Rapid identification of Atypical Mycobactria with PCR-RFLP Analysis(PRA) method

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Mycobacterium

- *Mycobacterium* ⟶ Actinobacteria ⟶ Mycobacteriaceae

- *Mycobacterium tuberculosis* complex
- Nontuberculous Mycobacteria (NTM)

DIAGNOSIS

Diagnosis base on

- Zheel-neelson staining
- Biochemical tests

➢ Expensive, time consuming, complex interpretation

Molecular methods

- PCR
- RFLP
- PCR-RFLP Analysis(PRA)

➢ Rapid, accurate, easy

Treatment

Material and methods

- Sampling
- Culture
- Colony morphology
- Biochemical tests
- Antibiotic sensitivity tests
- Pigment production

Molecular study

- **DNA extraction**
- **Approval of NTM with PCR**

Using *pncA1* and *pncA2*

- **PCR amplification of 644-bp hsp65**

Table1: PCR mix

PCR Buffer (10X)	2.5 µl
MgCL2 (1.5mM)	0.8 µl
dNTPs (1.2) (fermentas)	0.5 µl
*Forward primer (100pmol/ µl)	0.4 µl
*Reverse primer (100pmol/ µl)	0.4 µl
Taq DNA polymerase (2U/ µl) (fermentas)	0.5 µl
DD water	12.9µl
Final volum	18µl

Table2: Thermo cycler program

cycles	time	Temperature(C°)	PCR
35	60s	95	First denaturation
35	45s	62	Denaturation
35	90s	72	Annailing
	5Min	72	Extention

- Electrophoresed on 1.2% agarose

RFLP

- AvaII,HphI, and HpaII.

Digestion with AvaII

- 21.5ML PCR product+2.5ML enzyme buffer+1ML AvaII(10U/ML)=25ML
- Overnight Incubated in 37C
- Electrophoresed on 2.5% agarose

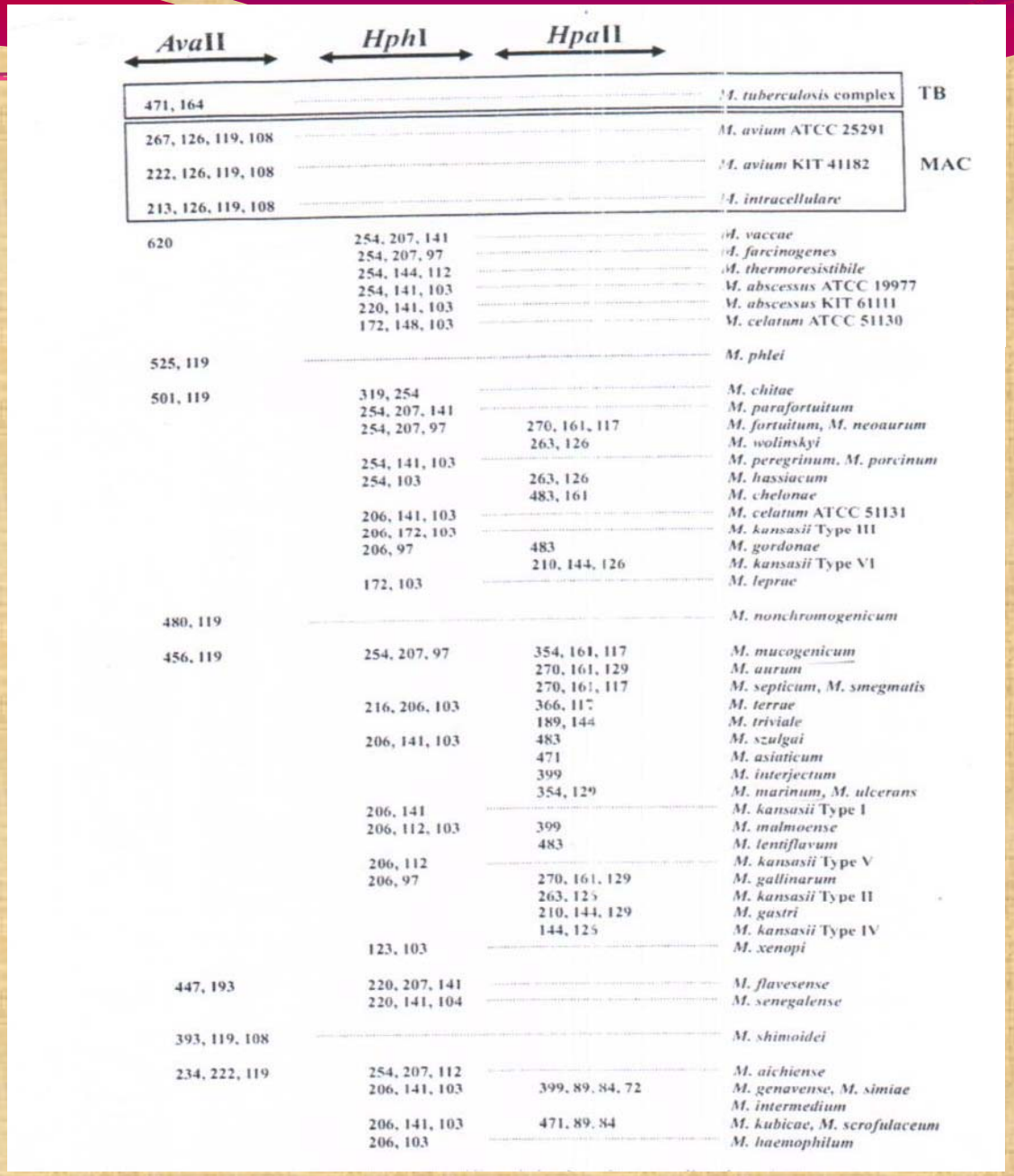
Digestion with HphI

- 21.5ML PCR product+2.5ML enzyme buffer+1ML HphI(10U/ML)=25ML
- Overnight Incubated in 37C
- Electrophoresed on 2.5% agarose for 2hours and 15min.using Ultra low range DNA ladder.
- Staining with ethidium bromide and analyzed with Gel documentation

Digestion with HpaII

- 21.5ML PCR product+2.5ML enzyme buffer+1ML HpaII (10U/ML)=25ML
- Overnight Incubated in 37C
- Electrophoresed on 2.5% agarose for 2hours and 15min.using Ultra low range DNA ladder.
- Staining with ethidium bromide and analyzed with Gel documentation

- Analysis of PRA profiles
- Compare PRA patterns of each enzyme with standard algorithm



*Ava*II	*Hph*I	*Hpa*II		
471, 164			*M. tuberculosis* complex	TB
267, 126, 119, 108			*M. avium* ATCC 25291	MAC
222, 126, 119, 108			*M. avium* KIT 41182	
213, 126, 119, 108			*M. intracellulare*	
620	254, 207, 141		*M. vaccae*	
	254, 207, 97		*M. farcinogenes*	
	254, 144, 112		*M. thermoresistibile*	
	254, 141, 103		*M. abscessus* ATCC 19977	
	220, 141, 103		*M. abscessus* KIT 61111	
	172, 148, 103		*M. celatum* ATCC 51130	
525, 119			*M. phlei*	
501, 119	319, 254		*M. chitae*	
	254, 207, 141		*M. parafortuitum*	
	254, 207, 97	270, 161, 117	*M. fortuitum, M. neoaurum*	
		263, 126	*M. wolinskyi*	
	254, 141, 103		*M. peregrinum, M. porcinum*	
	254, 103	263, 126	*M. hassiacum*	
		483, 161	*M. chelonae*	
	206, 141, 103		*M. celatum* ATCC 51131	
	206, 172, 103		*M. kansasii* Type III	
	206, 97	483	*M. gordonae*	
		210, 144, 126	*M. kansasii* Type VI	
	172, 103		*M. leprae*	
480, 119			*M. nonchromogenicum*	
456, 119	254, 207, 97	354, 161, 117	*M. mucogenicum*	
		270, 161, 129	*M. aurum*	
		270, 161, 117	*M. septicum, M. smegmatis*	
	216, 206, 103	366, 117	*M. terrae*	
		189, 144	*M. triviale*	
	206, 141, 103	483	*M. szulgai*	
		471	*M. asiaticum*	
		399	*M. interjectum*	
		354, 129	*M. marinum, M. ulcerans*	
	206, 141		*M. kansasii* Type I	
	206, 112, 103	399	*M. malmoense*	
		483	*M. lentiflavum*	
	206, 112		*M. kansasii* Type V	
	206, 97	270, 161, 129	*M. gallinarum*	
		263, 125	*M. kansasii* Type II	
		210, 144, 129	*M. gastri*	
		144, 125	*M. kansasii* Type IV	
	123, 103		*M. xenopi*	
447, 193	220, 207, 141		*M. flavescense*	
	220, 141, 104		*M. senegalense*	
393, 119, 108			*M. shimoidei*	
234, 222, 119	254, 207, 112		*M. aichiense*	
	206, 141, 103	399, 89, 84, 72	*M. genavense, M. simiae*	
			M. intermedium	
	206, 141, 103	471, 89, 84	*M. kubicae, M. scrofulaceum*	
	206, 103		*M. haemophilum*	

Fig1: standard algorithm

Results

Table3: Isolated samples each year

Year	Number	Percent
89	29	58%
90	21	42%
Total	50	100%

Table4: Isolated NTM by sex

Year	Sex	Number	Percent
89	Male	14	48%
	Female	15	52%
90	Male	15	71%
	Female	6	29%

Table5:Isolated NTM By age

Age range	Number	Number
Up to 15	5	10%
15-30	7	14%
30-45	11	22%
45-60	9	18%
Over 60	18	36%
Total	50	100%

Isolated NTM by place of sampling

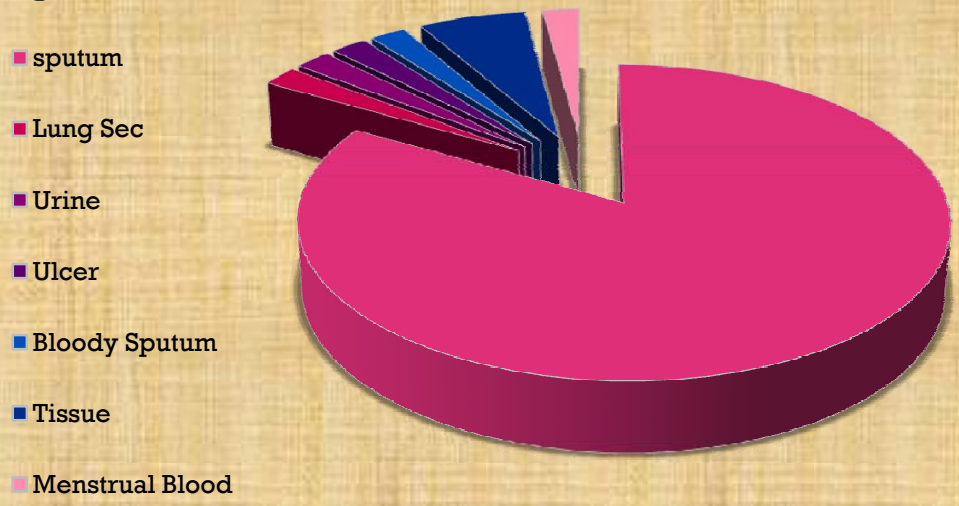
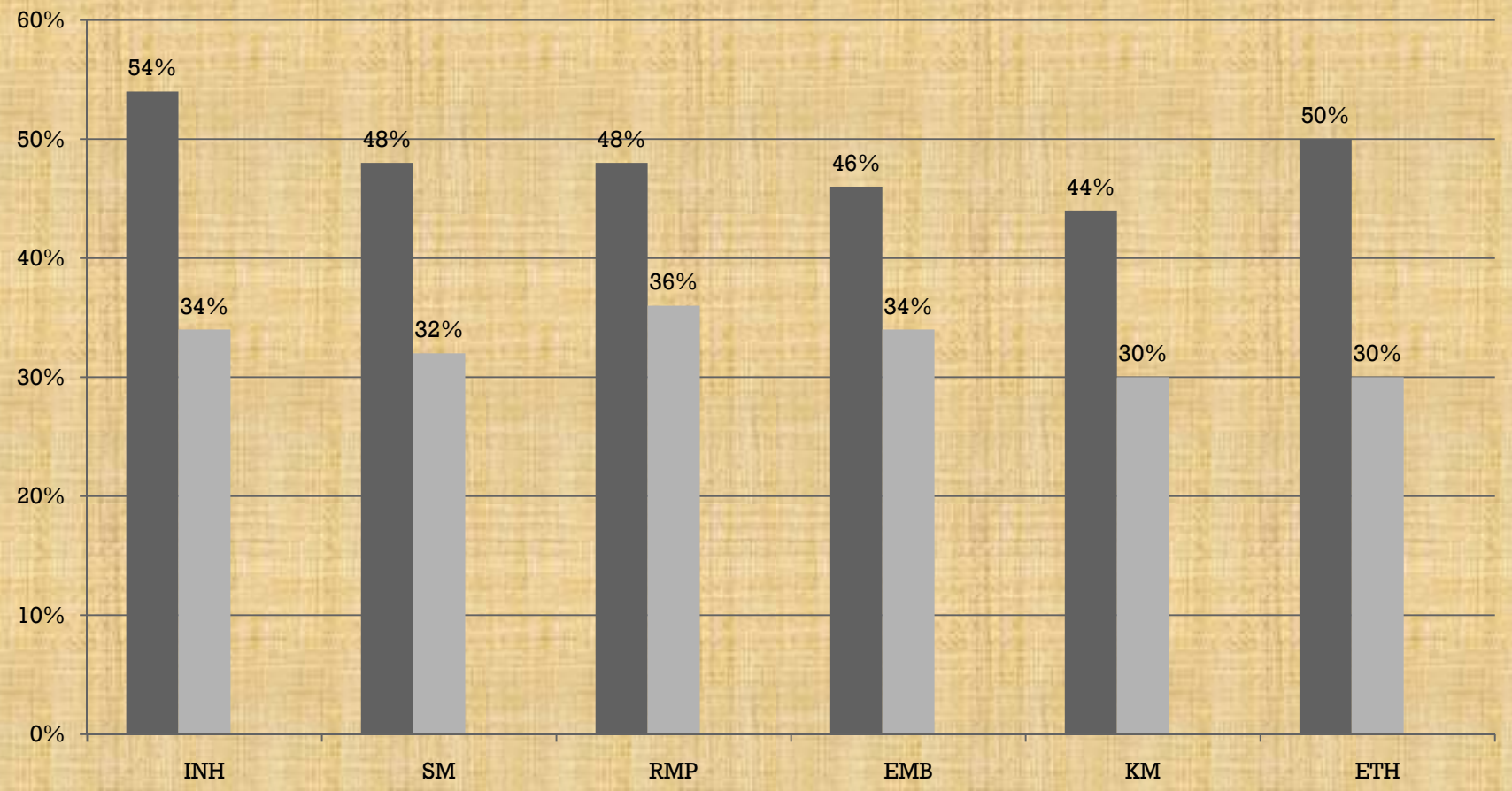


Table6: Analysis of smear

	Direct Smear	
Year	Negative	Positive
89	86%	14%
90	71%	33%
Total	٪۸۰	٪۲۰

- 80% of smears in this study had negative smear for acid-fast bacilli and just 20% were positive

Analysis of Antibiotic sensivity



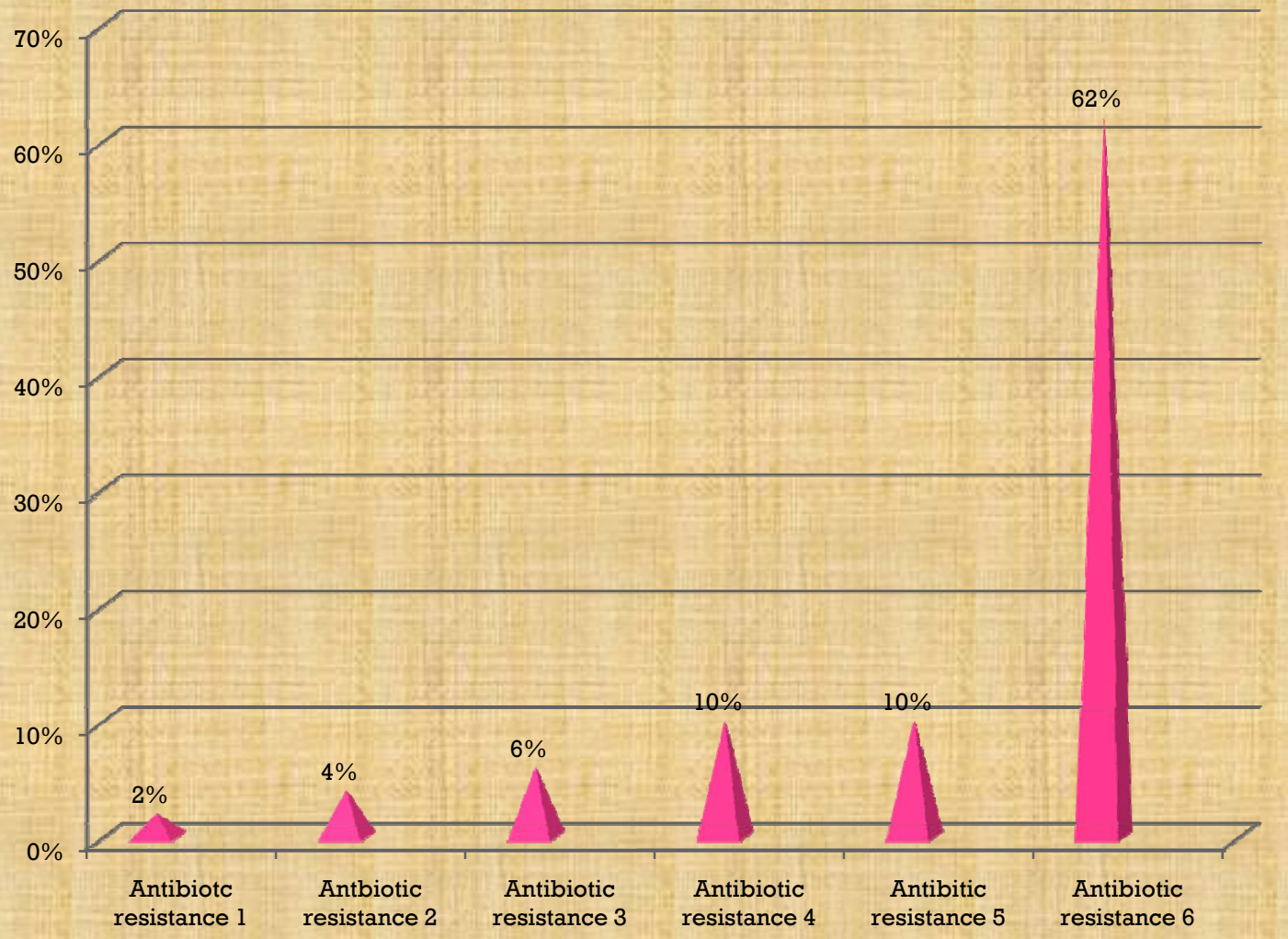

70%
60%
50%
40%
30%
20%
10%
0%
2%
4%
6%
10%
10%
62%
Antibiotc resistance 1
Antbiotic resistance 2
Antibiotic resistance 3
Antibiotic resistance 4
Antibitic resistance 5
Antibiotic resistance 6

Table7: resistance pattern

	89		90	
Resistance pattern	Antibiotypes	% of strains	Antibiotypes	% of strains
INH	1	3.5%	1	0%
INH,RMP	2	3.5%	2	5%
INH,RMP,EMB	3A	0%	3A	5%
INH,SM,EMB	3B	3.5%	3B	0%
SM,RMP,KM	3C	0%	3C	5%
INH,SM,RMP,ETH	4A	3.5%	4A	0%
INH,SM,EMB,ETH	4B	0%	4B	5%
INH,RMP,EMB,ETH	4C	7%	4C	0%
RMP,EMB,KM,ETH	4D	0%	4D	5%
INH,SM,EMB,KM,ETH	5A	3/5%	5A	0%
INH,SM,RMP,EMB,KM	5B	0%	5B	5%
INH,SM,RMP,EMB,ETH	5C	0%	5C	5%
INH,SM,RMP,KM,ETH	5D	7%	5D	0%
INH,SM,RMP,EMB,KM,ETH	6	66%	6	57%

Fig2: Analysis of PCR of hsp65 gene

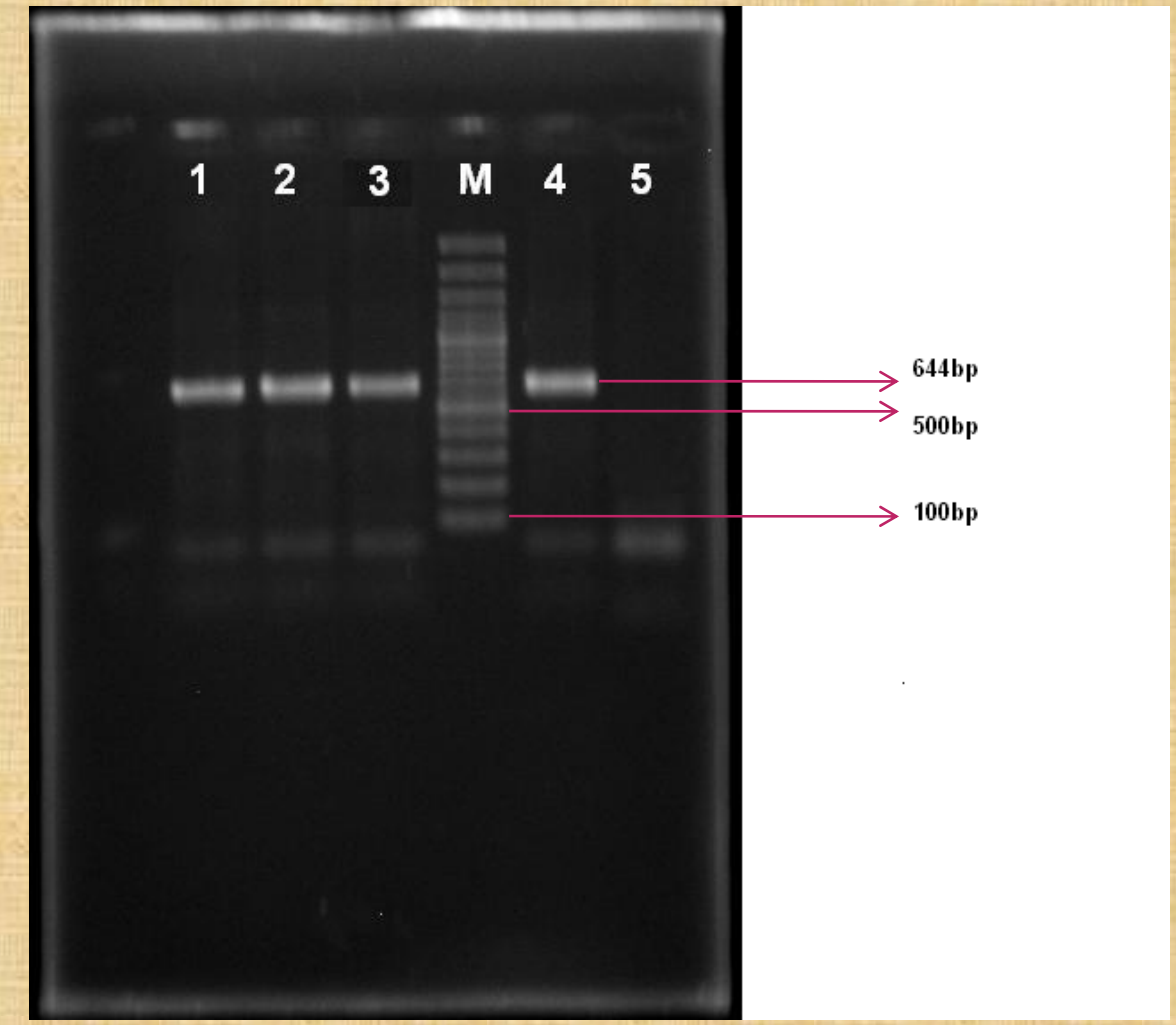


Fig3: Analysis of RFLP with AvaII

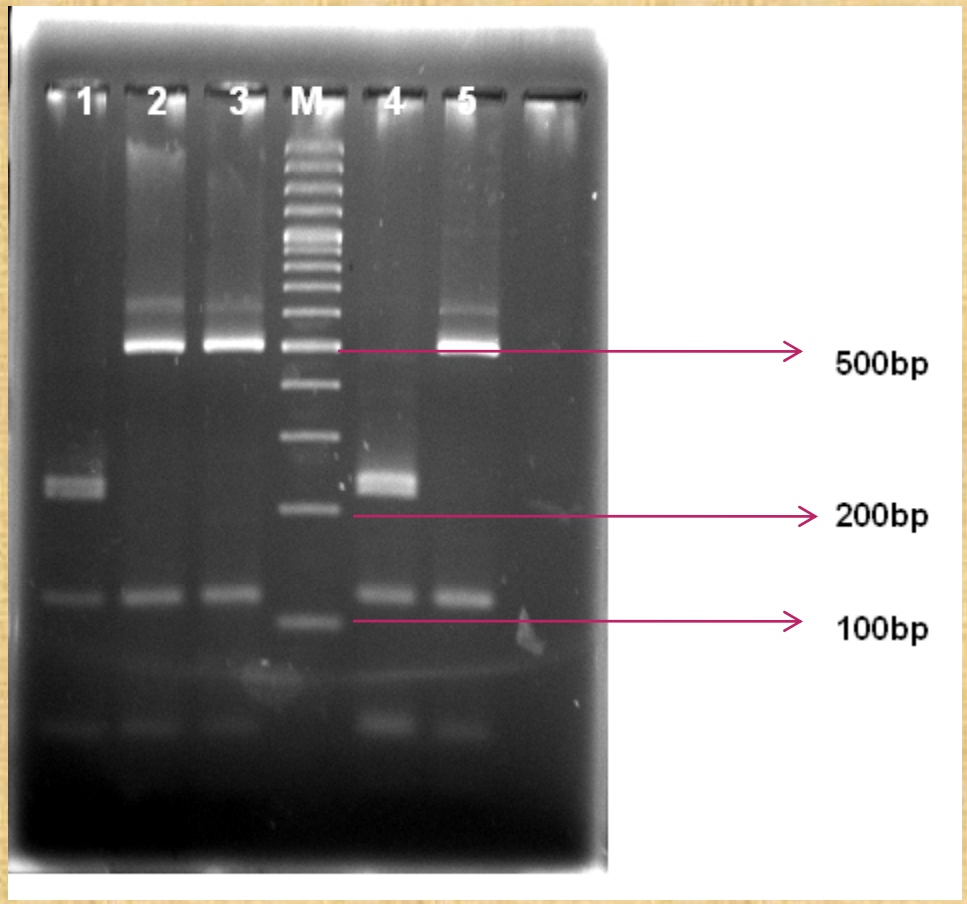


Fig4: Analysis of RFLP with HphI

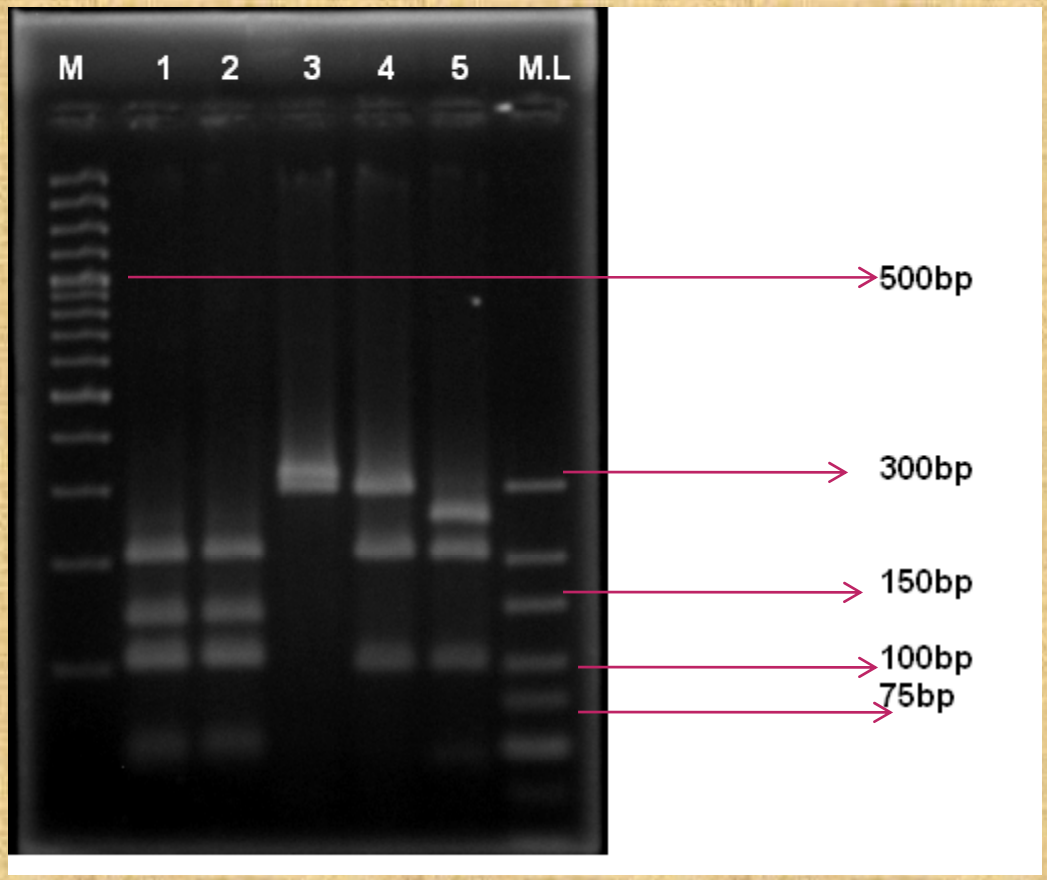
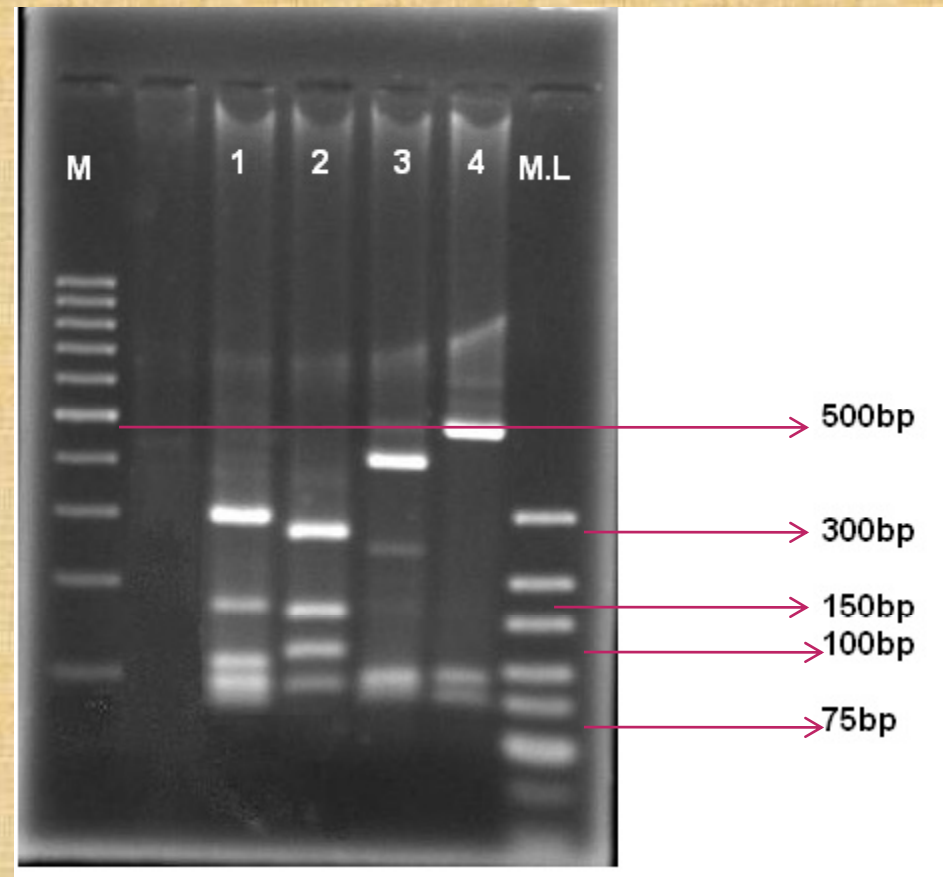


Fig5: Analysis of RFLP with HpaII



Analysis results of RFLP

- compared with standard algorithm
- acording to the algoritm 13 speciea were recognised:
- *M.fortuitum(30%), M.simiae(24%), M.kansasii(12%), M.s zulgae(6%), M.gordonae, M.gallinarum, M.triviale & M.aichiense(4%), M.hassiacum , M.malmoense, M.aurum, M.marinum & M.abscessus(2%)*
- 48% of isolates were recognized and 2% were not identified.

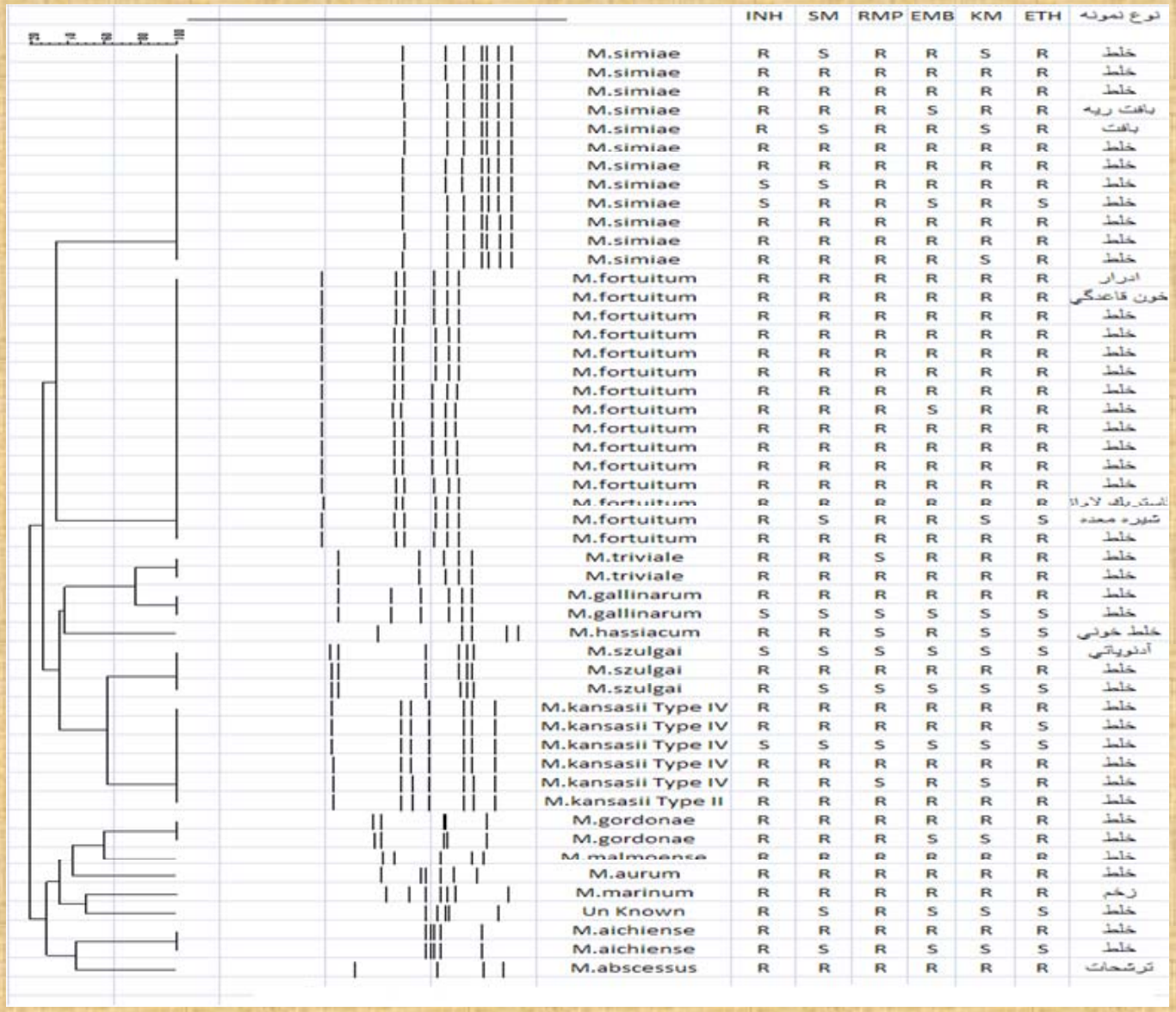
Table8: Recognized species by year

Species identified	Year: 89	Year: 90
M.fortuitum	31%	29%
M.simiae	24%	24%
M.kansasii	7%	19%
M.szulgae	10%	0%
M.gordonae	7%	0%
M.gallinarum	0%	10%
M.triviale	7%	0%
M.hassiacum	3%	0%
M.malmoense	0%	5%
M.aurum	0%	5%
M.marinum	0%	5%
M.aichiense	3%	5%
M.abscessus	3%	0%
Un Known	3%	0%

Dendrogram preparation

- By results of identification of NTM acording to fragment sizes of each enzymes in compare with markers and by using Gel compareII software dendrogram of NTM idenficatin was drawn.

Fig6: dendrogram of NTM



	INH	SM	RMP	EMB	KM	ETH	نوع نمونه
M.simiae	R	S	R	R	S	R	خلط
M.simiae	R	R	R	R	R	R	خلط
M.simiae	R	R	R	R	R	R	خلط
M.simiae	R	R	R	S	R	R	بافت ريه
M.simiae	R	S	R	R	S	R	بافت
M.simiae	R	R	R	R	R	R	خلط
M.simiae	R	R	R	R	R	R	خلط
M.simiae	S	S	R	R	R	R	خلط
M.simiae	S	R	R	S	R	S	خلط
M.simiae	R	R	R	R	R	R	خلط
M.simiae	R	R	R	R	R	R	خلط
M.simiae	R	R	R	R	S	R	خلط
M.fortuitum	R	R	R	R	R	R	ادرار
M.fortuitum	R	R	R	R	R	R	خون قاعدگي
M.fortuitum	R	R	R	R	R	R	خلط
M.fortuitum	R	R	R	R	R	R	خلط
M.fortuitum	R	R	R	R	R	R	خلط
M.fortuitum	R	R	R	R	R	R	خلط
M.fortuitum	R	R	R	R	R	R	خلط
M.fortuitum	R	R	R	S	R	R	خلط
M.fortuitum	R	R	R	R	R	R	خلط
M.fortuitum	R	R	R	R	R	R	خلط
M.fortuitum	R	R	R	R	R	R	خلط
M.fortuitum	R	R	R	R	R	R	خلط
M.fortuitum	R	R	R	R	R	R	خلط
M.fortuitum	R	S	R	R	S	S	شوره معده
M.fortuitum	R	R	R	R	R	R	خلط
M.triviale	R	R	S	R	R	R	خلط
M.triviale	R	R	R	R	R	R	خلط
M.gallinarum	R	R	R	R	R	R	خلط
M.gallinarum	S	S	S	S	S	S	خلط
M.hassiacum	R	R	S	R	S	S	خلط خوني
M.szulgai	S	S	S	S	S	S	آدنوپاتي
M.szulgai	R	R	R	R	R	R	خلط
M.szulgai	R	S	S	S	S	S	خلط
M.kansasii Type IV	R	R	R	R	R	R	خلط
M.kansasii Type IV	R	R	R	R	R	S	خلط
M.kansasii Type IV	S	S	S	S	S	S	خلط
M.kansasii Type IV	R	R	R	R	R	R	خلط
M.kansasii Type IV	R	R	S	R	S	R	خلط
M.kansasii Type II	R	R	R	R	R	R	خلط
M.gordonae	R	R	R	R	R	R	خلط
M.gordonae	R	R	R	S	S	R	خلط
M.malmoense	R	R	R	R	R	R	خلط
M.aurum	R	R	R	R	R	R	خلط
M.marinum	R	R	R	R	R	R	زخم
Un Known	R	S	R	S	S	S	خلط
M.aichiense	R	R	R	R	R	R	خلط
M.aichiense	R	S	R	S	S	S	خلط
M.abscessus	R	R	R	R	R	R	ترشحات

Discussion

Phenotypic methods

- Time consuming
- Inaccuracy
- Variable test

Molecular methods

- Rapid
- Accurate
- Affordable
- Species and subspecies identification

★ *M.kansasii* Type II(2%) and *M.kansasii* Type IV(10%)

- **Frequency comparison between two years**

  Similar prevalence which shows fixed Species distribution
- **The relationship between sex and NTM**

  Men
- **The relationship between age and NTM**

  immunosuppressed people
- **The difference between Direct smear & culture**

  TB(50-60%) via NTM(20%)

- **Comparison of anti Biotypes**

MDR(62%)

- **The most common multi-drug resistance**

  M.fortuitum(26%)

- **The most common antibiotic resistance pattern**

  4C(INH,RMP,EMB,ETH) & 5D(INH,SM,RMP,KM,ETH)

- **The Difference of TB treatment & NTM treatment**

- **The importance of early diagnosis**

  NTM ⟶ Binding to damaged mucus ⟶ Disorder of Peripheral immune ⟶ Increasing drug resistance

- Conventional methods based on culture and biochemical tests ⟶ Time consuming and complex
- Chromatographic techniques and analysis of DNA sequences in the 16S rRNA gene ⟶ arduous
- Simple genotyping methods(INNO-Lipa or Accoprobe) ⟶ small volumes of test
- PRA

✓ Distinguish TB from NTM

✓ Visual comparison

✓ Rapid identification

- PRA targeting 644bp Heat Shock Protein 65. (hsp65) gene for differentiation of Mycobacterium spp

Using equal condition & The reference culture strains

- ✓ High resolution
- ✓ Important clinical species(TB & MAC) diagnosis with only one enzyme
- ✓ No Need for computer analysis

Biodiversity

- Bahremand et al(1377):

  M.Fortuitum ,M.chelonae, M. Kansasii, M. Flavescence, M. agri, M. Phlei, M.smegmatis were the dominant species

- Ghazi saeedi et all(1386):

  M.marinum(1.76%)

- Fezeh et all in (1388):

  M.simiae(58%) and *M.kansasii*(19%)

- Kim et all(2005):

  M.szulgai(7/62%)، *M.abscessus*(10/48%)
  , *M.fortuitum*(4/76%) , *M.kansasii*(17/14%)

➡ *M.Fortitum* & *M.simiae* via MAC

An unknown type

??! Mutations in the enzyme cutting area/s

??! Possible emergence of new species

Conclusion

The results showed PCR-RFLP (PRA) using *Ava II*, *HphI* & *HpaII* is a simple, fast and accurate for the general grouping of atypical mycobacterium isolates.the method using only AvaII can identfy TB and MAC isolates.PRA with reducing time to diagnosis of atypical strains from *Mycobacterium tuberculosis* and identification of atypical mycobacterium into species or even sub species level can be effective in the treatment of mycobacterial infections

Suggestion

1/PRA targeting 644bp of hsp65 for identification of
#environmental NTM
BCG
#Compliance with clinical samples

2/PRA using other regions of *hsp* 65 gene or *hsp60*,*hsp90* and other consensus genes

3/Evaluation of Larger population in order to increase the precision

4/Annual survey In different geographical areas

5/ Evaluation of clinical samples directly

Thanks for Your
Attention