Grant Number: RG/2006/HS/07

Restriction Fragment Length Polymorphism (RFLP) and Spoligotyping on *Mycobacterium tuberculosis* strains isolated from patients attending the Central Chest Clinic Kandy

SECTION 1

i) Contract Number: RG/2006/HS/07

ii) Title of the project : Restriction Fragment Length Polymorphism (RFLP) and Spoligotyping on *Mycobacterium tuberculosis* strains isolated from patients attending the Central Chest Clinic Kandy.

iii) Principal Investigator: D.N Magana-Arachchi, PhD

Collaborators ; Prof. V. Thevanesam & Dr.D. Medagedara

iv) Co-Investigators; None

v) Institute where research was carried out: Institute of Fundamental Studies Hantana Road, Kandy & Department of Microbiology, Faculty of Medicine, University of Peradeniya.

vi) Date of award of the grant: 28.12.2006

vii) Date of Completion: 29.10.2010

viii) Total Allocation: Rs. 1,826,387.00

ix) Total spent: Rs. 1,826,387.00

x) Number of Research Students employed; One. From January 2007 to 26th November 2009

xi) Post Graduate degree completed with dates; Registered for a M.Phil but discontinued (see Annexure 1 for details)

xii) Number of Technical Assistants and /or labourers employed and period of Service; Technical assistants none. Labourers from Department of Microbiology for 2 years.

xiii) Publications /Communications;

1. Meegahakumbura M G K M, Ambalavanar V, Madegedara R M D, Thevanesam V, **Magana-Arachchi D N*** (2008) Socio -demographical features among the tuberculosis patients attending the Central Chest Clinic, Kandy – A Preliminary study, *Proceedings of the Kandy Society of Medicine, 30th Annual sessions,*30;Pages 95-96:47.

Publications; Two manuscripts are in preparation.

SECTION 2

Summary

To determine a detailed picture of tuberculosis (TB) epidemiology in Kandy, Sri Lanka, 110 *M.tuberculosis* complex isolates from TB Patients attending the Central Chest Clinic, Kandy, who were positive for acid fast bacilli on direct examination of sputum by Ziehl-Neelsen from 2007 -2009 were analyzed by IS *6110* DNA finger printing and by Spoligotyping. The software GeneDirectory was used to compare the IS *6110* hybridization patterns. Spoligotyping was carried out as previously described and spoligopatterns were analyzed using MS Excel data sheets and grouped together for any similarity. The data was further analyzed by comparing with the SPOTCLUST data base. In RFLP, the majority of the circulating *M .tuberculosis* strains in Kandy belong to a single family, but the degree of IS *6110* DNA polymorphism among strains was high. In total 71 distinct IS *6110* patterns were found with strains clustering into one main family and 10 distinct strains. Within the main family three isolates were grouped into one cluster, with closely related isolates while rest of the bacterial strains was grouped into one. The number of *IS 6110* DNA varied between 1 and 17. Strains containing a single copy of IS *6110* were predominant among the study population (15) and except for three, the location of the bands in fingerprints were different. Among the strains tested there were 25 strains that lacked the IS *6110* element. SPOTCLUST incorporates biological information on spoligotype evolution, and in spoligotyping of 110 *M.tuberculosis* isolates revealed a total of 24 families including the nine major families. The most predominant group corresponded to Family33. Strains were distributed among all three principal genetic groups PGG1, PGG 2 and PGG3. Segregation of *M.tuberculosis* into 'ancestral' versus 'modern' lineages based on PGG indicates that isolates from Kandy have originated from both lineages. In spoligotyping high strain diversity was seen and except for two strains 000000000003771 (ST1) and 000000000000031(ST 585) the rest were not defined in the latest spoligotype data bases SpolDB4/SITVIT. This is the first study in Sri Lanka in which the RFLP pattern of *M.tuberculosis* strains and the spoligotyping in a population has been examined. By using the genetic marker of IS *6110* it was possible to differentiate most of the *M.tuberculosis* isolates. The preliminary inferences from this study plead for a more extensive analysis of the data to study the variability of *M. tuberculosis* strains and their transmission dynamics.

SECTION 3

Restriction Fragment Length polymorphism (RFLP) analysis and Spoligotyping on *Mycobacterium tuberculosis* strains isolated from patients attending the Central Chest Clinic, Kandy

(i) Introduction

Tuberculosis (TB) had been a dreaded disease in Sri Lanka for centuries. Known as "Kshaya Rogaya" or "Kasa Rogum", these names aptly describe the disease as being associated with cough and wasting. TB, primarily affects young, between the ages 15 and 54, and the less affluent, who nevertheless, form the backbone of the economy of the country. The incidence of mortality, was 8.8 per 100,000 in 2005, and in global ranking 73 by estimation of number of cases.At present, TB patients in Sri Lanka get free treatment.

The strain classification or sub typing is important epidemiologically for recognizing outbreaks of infection, detecting the cross transmission of nosocomial pathogens, determining the source of infection, recognizing the particularly virulent strains of organisms and in monitoring vaccination programs (Olive and Bean, 1999). Sub typing has been accomplished by a number of different approaches, and if the method to be successful it has to satisfy several criteria. Mainly all the organisms within a species must be type able by the method used (Olive and Bean, 1999). Secondly, it must have high differentiation power and the methodologies should be reproducible (Arbeit, 1995).

Mycobacterial strain typing by means of molecular methods has become an important instrument for tuberculosis surveillance, control and prevention (van Soolingen, 1998). Among DNA fingerprinting methods which restriction fragment length polymorphism (RFLP) typing is the most common method used which, has permitted novel investigations of the epidemiology and pathogenesis of tuberculosis.

The use of IS *6110*, an insertion sequence which is present in *Mycobacterium tuberculosis*, is generally considered to be the gold standard for tuberculosis molecular epidemiology studies, but other molecular typing techniques could be used as adjuncts in selected circumstances (Cohn and O'Brien, 1998). Das *et al*, 1995 studied the utility of a standardized IS *6110 / Pvu* II, RFLP typing method for distinguishing between isolates *of M. tuberculosis*, and assess the potential for distinguishing between relapse versus re infection rates. They concluded that despite the high frequency of single- and zero-band isolates in the population, the discriminatory power of RFLP typing with IS *6110* is sufficiently high to be useful for clinical and epidemiological studies (Das *et al.*, 1995). Sahadevan *et al*, 1995 observed that *M.tuberculosis* isolates obtained from patients' sputa on diagnosis and during follow-up after short-course chemotherapy in Madras, had either no copy or only a single copy of IS *6110*. This posed a limitation for DNA fingerprinting with an IS *6110*-based probe to determine the frequency of exogenous re infection versus that of endogenous reactivation. They overcame this limitation by using an alternate probe, the direct-repeat element. Comparison of pre- and post treatment isolates by direct-repeat restriction fragment length polymorphism analysis indicated a high degree of endogenous reactivation among patients who had relapses after the successful completion of chemotherapy (Sahadevan *et*

al., 1995). Van Duin *et al*, investigated an episode of laboratory cross contamination using IS *6110* RFLP typing and it proved to be a useful tool to trace the source of contamination (Van Duin *et al.*, 1998).

Spoligotyping is a technique based on the polymorphism of the direct repeat (DR) locus present in *M.tuberculosis* DNA. The DR sequences are composed of multiple 36bp copies, interspersed by short non repetitive sequences (Kmerbeek *et al*, 1997). The presence or absence of each non repetitive sequence creates a pattern for each strain when analyzed by spoligotyping. A database of spoligotypes of *M.tuberculosis* has been created (Sola *et al*, 2001) containing the global distribution and phylogenetic analysis of worldwide spoligotypes (Dale *et al*, 2001). This database is useful for comparing the patterns found in different regions of the world, enabling a better understanding of the dynamics of disease distribution (Filliol *et al*, 2002; Borsuk *et al*, 2005).

A study done by P.Farnia *et al*, 2004 using RFLP and spoligotyping methods showed the importance of both typing methods in understanding the epidemiological factors that facilitates the spread of tuberculosis inside a country. Their study revealed that both transmission and reactivation are contributing to the spread of tuberculosis in Tehran. According to Krüüner *et al*, 2002 detection of super infection with a new *M.tuberculosis* strain during the treatment of an episode of active TB is possible only with Molecular epidemiology tools (Krüüner *et al*, 2002). Borsuk *et al,* results highlighted the importance of molecular epidemiology studies of tuberculosis in insufficiently studied regions with a high TB burden, in order to uncover the true extent of genetic diversity of the pathogen.

Therefore, the present study was focused on characterization of *M.tuberculosis* isolates, obtained from patients in the city of Kandy, by IS *6110* RFLP assay and spoligotyping and the use of RFLP in the study of person-to-person transmission of pulmonary TB among the general population.

(ii) Objectives

(1)To characterize the *M.tuberculosis* strains by IS *6110* - RFLP from first visit TB patients (N= 100) & recurrent tuberculosis patients (N=50) who attend the Central Clinic Kandy, for treatment.

(2) To characterize the *M.tuberculosis* strains by Spoligo typing from first visit TB patients (N= 100) & recurrent tuberculosis patients (N=50) who attend the Central Clinic Kandy, for treatment.

(3) To determine the use of RFLP in the study of person to person transmission of pulmonary TB among the general population (first visit and recurrent tuberculosis patients) and their effect on the epidemiology of TB.

(iii) Materials and Methods

Ethical clearance was obtained from Faculty of Medicine, University of Peradeniya

Study population

In total 215 patients attending the Central Chest Clinic, Kandy, during February 2007 to 2009 were selected as the study population.

Group I: Patients attending the Central Chest Clinic, Kandy, Sri Lanka who were positive for acid fast bacilli on direct examination of sputum by Ziehl- Neelsen stain and / or culture and / or had radiological findings suggestive of TB (n = 178).

Group II: Recurrent TB Patients attending the Central Chest Clinic, Kandy, Sri Lanka who are positive for acid fast bacilli on direct examination of sputum by Ziehl- Neelsen stain and / or culture and / or had radiological findings suggestive of TB (n = 12).

Group III: Patients attending the Central Chest Clinic, Kandy, Sri Lanka who were negative for acid fast bacilli on direct examination of sputum by Ziehl- Neelsen stain and / or culture and / or no radiological findings suggestive of TB but had symptoms of other pulmonary diseases. (n = 25).

Collection of data

A questionnaire was administered to patients who attend the Central Chest Clinic, Kandy

Collection of sputum samples

Two consecutive early morning sputum samples were collected from patients attending Central Chest Clinic, Kandy. Sputum samples were collected into autoclavable small wide mouth glass bottles and were stored at 4^0C until processed.

Specimen processing and culture

Decontamination of sputum was done using NaOH / Na citrate and N-acetyl L - cystine technique of Kubica and Dye (Kubica and Dye, 1963). Briefly, equal volumes of sodium hydroxide (4%), sodium citrate (2.9%) and N - acetyl L - cysteine (0.01g per 2.0ml of sodium hydroxide and sodium citrate), were added to the sputum specimens, in plastic sterilized tubes. Following vortexing (15 sec) the specimens were kept at room temperature for 15 min in a shaker (100rpm). Next, the tubes were filled with sterile distilled water and centrifuged at 7000 g for 15 min at 4^0 C. Supernatants were discarded and pellets were re suspended in 1.0 ml of distilled water. Lowenstein - Jensen media and Middle brook 7H-10 were inoculated with 0.5 ml of above suspension. Cultures were observed for 8 weeks at 37^0C until the appearance of colonies. The colonies were identified using the conventional biochemical tests.

Isolation of genomic DNA from Mycobacteria

Isolation of Genomic DNA and Southern blotting were performed as described by van Soolingen et al (van Soolingen et al,1991: van Embden et al, 1993) In brief, bacterial cells were scraped and transferred from Lowenstein-Jensen medium into a micro centrifuge tube containing 500 μl of

TE buffer. Bacterial cells were killed by heating at 80^{0}C (20 min). Next the tube was cooled to room temperature and the culture was centrifuged (5 min, 12,000g). The supernatant was discarded and the pellet was re suspended in 40 μl of extraction buffer (50mM Tris, pH 8.0, 2.2 mM EDTA) and 10 μl of lysozyme (50 mg/ml). The mixture was vortexed and incubated at 37^{0}C for 1hr. Proteinase k, 5 μl (20 mg/ml), 75 μl of TE buffer (10mM Tris HCl, pH 7.5; 1 mM EDTA, pH 7.9) and 20 μl of 37% N-lauryl sarcosine was added to the mixture, mixed and will be incubated for 1hr at 55^{0}C followed by 95^{0}C for 10 min. A 100μl aliquot of N-cetyl-N, N, N-trimethyl ammonium bromide (CTAB) was added and be incubated for 10 min at 65^{0}C. Finally the DNA was extracted with chloroform – isoamyl alcohol (24:1, v/v) and the precipitation of DNA was done by the addition of 1/10 volume of 3M sodium acetate, followed by three volumes of absolute ethanol at –20^0 C. The solution was incubated overnight at –20^0 C and DNA pellet was obtained by centrifuging the mixture at 12,000 g for 20 min. The DNA pellet was washed twice with 70% ethanol and air-dried. Finally, pellet was re dissolved in 20 μl of 0.1TE (0.001M Tris-HCl, 0.0001 M EDTA; pH 8.0). The DNA was stored at -20^0 C.

A) IS *6110* - RFLP

Digestion of chromosomal DNA for RFLP

Genomic DNA (5ug) per each sample / isolate (obtained from above procedure) was digested with restriction enzyme *Pvu* II in a final volume of 25 μl as recommended by the manufacturer (Pharmacia Biotech).

Separation of DNA fragments by electrophoresis

The *Pvu* II digested chromosomal DNA from samples was size fractionated on 1% agarose gels. Along with the samples a DNA marker (λ cleaved *Hind*III/PhiX174-*Hae*III) and DNA from the reference *M.tuberculosis* strain cleaved with *Pvu*II was included.

Southern blotting

Gel was soaked in HCl (0.25 M) for 20 minutes, followed by several volumes of gel soak I solution (1.5M NaCl, 0.5 M NaOH) for 30 minutes and next in several volumes of gel soak II solution (1 M Tris HCl, pH 8.0, 1.5 M NaCl) for 1 hr at room temperature with constant shaking. The gel was then Southern blotted onto nylon filters as described (Sambrook *et al.*, 1989).

Preparation of DNA probe by PCR

The IS *6110* – specific DNA probe of 245 bp was amplified by PCR using the oligonucleotide primers INS-1(5'CGTGAGGGCATCGAGGTGGC) and INS- 2 (GCGTAGGCGTCGGTGAC AAA)corresponding to bp 631 to 650 and 856 to 875 which are based on the positions of the IS *6110* sequence, respectively (Figure1).

Preparation of the labeled probe for RFLP

The probe was labelled by using a direct nucleic acid labeling and detection kit (ECL, Amersham, RPN 3001) according to the manufacturer's instructions. DNA to be labeled was diluted to a concentration of 10 ng / μl using water. DNA was denatured by heating for 5 min in a boiling water bath. The DNA sample was immediately cooled on ice for 5 min and centrifuged (2sec, 12000g). An equivalent volume of DNA labeling reagent was added to the cooled DNA and mixed thoroughly. An equivalent volume of glutaraldehyde was added to the solution, and

spun briefly in a micro centrifuge. Next the DNA was incubated for 10 min at 37^0C. The labeled DNA probe was stored in 50 %(v/v) glycerol at –20^0C until used.

Hybridization and detection

Hybridization
The nylon filter was pre hybridized with hybridization buffer (0.125ml/cm^2) in a sealed plastic bag for 1 hour at 42^0C. Labeled probe was mixed with the hybridization buffer, and was added to the solution containing the filter. The nylon filter was hybridized overnight at 42^0C with shaking.

Post hybridization washings
The hybridized filter was removed from the plastic bag and placed in a clean plastic box. The filter was washed twice (2x10 min) with the pre warmed (55^0C) primary wash buffer at 55^0C. Next the filter was placed in a clean plastic box and washed twice with the secondary wash buffer for 5 min at room temperature on a shaking platform.

Detection
The filters were treated with detection reagents as per manufacturers instructions in a dark room and then exposed to Kodak XAR-5 film for overnight at room temperature. The nylon filters were stored under moist conditions at 4 ^{0}C for further use.

B) Spoligotyping

Spoligotyping was carried out as previously described by J.Kmerbeek et al, 1997.

Preparation of the membrane containing the spacer-oligonucleotides
Standard spacer oligonucleotides (Table 1, supplementary data) were diluted to the optimized concentrations in 150 µl 500mM $NaHCO_3$, pH 8.4. Next Biodyne C membrane was activated by 10 min incubation in 10ml freshly prepared 16% (w/v) 1-ethyl -3-(3-dimethyl aminopropyl)carbodiimide (EDAC) in demineralized water, in a rolling bottle at room temperature. Membrane was rinsed with water for 2 min, placed on the mini blotter, and filled the slots with diluted oligonucleotides. Next membrane was incubated for 1 min at room temperature and then oligonucleotide solutions were removed by aspiration. Next blot was incubated in 100mM NaOH for 10 min in a sealed bag to in activate the membrane. Membrane was washed in 250 ml 2xSSPE/ 0.1%SDS for 5 min at 60^0C and then in 100ml 20mM EDTA pH 8.0 for 15 min at room tem. Membrane was stored at 4^0C until used.

PCR for DR
PCR was performed with primers Dra 5'- GGTTTTGGGTCTGACGAC-3' (biotinylated 3'end) and Drb 5'- CCGAGAGGGGACGGAAAC-3'. The PCR reaction contained 10ng of DNA, 1U of *Taq* DNA polymerase, 20pmol of each primer and 200µm dNTPs. The cycling parameters were 3 min at 96 ^{0}C, followed by 1 min at 96 ^{0}C, 1 min at 55 ^{0}C and 30sec at 72 ^{0}C for 30 cycles.

Hybridization with PCR product and detection

Membrane was washed in 250 ml 2xSSPE/ 0.1%SDS for 5 min at 60^0C and was placed in the mini blotter in a way that the slots were perpendicular to the line pattern of the applied oligonucleotides. 20μl of the PCR product was added into 150μl 2xSSPE/01%SDS and the diluted product was denatured by heating for 10 min at 100 ^{0}C and was immediately cooled on ice. Next residual fluid was removed from the slots and the slots were filled with diluted PCR product and incubated for 1hr at 60 ^{0}C. Next samples were removed and the membrane was washed twice in 250 ml 2xSSPE/ 0.5%SDS for 10 min at 60^0C. Membrane was placed in a sealed bag and allowed it cool to prevent inactivation of the peroxidase. Membrane was incubated in 1:4000 diluted streptavidin-peroxidase conjugate: (2.5μl streptavidin-peroxidase conjugate in 10 ml of 2xSSPE/ 0.5%SDS for 45-60 min in a sealed bag). Next membrane was washed twice in 250 ml 2xSSPE/ 0.5%SDS for 10 min at 42^0C. Then membrane was rinsed twice in 250 ml 2xSSPE for 5 min at room temperature. For chemiluminiscent detection of hybridizing DNA the membrane was incubated for 1 min in 20ml ECL detection liquid. Membrane was covered with a Saran-wrap and was exposed to an X-ray film overnight at room temperature. Finally, the X-ray film was developed using Kodak developer (1min) and fixer (3min).

Analysis of DNA RFLP patterns

The software GeneDirectory from SYNGENE was used to compare hybridization patterns, using the Dice Coefficient of similarity and the UPGMA algorithm, with a 1% band position tolerance.

Analysis of spoligotypes

Spoligopatterns were analyzed using MS Excel data sheets and grouped together for any similarity. The data was further analyzed by comparing with the SPOTCLUST data base which has been created and maintained by I. Vitol, J. Driscoll, B. Kreiswirth, N. Kurepina, and K.P. Bennett.

Analysis of questionnaires

Epi Info 6.0 was used for statistical analysis of the questionnaires.

(iv) Results /Output

A total of 190 sputum specimens were obtained from first visit and recurrent tuberculosis patients attending the Central Chest Clinic, Kandy who were positive for acid fast bacilli. In addition, as control population 25 sputum samples were collected from patients attending the same clinic, who were negative for acid fast bacilli on direct examination of sputum by Ziehl-Neelsen stain and / or culture and / or no radiological findings suggestive of TB but had symptoms of other pulmonary diseases. Sample collection took place over a period of 2 years from February 2007 to March 2009. Of the 178 specimens (from the first visit patients) inoculated in Lowenstein-Jensen media, 122 (68.5%) grew within eight weeks of incubation. The remaining 56 did not grow or got contaminated and were excluded from typing. Only one of the 12 specimens from patients with recurrent tuberculosis grew during the eight week incubation

period. Of the 25 specimens (from control population) inoculated in Lowenstein-Jensen media, four grew within five days of incubation.

Biochemical identification

Biochemical tests were successfully carried out to differentiate *Mycobacterium tuberculosis* complex from mycobacteria other than tuberculosis in 78 of 103 isolates from the first visit patients. Of these 78 isolates, 76 (97.4%) were identified as either *M. tuberculosis*, or *M .tuberculosis* complex and 2 (2.6%) as MOTT (Table 1). The isolate from the recurrent patient and the four isolates from control population were identified as MOTT. Due to contamination or difficulties in re-culturing the organism it was not possible to perform all tests on all isolates and the different numbers reflect these difficulties.

Table 1: The results of the biochemical tests of 96 study isolates

Isolate	MTB/MTC/MOTT
M2	MTB
M5	MTB
M8	MTB
M9	MTB
M17	MTC
M18	MTB
M19	NS
M20	MTB
M22	MTB
M23	MTB
M24	MTB
M25	MTB
M26	MTB
M27	NS
M30	MTB
M31	MTB
M32	MTB
M34	MTB
M35	MTB
M37	MTB

M41	MTB
M42	MTC
M43	MTC
M48	MTB
M50	MTB
M51	NS
M52	NS
M53	MTB
M54	MTB
M59	MTB
M60	MTC
M62	MTB
M64	MTB
M66	MTB
M69	MTB
M70	MTB
M72	MTB
M73	MTC
M76	MOTT
M77	MTC
M78	NS
M83	MTB
M84	MTB
M85	MTB
M87	NS
M88	MTB
M110	MTB
M111	MTB
M114	NS
M115	MTB

M116	MTB
M117	MTB
M118	MTC
M119	MTB
M120	MTB
M123	MTC
M124	NS
M125	NS
M128	MTB
M129	MTB
M130	NS
M131	MTB
M132	MTB
M133	NS
M134	MTB
M136	NS
M137	NS
M138	NS
M139	MTB
M140	MTB
M141	MTB
M142	NS
M143	MTB
M144	NS
M145	NS
M146	MTB
M147	MTB
M148	MTB
M149	MTC
M150	MTB

M151	MTB
M152	MTB
M153	MTB
M155[a]	MTB
M156	MTB
M157	MTC
M163	MTB
M164	MTB
M168	MOTT
M169	MTB
M170	MTB
M171	MTB
M174	MTC
M175	MTB
M177	MTB
M183[a]	MOTT

[a] **From recurrent patient; MTB - *Mycobacterium tuberculosis;* MTC- *Mycobacterium tuberculosis* complex; MOTT - Mycobacteria other than tuberculosis; R - resistant; S - sensitive; NS - not successful**

B) Restriction Fragment Length Polymorphism Analysis

For DNA fingerprinting the restriction enzyme *Pvu* II was used to cleave chromosomal DNA of the mycobacterial strains. The enzyme cleaves the 1.35 kb IS *6110* element at a single site (McAdam, *et al*, 1990). One hundred and twenty *M tuberculosis* strains were subjected to Southern blot analysis with labeled IS *6110* DNA as a probe, following *Pvu* II digestion. RFLP analysis was successfully carried out to differentiate *Mycobacterium tuberculosis* complex from mycobacteria other than tuberculosis in 100 of 122. isolates from the first visit patients. Supplementary figures 1 to 6 are the Southern blots showing the fingerprints of *Pvu* II restricted chromosomal DNA of *M. tuberculosis* isolated from the sputum of the patients. These figures illustrate the high degree of DNA polymorphism in both banding patterns and number of copies of IS *6110* among strains. None of the isolates had an identical banding pattern except for the three strains with a single copy of IS*6110*.

The number of *IS 6110* DNA containing *Pvu* II fragments in strains varied between 1 and 17 indicating that these strains contain 1 to 17 copies of the IS *6110* element. Table 3 summarizes the number of IS copies found in the strains that were investigated among the study group.

Strains containing a single copy of IS *6110* were predominant among the study population (15) and except for three strains, the location of the bands in fingerprints were different and therefore the location of IS *6110* elements in the chromosomal DNA.

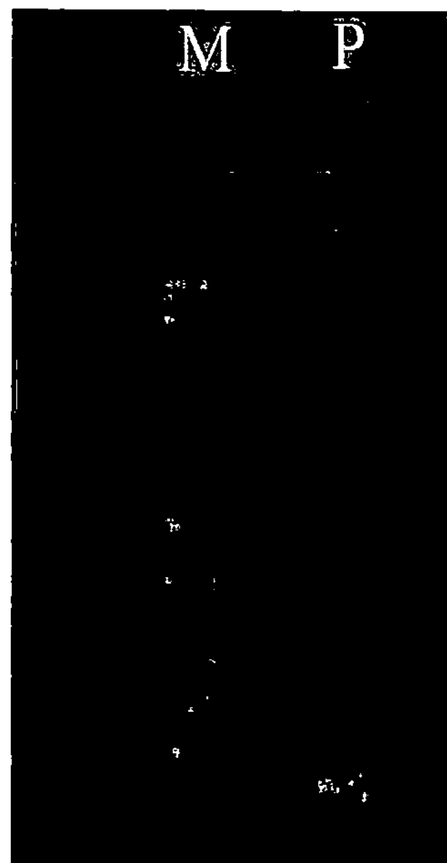

Figure 1: The IS *6110* – specific DNA probe of 245 bp for RFLP analysis

Table 2 : IS element copies (IS *6110*) found in study groups

Number of IS *6110* copies	**Number of strains**	**% of strains**
0	$15^a + 10^b = 25$	25
1	12	12.2
2	3	3.1
3	3	3.1
4	5	5.1
5	4	4.1
6	9	9.2
7	3	3.1
8	4	4.1
9	6	6.1
10	6	6.1
11	8	8.2
12	2	2.04
13	1	1.02
14	3	3.1
15	1	1.02
16	2	2.04
17	1	1.02

[a] MTC / MTb *Mycobacterium tuberculosis,* [b] not successful (~ MOTT)

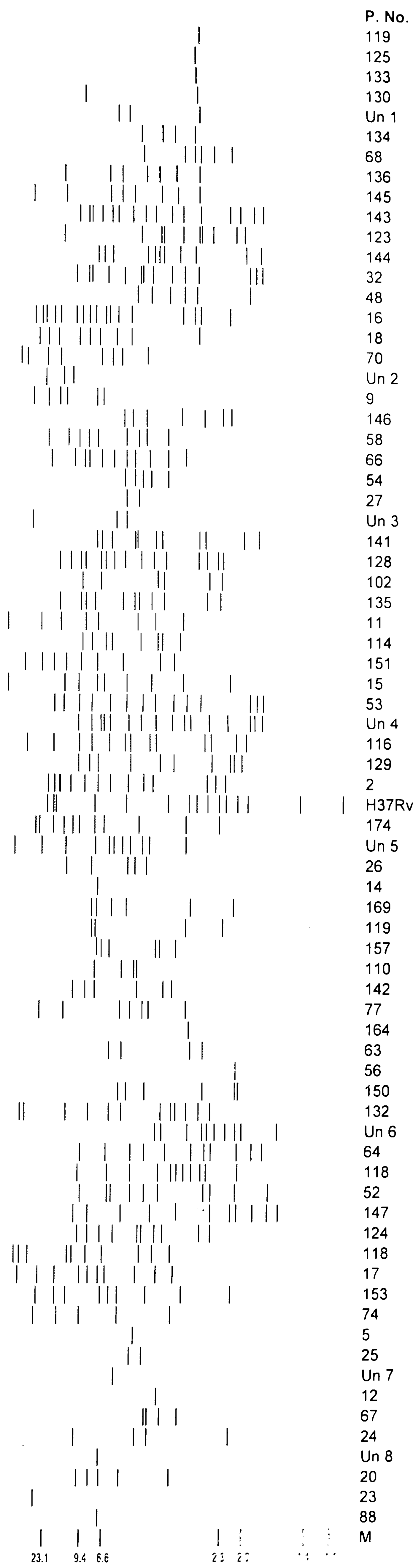

Figure 2: The degree of relatedness among the mycobacterial strains.
GeneDirectory software from SYNGENE was used to compare hybridization patterns, using the Dice coefficient of similarity and the UPGMA algorithm, with a 1% band position tolerance.

Computer analysis of DNA fingerprints among the patients

The fingerprints of the 73 strains were subjected to similarity analysis by using the software programme GeneDirectory from SYNGENE. Figure 2 demonstrates the degree of relatedness among the mycobacterial strains. In total 71 distinct IS *6110* patterns were found with strains clustering into one main family (63) and 10 distinct strains. Within the main family three isolates were grouped into one cluster, with closely related isolates while rest of the bacterial strains (60) were grouped into one. Sub clustering pattern of the main family was interesting with total 57 bacterial strains clustering into 3main groups with 19, 27 and 11 strains respectively.

C) Spoligotyping

A total of 110*M.tuberculosis* isolates were analyzed by spoligotyping. Figures 1 to 2 are the Spoligo patterns obtained for the *M. tuberculosis* isolated from the sputum of the patients. When spoligo patterns were compared from SPOTCLUST which was based on the SpolDB3 model, 24 distinct families were identified including the nine major spoligotyping-based families; *Mycobacterium africanum, M.bovis,* East African-Indian (EAI) Beijing, Haarlem, Latin American and Mediterranean (LAM), Central and Middle Eastern Asian (CAS), a European family X, and a default family T (Table 3).

Table 3: Spoligotyping – based families that observed in *M. tuberculosis* isolates in patients with tuberculosis in Kandy by SPOTCLUST

Spoligotyping – based family	Total	* Country of Origin
Family33 family with any probability	45	USA/India/Zambia/Russia/Egypt/NewZealand
Family36 family with probability	13	USA
M. tuberculosis EAI1 family with probability	7	USA
M. tuberculosis Beijing family with probability	7	USA/Russia/Czech/Singapore/NewZealand /SouthAfrica
M. africanum family with probability	5	USA/Nigeria/SierraLeone/ Guinea
Family35 family with probability	5	USA/Russia/ Czech
M. tuberculosis LAM7 family with probability	5	USA/NewZealand/ Czech
M. tuberculosis T3 family with probability	3	USA/Russia/
M. bovis-BCG family with probability	2	USA/Zambia/Russia/Czech/NewZealand/Malawi
M. tuberculosis T1 family with probability	2	USA/Russia/India/Czech/Zambia/NewZealand /SouthAfrica/Uganda
M. microti family with probability	2	USA
M. tuberculosis T2 family with probability	2	USA
M. tuberculosis CAS family with probability	1	USA/Czech/SouthAfrica/ India/Zambia/Uganda
M. tuberculosis LAM8 family with probability	1	USA/Czech/Zambia

M. tuberculosis Haarlem3 family with probability	1	USA/Czech/Russia/NewZealand/SouthAfrica
M. tuberculosis Haarlem1 family with probability	1	USA/Czech/Russia/India
M. tuberculosis X3 family with probability	1	USA/NewZealand/SouthAfrica/ India/Nigeria
M. tuberculosis H37Rv family with probability	1	USA/Russia
M. tuberculosis LAM3 family with probability	1	USA/SouthAfrica
M. tuberculosis LAM1 family with probability	1	USA/SouthAfrica
M. tuberculosis X2 family with probability	1	USA/NewZealand/SouthAfrica
M. tuberculosis EAI5 family with probability	1	USA/Tanzania/Zambia/India /NewZealand/India/Malawi
M. tuberculosis T4 family with probability	1	USA/NewZealand/Russia
M. tuberculosis Haarlem2 family with probability	1	USA/Czech

Except for two strains 000000000003771 (ST1) and 000000000000031(ST 585) the tested strains were not defined in the latest spoligotype data bases SpolDB4/SITVIT.

Analysis of Socio - Demographic data

Data were available for 205 patients. Of these, 180 were positive for acid fast bacilli on direct examination of sputum by Ziehl- Neelsen stain and / or culture and / or had radiological findings suggestive of TB while rest 25 were from control population. The results of the different parameters analyzed were summarized in Table 4.

In AFB positive category the majority were males (69.4%) with 30.6% females. How ever in control population this was different with both females and males were equal in ratio. In Category I, 90% of the population was in the age group of between the ages 16 and 60, and the less affluent, which nevertheless, form the backbone of the economy of the country. There were no significant differences among two categories except for the two parameters contact history of TB and being out of the country (travel- abroad) by the patient.

Table 4: Analysis of variables in study population

Category I [AFB (+)ve] Total = 180		Category II [AFB (-)ve] Total = 25		P Value	
Sex	Female- **55** - 30.6%	Sex	Female - **13** - 52%	0.042	
	Male- **125** – 69.4%		Male - **12** – 48%	0.042	
Age	0-15 - **1** – 0.6%	Age	0-15 - **0** – 0%	0.316	**1.000
	16-60 - **163** – 90.6%		16-60 - **16** – 64%	0.007	
	61/ above- **16** - 8.8%		61/ above- **9** - 36%	0.006	
Drinkers	Total - **97** – 77.6%	Drinkers	Total- **6** – 50%	0.064	
Type	Toddy 1 – **2**	Type	Toddy 1- **0**	0.153	**1.000
	Kasippu 2 – **29**		Kasippu 2- **1**	0.406	**0.669
	Arrack 3 – **33**		Arrack 3- **3**	0.446	**0.419
	Any other 4 – **2**		Any other 4 - **0**	0.153	**1.000
	> than two 5 – **28**		> than two 5 - **2**	0.821	**1.000
Quantity **(known)**	¼ bottle 1 – **22**	Quantity	¼ bottle 1 -**2**	0.589	**0.622
	½ bottle 2 - **35**		½ bottle 2 -**4**	0.123	**0.196
	1 bottle 3 - **9**		1 bottle 3 -**0**	0.002	**1.000
	> than one 4 - **0**		> than one 4- **0**	*	
Smokers	Total - **99** - 79.2%	Smokers	Total- **10** – 83.3%	0.716	**1.000
Type	Beedi 1 - **16**	Type	Beedi 1- **1**	0.545	**1.000
	Cigarettes 2 - **48**		Cigarettes 2- **0**	0.000	**0.002
	Cigars 3 - **3**		Cigars 3- **2**	0.184	**0.066
	Ganja 4 - **3**		Ganja 4- **0**	0.079	**1.000
	Any other 5 - **1**		Any other 5- **0**	0.315	**1.000
	> than two 6 - **28**		> than two 6- **7**	0.005	**0.010
Contact History	- **49** – 27.2%	Contact History	- **1** – 4%	0.000	**0.011
Past History of TB	Unknown - 3	Past History of TB	- **1**- 4%	*	
Travel Abroad	- **15** – 8.3%	Travel Abroad	- **0** - 0%	0.000	**0.224
Level of Education	Total- **180** – 100%	Level of Education	Total- **25** – 100%	*	**1.000
	1 - **6** – 3.3%		1 – **0**	0.013	**1.000
	2 – **54** – 30%		2 – **1**	0.000	**0.004
	3 – **45** – 25%		3 – **14**	0.003	
	4 – **59** – 32.7%		4 - **7**	0.620	
	5 – **16** – 9%		5 - **3**	0.649	**0.710
	6 – **0**		6 - **0**	*	**1.000
Being imprisoned	- **8** – 4.4%	Being imprisoned	- **0** – 0%	0.004	**0.599
Culture +ve	- **121** – 67.2%	Culture +ve	- **0**- 0%	0.000	**0.000
Copy No.	Total - **59** – 48.76%	Copy No.	Total - 0		
	Less than 6 - **29**- 23.96%		Less than 6 - 0		
	More than 6 - **30** –24.79%		More than 6 - 0		

** Fisher's exact test: P-Value

(v) Discussion

In the study presented, a detailed picture of TB, in the district of Kandy, Sri Lanka has been attained by combining IS*6110* RFLP typing, spoligotyping, biochemical analysis and classical epidemiological methods. The data obtained are based on a study period of 2 years in which total 215 patients who attend the Central Chest Clinic, Kandy, for pulmonary treatment. Due to the lesser number of recurrent tuberculosis patients, the number of first visit patients were increased to 178.

Two of the isolates from the first visit patients in this study (who were treated as having tuberculosis in the Central Chest Clinic, Kandy) were identified as mycobacteria other than tuberculosis (MOTT) biochemically (Table 2). This finding is comparable to the study by Elwitigala, J. *et al.* from mycobacterial isolates obtained from patients throughout Sri Lanka excluding the districts Mullaitivu and Kilinochchi, cultured during the period of 2005-2007. In their study, MOTT accounted for 3.27% of the total isolates (Elwitigala, J. *et al.* 2008). The isolate from the recurrent tuberculosis patient was found to be a MOTT strain with rifampin resistance (unpublished data), which explains the reason for the treatment failure in that patient. In RFLP analysis all three strains did not produce any banding pattern with IS*6110* confirming their species variation.

The epidemiological analysis of TB using IS*6110* is based on the observation that the polymorphism of IS*6110* RFLP patterns among unrelated clinical isolates is high, where as epidemiologically related *M.tuberculosis* strains show identical or similar (one band variation) finger prints (Van Soolingen *et al.* 1995). This study shows that the majority of circulating *M.tuberculosis* strains in Kandy belong to a single family, but the degree of IS *6110* DNA polymorphism among strains were high. Interpretation of the clustering of the isolates in the family is complex and the explanation for the high degree of polymorphism in DNA fingerprints can be due to the different origins. Without performing DNA sequencing analysis definite conclusions cannot be made whether the isolates underwent any genetic changes within a given time.

Among the strains tested there were 25 strains that lacked the IS *6110* element. Among these 15 strains were confirmed as *M.tuberculosis* while three were identified as MOTT with DNA sequencing and biochemical analysis. This has implications for diagnosis of infection when IS *6110* is used as the sequence for DNA amplification. Previous studies showed that *M.tuberculosis* strains carrying one or few IS *6110* copies are often difficult to differentiate by IS *6110* standard RFLP analysis because of a site specific preference for insertion of the IS element. Therefore to further differentiate the strains other genetic markers such as polymorphic rich GC repetitive sequence (PGRS) and direct repeats (DR) have been used (Soolingen *et al,* 1993; Bauer *et al*, 1999). In this study for DNA fingerprinting restriction enzyme *Pvu* II was used to cleave the chromosomal DNA of the mycobacterial strains. The enzyme cleaves the 1.35 – kb IS *6110* element at a single site. In the present study except for three strains, the location of the bands in fingerprints were different and therefore the location of IS *6110* elements in the chromosomal DNA. Therefore *M.tuberculosis* strains carrying one or few IS *6110* copies were differentiated without difficulty. In this study 52% of the isolates had five or less than five

copies. This pattern is similar to a previous study in which 68% was rescored from recurrent TB patients (Magana-arachchi *et al, 2010*) and also from other countries in the Asian region, such as India, Malaysia, Oman and Hong Kong (Soolingen, 1996).

We included only culture-positive patients to enhance the possibility of typing actively transmitting strains. Although the exclusion of culture-negative cases could potentially have introduced a bias in the strain composition, we were unable to perform RFLP on all patients due to study constraints. Additionally, we found high strain diversity, with a large number of small clusters, as well as a significant proportion of strains hitherto unreported in the global databases. It has also been noted that the DNA polymorphism could be made use of to identify transmission rates of drug resistance and drug sensitive strains. RFLP typing can be carried out on primary isolates to determine drug resistance. By comparison of these isolates with the existing RFLP patterns of the drug resistance isolates the time taken for determining drug resistance may be much shorter compared to the conventional antibiotic sensitivity testing which takes more than four weeks.

In this study we used the algorithm, SPOTCLUST which incorporates biological information on spoligotype evolution, without attempting to derive the full phylogeny of *M.tuberculosis* complex. Spoligotyping of 110 *M.tuberculosis* isolates revealed a total of 24 families including the nine major families. The most predominant group among the isolates of *M.tuberculosis* corresponded to Family33. In this family, only spacers 33-34 are absent and recently described clade MANU of Indian origin belongs to this family. According to the analysis, bacterial strains were distributed among all three principal genetic groups PGG1, PGG 2 and PGG3. Segregation of *M.tuberculosis* into 'ancestral' versus 'modern' lineages based on PGG indicates that isolates from Kandy have originated from both lineages. In our spoligotyping patterns we found high strain diversity and except for two strains 000000000003771 (ST1) and 000000000000031(ST 585) the tested strains were not defined in the latest spoligotype data bases SpolDB4/SITVIT. Due to study constraints (limited time), we could not complete the cluster analysis on spoligotyping and after completing it in due course we will be able to identify the risk factors associated with TB transmission as well as the evolution of *M.tuberculosis* in Sri Lanka.

(vi) Conclusions and recommendations

In conclusion a high degree of polymorphism was observed both in the DNA fingerprinting patterns of *M.tuberculosis* isolates, with a copy number varying from 1-17, while in spoligotyping 24 distinct families were identified including the nine major spoligotyping-based families. However in the absence of DNA sequencing analysis, conclusions cannot be made whether these isolates were genetically different or they underwent any genetic changes within a given time even though the origin was from a common ancestor. This is the first study in Sri Lanka in which the RFLP pattern of *M.tuberculosis* strains and the spoligotyping in a population has been examined. By using the genetic marker of IS *6110* it was possible to differentiate most of the *M.tuberculosis* isolates. The preliminary inferences from this study plead for a more extensive analysis of the data to study the variability of *M. tuberculosis* strains and their transmission dynamics. In most of the countries the RFLP and spoligotyping patterns are recorded from each tuberculosis patient and the strains are deposited for future reference in treatment.

Therefore establishment of molecular typing methods in Sri Lanka will not only be useful to study strain variations in epidemiological analysis but may be used beneficially in treatment of patients as well as identifying patients carrying drug resistance strains and on reactivation of the disease.

(vii) References

Arbeit RD. Laboratory procedures for the epidemiologic analysis of microorganisms. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, and Yolken RH, eds. Manual of Clinical Microbiology, 6th ed. American Society for Microbiology, Washington, D.C. 1995: 190-208.

Borsuk S, Dellagostin MM, de Goes Maderia S, *et al.* Molecular characterization of *Mycobacterium tuberculosis* Isolates in a region of brazil with a high incidence of tuberculsis. Microbes and Infection. 2005;7: 1338-1344.

Cohn DL and O'Brien RJ. The use of restriction fragment length polymorphism (RFLP) analysis for epidemiological studies of tuberculosis in developing countries. Int. J.Tuberc.Lung Dis 1998; 2(1): 16-26.

Das S, Paramasivan CN, Lowrie DB, Prabhakar R and Narayanan PR. IS *6110* restriction fragment length polymorphism typing of clinical isolates of *Mycobacterium tuberculosis* from patients with pulmonary tuberculosis in Madras, South India. Tubercle and Lung Disease. 1995; 76(6): 550-4.

Dale JW, Brittain D, Cataldi AA, *et al.* Spacer oligonucleotide typing of bacteria of the *Mycobacterium tuberculosis* Complex: recommendations for standardised nomenclature. Int. J.Tuberc.Lung Dis. 2001; 5: 216-219.

Farina P, Mohammadi F, Masjedi MR, *et al.* Evaluation of tuberculosis transmission in Tehran:using RFLP and spoligotyping methods. Journal of Infection.2004,49;94-101.

Filliol I, Ferdinand S, Sola C, Thonnon, J and Rastogi N. Spoligotyping and IS6110 –RFLP typing of *Mycobacterium tuberculosis* from French Guiana: a comparison of results with international databases underlines interregional transmission from neighboring countries. Res.Microbiol. 2002;153 :81-88.

Kamerbeek J, Schouls L, Kolk A,et al,. Simultaneous detection and strain differentiation of *M.tuberculosis* for diagnosis and epidemiology. Journal of Clinical Microbiology. 1997. 35; 907-914.

Krüüner A, Pehme L, Ghebremichael S, *et al,*. Use of Molecular techniques to distinguish between treatment failure and exogenous reinfection with *Mycobacterium tuberculosis* . CID. 2002;35: 146-155.

Kubica GP, Dye WE, Cohn ML and Middlebrook G. Sputum digestion and decontamina- tion with N-acetyl L-cysteine as a sputum digestant for the isolation of mycobacteria. American Review of Respiratory Disease 1963; 89 ; 284-86.

Magana-Arachchi DN*, Perera AJ , Senaratne V and Chandrasekaran NV (2010)Pattern of Drug Resistance and RFLP Analysis on Mycobacterium Tuberculosis Strains Isolated From Recurrent Tuberculosis Patients. *Southeast Asian Journal of Tropical Medicine and Public Health.41*(3); 583-589.

Olive DM and Bean P. Principles and applications of methods for DNA based Typing of Microbial organisms. Journal of Clinical Microbiology 1999; 37 (6): 1661-69.

Sahadevan R, Narayanan S, Paramasivan CN, Prabhakar R and Narayanan PR. Restriction fragment length polymorphism typing of clinical isolates *of Mycobacterium tuberculosis* from patients with pulmonary tuberculosis in Madras, India, by use of direct-repeat probe. Journal of Clinical Microbiology 1995; 33(11): 3037-9.

Sola C, filliol I, Gutierrez I, *et al.* Spoligotype database of *Mycobacterium tuberculosis*: biogeographic distribution of shared types and epidemiologic and phylogenetic perspectives. Emerg.Infect.Dis. 2001; 7: 390-396.

Van Duin JM, Pijnenburg JE, van Rijswoud CM, de Haas PE, Hendriks WD and van Soolingen D. Investigation of cross contamination in a *Mycobacterium tuberculosis* laboratory using IS *6110* DNA fingerprinting. International Journal of Tuberculosis and Lung Disease 1998 May; 2(5): 425-9

van Soolingen D, de Haas PE, Hermans PW, Soll DR and van Embden JD, Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* tuberculosis complex strains: Evaluation of an insertion sequence dependent DNA polymorphism as a tool in the epidemiology of tuberculosis.J.Clin.Microbiol.1991: 29;2578-2586.

van Embden JDA, Cave MD, Crawford JT *et al*, Strain identification of *Mycobacterium tuberculosist* by DNA fingerprinting : recommendation for a standardized methodology. J.Clin.Microbiol.1993: 31; 406-409.

van Soolingen D. Utility of molecular epidemiology of tuberculosis. The European respiratory Journal. 1998; 11: 795-797.

van Soolingen D, and, Hermans PW, Epidemiology of tuberculosis by DNA fingerprinting. The European respiratory Journal. 1995; 8(Suppl.20):649s-656s.

" Vitol I, Driscoll J, Kreiswirth B, Kurepina N and K.P. Bennett. Identifying Mycobacterium tuberculosis Complex Strain Families using Spoligotypes"

(viii) Constrains

1) Due to the misconduct in data handling, the research assistant Ms. Meegahakumbura M G K M, had to be removed from the Institute and as a result data verification and data analysis was delayed (See Annexure 1).
2) Due to the unavailability of software (funds were not sufficient to purchase) for RFLP and spoligotyping analysis, data analysis were delayed until the purchasing of the GeneDirectory software in 2010 April through another grant (NRC 07-47).

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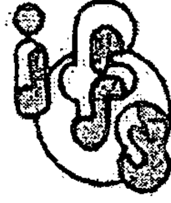

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INSTITUTE OF FUNDAMENTAL STUDIES
SRI LANKA

Hantana Road, Kandy, Sri Lanka.

Higher Degrees
26/03/10

26th March 2010

Dean
Faculty of Medicine,
University of Peradeniya
Peradeniya

Dear Sir,

Discontinuation of M.Phil Degree

We regret to inform that Ms. Meegahakumbure Gedara Krishanthi Madhavi, who registered as a M.Phil student in Faculty of Medicine, University of Peradeniya under the title "Restriction Fragment Length polymorphism (RFLP) analysis and Spoligotyping on *Mycobacterium tuberculosis* strains isolated from patients attending the Central Chest Clinic, Kandy" will not complete her M.Phil. Due to unavoidable circumstances she had to leave Institute of Fundamental Studies and as such she is not in a position to complete her higher degree.

Thank you

Yours Sincerely

Supervisors;

D N Magana Arachchi

D.N.Magana-Arachchi, PhD
Research Fellow
Institute of Fundamental Studies,
Kandy

V.Thevanesam

Prof. V. Thevanesam MBBS, DM, MRCP
Professor, Department of Microbiology,
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University of Peradeniya

HEAD OF DEPARTMENT
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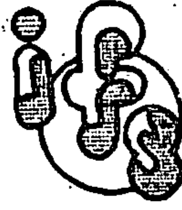

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INSTITUTE OF FUNDAMENTAL STUDIES SRI LANKA

Hantana Road, Kandy, Sri Lanka.

24th November 2009

Director,
Institute of Fundamental Studies,
Hantana Road,
Kandy.

Dear Sir,

Removal of Research Assistant Ms. MGKM Meegahakumbura from Cell Biology (Tuberculosis) Project

Ms. Meegahakumbura initially worked as a Research assistant in NSF grant RG/2006/HS/07 project titled " Restriction Fragment Length Polymorphism (RFLP) and Spoligotyping on *Mycobacterium tuberculosis* strains isolated from patients attending the Central Chest Clinic Kandy". Presently she is working as a Research Assistant (Grade I) in Cell Biology (Tuberculosis) Project.

After completing the grant, I was given six months to submit my Final Report and therefore I requested Ms. Meegahakumbura to submit the results to me for analysis. When I was going through the results, I observed a discrepancy. When I questioned her, she admitted that she cooked up the results.

She has let down me, her other M.Phil supervisor Prof. Thevanesam, Dr. Madegedara, NSF and IFS. I am disgusted with her and due to this deception; I do not want to keep such person in my laboratory in future. Therefore, I am ordering her to vacate my laboratory with immediate effect.

Thank you,

Yours truly,

D.N.Magana-Arachchi, PhD
Research Fellow

Project Leader ; Cell Biology

Mrs Pradeepa

Pl. prepare the letter

26/11/2009

From

M.G.K.Meegahakumbura,

Meegahakumbure Gedera,

Kehelpannala.

To

Director,

Institute of Fundamental Studies,

Hantana,

Kandy.

Dear Sir,

Resignation from the post of Research Assistant-Grade I

I'm M.G.K.Meegahakumbura worked as a Research Assistant Grade I since 2009/01/01 at the Department of Plant Cell Biology, IFS would like to resign from the above post effective from 26/11/2009.

Thank you.

M.G.K.Meegahakumbura,

2009/11/26

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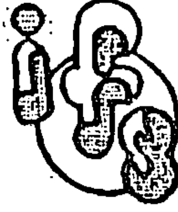
: 081 2234734
: 081 2232106-7
: 081 2200253-5



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INSTITUTE OF FUNDAMENTAL STUDIES SRI LANKA

Hantana Road, Kandy, Sri Lanka.

Our ref. No.: AD/RESIG/2009-09

30.11.2009

Ms. M.G.K.M.Meegahakumbura
Meegahakumbure Gedera
Kehelpannala.

Dear Ms. Meegahakumbura,

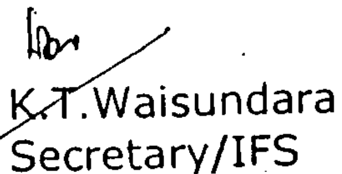
Reference your resignation letter dated 26.11.2009. I would like to inform you that the Director has been accepted your resignation from the IFS with effect from 26.11.2009. However, as you have not given one months' notice of resignation as per conditions of service agreed by you, you should pay one months' salary to IFS in lieu of notice.

According to the rules and regulations of the Institute you are requested to hand over all documents, equipment and other inventory items that are in your custody to Dr. D.N.Magana Arachchi.

Action EPF, ETF and gratuity payable would be initiated on receipt of the relevant clearances (check-out form).

Thank you for your valuable service that you have rendered to the Institute and I wish you all success in your future endeavors.

Yours sincerely,



K.T.Waisundara
Secretary/IFS

Cc: Dr. D.N.Magana Arachchi
Sr. Asst. Accountant
P.F.

Supplementary Data

Table 1: Sequences of the oligonucleotides used in this study

no. Oligonucleotide sequence Spacer	no. Oligonucleotide sequence
1...	ATAGAGGGTCGCCGGTTCTGGATCA
2...	CCTCATAATTGGGCGACAGCTTTTG
3...	CCGTGCTTCCAGTGATCGCCTTCTA
4...	ACGTCATACGCCGACCAATCATCAG
5...	TTTTCTGACCACTTGTGCGGGATTA
6...	CGTCGTCATTTCCGGCTTCAATTTC
7...	GAGGAGAGCGAGTACTCGGGGCTGC
8...	CGTGAAACCGCCCCCAGCCTCGCCG
9...	ACTCGGAATCCCATGTGCTGACAGC
10...	TCGACACCCGCTCTAGTTGACTTCC
11...	GTGAGCAACGGCGGCGGCAACCTGG
12...	ATATCTGCTGCCCGCCCGGGGAGAT
13...	GACCATCATTGCCATTCCCTCTCCC
14...	GGTGTGATGCGGATGGTCGGCTCGG
15...	CTTGAATAACGCGCAGTGAATTTCG
16...	CGAGTTCCCGTCAGCGTCGTAAATC
17...	GCGCCGGCCCGCGCGGATGACTCCG
18...	CATGGACCCGGGCGAGCTGCAGATG
19...	TAACTGGCTTGGCGCTGATCCTGGT
20...	TTGACCTCGCCAGGAGAGAAGATCA
22...	ACCGCAGACGGCACGATTGAGACAA
21...	TCGATGTCGATGTCCCAATCGTCGA
23	AGCATCGCTGATGCGGTCCAGCTCG
24	CCGCCTGCTGGGTGAGACGTGCTCG
25	GATCAGCGACCACCGCACCCTGTCA
26	CTTCAGCACCACCATCATCCGGCGC
27	GGATTCGTGATCTCTTCCCGCGGAT
28	TGCCCCGGCGTTTAGCGATCACAAC
29	AAATACAGGCTCCACGACACGACCA
30	GGTTGCCCCGCGCCCTTTTCCAGCC
31	TCAGACAGGTTCGCGTCGATCAAGT
32	GACCAAATAGGTATCGGCGTGTTCA
33	GACATGACGGCGGTGCCGCACTTGA
34	AAGTCACCTCGCCCACACCGTCGAA
35	TCCGTACGCTCGAAACGCTTCCAAC
36	CGAAATCCAGCACCACATCCGCAGC
37	CGCGAACTCGTCCACAGTCCCCCTT
38	CGTGGATGGCGGATGCGTTGTGCGC
39	GACGATGGCCAGTAAATCGGCGTGG
40	CGCCATCTGTGCCTCATACAGGTCC
41	GGAGCTTTCCGGCTTCTATCAGGTA
42	ATGGTGGGACATGGACGAGCGCGAC
43	CGCAGAATCGCACCGGGTGCGGGAG

Supplementary Data

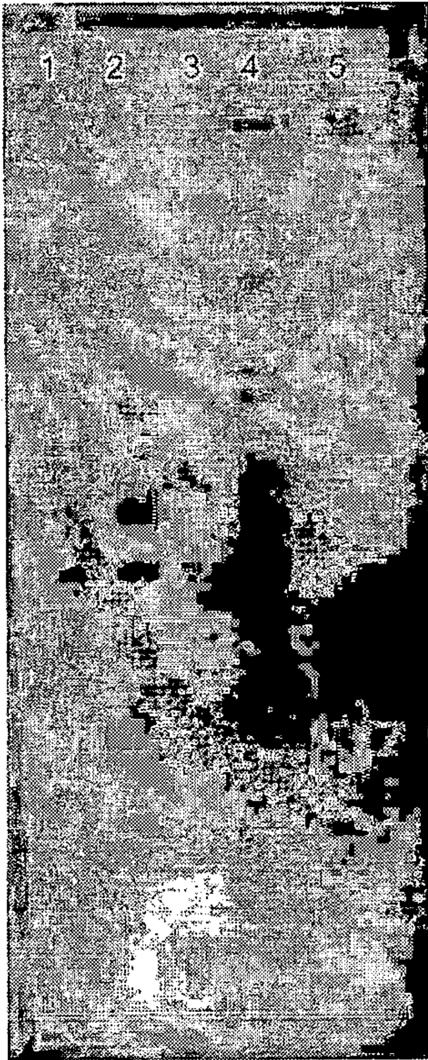


Figure 1; IS *6110* DNA fingerprints of *M.tuberculosis* strains originating from Study population.

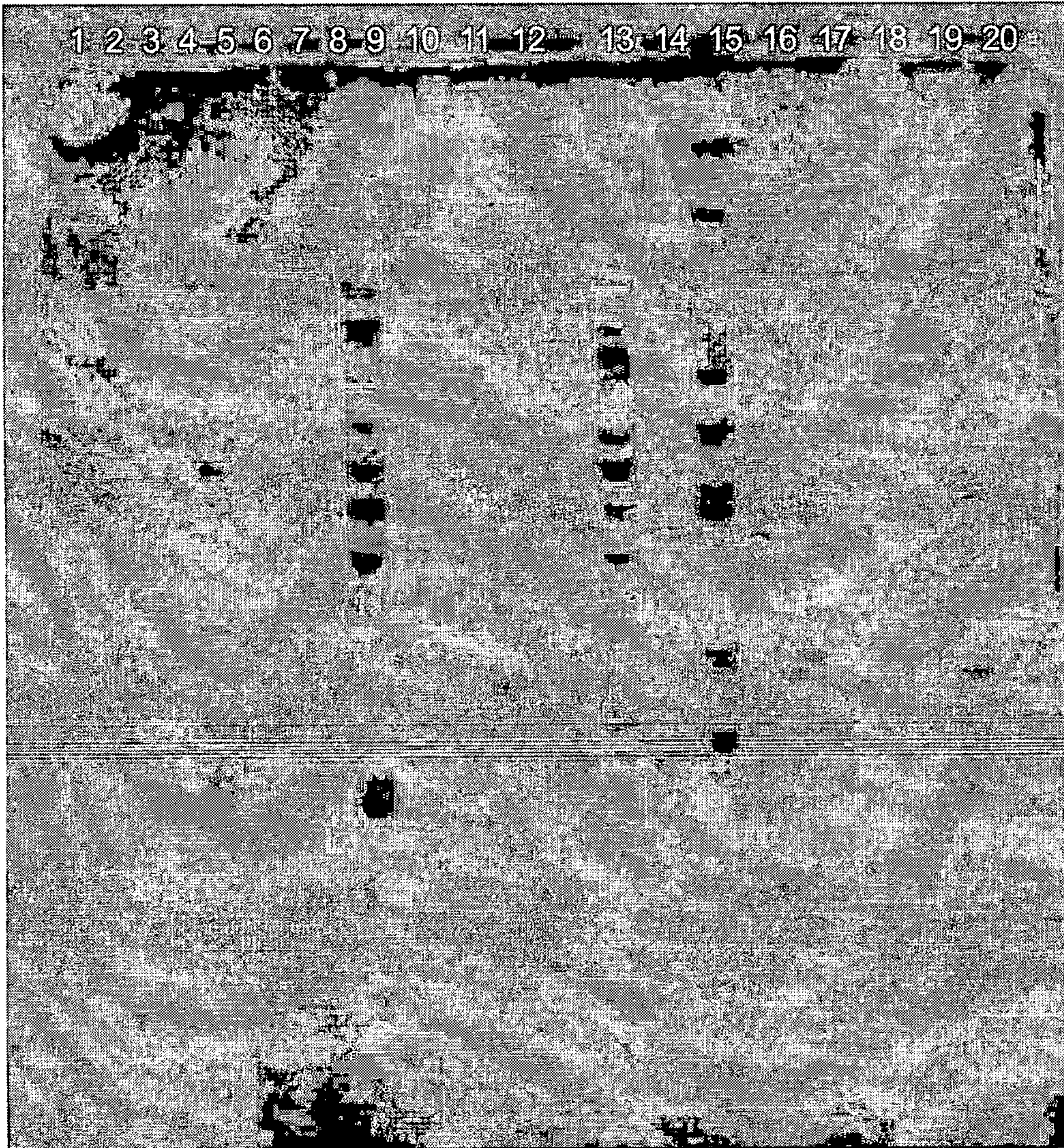


Figure 2; IS *6110* DNA fingerprints of *M.tuberculosis* strains originating from Study population.

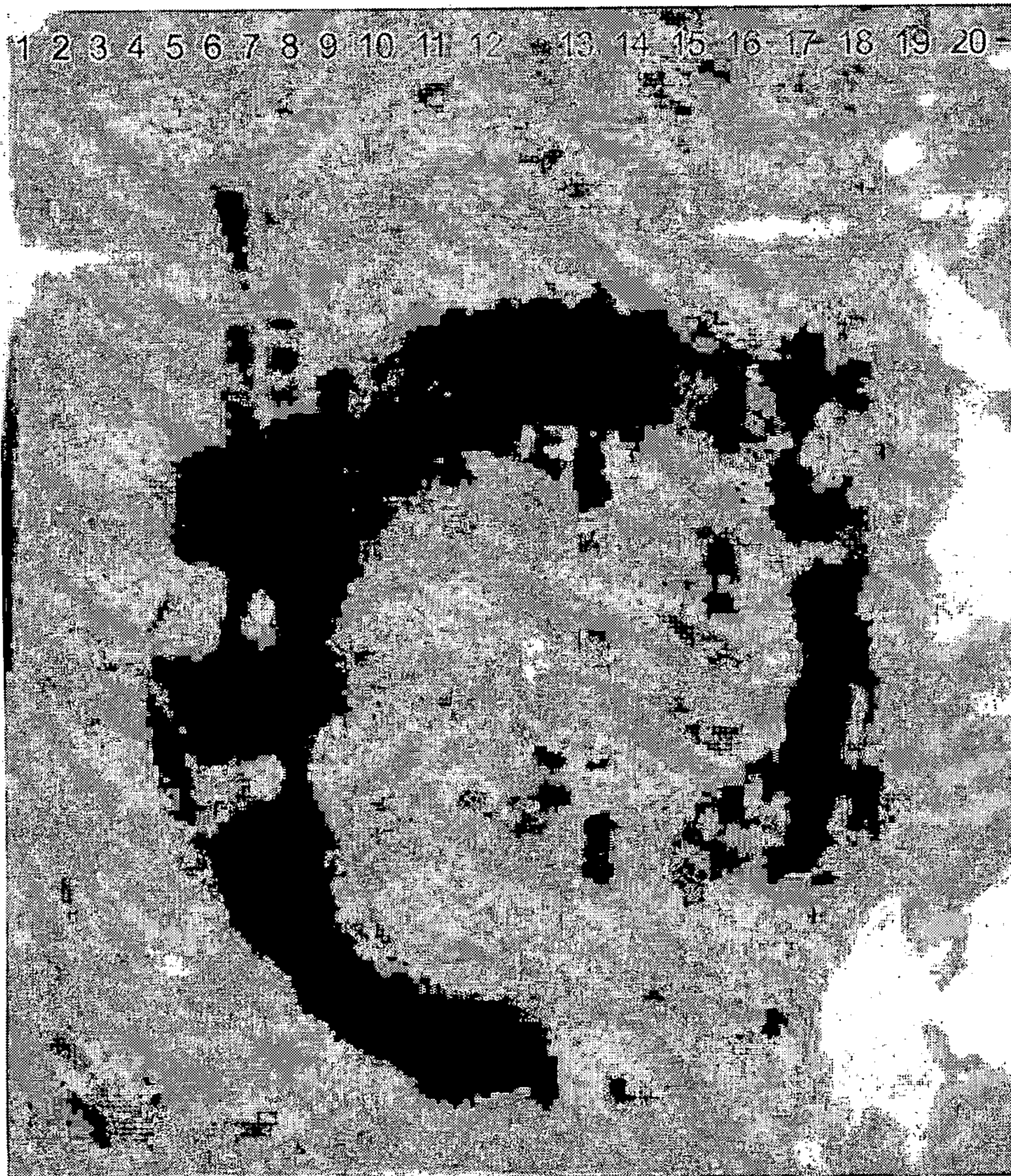


Figure 3; IS *6110* DNA fingerprints of *M.tuberculosis* strains originating from Study population.

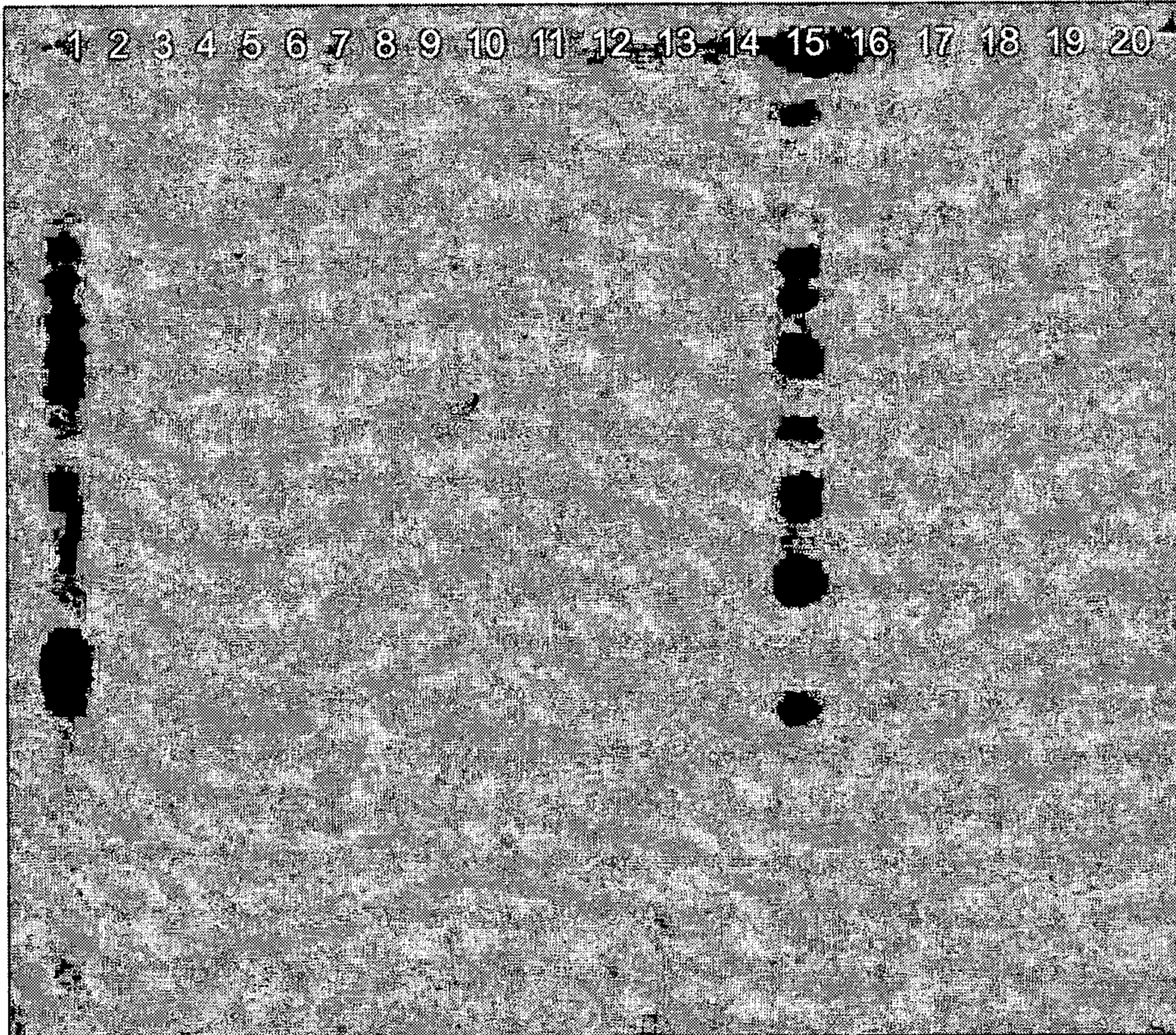


Figure 4; IS *6110* DNA fingerprints of *M.tuberculosis* strains originating from Study population.

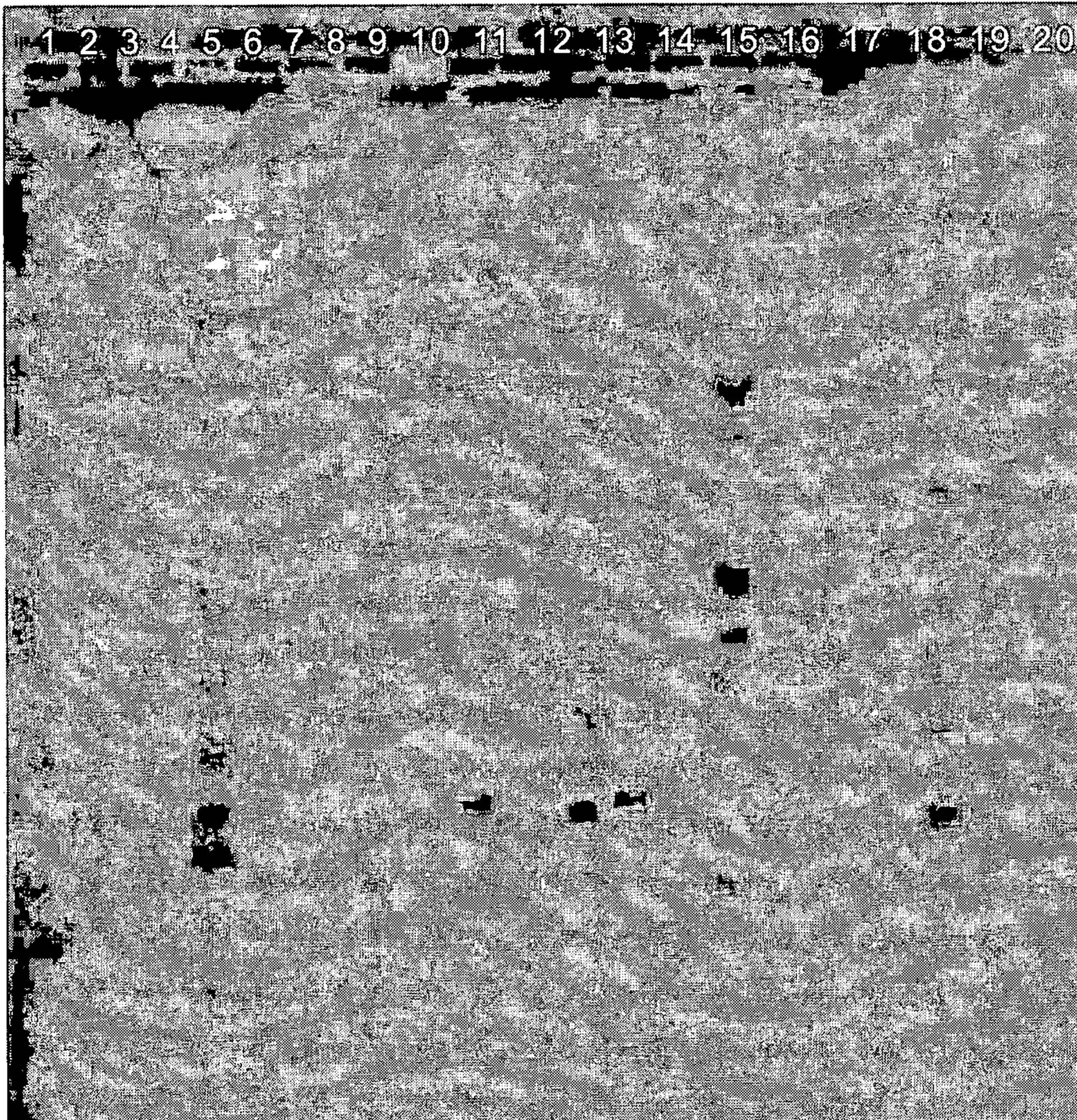


Figure 5; IS *6110* DNA fingerprints of *M.tuberculosis* strains originating from Study population.

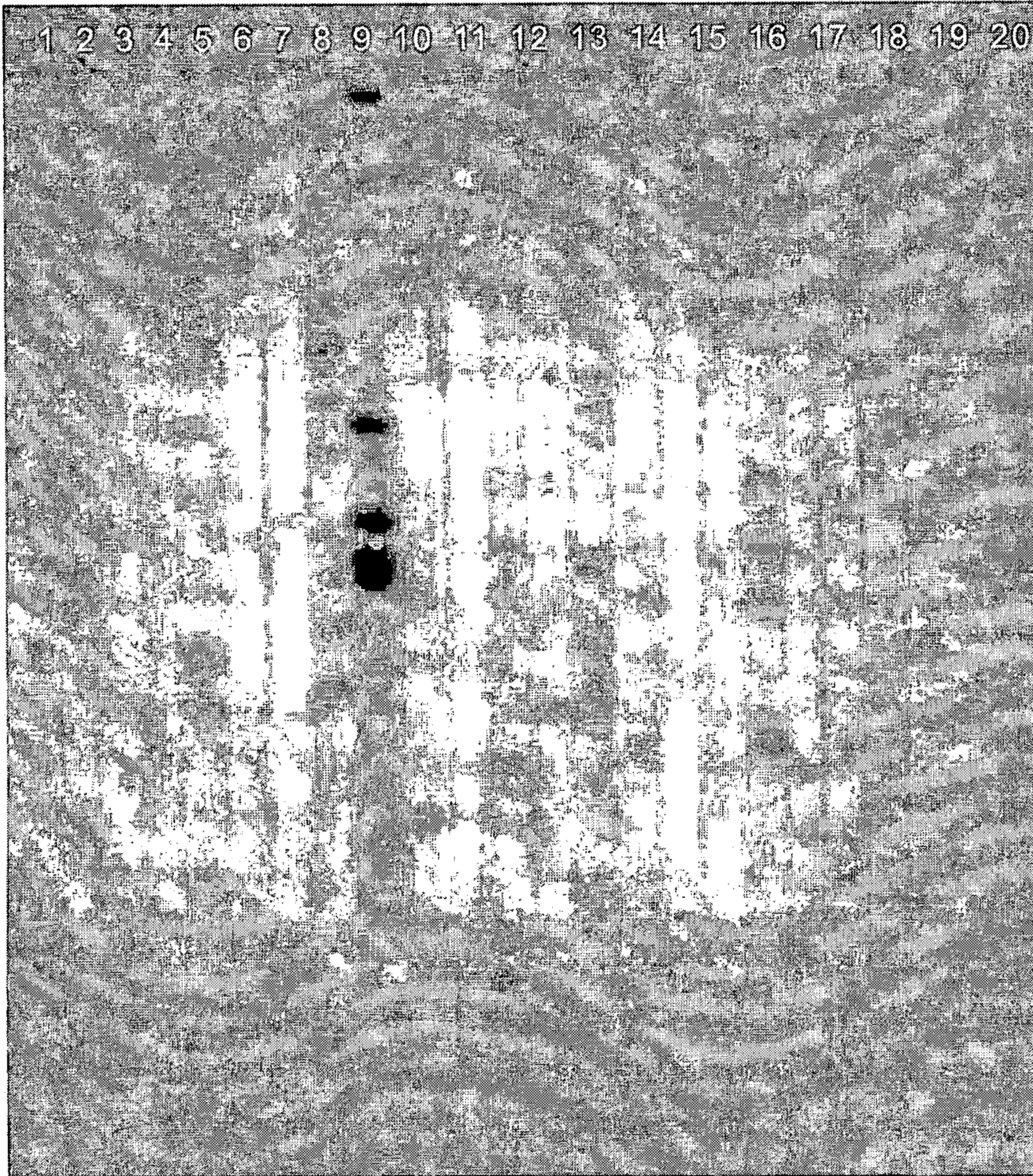


Figure 6; IS *6110* DNA fingerprints of *M.tuberculosis* strains originating from Study population.

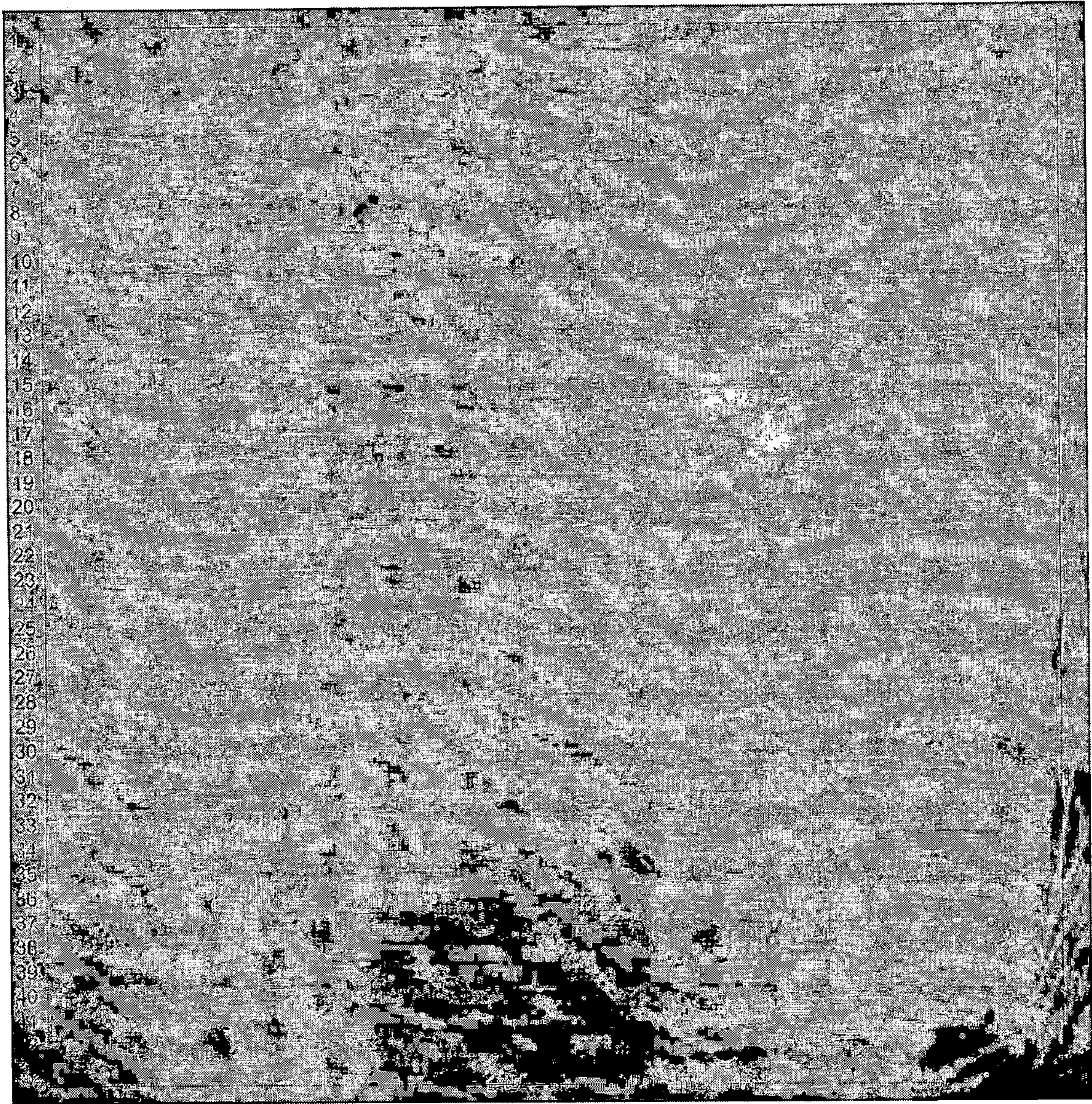


Figure 1; Spoligotyping patterns obtained from study population (A1-A45)

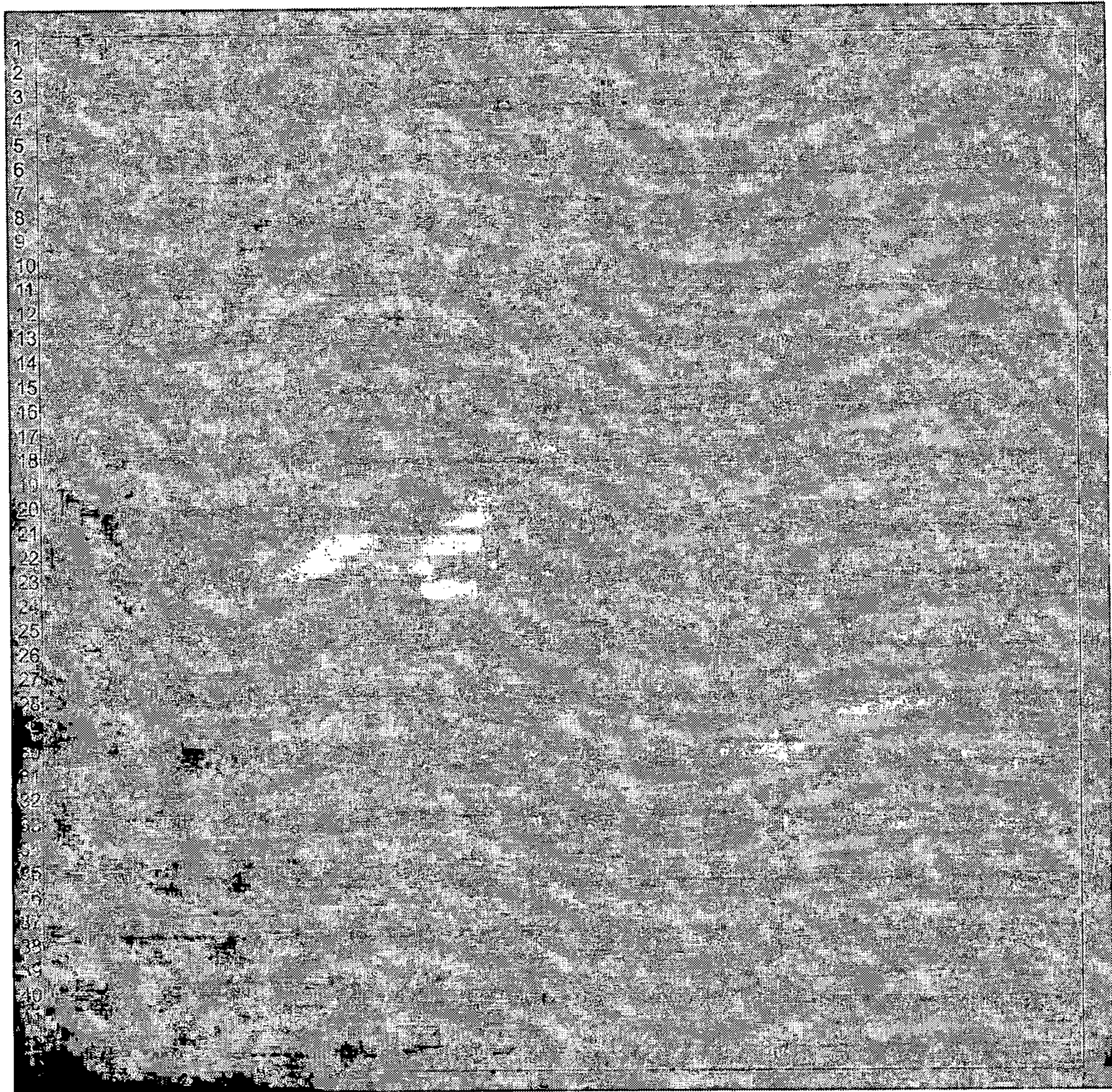


Figure 2: Spoligotyping patterns obtained from study population (B1-B45)

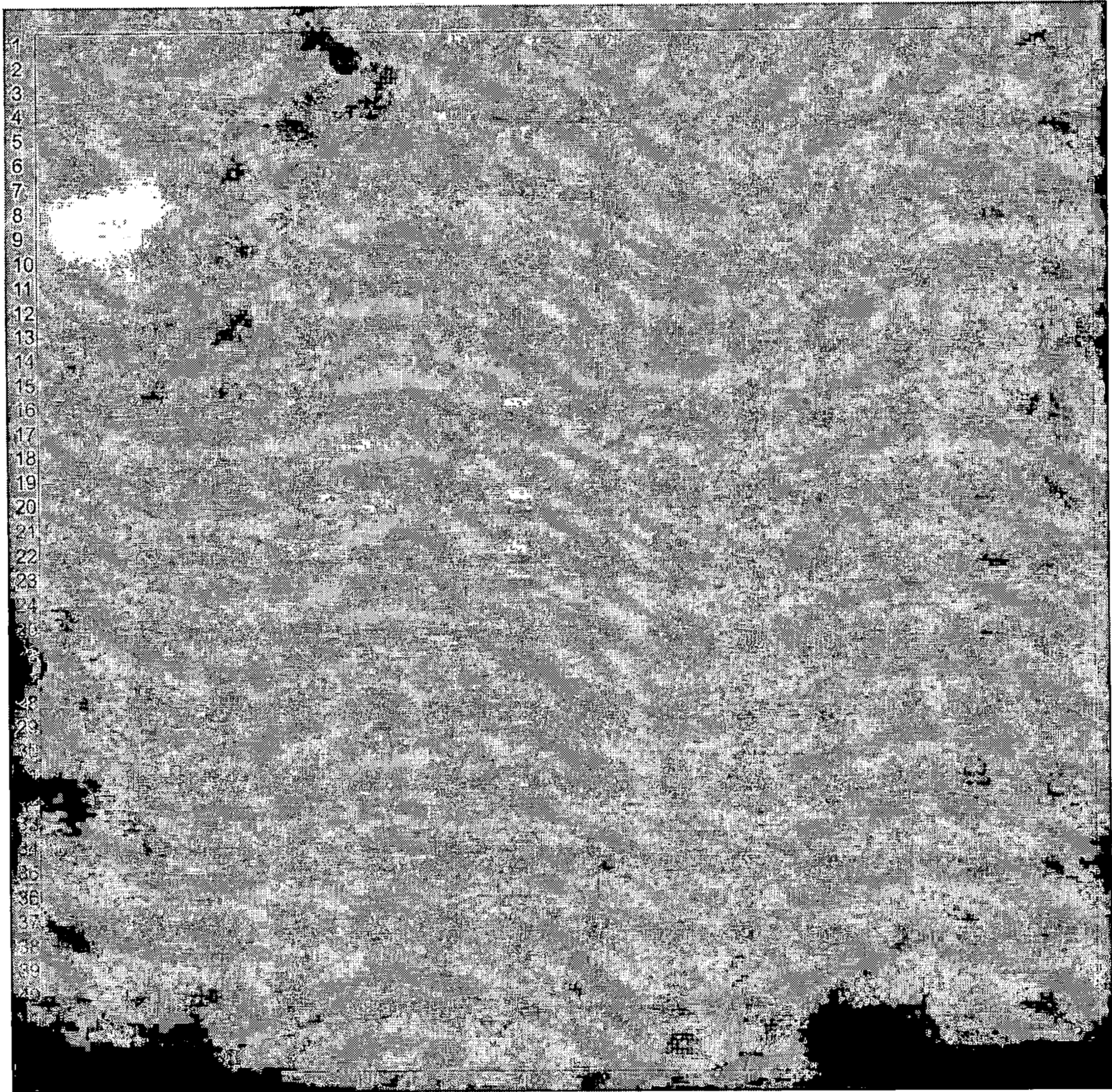


Figure 3: Spoligotyping patterns obtained from study population (C1-C45)

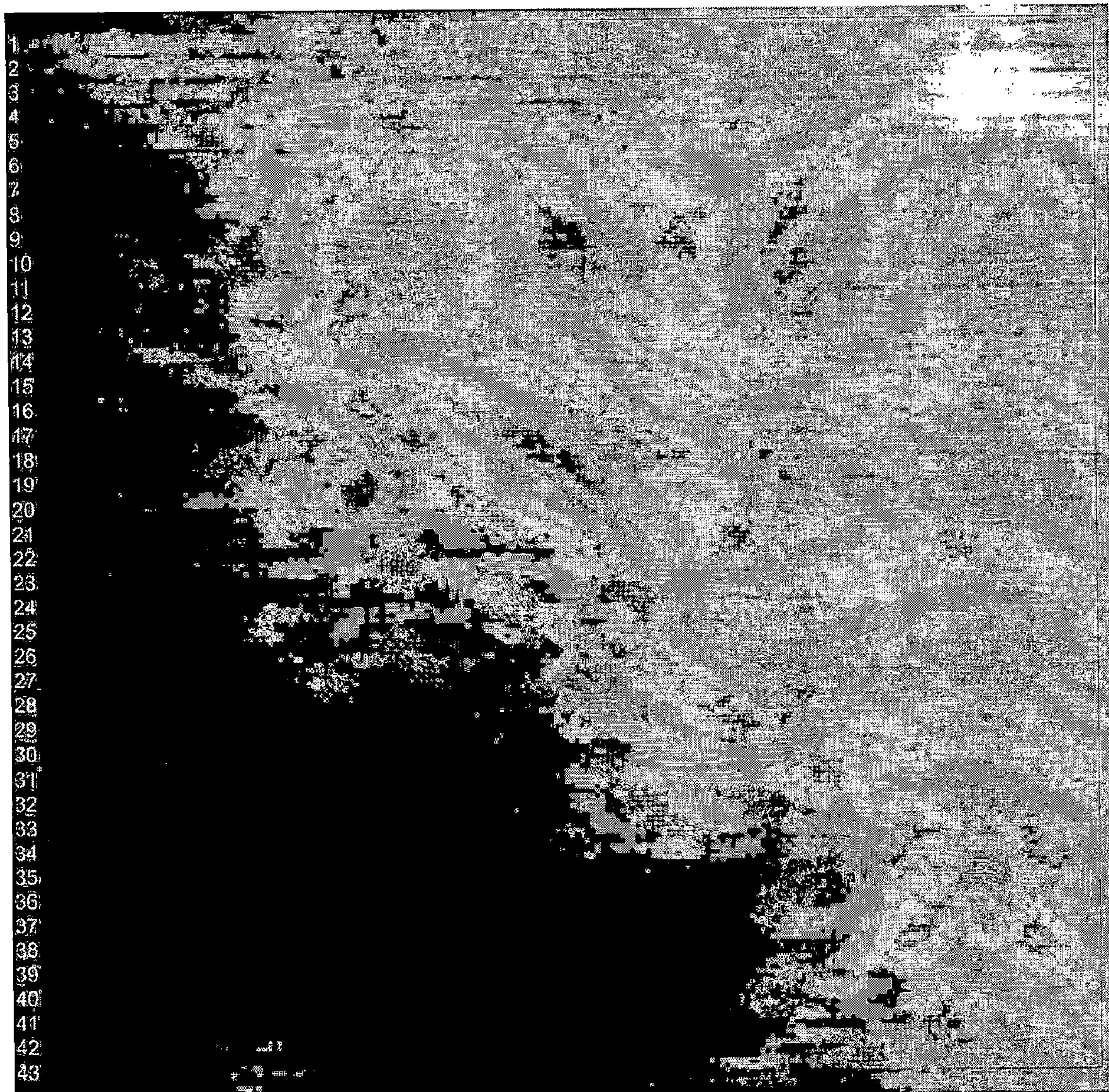


Figure 4: Spoligotyping patterns obtained from study population (D1-D45)

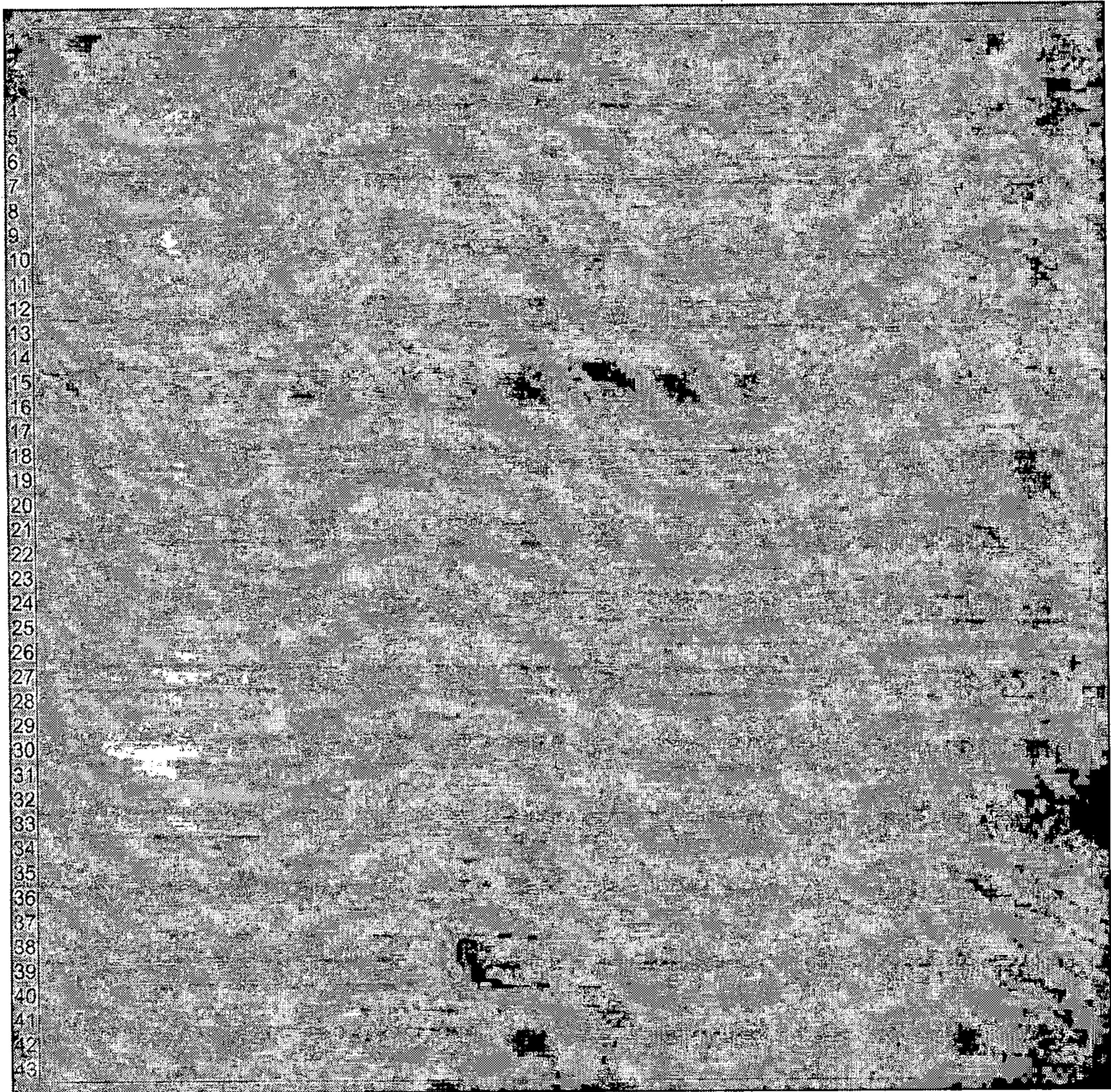


Figure 5: Spoligotyping patterns obtained from study population (E1-E45)

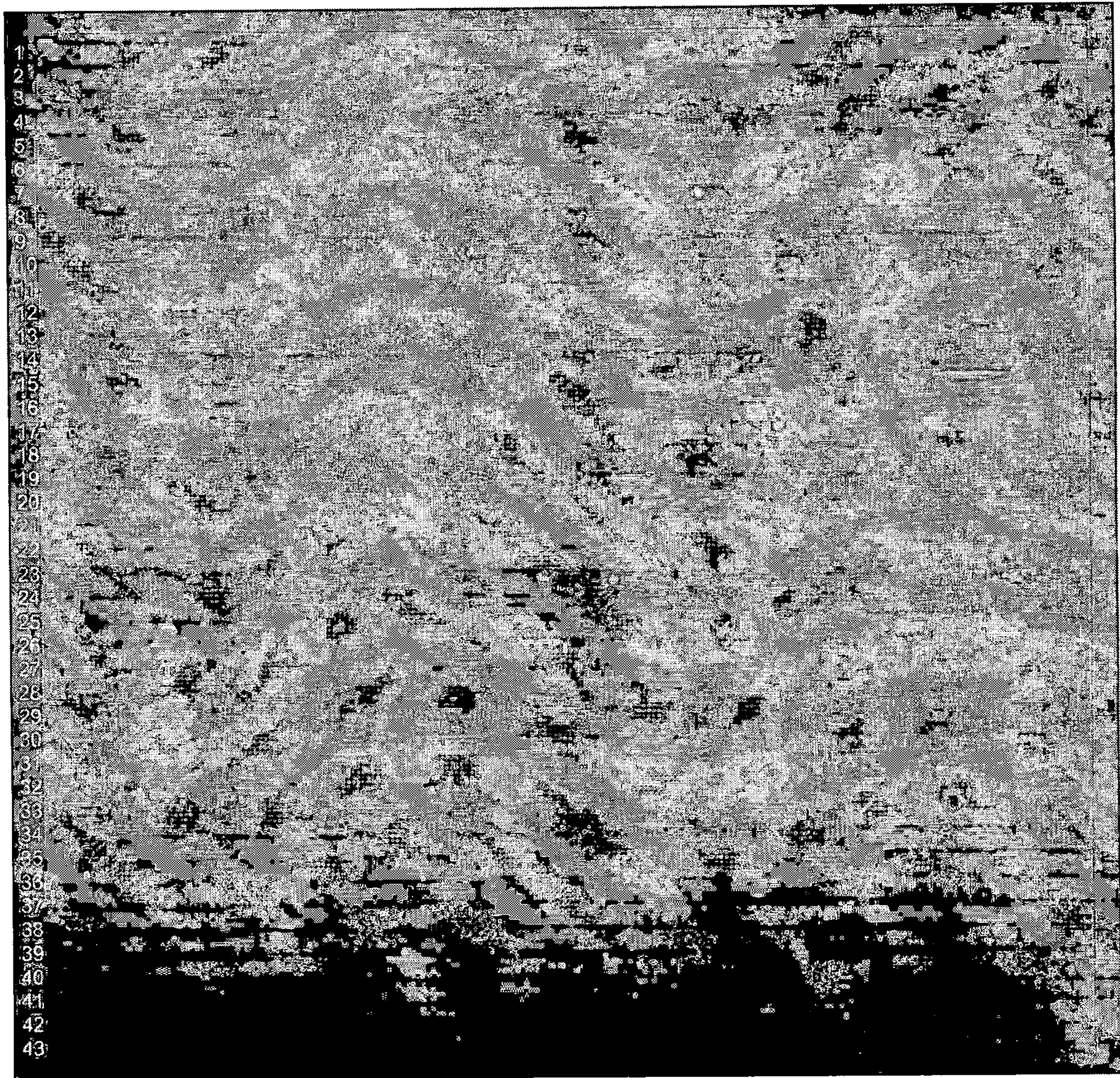


Figure 6: Spoligotyping patterns obtained from study population (F1-F45)

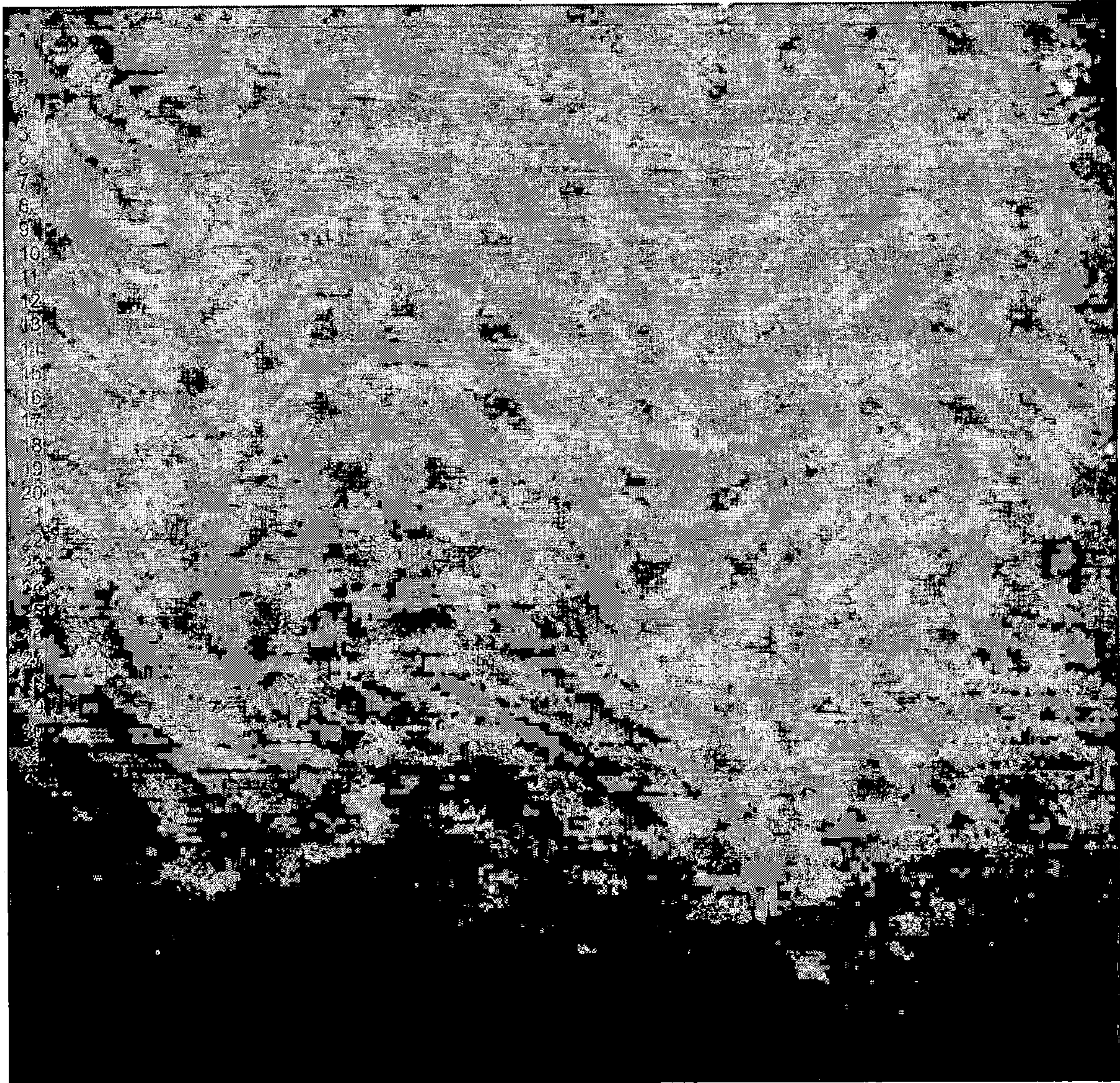


Figure 7 : Spoligotyping patterns obtained from study population (G1-G45)

Table 2: Octal Codes for the Spoligotyping patterns obtained from study population Figures A-G

	Octal Code	**Spoligotyping-based familie**
A1	034660677777031	M. africanum family with probability 0.999993966098557
G34	777740000007771	Family33 family with probability 0.712570256284271
D43	760000077777770	Family33 family with probability 0.935856698427808
A27	776274707771770	Family33 family with probability 0.951386414236296
G6	707777617771371	Family33 family with probability 0.951386419194889
G33	777760000007771	Family33 family with probability 0.970463277055791
G29	700377000177771	Family33 family with probability 0.988861945614464
A43	763717177771000	Family33 family with probability 0.991672740805973
B26	137000000376011	Family33 family with probability 0.995907388835762
B39	603140004017771	Family33 family with probability 0.996285542352182
G28	774000000616171	Family33 family with probability 0.997011141184012
G1	700600337777771	Family33 family with probability 0.997479253134596
B13	570177740037150	Family33 family with probability 0.998898846007296
D44	600414077777700	Family33 family with probability 0.99893010810634
G32	773000000017071	Family33 family with probability 0.999206180531312
D1	000004177773740	Family33 family with probability 0.999320851511222
A17	374077160603710	Family33 family with probability 0.999488291781706
A18	370077474503770	Family33 family with probability 0.999726007250184
B7	772300343776601	Family33 family with probability 0.999777867600623
G30	776676000017771	Family33 family with probability 0.999800749037297
B15	300037434037341	Family33 family with probability 0.999867803794113
A14	363003017763610	Family33 family with probability 0.999914404880324
C37	777407730007771	Family33 family with probability 0.99992734004176
F30	200777777777771	Family33 family with probability 0.999957624424991
C41	030777777777771	Family33 family with probability 0.99996470760368
F29	000777777777771	Family33 family with probability 0.99996470760368
A22	770474757655700	Family33 family with probability 0.999970653297891
A42	773617370074140	Family33 family with probability 0.99998026308869
C32	777403777603771	Family33 family with probability 0.999987571469228
D21	377777674007000	Family33 family with probability 0.99998956102134
C36	774000776077771	Family33 family with probability 0.999989785365353
A41	771717514475400	Family33 family with probability 0.999991574266125
A2	000750703437771	Family33 family with probability 0.999994548448783
C20	776003777774171	Family33 family with probability 0.999998013285857
A40	775741537755401	Family33 family with probability 0.999998436051131
C27	773601776774771	Family33 family with probability 0.999998577511626

A36	176377777763600	Family33 family with probability 0.999999151433408
A37	377770777763000	Family33 family with probability 0.999999254459057
C19	717777777774000	Family33 family with probability 0.999999554885879
A31	777577733774341	Family33 family with probability 0.999999777800286
A32	767177577774341	Family33 family with probability 0.999999777800286
B8	547300301774701	Family33 family with probability 0.999999804148486
A8	174173774077740	Family33 family with probability 0.999999885725758
A16	374177760607700	Family33 family with probability 0.999999923728999
C29	777001777637600	Family33 family with probability 0.999999945699194
A4	303741403677400	Family33 family with probability 0.999999961063528
A6	764370777467760	Family33 family with probability 0.999999976706119
A30	737147777714371	Family33 family with probability 0.999999986025088
D32	037777770377400	Family33 family with probability 0.999999993057199
C44	777477607777000	Family33 family with probability 0.999999993875425
A20	376037477773770	Family33 family with probability 0.999999999415542
G14	677177777773771	Family33 family with probability 0.999999999518895
B10	643202000077041	Family33 family with probability 0.999999999565984
A21	777737447675631	Family33 family with probability 0.999999999740715
A28	776377743775740	Family33 family with probability 0.999999999761326
A13	367003416375700	Family33 family with probability 0.99999999979043
A3	037741401475700	Family33 family with probability 0.99999999987054
G2	741610144377771	Family33 family with probability 0.999999999897981
A26	757474717773740	Family33 family with probability 0.999999999924636
B2	162076347633771	Family33 family with probability 0.999999999948708
A15	777007637677610	Family33 family with probability 0.99999999996475
G38	113000077037471	Family33 family with probability 0.999999999980856
G10	601571777777731	Family33 family with probability 0.999999999996688
G5	741774367626771	Family33 family with probability 0.999999999997323
C35	777601476037771	Family33 family with probability 0.999999999998722
A24	756174717677700	Family33 family with probability 0.999999999998888
A35	776377747767740	Family33 family with probability 0.999999999998971
C25	636377077737371	Family33 family with probability 0.999999999999684
A23	776277717657700	Family33 family with probability 0.999999999999865
G8	777757077777611	Family33 family with probability 0.99999999999989
A10	376077574177720	Family33 family with probability 0.999999999999895
A25	776163717717740	Family33 family with probability 0.999999999999917
G12	766367777777761	Family33 family with probability 0.999999999999962
G15	037417777767771	Family33 family with probability 0.999999999999966
A12	367073410177700	Family33 family with probability 0.999999999999979
C26	317607037776371	Family33 family with probability 0.999999999999981
C31	777777477777600	Family33 family with probability 0.999999999999985

B1	357767371637011	Family33 family with probability 0.999999999999991
A9	357003574077700	Family33 family with probability 0.999999999999998
C28	777601777777700	Family33 family with probability 0.999999999999999
A5	007774577777540	Family33 family with probability 1
A11	343017414177700	Family33 family with probability 1
A19	377606740077700	Family33 family with probability 1
A29	777777703716770	Family33 family with probability 1
A33	777177777717741	Family33 family with probability 1
A34	777377577707740	Family33 family with probability 1
C18	777777477777771	Family33 family with probability 1
C21	007777777777771	Family33 family with probability 1
C22	017707177777771	Family33 family with probability 1
C23	777707477777360	Family33 family with probability 1
C24	777606077777160	Family33 family with probability 1
C30	777777176077400	Family33 family with probability 1
C38	777777037777771	Family33 family with probability 1
C39	007037577777741	Family33 family with probability 1
C40	767777777377771	Family33 family with probability 1
D36	377777377777771	Family33 family with probability 1
G3	777436160177771	Family33 family with probability 1
G4	577637173737771	Family33 family with probability 1
G7	777001477777571	Family33 family with probability 1
G11	617463777777751	Family33 family with probability 1
G17	777713777757771	Family33 family with probability 1
G18	777750777737771	Family33 family with probability 1
G19	777777037677771	Family33 family with probability 1
G20	777773744377770	Family33 family with probability 1
G21	773777761777771	Family33 family with probability 1
G22	763777741777771	Family33 family with probability 1
G23	433777774777771	Family33 family with probability 1
G24	767776777377771	Family33 family with probability 1
G25	717677777777771	Family33 family with probability 1
G26	777577773777771	Family33 family with probability 1
E33	016000000000171	Family35 family with probability 0.801314900375674
F12	034000000000000	Family35 family with probability 0.847327652857628
F25	600000000000000	Family35 family with probability 0.84805584013257
F26	600000000000000	Family35 family with probability 0.84805584013257
D35	006000000000001	Family35 family with probability 0.926823317956318
E11	400400000000000	Family35 family with probability 0.999999229060464
G27	777400000000000	Family35 family with probability 0.999999791058311
G35	777600000000771	Family35 family with probability 0.999999930714249

G37	777400000001771	Family35 family with probability 0.999999992509192
G36	637400000000771	Family35 family with probability 0.999999998494107
D17	777600000001740	Family35 family with probability 0.999999998939708
B17	603000000000360	Family35 family with probability 0.999999999824544
B28	707400000000200	Family35 family with probability 0.999999999997909
F8	000000016000000	Family36 family with probability 0.513294498250952
G41	000000000400071	Family36 family with probability 0.888468874180851
G43	000000000060010	Family36 family with probability 0.941069856075252
E27	000000006000000	Family36 family with probability 0.990997027817074
E34	000000014600771	Family36 family with probability 0.996270565323266
G40	100000000010031	Family36 family with probability 0.998336930783021
B30	600000006000011	Family36 family with probability 0.999815105225541
D39	000020403777770	Family36 family with probability 0.99994665021043
D9	000000011407031	Family36 family with probability 0.999998995533959
E42	400000001400000	Family36 family with probability 0.999999462540899
F14	200000001600000	Family36 family with probability 0.999999966154745
D4	000000006010000	Family36 family with probability 0.999999992550466
D6	000000007760071	Family36 family with probability 0.99999999684081
E35	000000003400000	Family36 family with probability 0.999999997676422
D16	000000000300700	Family36 family with probability 0.999999998402267
D10	000000001400371	Family36 family with probability 0.999999998404676
D7	000010007770770	Family36 family with probability 0.999999999913632
D8	000000007777020	Family36 family with probability 0.999999999983061
D12	000000007774000	Family36 family with probability 0.999999999992924
B40	000000017777771	Family36 family with probability 0.999999999999858
D38	000000017777771	Family36 family with probability 0.999999999999858
B38	000100001777771	Family36 family with probability 0.999999999999875
D5	000000003764000	Family36 family with probability 0.999999999999901
D11	000000007770000	Family36 family with probability 0.999999999999901
D20	200000007770771	Family36 family with probability 0.999999999999916
D42	700000001037771	Family36 family with probability 0.999999999999918
D13	000000007774071	Family36 family with probability 0.999999999999997
D14	000000007774171	Family36 family with probability 1
D15	000000003704760	Family36 family with probability 1
F44	000000000777771	Family36 family with probability 1
G39	000000001774371	Family36 family with probability 1
D40	000016000777760	M. africanum family with probability 0.501119130161316
B44	200777000016000	*M. africanum* family with probability 0.983495322273467
D41	000020077066401	M. africanum family with probability 0.989160706282127
A39	774776777777400	M. africanum family with probability 0.995485337997018
B5	164163747477000	*M. africanum* family with probability 0.998426221602636

B4	760176377316000	*M. africanum* family with probability 0.998882069460612
B14	374677777177411	*M. africanum* family with probability 0.998903703348011
B3	370176377716030	*M. africanum* family with probability 0.999875801507415
A38	774770147773411	M. africanum family with probability 0.999947357629857
C45	774736603777600	M. africanum family with probability 0.999960860790053
C43	574377347777000	M. africanum family with probability 0.999996330445201
B12	770034740776021	*M. africanum* family with probability 0.999999964431919
C42	031761777777400	M. bovis-BCG family with probability 0.892068002063187
A7	070170416077600	M. bovis-BCG family with probability 0.999945374025301
E9	004000000000000	M. microti family with probability 0.989824772902669
E38	000000001000000	M. microti family with probability 0.99968358439212
E41	000000000200000	M. microti family with probability 0.999683584444811
F4	000010000000000	M. microti family with probability 0.999712727851109
F36	000000000000200	M. microti family with probability 0.999964699800036
B41	000000140000100	*M. tuberculosis* Beijing family with probability 0.869484347747094
E31	000000000000020	M. tuberculosis Beijing family with probability 0.927646980105896
F3	000000000000001	M. tuberculosis Beijing family with probability 0.927650337782549
E19	000000000000060	M. tuberculosis Beijing family with probability 0.927942175929571
E20	000000000000060	M. tuberculosis Beijing family with probability 0.927942175929571
F33	000000000000030	M. tuberculosis Beijing family with probability 0.92794276454255
F35	000000000000030	M. tuberculosis Beijing family with probability 0.92794276454255
G42	000000000000031	*M. tuberculosis* Beijing family with probability 0.927943826902957
F41	000000000000071	M. tuberculosis Beijing family with probability 0.92794407743198
F42	000000000000071	M. tuberculosis Beijing family with probability 0.92794407743198
B33	000000000001600	*M. tuberculosis* Beijing family with probability 0.999660917952642
F34	000000000002000	M. tuberculosis Beijing family with probability 0.999660921566655
D3	000010000007000	M. tuberculosis Beijing family with probability 0.999978497128319
D23	000000000007371	M. tuberculosis Beijing family with probability 0.999978642844673
E32	000000400002031	M. tuberculosis Beijing family with probability 0.999999815290408
D19	000000000003011	M. tuberculosis Beijing family with probability 0.999999999982487
F43	000000000003771	M. tuberculosis Beijing family with probability 0.999999999982487
G9	700376740100271	M. tuberculosis CAS family with probability 0.99531244875026
B42	000007700000431	*M. tuberculosis* CAS family with probability 0.999633831897278
B43	000773600002000	*M. tuberculosis* CAS family with probability 0.999890597300842
B18	702027700000170	*M. tuberculosis* CAS family with probability 0.999980282296159
E17	340001760000000	M. tuberculosis EAI1 family with probability 0.560600769307378
D27	000000304000020	M. tuberculosis EAI1 family with probability 0.577273211743168
F1	000200340000000	M. tuberculosis EAI1 family with probability 0.761710857918745
E36	000001003000000	M. tuberculosis EAI1 family with probability 0.764746559858998
D37	003776000000000	M. tuberculosis EAI1 family with probability 0.852674330246765
E43	000004300000000	M. tuberculosis EAI1 family with probability 0.905718708795168

F2	000000070400000	M. tuberculosis EAI1 family with probability 0.987628413139816
E13	000070040000000	M. tuberculosis EAI1 family with probability 0.987876158736076
F11	000034600000000	M. tuberculosis EAI1 family with probability 0.991368696311718
B24	107014000000011	*M. tuberculosis* EAI1 family with probability 0.996598954895583
E37	000001600000000	M. tuberculosis EAI1 family with probability 0.99671928320734
F5	000020036000000	M. tuberculosis EAI1 family with probability 0.998740076690006
E14	000034034000001	M. tuberculosis EAI1 family with probability 0.999511225493188
B23	355700174000000	*M. tuberculosis* EAI1 family with probability 0.999621413269238
D29	377002002000000	M. tuberculosis EAI1 family with probability 0.999736343881002
D31	161777774000000	M. tuberculosis EAI1 family with probability 0.999770400290389
D28	177001401000000	M. tuberculosis EAI1 family with probability 0.999993631876183
F6	000063203000000	M. tuberculosis EAI1 family with probability 0.999999922395467
C34	777001777003571	M. tuberculosis EAI5 family with probability 0.992704821063277
B20	775607457720360	*M. tuberculosis* H37Rv family with probability 0.488319703510718
B22	047472176000171	*M. tuberculosis* Haarlem1 family with probability 0.906739123842075
B32	007354060000300	*M. tuberculosis* Haarlem1 family with probability 0.990064115830236
F13	000000004000000	M. tuberculosis Haarlem2 family with probability 0.99564928126573
D33	001777770300060	M. tuberculosis Haarlem3 family with probability 0.769410323785737
C33	777407777600771	M. tuberculosis Haarlem3 family with probability 0.771058984595225
D22	377777777700771	M. tuberculosis Haarlem3 family with probability 0.772435628564132
B29	617777000100060	*M. tuberculosis* LAM1 family with probability 0.501141009412426
B21	376006003000171	*M. tuberculosis* LAM3 family with probability 0.765661540445343
G44	000140000070021	*M. tuberculosis* LAM7 family with probability 0.639010520584711
B34	740000000006000	*M. tuberculosis* LAM7 family with probability 0.794458709410681
E10	140000000000000	M. tuberculosis LAM7 family with probability 0.795502577290285
E12	140000000000000	M. tuberculosis LAM7 family with probability 0.795502577290285
D34	360000000210000	M. tuberculosis LAM7 family with probability 0.908057175593716
E30	000017000000000	M. tuberculosis LAM7 family with probability 0.996160132987302
F9	000003000000000	M. tuberculosis LAM7 family with probability 0.999673024712485
F10	000007000000000	M. tuberculosis LAM7 family with probability 0.999674206671001
D2	000061400010000	M. tuberculosis LAM7 family with probability 0.999751420413527
F17	000003400000000	M. tuberculosis LAM7 family with probability 0.999782859256213
B31	014120000000000	*M. tuberculosis* LAM7 family with probability 0.999997094239657
B11	574317700000140	*M. tuberculosis* LAM8 family with probability 0.996210328093751
B35	077370000000730	*M. tuberculosis* LAM8 family with probability 0.9998953357088
G31	775774000000171	*M. tuberculosis* LAM8 family with probability 0.999908688971885
G16	777603777760011	M. tuberculosis T1 family with probability 0.307749214486593
E15	377777777770000	M. tuberculosis T1 family with probability 0.572939570419449
B6	362706000370411	*M. tuberculosis* T1 family with probability 0.754009511335925
B9	706307777770100	*M. tuberculosis* T1 family with probability 0.819745716206777
G13	742035777770761	M. tuberculosis T1 family with probability 0.852427627574695

D24	377777377770071	M. tuberculosis T1 family with probability 0.979525663474976
A44	763717377741000	M. tuberculosis T2 family with probability 0.685664816184957
B25	160777777600011	*M. tuberculosis* T2 family with probability 0.892775696479356
D30	000377777740000	M. tuberculosis T2 family with probability 0.999841235508047
E16	377777777760000	M. tuberculosis T2 family with probability 0.999884499331622
F31	000000740340000	M. tuberculosis T3 family with probability 0.634833229291429
E39	000000613300000	M. tuberculosis T3 family with probability 0.705246382584128
B16	200000760020161	*M. tuberculosis* T3 family with probability 0.777500768546776
E44	000000160020000	M. tuberculosis T3 family with probability 0.869144363261653
E40	000001600100000	M. tuberculosis T3 family with probability 0.949048671282111
F28	000000037600171	M. tuberculosis T3 family with probability 0.988849215427887
B19	574000600170771	*M. tuberculosis* T3 family with probability 0.999928931318462
D18	777400003743740	M. tuberculosis T4 family with probability 0.999999999065663
B36	001604160060600	*M. tuberculosis* X2 family with probability 0.779243000248544
B37	000003766300060	*M. tuberculosis* X3 family with probability 0.778038571566262
B27	000030003000171	*M. tuberculosis* X3 family with probability 0.999112713289289

SECTION 4

Impact of Research Results

i) Relevance of results achieved to scientific advancement

It is widely accepted that TB is one of the most important threats to human health on global scale. Infection with *M.tuberculosis* complex causes the greatest number of deaths by a single infection agent. Molecular Epidemiology is a field that has emerged largely from the integration of molecular biology, clinical medicine, statistics and epidemiology. In essence, molecular epidemiology focuses on the role of genetic and environmental risk factors, at the molecular /cellular or biochemical level in disease aetiology and distribution among populations.

IS *6110* RFLP analysis is the Gold standard for the molecular epidemiology of *M.tuberculosis* complex strains. The epidemiological analysis of TB using IS*6110* is based on the observation that the polymorphism of IS*6110* RFLP patterns among unrelated clinical isolates is high, where as epidemiologically related *M.tuberculosis* strains show identical or similar (one band variation) finger prints. In this study an extensive diversity in patterns for isolates with both > 6 < IS *6110* insertions were seen. Previous studies showed that *M.tuberculosis* strains carrying one or few IS *6110* copies are often difficult to differentiate by IS *6110* standard RFLP analysis because of a site specific preference for insertion of the IS element. In the present study except for three strains, the location of the bands in fingerprints were different and therefore the location of IS *6110* elements in the chromosomal DNA. Therefore *M.tuberculosis* strains carrying one or few IS *6110* copies were differentiated without difficulty. That means none of the isolates were clinically related and IS *6110* still remains the single most discriminatory technique for the analysis of *M.tuberculosis* isolates. Spoligotyping is the simplest technique for *M.tuberculosis* complex genotyping. This technique is ideal for a first step analysis of *M.tuberculosis* particularly in regions with diverse populations. Spoligotyping revealed a total of 24 families including the nine major families. The most predominant group among the isolates of *M.tuberculosis* corresponded to Family33. In this family, only spacers 33-34 were absent and recently described clade MANU of Indian origin also belongs to this family. According to the analysis, bacterial strains were distributed among all three principal genetic groups PGG1, PGG 2 and PGG3. Segregation of *M.tuberculosis* into 'ancestral' versus 'modern' lineages based on PGG indicates that isolates from Kandy have originated from both lineages. Spoligotyping patterns had a high strain diversity and except for two strains 000000000003771 (ST1) and 000000000000031(ST 585) the tested strains were not defined in the latest spoligotype data bases SpolDB4/SITVIT. These two techniques have enabled both short term (local epidemiological) such as outbreaks, and long term (global epidemiological) investigations such as understanding spatiotemporal transmissions and evolutionary dynamics.

Due to study constraints, we could not complete the cluster analysis on RFLP & spoligotyping and after completion of it in due course we will be able to identify the risk factors associated with TB transmission as well as the evolution of *M.tuberculosis* in Kandy, Sri Lanka.

ii) **Relevance of results achieved to national / socio-economic development**

TB, primarily affects young, between the ages 15 and 54, and the less affluent, who nevertheless, form the backbone of the economy of the country. According to our results 90% of the population was in the age group of between the ages 16 and 60. WHO estimated sputum smear positive pulmonary TB rate for Sri Lanka is 32 (3 year average), 2006, 2007, 2008. One of the most important challenges in the control of TB is a rapid diagnosis of cases and the optimization of anti tuberculous treatment, mainly to prevent the development of resistance and the dissemination of resistant strains. It has been noted that the DNA polymorphism could be made use of to identify transmission rates of drug resistance and drug sensitive strains. RFLP typing can be carried out on primary isolates to determine drug resistance. By comparison of these isolates with the existing RFLP patterns of the drug resistance isolates the time taken for determining drug resistance may be much shorter compared to the conventional antibiotic sensitivity testing which takes more than four weeks.

Spoligotyping, a new method for simultaneous detection and typing of *M.tuberculosis* complex bacteria is based on PCR technique and avoids the timing problems associated with the slow growth of the bacteria. Being a rapid method spoligotyping can be used in clinical setting for diagnostic purposes as well as for epidemiologic studies for TB transmission.

This is the first study in Sri Lanka in which both the RFLP pattern of *M.tuberculosis* strains and the spoligotyping in a population has been examined. In most of the countries the RFLP and spoligotyping patterns are recorded from each tuberculosis patient and data are deposited for future reference in treatment. **In this study we have demonstrated the feasibility of establishing molecular typing methods in Sri Lanka specially in spoligotyping without using any commercial kits. At present, TB patients in Sri Lanka get free treatment. If chronic cases could be prevented and/or cured this could lead to considerable savings in the national economy. It is now widely appreciated that better health has an important role in reducing poverty and promoting economic growth.**

iii) Dissemination / application of research output

If necessary, the spoligotyping can be established at Institute of Fundamental Studies, Kandy and services can be provided to any interested Institute either in Government or private sector.

SECTION 5

Miscellaneous

i) List of Major equipment acquired during the project period and their functionality

1) DNA Thermal Cycler - Functioning well.

2) Mini Blotter - Functioning well.

ii) List of publications / communications arising from the project and / or presentations made at seminars, workshops etc.

1. Meegahakumbura M G K M, Ambalavanar V, Madegedara R M D, Thevanesam V, **Magana-Arachchi D N*** (2008) Socio -demographical features among the tuberculosis patients attending the Central Chest Clinic, Kandy – A Preliminary study, *Proceedings of the Kandy Society of Medicine, 30th Annual sessions,* 30;Pages 95-96:47.

Publications; Two manuscripts are in preparation.

47. SOCIO -DEMOGRAPHICAL FEATURES AMONG THE TUBERCULOSIS PATIENTS ATTENDING THE CENTRAL CHEST CLINIC, KANDY – A PRELIMINARY STUDY

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Background: *Mycobacterium tuberculosis*, the etiological agent of Tuberculosis (TB) is one of the most effective human pathogens and is responsible for 3 million deaths every year. It is estimated that a total of 225 million new cases and 79 million deaths will occur between 1998-2030. TB had been a dreaded disease in Sri Lanka for centuries. In 2005, 9248 new cases of tuberculosis have been reported in Sri Lanka.

Objectives: The purpose of our survey was to study the relationship between AFB smear positivity on direct examination of sputum with culture positivity and to determine the risk factors for the development of TB among the sputum positive tuberculosis patients.

Methodology: In the present study, 57 Questionnaires were administered to patients who were positive for acid fast bacilli, on direct examination of sputum by Ziehl – Neelsen stain attending the Central Chest Clinic Kandy from February till August 2007. Early morning sputum samples were collected and decontamination of specimens was carried out using 4% NaOH/ 2.9% NaCitrate. *M. tuberculosis* strains were grown on LJ slopes for 2 months at 37°C. Statistical analysis was done using the Epi Info 6.0 package.

Results: Of the 57 specimens, 71.9% became culture positive. The patient population consisted of 63.8% males and 36.2% females. There were no patients in the age group of 0-15 years and the findings revealed that the peak age group of patients (49.1%) was in the range of 16-45years. The analyzed data showed that the level of education of 59.7% of the patients was between Grade 1-9 and 61.1% of them belonged to Social class V (non skilled manual). None of the patients were professionals. Of the 57 patients, 22.8% had a positive contact history of TB. 80.7% of the population did not have any other illness and 17.5% had diabetes mellitus. Among the male population 89.2% were smokers and 94.6% had the habit of drinking.

Conclusions: The findings revealed that only 71.9% of the sputum positive specimens were culture positive which might be due to the dry sputum, false sputum positivity on direct examination by Ziehl – Neelsen, or over decontamination of sputum. This study showed that tuberculosis primarily affects young, otherwise healthy males, who are the backbone of the economy of this community and there was a correlation between smoking and drinking habit among the male population and the development of TB. Diabetes mellitus was another risk factor for the development of TB.

Key words: Mycobacterium tuberculosis, Social class V

ETHICAL CLEARANCE CERTIFICATE

This is to certify that the Committee on Research and Ethical Review,
Faculty of Medicine, University Peradeniya
received
the research proposal on

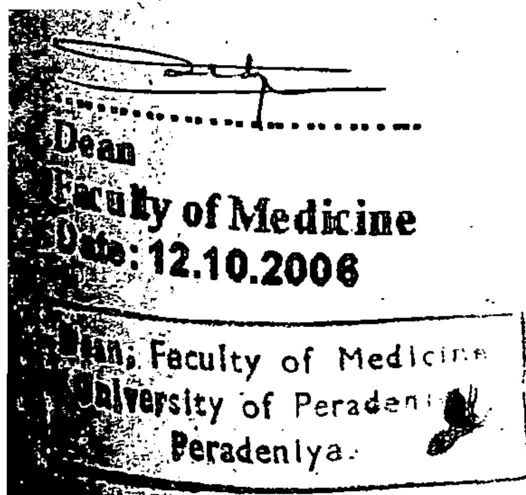
"Restriction Fragement length polymorphism (RFLP) analysis & spoligotyping on M. Tuberculosis strains isolated from patients attending the Central Chest Clinic Kandy"

submitted by

Dr. D.N Maganaarachchi
of

Institute of Fundamental Studies
on
12th July 2006

The Committee is satisfied that the said study has taken into consideration all ethical aspects in its implementation and granted Ethical Clearance
at it's meeting held on
12th October 2006.



Dean
Faculty of Medicine
Date: 12.10.2006

Chairperson
Committee on Research and Ethical Review
Date: 12.10.2006

SECTION 6

Summary Statement of Expenditure (indicate under Personnel, Equipment, Consumables, Travel and subsistence and Miscellaneous)

The financial position of grant No.NSF/ RG/2006/HS/07 as at 31/07/2009 awarded to Dr.D .N. Magana Arachchi by National Science Foundation is as follows.

		Funds received by the Univ./ Institution	Total expenditure Rs.	Balance available Rs
Personnel -	Research Student	570,000.00	571,717.37	(1,717.37)
	Technical Assistant	-	-	
	Other	23,507.00	23,505.12	1.88
Equipment -	Foreign	503,165.00	854,231.96	(351,066.96)
* Mini Blotter				
* Thermal Cycler Block				
	Local	-	-	-
Consumables -	Foreign	714,840.00	364,172.34	350,667.66
	Local	-	-	-
Travel & Subsistence		7,875.00	7,437.50	437.50
Miscellaneous		7,000.00	5,322.71	1,677.29
	Total	1,826,387.00	1,826,387.00	NIL

Unspent balance of the funds received -

Fund received	Rs.	1,826,387.00
Actual expenditure		1,826,387.00
Balance		-
Cash Imprest/Cash advance		-
Balanceas at 31/07/2009		NIL

True Copy

ACCOUNTANT
Institute of Fundamental Studies
HANTANA ROAD,
KANDY
29/10/2010

....
Bursar/Accountant
ACCOUNTANT
Institute of Fundamental Studies
KANDY

Date: 17/08/09

* Mini Blotter 192,981.96
* Thermal Cycler Block 661,250.00

SECTION 7

i) Grantee's signature; D. N. Magana Arachchi

ii) Comments of the Head of the Department / signature;

iii) Head of the Institution's signature;

Director
Institute of Fundamental Studies
Hantana Road
Kandy

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