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The Use of DNA Extracts from Ziehl-Neelsen Stained Slides for Spoligotyping in National Reference TB Laboratory of Iran

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Spoligotyping is a molecular typing method that is used to differentiate *M.tuberculosis* complex. The method is based on polymorphism of the chromosomal DR locus consisting of identical 36-bp DRs alternating with 35-41 unique spacers. In this study, the spoligotyping was applied to Ziehl-Neelsen stained smears and its utility in smears with different positivity rate was assessed. DNA were extracted from smears (n=115) with different positivity rate [3+ (39; 33%), 2+ (28; 24), 1+ (33; 28%) and Scanty (15; 13%)]. Thirty negative smear slides were also used as a control. PCR was performed with primers specific for direct repeats and the product was detected by ECL hybridization kit. The obtained data entered the X-file and were analyzed with previously published database (spolDB4).

The utility rate in 3+, 2+ and 1+ smears were 84.6%, 71.4%, 36.4% respectively ($p<0.01$). The smears with scanty results could not produce any patterns. In overall, the spoligopatterns was observed in 56.5% of samples. The predominant spoligopatterns identified as Haarlem (47.6%), CAS (26%) and T (15.3%). Spoligotyping can be reliable in smears with high positivity rate. Therefore, the method may be the best option to identify the circulating strains within limited resources laboratories.

Key words: Ziehl-Neelsen slides, Spoligotyping, Molecular epidemiolog.

The last decade has seen a dramatic recovery in the incidence of tuberculosis (TB) all over the world and an increase need for more rapid methods to diagnose and prevent distribution of this disease (1). According to the world health organization (WHO) the estimated incident rate of TB in Iran is 22 cases per 100,000 populations (2). Whereas, the notification rate is less than 75%. This means a large number of cases are missed in the early stages of diseases. Laboratory diagnosis relies on direct examination of smears by the Ziehl-Neelsen (ZN) staining and it is used in most laboratories where culturing is usually impossible. Early diagnosis, effective treatment and successful cessation of transmission are major strategies in the control of TB[3]. For molecular investigation the sputum samples generally transported to National Reference TB Laboratory (NRL) in Tehran. Although, due to large distance and improper transport systems, the samples from remote cities near the border either get contaminated or never reaches the NRL. As a consequence, the clonal structure of *M.tuberculosis* especially in these provinces is not very clear[4]. Recently, it was shown

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that spoligotyping is sensitive to detect mycobacteria from extract of ZN-stained slides[3,5,6]. Spoligotyping permits rapid identification and differentiation of *M.tuberculosis* complex strains. The method requires minimal amounts of DNA and is based on polymorphism of the chromosomal DR locus consisting of identical 36-bp DRs alternating with 35-41 unique spacers[7]. Previously, we used spoligotyping for epidemiological studies, but we did not know its utility in stained slides. The purpose of the present study was to carry out Spoligotyping on DNA samples extracted from ZN-stained slides. This technique may have application in identification of spoligotypes in areas remote from research laboratory and would also increase our knowledge about the clonal structure of M.tuberculosis in the population when applied to old samples in different locations.

MATERIAL AND METHODS

Ziehl-Neelsen (ZN)- stained slides

Hundred fifteen positive ZN-stained smears [3+ (39), 2+ (28) and 1+ (33) scanty (15)] from sputum samples of patients who attended National Research Institute of Tuberculosis and lung diseases, Tehran, were used for this study (2009-2010). Thirty negative smears included as a control. The slides were randomly picked up from boxes which stored over one year old.

Extraction of DNA

First the slides were rinsed with sterile distilled water (500µl/per slide) and then the ZN-stained layer were scraped off from the microscopic slides. The content was transfer into microtubes and 1000 µl of 50% sucrose was added and the mixture incubated at 100°C for 30 min. Thereafter, the tubes centrifuged at 14500×g for 10 min, supernatant removed and 200µl of distilled water was added to each sediment. DNA extraction followed on sediment using QIAamp® DNA Mini Kit (Qiagen, Germany) according to company protocol.

Spoligotyping

Spoligotyping was performed as previously described by Kamerbeek et al (7). In brief DR region was amplified by PCR using 20 pmol of primers DRa (5'-CCG AGA GGG GAC GGA AAC- 3') and DRb (5'- GGT TTT GGG TCT GAC GAC-3'), 20-50ng of DNA and 0.5 U of Hot Start Taq plus DNA polymerase (Qiagen). The PCR condition was: 40 cycles of 1 min at 95°C, 1 min at 55°C and 30 sec at 72°C. The first denaturation and final extension steps were held for 10 min. The amplified DNA was hybridized to a set of 43 immobilized oligonucleotides derived from the spacer sequences of MTB H37Rv and M.bovis BCG P3 by reverse line blotting. Hybridized DNA was detected with ECL detection liquid (Amersham, Buckinghamshire, UK) and by exposing ECL-Hyper film (Amersham) to the membrane for 1 h.

Statistical analysis

The result obtained were entered into a binary format as excel spreadsheets (Microsoft) and compared with published data[8-11].

RESULTS

In overall, sixty five samples (56.5%) yielded reproducible spoligopatterns whereas 50 samples (43.5%) showed incomplete or no patterns. All obtained spoligoptterns clustered into 5 *Mycobacterium tuberculosis* super families [Haarlem (47.6%), CAS (26%), T (15.3%), Beijing (7.7%), and EAI (3%)].

Table 1. Number and percentage of reliable and incomplete patterns obtained in this study

Positivity rate	Number	Reliable patterns	Incomplete or no patterns
Scanty	15	0 (0%)	15(100%)
1+	33	12(36.4%)	21(63.6%)
2+	28	20(71.4%)	8(28.6%)
3+	39	33(84.6%)	6(15.4%)
Sum	115	65 (56.5%)	50(43.5%)

All Shared types obtained in this study are demonstrated in Table 2. The first and second predominant spoligotype were shared type 127 (32.3%) and ST 294 (15.3%), both belonged to Haarlem family, followed by ST 53 belonged to T calde (15.3%), ST 381 and 428 together belonged to CAS family (15.3%), ST 1 belonged to Beijing family (7.7%), ST 26 belonged to CAS family (6.1%), ST 288 belonged to CAS family (4.6%) and ST 236 belonged to EAI family. H3, CAS1_DELHI, and Beijing strains was not found in 1+ smears whereas CAS2 and CAS1_KILI spoligotypes was not found in 2+ and 3+ smears, respectively.

Moreover, EAI5 strains was found only in 3+ smears. In overall, prevalence of H4 strains in all slides with 1+, 2+ and 3+ positivity was higher than other strains (Table 2).

Table 2. Number and percentage of M.TB families obtained in this study

Spoligotype	ST	Spoligopattern	No. of isolates (%)	1+	2+	3+
H4	127	nonnnnnnnnnnnnnnnnnnnnnnnnnnnnnnooonoooonnnnnnn	21(32.3)	5	6	10
H3	294	nonnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnonoooonnnnnnn	10(15.3)	0	2	8
T1	53	nnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnooonnnnnnn	10(15.3)	3	3	4
CAS1-DELHI	428	nnnoooonnnnnnnnnnnnnnnnnooooooooooooonnonnnnnn	10 (15.3)	0	4	6
	381	nnnoooonnnnnnnnnnnnnnnnnooooooooooooonnooonnnn				
Beijing	1	ooooooooooooooooooooooooooooooooooonnnnnnnnn	5 (7.7)	0	3	2
CAS1-KILI	26	nnnoooonnnnnnnnnnnnnnnnnooooooooooooonnnnnnnnn	4 (6.1)	2	2	0
CAS2	288	nnnooooooonnnnnnnnnnnnnooooooooooooonnnnnnnnn	3 (4.6)	2	0	1
EAI5	236	nnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnooooonnnnnnnnn	2 (3)	0	0	2

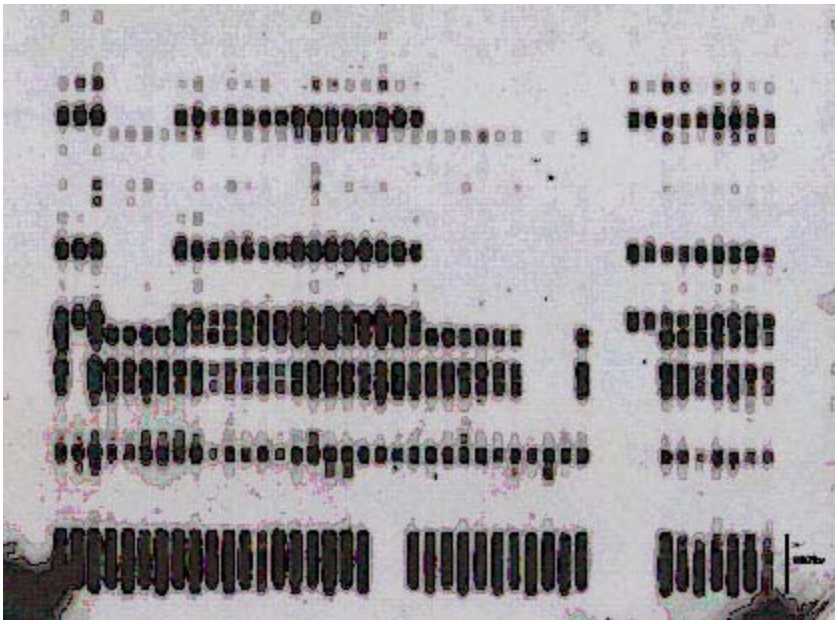

Fig. 1. Spoligo patterns obtained from extracted DNA of ZN-slides

DISCUSSION

The accessibility of Ziehl-Neelsen slides in routine diagnostic laboratories, and the ease of storage and transporting the slides, makes spoligotyping applicable method in areas where *M.tuberculosis* is not routinely cultured[5]. At present 345 peripheral and intermediate TB laboratories are functioning within the country. The main diagnostic tools in these laboratories are smear microscopy.

This study was carried out to optimize spoligotyping method on smear slides. As a consequence we may use the slides for further epidemiological studies. For this purpose, one year old ZN-stained positive slides were selected. In the study by Swarnkar *et al.* spoligotyping was applied to 3-4 month old ZN-slides, out of 42 slides, 41 samples yielded a PCR product. In mentioned study, all slides had 2+ and 3+ positivity rate and no 1+ or scanty smears were examined[6]. In our study, 36.4% of 1+ smears yielded a PCR product (table 1) but we also could not get any pattern from scanty smears. Therefore, the scanty smears are not suitable for typing or DNA extraction. Although the utility rate was increased with smear positivity rate *i.e.* in 3+ smears the possibility of reproducible patterns was 84.6% In contrast to 36.4% for 1+ smears ($p<0.05$). Therefore, the microbial load of *M.tuberculosis* is a key factor for better amplification. Similarly, Suresh *et al.*,[5] reported high signal from 2+ and 3+ smears for spoligotyping. Spoligotyping has been also applied successfully in paraffin wax embedded tissues, freshly prepared slides and decayed lung[12,13,14]. This technique is cost effective, easy to perform and provides the rapid typing of *M.tuberculosis* complex[15]. And it might be of practical use for the rapid diagnosis of infections caused by Beijing or some other strains related to drug resistance[12]. Our results showed the high prevalence of Haarlem super families (48%) The second and the third predominant families were CAS and T with 26% and 15.3% respectively (table 2). Previously, Velayati *et al* reported the CAS and T clade as the circulating strains of Tehran[16,17]. Although, due to improper laboratory facilities we have no clear estimation about different sub families prevalence within the country, that is why we may proposed to used Ziehl-Neelsen slides as a based material for spoligotyping.

CONCLUSION

In conclusion, spoligotyping from smear slides would help the national tuberculosis control program (NTP) to identify the clonal structure of *M.tuberculosis* strains in areas which do not routinely culture this bacteria.

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REFERENCES

1. Gori, A., Bandera, A., Marchetti, G. Spoligotyping and *Mycobacterium tuberculosis. Emerg Infect Dis.,* 2005; **11**(4): 1242-1248.
2. World Health Organization (WHO). 2009. Global Health Atlas. Geneva: WHO. Available online at: http://www.who.int/globalatlas/dataQuery/default.asp.
3. Van der Zanden, A.G.M., Te Koppele-Vije, E.M., Vijaya Bhanu, N. Use of DNA Extracts From Ziehl-Neelsen-stained slides for molecular detection of rifampin resistance and spoligotyping of *Mycobacterium tuberculosis. J Clin Microbiol.* 2003; 1101-1108.
4. Rohani, M., Farnia, p., Naderi Nasab, M., Beijing Genotype and Other Predominant *Mycobacterium tuberculosis* Spoligotypes Obsereved in Mashhad City, Iran. *IJMM.,* 2009; **27**(4): 306-10.
5. Suresh, N., Arora, J., Pant, H. Spoligotyping of *Mycobacterium tuberculosis* DNA from archival Ziehl-Neelsen-stained sputum smears, *Journal of Microbiological Methods.* 2007; **68**: 291-295
6. Swarnkar, p., Katoch, V.M., Soni, H., Spoligotyping of *Mycobacterium tuberculosis* Strains from Ziehl-Neelsen Stained Sputum Slides from DTC, Satna (M.P.). *International Journal of Research in Pharmaceutical and Biomedical Sciences.,* 2010; **1**(2) : 97-101
7. Kamerbeek, J., Schouls, L., Kolk, A. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *J Clin Microbiol.* 1997; **35**:

907-14.

8. Sola, C., Filliol, I., Gutierrez, CM., Morkrousouv, I., Vincent, V., Rastogi, N., et al. Spoligotype database of *Mycobacterium tuberculosis*: biogeographical distribution of shared types and epidemiological and phylogenetic perspectives. *Emerg Infect Dis.*, 2001; **7**: 390-6
9. Soini, H., Pan, X., Amin, A. Characterization of *Mycobacterium tuberculosis* isolates from patient in Houston, Texas, by spoligotyping. *J Clin Microbiology.,* 2000; **38**: 669-79
10. Filliol, I., Discroll, JR., van Soolingen, D.Snapshot of moving and expanding clones of *M.tuberculosis* and their global distribution assessed by spoligotyping in an international study. *J Clin Microbiol,*. 2003; **41**: 1963-70
11. Niobe-Eyangoh, SN., Kuaban, C., Sorlin, P. Genetic biodiversity of *M.tuberculosis* complex strains from patients with pulmonary tuberculosis in Cameroon. *J Clin Microbiol,* 2003; **41**: 2547-53.
12. Van der Zanden, A.G.M., Hoentjen, A.H., Hielmann, F.G.C. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* complex in paraffin wax embedded tissues and in stained microscopic preparations. *Mol Pathol.* 1998; **51**: 209-214.
13. Qian, L., Van Embden, J.D.A., Van de Zanden, A.G.M. Retrospective analysis of the Beijing family of *Mycobacterium tuberculosis* in preserved lung tissues, *J clin Microbiol* 1999; **37**: 471-474.
14. Zink, A.R., Sola, C., Reischl, U. Characterization of *Mycobacterium tuberculosis* complex DNAs from Egyptian mummies by spoligotyping. *J Clin Microbiol.* 2003; **41**; 359-367.
15. Burgos, M.V., Pym, A.S. Molecular epidemiology of tuberculosis, *Eur respire J*, 2002, **20**; Suppl. 36, 54s-65s.
16. Velayati, A.A., Farnia, P., Mirsaedi, M. The most prevalent *Mycobacterium tuberculosis* superfamilies among Iranian and afghan TB cases. *Scand J Infect Dis* 2006; **38**(6-7): 463-8.
17. Farnia, P., Masjedi, M.R., Mirsaeidi, M. Prevalence of Haarlem I and Beijing types of *Mycobacterium tuberculosis* Strains in Iranian and afghan MDR-TB Patients. *J Infect.* 2006; **53**(5): 331-6.