Laser-Raman spectroscopy: a novel approach for sensitive molecular characterization of the *Mycobacterium* genomic DNAs

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ABSTRACT

In the present work we give a detailed analysis on the key Raman markers of genomic DNA for four species of Mycobacterium namely: Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium avium and Mycobacterium phlei. The fundamental groups of bands have done for the first time to these Mycobacterium genomic DNAs. Molecular Laser Raman Spectroscopy (MLRS); a novel and highly sensitive method, was used for whole genome characterization and comparison of the four species under investigation. The Raman spectroscopic analysis showed, in the M. avium and M. bovis the DNA is more rich in A+T as compared with the others, while the M. phlei and M tuberculosis are more rich in G+C. Moreover, a detailed analysis of all the bands diagnostic of B-form backbone geometry; the phosphate groups, bands diagnostic of deoxynucleoside conformation, ring vibrations of the bases, carbonyl stretching modes of thymine, guanine and cytosine was given here for the four mycobacterial species DNAs. The present study describes the use of (MLRS), as a novel promising assay, for rapid and discriminative detection of a mycobacterial genome with no confusion with other bacteria, utilizing genomes of four different mycobacteria species. In addition, Molecular Polymorphic Laser Raman Spectroscopy fingerprints and / or sequences may serve as markers that provide a basis for virulence, phylogenetic and evolutionary studies.

Key words: *Mycobacterium tuberculosis, DNA, molecular Laser Raman spectroscopy (MLRS).*

INTRODUCTION

The mycobacterial genomic DNA contains a high guanine plus cytosine content that ranges from 58 to 69%. Mycobacteria are particularly resistant to normal methods of chemical lysis owing to the nature of their cell wall. Also, the high content of polysaccharides can contaminate preparations of their nucleic acids (Clark-Curtiss, 1990). Most mycobacteria are non-pathogenic, while some species are highly pathogenic to human, mammals, birds, reptiles, amphibians and fish. The most important species of this genus is *M.*

tuberculosis, the causative agent of tuberculosis (TB).

Mycobacteria, particularly *M.bovis* and *M. avium* complex, are widely distributed both nationally and internationally among domestic ruminants. Thus, a diagnostic assay for mycobacteria should accurately encompass precise identification of both tuberculous and nontuberculous mycobacteria. The emergence of multidrug-resistant strains and its association with outbreaks inside and outside hospitals illustrates that rapid diagnosis is essential (WHO, 2000).

Diagnosis of a mycobacterial infection is made on clinical grounds confirmed by the intradermal test (IDT) with bovine PPD tuberculin and the demonstration of the etiologic Mycobacterium in clinical specimen by microscopy, culture, or by the use of DNA probes and the polymerase chain reaction. The latter methods can potentially allow detection of mycobacterial DNA, RNA or proteins directly from the specimen before the culture results are available and can differentiate mycobacteria to the species level. Such methods as high-performance, gas-liquid, and thin-layer chromatographies, rapid-cycle PCR fluorimetry and DNA sequence analysis of the 16S rRNA gene (rDNA) region are either too labor-intensive, difficult, or expensive for routine use (Lachnik *et al.*, 2002) .

PCR-restriction fragment length polymorphism analysis is simple to perform, rapid, and economical that makes it highly attractive for routine clinical laboratories. However, assays for PRA have often been criticized as being difficult to read because of minor differences in band size patterns between some species that are made worse by gel-to-gel variations and the occurrence of new patterns that have not been previously reported (Richter et *al.*, 1999).

Molecular Laser Raman Spectroscopy (MLRS) offers certain advantages for the investigation of structural thermodynamic and kinetic properties of DNA. The Raman method is not limited by size or state of aggregation of the DNA specimen, and in the case of aqueous solutions, it is favorable to use either normal (H_2O) or heavy water (D_2O) as the solvent. In recent applications, utilization of MLRS method to distinguish specific and non-specific binding of divalent cations to DNA has been reported by Duguid *et al.* (1993). Besides, monitoring thermal denaturation of DNA and DNA/metal –ion complexes (Duguid *et al.*, 1995 and 1996), measuring the kinetics of proton/deuteron exchange of genomic DNA in condensed and uncondensed states, were demonstrated (Reilly *et al.*, 1994 and Tuma *et al.*, 1996).

The aim of the present study is to apply Molecular Laser Raman Spectroscopy (MLRS), as a highly sensitive method for identification and differentiation of four different *Mycobacterium* spp. namely; *M. tuberculosis*, *M. avium*, *M. bovis* BCG and *M. phlei*. This can offer an excellent alternative to previously established nucleic-acid amplification-based techniques for the diagnostic mycobacterial laboratory. Also, MLRS is prepondrant, simple, rapid, and more accurate than conventional procedures for differentiating Mycobacterium species, and a recent tool for fingerprinting of their genomic DNAs.

MATERIALS AND METHODS

Raman spectroscopy

An FTIR "Raman" spectrometer (Bruker) IFS-bb was used with Nd-YAG laser, $\lambda = 1064$ nm laser line as a source of excitation with a power of 0-1500mW. Liquid nitrogen cooled Ge detector (solid state-detection which is highly sensitive in the region of our measurements) was used. A suitable software (OPUS 3) was used for data acquisitions in the

region -2000 up to 3600 cm^{-1}. The Notch filter allows to go as close to the excitation line as possible. The effective resolution of the system is less than 2 cm^{-1}. Aqueous solutions of genomic DNA of the four mycobacterial species: *M. tuberculosis* (T), *M. avium* (A), *M. bovis* (B) and *M. phlei* (P) were used in this study. A special cell was used for aqueous solutions (Badr *et al.*, 2005) and a special lens was used to collect the light scattered back from the sample; i.e., back scattering or 180° geometry.

Bacterial strains

A total of four mycobacterial reference strains, (Table 1) representing four mycobacteria species namely, *Mycobacterium tuberculosis* (a tuberculous Mycobacterium), *Mycobacterium bovis* bacillus Calmette-Guérin (BCG, Copenhagen strain, an avirulent vaccinal strain of *M. bovis*), *Mycobacterium avium* (a representative of *M. avium* complex) and *Mycobacterium phlei* (a typical saprophytic molecular species) were used to develop the new comparative analytical Raman Laser Spectroscopic (MLRS) method in this study. All strains were colony purified before growing them in an appropriate liquid medium, Middlebrook 7H9 media (Difco) supplementes when needed with 0.2% glycerol, 0.05% Tween 80, and 10% ADS (0.5% bovine albumin fraction V, 0.2% dextrose, 140 mM NaCl) for all mycobacteria. All strains were kindly obtained from the Bacterial Diagnostics Dept., Veterinary Serum and Vaccine Researches Institute, Abbassia, Cairo, Egypt.

Genomic DNA extraction

Genomic DNA was extracted following the procedure described by **Juana *et al.*, (1998)** with some modifications. Briefly, the four were individually pelleted at 3000 rpm in a cooling centrifuge for 20 minutes. A loop of the bacterial cell pellet (containing $3x10^8$ cells, matching Mcferland 3) was transferred to a clean, sterile screw-cap microfuge tube and lysed in 400 µl of a pre-lytic buffer (containing 20 mM citrate phosphate buffer, pH5.6; 100 mM Tris HCl, pH 8.0; 50 mM EDTA; 20 mg / ml of lysozyme, and Tween-80 0.1%, w/v) and were then incubated for 2 h at 37°C. A 300 µl of a lysis buffer (containing 500 µg / per ml of proteinase K, 150 mM NaCl; and 10% sodium dodecyl sulfate, SDS) was added to the suspension and incubated at 56°C for 1 h. The lysate was centrifuged (14,000 rpm for 20 min) and the supernatant was subjected to phenol-chloroform extraction. The DNA was precipitated with isopropanol, resuspended, treated with RNase, re-extracted with phenol-chloroform and chloroform, re-precipitated with 100% ethanol and washed in 70% ethanol. The final pellet was resuspended in 30 µl of nuclease-free water. The concentration of the DNA was estimated by determining the optical density at 260 nm and was generally brought to about 0.33 µg/µl.

Table (1): Features of the mycobacteria under investigation according to their pathogenicity, rate of growth and tubercuolous lesion.

Species	Pathogenicity	Rate of growth	Tuberculous lesion
1- *M . tuberculosis*	++	Slow	+
2- *M. . avium* complex	+	Slow	-
3- *M. bovis* BCG*	-	Slow	-
4- *M. phlei*	-	Fast	-

(++): highly pathogenic (-): nonpathogenic (+): pathogenic *Avirulent vaccinal strain

RESULTS AND DISCUSSION

I. Characterization of DNA of mycobacteria using molecular laser Raman spectroscopy:

FT Raman spectra of the four species; *M. avium complex, M. bovis, M. phlei* and *M. tuberculosis*; are given in Fig.(2 a and b) and Fig.(3).The obtained bands and the corresponding assignment are given in (Table2).It is well known that the spectral bands are characterized by four parameters:

The band shape which could be: Gaussian, Lorentzian or linear combination of both.Second, the band position or peak position or frequency, represented in wave numbers (cm^{-1}), its value characterizes the mode of vibration, the bond strength and the reduced mass of the vibrating atoms of the molecule or molecular segment. This value is sensitive to variations in the internal structure of the vibrating molecule or molecular segment, displacive or rotational.etc, as well as the position of the surrounding segments or molecules. Third, the band intensity (I) which could be absolute, or relative as well as integrated. The intensity of certain vibrational band reflects the amount or the number of vibrating molecules. It is very senstive to any increase or decrease of certain type of molecular bands. It decreases linearly with band rupture and vice versa. This makes Raman spectroscopy a good analytical tool which can detect even traces of certain molecule and could be used to evaluate e.g. the amount of protein in any compound...etc. Finally the band width at half its maximum intensity or the half band width ($\Delta\upsilon$ cm^{-1}). This parameter could be essentially related to the life time of the corresponding energy level. Abnormal broadening could be used to characterize the material under investigation. The used software allows to obtain the band parameters: I, υ cm^{-1} and $\Delta\upsilon$ cm^{-1} for the four DNAs in separate reports which could be used much easier to carry on all the estimations and calculations given below. To characterize the DNA of the four Mycobacteriutatic species using the obtained Raman spectra, It is possible to use the Raman markers of the well known four deoxynucleotide bases of the DNA. To do this, it is important to normalize the four spectra in such a way that we obtain the same intensity for the band at 1092$cm^{1}$of the phosphate group (Deng *et al.*, 1999), which originates from the localized symmetric stretching vibration of the phosphodioxy (PO_2^-) moiety and manifests the same intensity in all DNAs irrespective of base composition or sequence.

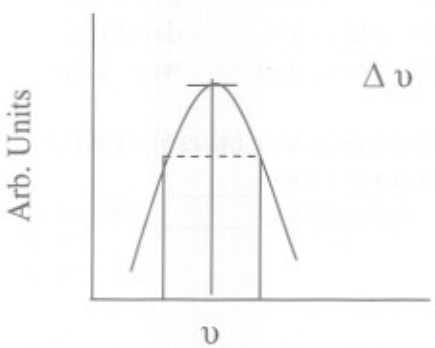

Fig. (1): The parameters of Raman vibrational bands.

Table (2): Raman bands, relative intensities and the corresponding assignment for the four mycobacterium species DNAs.

	DNA		DNA		DNA		DNA	
Assignment	**T**	**RI**	**A**	**RI**	**B**	**RI**	**P**	**RI**
	613	0.8571	610	1	610	0.85	618	0.8182
Ring breathing	622	0.8571	626	1	625	0.85		
Ring breathing	632	0.9286			639	0.85	635	0.8182
dC	640	0.8571	640	1			645	0.7273
dC	652	0.8571	657	1	651	0.85	654	0.7273
dT	662	0.9286			657	0.85		
dG	679	0.8571	670	0.8889			670	0.8182
			679	0.8889				
			682	1	690		684	0.8182
Ring breathing	692	0.8571	695	1	690	0.85		
Ring breathing	710	0.7857	706	1	709	0.85	705	0.7273
dA	722	0.7857	721	0.8889	724	0.85	718	0.7273
dA			727	0.8889	732	0.85	730	0.7273
Ring breathing	734	0.7857	738	1			736	0.7273
Ring breathing					743	0.8		
dT	748	0.8571	749	1	751	0.8	752	0.7273
Ring breathing			765	1	758	0.8	762	0.6364
Ring breathing	774	0.7857	773	1	779	0.85	778	0.7273
dT,dC,bk	783	0.7857	788	1				
Ring breathing	795	0.7857			791	0.8	793	0.7273
A-DNA			808	1	806	0.85	807	0.8182
dA	813	0.7857			813	0.85	819	0.7273
νOPO	834	0.7857	828	1	827	0.85	832	0.7273
	848	0.8571	851	0.8889	851	0.85	845	0.8182
bk			865	0.8889	864	0.9		
bk	879	1.1429	872	0.8889	878	1	879	1.1818
d			889	0.8889				
d					894	0.9	898	0.7273
	905	0.8571	901	1				
	917	0.7857	910	1	913	0.9	914	0.7273
d					924	0.9	920	0.8182
d	930	0.7857					927	0.8182
			937	1.1111	937	0.85		
	943	0.7857					940	0.7273
Sugar residues	960	0.7857	950	1.1111	956	0.9	953	0.7273
Sugar residues	966	0.7857	966	1			966	0.8182
d			975	1	971	0.9	976	0.8182
Sugar residues					984	0.95	988	0.8182
d	992	0.7857	990	1	997	0.9		
Sugar residues	1005	0.7857			1008	0.9	1000	0.8182
dT,dG,dC	1021	0.8571	1016	1	1014	0.9	1018	0.8182
Sugar residues	1028	0.8571	1025	1	1026	0.95	1027	0.8182
Sugar residues	1035	0.8571						

Table (3) summarizes the comparison between the four genomic DNAs, where it is clearly seen that the Raman marker of the dA reflects that the bands that are more intense in the species *M. bovis* then, *M. avium* complex, *M. tuberculosis* and *M. phlei.* And so for the four deoxynucleosides (dA, dT, dC and dG).

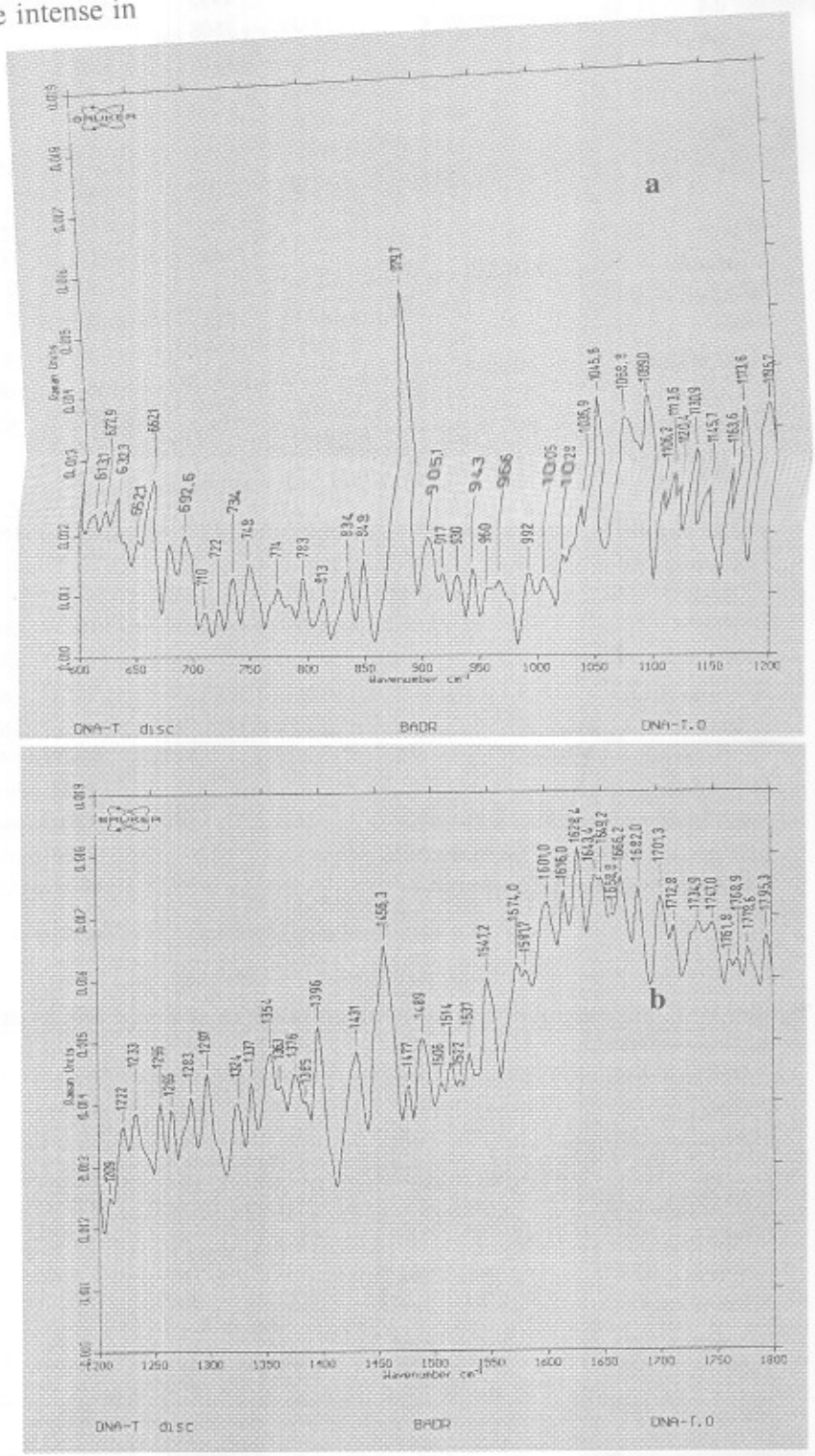

Fig. (2): FT Raman spectra of the genomic DNA of mycobacterium M.tuberculosis (T):
(a) 600 – 1200 cm^{-1}
(b)1200 – 1800 cm^{-1}

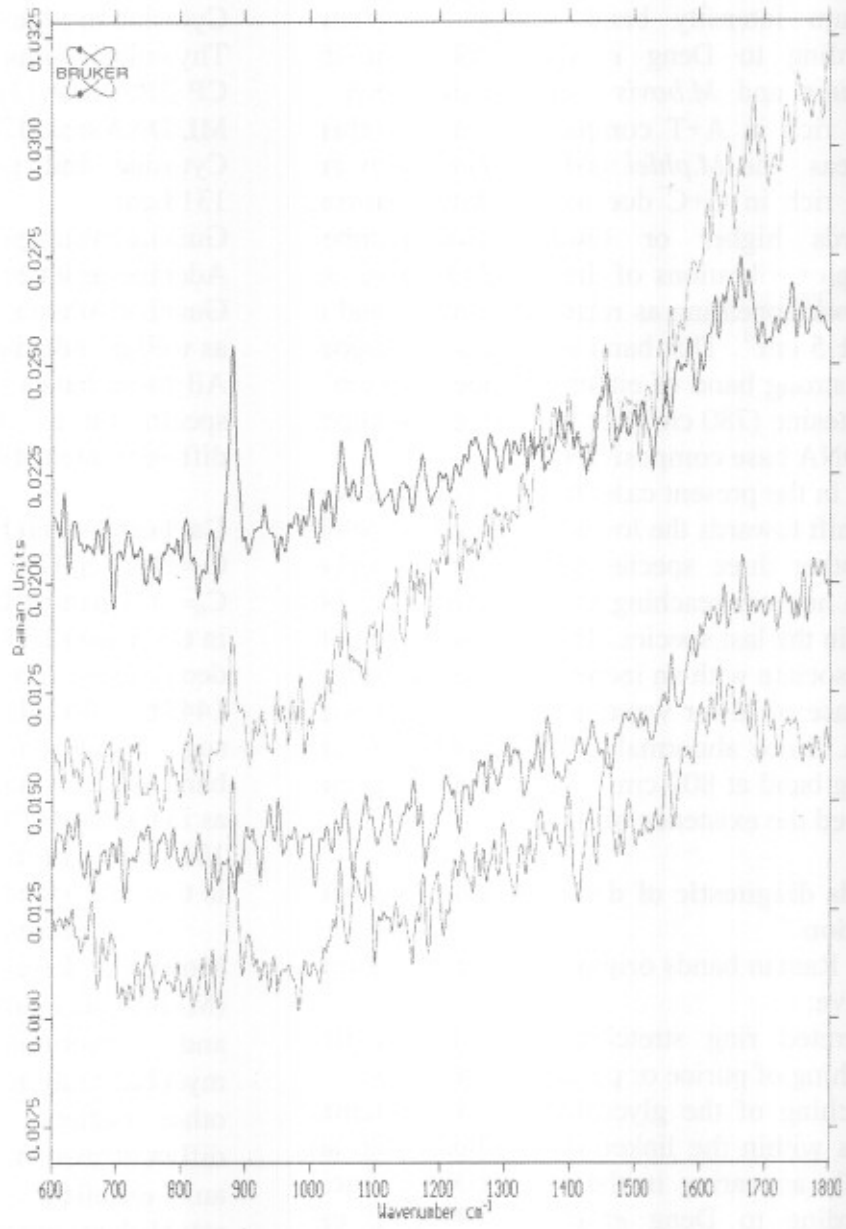

Fig.(3): FT Raman spectra of the genomic DNAs four species; M. tuberculosis (T), M .avium complex (A), M.bovis (B) and M. phlei (P)

Bands diagnostic of B-form backbone geometry (The phosphate groups)

These groups give three prominent Raman band which indicate the global B-form structure (Deng *et al.*, 1999).Symmetric stretching vibration of (PO_2^-) moiety (mentioned above) appearing as relatively strong band at 1092 $cm^{-1.}$ It is invaried to differences in base composition. It is well known that this band is shifted to 1100 cm^{-1}in abnormal DNA and RNA (Deng *et al.*,1999). In our case only the first species *M.avium* showed the existence of the A-form DNA. Complex vibrational mode involving the

deoxyribose-linked phosphodiester network. (5´ C-O-P-O-C 3`) of B-DNA appearing as a medium intensity band at 835 ± 5 cm^{-1} according to Deng *et al.*, (1999). In the *M.avium* and *M.bovis* species the DNA is more rich in A+T compared with the others whereas the *M.phlei* and *M.tuberculosis* are more rich in G+C due to the shift occurring towards higher or lower wave number. Complex vibrations of the (5´ C-O-P-O-C 3`) network appearing as relatively strong band at 790 ± 5 cm^{-1}. This band is always overlapped by a strong band of either thymine (790 cm^{-1}) or cytosine (780 cm^{-1}) or both depending upon the DNA base composition.

In the present case, the B species showed the shift towards the lower wave number while the other three species are shifted to higher wave number reaching the upper limit of 795 cm^{-1} in the last species. This shift was found to be associate with an increase in the intensity in the case of lower wave number shift and vice versa. Since abnormal DNA generates a very strong band at 807 cm^{-1}, the first three species showed the existence of A-form.

Bands diagnostic of deoxynucleoside conformation

Raman bands originating from vibrations involve:

Concreted ring stretching motion, i.e, ring breathing of purine or pyrimidine residues.

Stretching of the glycosidic band. Stretching bands within the linked deoxyribose ring. are usually appearing in the range 600-800 cm^{-1}, according to Deng *et al* ., (1999). It can concluded that each of the four DNAs might contain deoxynucleotides with C 2´- endo sugar and anti- glycosyl torsion.

Ring vibrations of the bases

Most of the bands originating from the in-plane vibrations of base residues are involved in spectral region 1150-1600 cm^{-1} which contains many overlapping bands arising from:

Cytosine residues near 1178 cm^{-1}
Thyamine residues near 1190 cm^{-1}
CP DNA near 1187 cm^{-1}
ML DNA near 1270 cm^{-1}
Cytosine and guanine residues near 1293, 1318 cm^{-1}
Guanine residues near 1989 cm^{-1}
Adenine residues near 1482 cm^{-1}, 1511 cm^{-1}
Guanine/Adenine pair of 1489/1982 cm^{-1}
as well as: adenine pair 1578±1 cm^{-1}
All these bands are appearing in the obtained spectra for the four species: examined with different intensities.

Carbonyl stretching modes of the following.

C_2=O & C_4=O .Thymine .
C_6=O Guanine C_6=O Cytosine are appearing in the region 1600-1750 cm^{-1}
deoxyribose moiety ≡895±1, 922±1, 1053±2, 1444±2, 1462±1 cm^{-1}
sugar residues of DNA 950 - 1050cm^{-1}
band 998cm^{-1} is predominant in GC rich DNA, as in the case of the M *bovis* species.
1016 cm^{-1} is predominant in AT rich DNA, as in the case o the M. *avium.*

The present study describes the use of Molecular Laser-based Raman Spectroscopy (MLRS), as a novel promising assay, for rapid and discriminative detection of a mycobacterial genome with no confusion with other bacteria, utilizing genomes of four different mycobacteria species. This can offer an excellent alternative to previously established nucleic acid amplification-based techniques for the diagnostic mycobacterial laboratory. This approach will be so useful for epidemiological studies and control programs of TB in man and animals.

Several studies of the *Mycobacterium* genus describe large-sequence polymorphisms (LSPs) among the *M. bovis* BCG vaccine

strains and virulent *M.* bovis as well as among other mycobacterial species and *strains (Gordon et al.*, 1999 and Kato-Maeda et al., 2001). The live attenuated bacillus Calmette-Guérin (BCG) vaccine for the prevention of disease associated with *Mycobacterium tuberculosis* was derived from the closely related virulent tubercle bacillus, *Mycobacterium bovis*. Three distinct genomic regions of difference (designated RD1 to RD3) were found to be deleted from BCG, and were conserved in all virulent laboratory and clinical isolates of *M. bovis* and *M. tuberculosis* tested. These findings may be applicable to the rational development of new diagnostic tests to distinguish BCG vaccineation from tuberculosis infection (Mahairas *et al.*, 1996).

MLRS whole-genome comparison of the four mycobacteria (*M. tuberculosis, M. avium subspp. avium, M. bovis BCG and M. phlei*) characterized the polymorphic molecular thermodynamic structures, with potential relevance to their nucleotide sequence content, pathogenicity and evolution. Some polymorphic loci included higher nucleotide markers (dA, dT, dG and dC) with variable frequencies compared to other genomes. These MLRS – Laser based analysis could be correlated to polygenetic relationships among the four Myobacterium species. Our results coincide relatively with that obtained by Fleischmann *et al.* (1999), where molecular polymorphisms at many genetic loci among Mycobacterium spp. are extensive. Polymorphic MLRS fingerprints and / or sequences may also serve as markers that provide a basis for virulence, phylogenetic and evolutionary studies.

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المستخلص العربى

"مطيافية ليزر الرامن": اتجاه جديد للتوصيف ألجزيئي الدقيق لجينوم الحمض النووي "الدنا" لميكروب السل

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توضح الدراسة الحالية التحاليل التفصيلية و مناقشة مفاتيح معلمات الرامان للحمض النووي DNA" الدنا" الجينومي لكل من الميكروبات الأربعة تحت الفحص : ميكوبكتريا التيوبركيلوزيس (السل الآدمي) و ميكوبكتريا البوفيس (السل البقرى) عترة اللقاح المضعفة كالميت–جيورين (بى.سى.جى) و ميكوبكتريا الأفيام (سل الطيور) و ميكوبكتريا فيلياي باستخدام مطيافية ليزر– رامان الجزيئية (MLRS) ، كما تم للمرة الأولى تسجيل المجاميع الأساسية لمناطق طيف رامان للحمض النووي DNA" الدنا" الجينومي للأنواع الأربعة من الميكوبكتريا مع توصيف التركيبات الجزيئية عديدة الأشكال (البولي مورفية) الديناميكية الحرارية مع القدرة الكبيرة على ربطها بمحتوى التتابع النيوكليوتيدى و قابلية إحداث المرض و العدوى بالمرض و التطور (الارتقاء). و تحتوي بعض المواقع متعددة الأشكال (البولي مورفية) على علامات نيوكلوتيدية عالية من محتوى الأدينين ، الثيامين ، الجوانين ، السيتوسين (dA, dT, dG & dC) بتكرارات خاصة بكل جينوم مختلفة عن الجينومات الأخرى. و قد أظهرت الدراسة المقارنة لأطياف DNA " الدنا" أن جينومات ميكوبكتريا الأفيام وميكوبكتريا البوفيس تتميز بمحتوى أكثر من أل (A+T) عن الجينومات الأخرى و أن جينومات ميكوبكتريا التيوبركيلوزيس و ميكوبكتريا فيلياي الأكثر غنى في محتوى (G+C) . و بعد ذلك أظهرت التحاليل التفصيلية لكل المناطق التشخيصية للعمود الفقري للشكل الهندسي للنوع الطيفي"الدنا" :B-form DNA ، مجاميع الفوسفات ، مجاميع الشكلية للنيوكلوسيدات ، الاهتزازات الحلقية للقواعد النيتروجينية ، ترددات الاهتزازات الأستطالية لمجموعات الكربونيل لنماذج الأدينين و الثيامين و الجوانين و السيتوسين ، الخصائص المميزة للحامض النووي الجينومي DNA "الدنا" لكل من الأربعة الميكوبكتريا . وأكدت الدراسة الحالية مقدرة آلية MLRS علي تحديد التمييز السريع للجينوم دون الخلط مع ميكوبكتريات أخري . مما يعطي بديلا حقيقيا دقيقا للآليات الراسخة من قبل والمبنية علي تضاعف المادة الوراثية بالـ بى. سى. آر. والأبعد من هذا، أنه يمكن لهذه الآلية الجزيئية عمل بصمة متعددة الأشكال بالليزر رامان MLRS و/أو التتابع النيوكليوتيدى و التي يمكن أن تستخدم كمعلمات أو دلالات تسمح بإرساء أسسا لدراسات الضراوة و التقسيم الوراثي و التطور للميكروبات .