overnight.

2. Termination of reaction:

1.5µl of EDTA (0.5M) was added to eppendrof tube to terminate the reaction.

3. Precipitation of probe:

2µl of 3M CH_3COONa and 3 volume chilled alcohol (75µl) were added in the tube to precipitate the probe. Then the reaction mixture was incubated at -70ºC for 30 minutes.

4. Centrifugation: reaction mixture was centrifuged at 13000g for 15 minutes at 4ºC.

5. Washing: Supernatant was decanted & 70% ethanol (150µl) was added to pellet & then centrifuged at 13000g at 4ºC for 15 minutes.

6. Addition of T.E. Buffer: The pellet in the test tube contains random DIG labeled probe & that was suspended in 50µl TE buffer.

iii) Restriction of DNA:

- Restriction of the DNA samples was done using the restriction enzyme PvuII.
- PvuII cut M.tuberculosis genome at a specific site with insertion sequence
  5'-CAG/CTG-3'
  3'-GTC/GAC-5'
- 5µl of intact DNA was taken in different eppendrof tubes.
- 3µl of restriction buffer (10 xs) was added as required by 2µl enzyme PvuII.
- By adding 20µl of distilled water total volume was made up to 30 µl.
- Tubes were centrifuged for 30 seconds & were kept at 37ºC for 4 hours. To stop the reaction 6µl of mounting dye Xylene – cyanol containing EDTA was added to the tubes, mixture was heated at 68ºC for 10 minutes to inactivate the enzymes.

iv) Electrophoresis:

For resolution of the restricted DNA, agarose gel electrophoresis was performed with 1% agarose gel.

Restricted DNA samples were loaded in the wells and electrophoresis was performed for 8 hours at 25 volts (1.2 volts/cm).

v) Southern transfer:

(a) Acidic washing: - The gel with electrophoresis DNA was treated with acidic buffer (0.25 M HCl) for 15 minutes with continuous shaking. Gel was washed twice with distil water.

(b) Alkaline washing: - The gel was treated with alkaline buffer (0.5M NaOH & 1.5M NaCl) for 15 minutes with continuous shaking. DNA was denatured by alkali. The gel was washed twice with sterile distil water.

(c) Neutral washing: - The gel was treated with neutral buffer (3M NaCl & 0.5M Tris HCl) for 15 minutes with continuous shaking. This step restores pH of the gel.

(d) Assembly of apparatus :-

- 15 x 20 cm^2 glass plate was placed on the top of glass tray.
- Whatmann filter paper wetted in 20x SSC buffer was placed over glass plate with ends hanging down in the tray containing 20x SSC.
- The gel was placed over Whatmann filter paper, & above gel a positively charged nylon membrane was placed.
- Three to four Whatmann filter paper (6x SSC) along with a stack of dry filter paper was kept over the membrane & 500gm of weight was kept on the top of glass plate's
- The whole arrangement kept at room temperature for overnight so that the DNA from the gel was transferred to the nylon membrane.
- Nylon membrane was treated with 6x SSC, air dried and gel was discarded.

(e) Fixation of DNA on the membrane: The DNA transferred to the nylon membrane was fixed by baking it at 120ºC for 30 minutes. Before and after baking the membrane was treated with 6 x SSC solutions for 2 minutes and dried with filter paper.

(f) Prehybridization: Nylon membrane was packed in a polythene bag, to which 15 ml of hybridization fluid containing 100µl of tRNA (10mg/ml) was added. Then it was incubated at 42º C for 2 hours in a shaking bath. The tRNA prevents the non specific binding of DNA probe to the nylon membrane. After 2 hour hybridization fluid was discarded.

(g) Probe addition: - 100µl of hybridization fluid and 6µl of denatured probe (probe was denatured at 95ºC for 5-10 minutes). This probe was mixed in hybridization fluid.

(h) Hybridization: - To the polythene bag 15 ml of hybridization fluid with 6µl of DIG labelled probe was added. The membrane was incubated at 42ºC for overnight in a shaking water bath.

(i) Post hybridization washing: - the membrane was taken out from the polythene bag and subjected to washing with different concentration of SSC solution.

- I washing: The membrane was washed with 4x SSC at 42ºC for 15 minutes in shaking water bath. Then the fluid was decanted.
- II washing: The membrane was washed with 2X SSC at 42ºC for 15 minutes in shaking water bath & fluid was decanted.
- III washing: The membrane was washed with 1x SSC at 42ºC for 15 minutes in shaking water bath & fluid was decanted.
- IV washing: The membrane was washed with 0.5x SSC at 42ºC for 15 minutes in shaking water bath and fluid was decanted.

(j) Detection:-

- Equilibration of membrane: Nylon membrane was transferred to glass tray & 15ml of DIG Buffer No.1 was added to it. Membrane was kept for 2 minutes and then the fluid was discarded.
- Blocking: DIG Buffer No.2 containing the blocking reagent was added to the membrane and incubated for 30 minutes at room temperature. The buffer was discarded later.

- Antibody binding: 30 ml of DIG Buffer no.2 with 6µl of anti-DIG alkaline phosphates (1:1500) was added to the membrane and incubated for30 minutes at 37ºC in a shaking water bath. The fluid was discarded later.
- Washing: Membrane was washed twice with DIG Buffer No.1 for 15 minutes each. This washing removes unbound antibodies.
- Equilibration of membrane: 20 ml of DIG Buffer No.3 was added to the glass tray & membrane was kept for 2 minutes in it. The fluid was discarded later.
- Colour reaction: Membrane was packed in polythene bag & 20 ml DIG Buffer No.3 with 250µl of coloured substrate solution (NBT/BCIP) was added to it. Membrane was incubated at 37ºC for 30 minutes in dark. Then membrane was exposed to light for few minutes, if bands are not observed then keep overnight at 37ºC in coloured substrate solution. Fluid was discarded later & 10 ml of Buffer No.1 was added to stop the reaction.
- Observation & storage of membrane: The bands of IS*6110* were observed visually. Membrane was sealed in a polythene bag. Then 20 ml of TE buffer was added to it & stored.

Result

A total of 22 isolates of *Mycobacterium tuberculosis* recovered from sputum samples of patients suspected for MDR-TB were taken from mycobacterial repository, National JALMA Institute for Leprosy, Agra for this study. These isolates were sub-culture further to maintain original isolates in repository. All the isolates were subjected to DNA isolation. Out of 22 isolates, the intact DNA was obtained from 14 isolates. Therefore, IS6110 RFLP was performed for 14 isolates. The results of IS*6110* of *M. tuberculosis* isolates can be classified into three groups namely A**, B** & **C**. Group **A** resulted with low copies of IS*6110* having 0-5 bands. Group **B** resulted with intermediate copies of IS*6110* having 6-15 bands. Group **C** resulted with high copies of IS*6110* having more than 15. Out of 14 isolates, 12 and 2 isolates were low and intermediate copy number, respectively (Table 1, Fig 1). The detailed results of the study are presented in table 2.

Table 5: IS6110 copy number among the isolates of MDR-TB.

Group	**Copy Number**	**Number of Isolates**
Group A	0 to 5 copy number	12
Group B	6 to 15 copy number	2
Group C	More than 15 copy number	

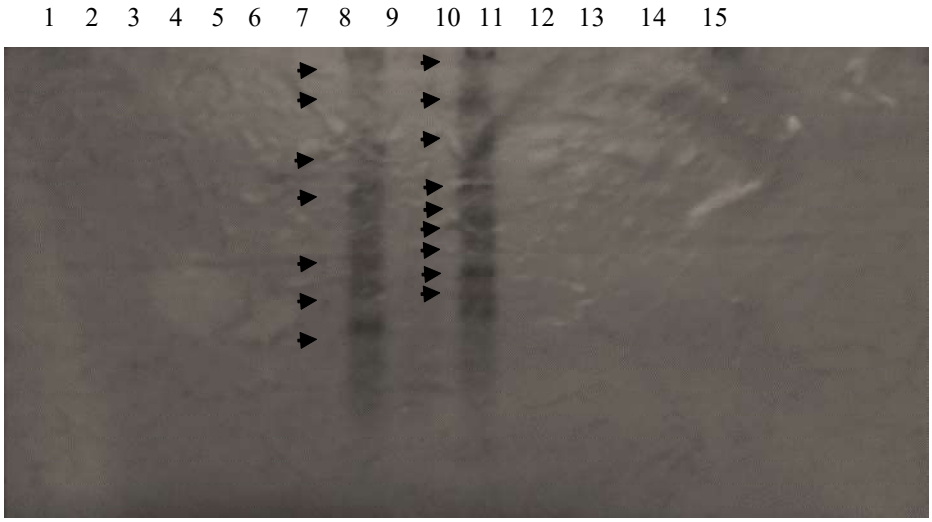

Fig 1: RFLP patterns of IS6110 copy no. of M. tuberculosis isolates included in this study

Table 6: Detailed Results of the Study.

S. No.	Lane No.	Isolates No.	Copy No.
1.	Lane No. 1	Ladder	-
2.	Lane No. 2	H37Rv	Copy No. 0
3.	Lane No. 3	JAL-16993	Copy No. 0
4.	Lane No. 4	JAL-16992	Copy No. 0
5.	Lane No. 5	JAL-16878	Copy No. 0
6.	Lane No. 6	JAL-16681	Copy No. 0
7.	Lane No. 7	JAL-17050	Copy No. 7
8.	Lane No. 8	JAL-16851	Copy No. 0
9.	Lane No. 9	JAL-16706	Copy No. 9
10.	Lane No. 10	JAL-16726	Copy No. 0
11.	Lane No. 11	JAL-16946	Copy No. 0
12.	Lane No. 12	JAL-16800	Copy No. 0
13.	Lane No. 13	JAL-16900	Copy No. 0
14.	Lane No. 14	JAL-16898	Copy No. 0
15.	Lane No. 15	Ladder	-

Discussion

The modern technology has resulted in the breakthrough of an advanced molecular typing method called Restriction Fragment Length Polymorphism (RFLP) analysis which is based on the insertion sequence IS*6110*. This has brought a new dimension to the study of tuberculosis and is now proving to be a boon and a new appreciation of the ecological complexities. Today, it is considered as a gold standard to which other methods are compared (Narayanan 2004, Chauhan et al 2007).

Although, the number of copies of IS*6110* can range 9, population based molecular epidemiological studies report that most strains contain 0-9 copies, a number sufficient to enable discrimination between the majorities of strains (Burgos and Pym 2002) .In the present study IS*6110* DNA fingerprinting was done and we have taken 14 isolates from the Mycobacterial Repository Center at NJIL & OMD Agra, belonged to Agra region, different hybridization patterns were obtained suggesting differences in copy number and genomic location of element. Results show that maximum number of isolates having multiple IS*6110* copies where as few were having less copies. Our observations are in concordance with previous studies published from JALMA (Chauhan et al 2004 and 2007).

Presence of low copy numbers of IS*6110* or no copies does not correlate with earlier studies carried out in south India, with a difference of higher percentage of low copy number in south Indian strains (Das et al 1995, Sahadevan *et al.,* 1995 and Radhakrishnan *et al.,* 2001).

It is well established that traditional DNA fingerprinting using IS*6110* as a probe is not sensitive in differentiating among *M. tuberculosis* isolates. The results further demonstrate the importance of secondary typing for investigating the relatedness of clinical isolates of *M. tuberculosis* obtained from widely separated geographic regions. The limitation of the study is that we sampled only few isolates from this area. This observation suggests that a combination of IS*6110* and other fingerprinting methods will increase the reliability of IS*6110* fingerprinting in studying the relationship of *M. tuberculosis* isolates might serve as a more appropriate primary typing method for a genotyping network that compares isolates from different geographic regions.

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