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Original Article
Özgün Araştırma

Typing of *Mycobacterium bovis* isolates from cattle using MIRU-VNTR analysis

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Abstract: Bovine tuberculosis, as a significant threat to both animal and human health, is a common global zoonotic disease. The emergence of molecular epidemiology has made it possible to gain a better understanding of the dynamics of disease transmission, and consequently, to come up with more effective control methods. The present study seeks to identify *Mycobacterium bovis* isolates in our region at a genotype level. To this end, the molecular epidemiological characteristics of *Mycobacterium bovis* strains isolated using classical methods and identified using molecular methods from the tissues and organs of cattle with suspected tuberculosis, obtained from slaughterhouses in the Konya province, or from those sent to Konya Veterinary Control Institute, were determined through genotyping. In the analysis of a total of 70 *Mycobacterium bovis* isolates, carried out using the MIRU-VNTR method, it was found that the repeat numbers for MIRU2, MIRU4, MIRU20, MIRU23, MIRU24, MIRU27, and MIRU39 loci did not vary between strains, while the repeat numbers for MIRU10, MIRU16, MIRU26, MIRU31, and MIRU40 loci varied between strains and had a high discriminatory power (0.25≤h). It was further observed that 29 subgroups between 1-14 isolates formed. The movement of animals in our region, which occurs for several reasons, is considered to cause *Mycobacterium bovis* strains to vary between herds, and the fact that the cattle from which the isolates were sourced for our study came from different herds was considered to cause a variation in the discriminatory power in the MIRU loci.

Keywords : Bovine tuberculosis, MIRU-VNTR, molecular typing, *Mycobacterium bovis*.

Sığırlardan izole edilen *Mycobacterium bovis* izolatlarının MIRU-VNTR analizi ile tiplendirilmesi

Özet: Sadece hayvan sağlığı değil aynı zamanda halk sağlığı açısından da önem arz eden sığır tüberkülozu, dünya genelinde yaygın olan zoonotik bir hastalıktır. Moleküler epidemiyolojinin ortaya çıkışıyla, hastalığın yayılma dinamiğinin anlaşılması ve daha etkili mücadele yöntemlerinin geliştirilebilmesinin temelleri atılmıştır. Ancak, sığır tüberkülozunun moleküler epidemiyolojisine yönelik, ülkemizde yapılan çalışma sayısı sınırlıdır. Bu çalışmada, bölgemizdeki *Mycobacterium bovis* izolatlarının genotip düzeyinde tanımlanması amaçlandı. Çalışmada, Konya'daki kesimhanelerden alınan ya da Konya Veteriner Kontrol Enstitüsü'ne teşhis için gönderilen tüberküloz şüpheli sığır doku ve organlarından klasik metotlarla izole edilen ve moleküler metotlar ile tanımlanan *M. bovis* suşlarının genotiplendirilmesi sonucu moleküler epidemiyolojik özellikleri belirlendi. Toplam 70 *M. bovis* izolatının, MIRU-VNTR yöntemi ile yapılan analizi sonucu; MIRU2, MIRU4, MIRU20, MIRU23, MIRU24, MIRU27 ve MIRU39 lokuslarındaki tekrar sayılarının suşlar arasında farklılık göstermediği; MIRU10, MIRU16, MIRU26, MIRU31 ve MIRU40 lokuslarındaki tekrar sayılarının ise suşlar arasında farklılık gösterdiği ve yüksek ayrım gücüne sahip olduğu (0.25≤h) ortaya konuldu. Ayrıca, izolat sayısı 1-14 arasında değişen 29 altgrup oluştuğu görüldü. Bölgemizde farklı sebepler ile yaşanan hayvan hareketliliğinin, *M. bovis* suşlarının sürüler arasında farklılık göstermesine, çalışmamızda ki izolatların kaynağı olan sığırların da farklı sürülere ait olmasının MIRU lokuslarında ayrım gücü çeşitliliğinin ortaya çıkmasına neden olduğu kanaatine varılmıştır.

Anahtar kelimeler: MIRU-VNTR, *Mycobacterium bovis*, moleküler tiplendirme, sığır tüberkülozu

Introduction

Mycobacterium bovis, as the main causative agent of bovine tuberculosis, is a zoonotic bacterium that also causes tuberculosis in many domestic and wild mammalian species, aside from in humans (Joen et al. 2008; Boledo-Martinez et al. 2015). In addition to being a significant global animal health concern, the disease affects animal production and international trade, and more importantly, threatens public health (Wedlock et al. 2002; Duarte et al. 2010). For this reason, the diagnosis of bovine tuberculosis is not only valuable for the prevention of the transmission of the disease among animals, but also for the prevention of transmission from animals to humans. For the control of the disease, the molecular characterization of the strains that circulate in the field is

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important (Joen et al. 2008). Classical bacteriological methods are important for the identification of the agent in the sample but are insufficient for the characterization of the strains (Boledo-Martinez et al. 2015). For this reason, the genotyping methods used for the clinical strains are considered valuable molecular tools for the epidemiology of the disease, as well as for control studies (Matos et al. 2010). The use of these methods allows the identification of the most common *Mycobacterium bovis (M. bovis)* genotypes in a region, permits the monitoring and control of the occurrence of multiple disease foci, and increases the effectiveness of disease control programs (Carvalho et al. 2016).

The first gene region that is used to identify the similarities or differences in the distribution of Mycobacterium strains is IS6110, although this method is insufficient when there is a need to type a large number of strains, requiring intensive labor. Spacer oligonucleotide typing (spoligotyping) and Variable Number Tandem Repeats of Mycobacterial Interspersed Repetitive Units (MIRU-VNTR) analyses offer a good alternative approach to the characterization of a high number of strains, giving faster results than IS6110-Restriction Fragment Length Polymorphism (Köksalan 2010).

Forty one different VNTR regions are present within the genomes of the complex members of *M. tuberculosis,* and vary between 50-100 bp are defined as MIRU. The sizes of bands that are determined using the primers that include MIRU regions and that recognize neighboring regions permit the determination of repeat numbers of MIRU locus. Afterward, the VNTR profile of each strain is obtained based on the numerical coding that is carried out using the copy numbers. While 12 loci MIRU primers were used in the epidemiological studies in the past, MIRU sets comprising 15 and 24 loci were used in later studies (Supply et al. 2006; Boledo-Martinez ve ark. 2015).

With the emergence of molecular epidemiology, faster and more reliable diagnostic methods were developed that provided information about the relationships between the strains as well as the genetic structure. MIRU-VNTR is a molecular tool that makes use of fast, objective, and high-resolution monitoring procedures for the identification of outbreak dynamics and epidemiology (Vargas et al. 2016). The goal of the present study is to determine the epidemiological characteristics of the clinical isolates of *M. bovis* in Konya province at a molecular level.

Materials and Method

Bacterial strains used in the study

For the present study, *M. bovis* strains that were isolated and identified using classical and molecular methods from the tissues and organs of 70 tuberculosis-suspected cattle obtained from slaughterhouses in Konya province, or samples sent to the Konya Veterinary Control Institute, were tested using 12 loci MIRU-VNTR to investigate their molecular epidemiological characteristics.

DNA Extraction

The DNA extraction of the mycobacteria that were isolated from the tuberculosis-suspected tissues and organs was performed using a DNeasy Blood & Tissue kit (QIAGEN-69506, Germany), following the instructions of the manufacturer.

MIRU-VNTR Analysis

The primers given in Table 1. were used in the targeted MIRU loci of *M. bovis* (02, 04, 10, 16, 20, 23, 24, 26, 27, 31, 39, 40). PCR was performed to determine the VNTR number of the 21 MIRU loci specific to each strain (Oral Zeytinli and Köksal 2012). The PCR mixture was prepared to include: 0.25 µl DMSO (Sigma), 2×PCR mixture (Fermentas), 3.125 µl [4mM $MgCl_2$, 1.6 mM dNTP mix (0.4 mM from each dNTP) containing 0.05 µl Taq DNA polimerase], "forward" primer 0.040 µl, "reverse" primer 0.040 µl, DNA 0.625 µl and dH2O 2.170 µl, totaling 6.250 µl.

Table 1. Primers used for the 12-Locus MIRU VNTR method.

Target Gene	Sequence of PCR primer (5′ to 3′)
MIRU 2a	5′-TGG ACT TGC AGC AAT GGA CCA ACT-3′
MIRU 2b	5′-TAC TCG GAC GCC GGC TCA AAA T-3′
MIRU 4a	5′-GCG CGA GAG CCC GAA CTG C-3′
MIRU 4b	5′-GCG CAG CAG AAA CGT CAG C-3′
MIRU 10a	5′-GTT CTT GAC CAA CTG CAG TCG TCC-3′
MIRU 10b	5′-GCC ACC TTG GTG ATC AGC TAC CT-3′
MIRU 16a	5′-TCG GTG ATC GGG TCC AGT CCA AGT A-3′
MIRU 16b	5′-CCC GTC GTG CAG CCC TGG TAC-3′
MIRU 20a	5′-TCG GAG AGA TGC CCT TCG AGT TAG-3′
MIRU 20b	5′-GGA GAC CGC GAC CAG GTA CTT GTA-3′
MIRU 23a	5′-CAG CGA AAC GAA CTG TGC TAT CAC-3′
MIRU 23b	5′-CGT GTC CGA GCA GAA AAG GGT AT-3′
MIRU 24a	5′-CGA CCA AGA TGT GCA GGA ATA CAT-3′
MIRU 24b	5′-GGG CGA GTT GAG CTC ACA GAA-3′
MIRU 26a	5′-CCC GCC TTC GAA ACG TCG CT-3′
MIRU 26b	5′-TGG ACA TAG GCG ACC AGG CGA ATA-3′
MIRU 27a	5′-TCG AAA GCC TCT GCG TGC CAG TAA-3′
MIRU 27b	5′-GCG ATG TGA GCG TGC CAC TCA A-3′
MIRU 31a	5′-ACT GAT TGG CTT CAT ACG GCT TTA-3′
MIRU 31b	5′-GTG CCG ACG TGG TCT TGA T-3′
MIRU 39a	5′-CGC ATC GAC AAA CTG GAG CCA AAC-3′
MIRU 39b	5′-CGG AAA CGT CTA CGC CCC ACA CAT-3′
MIRU 40a	5′-GGG TTG CTG GAT GAC AAC GTG T-3′
MIRU 40b	5′-GGG TGA TCT CGG CGA AAT CAG ATA-3′

Amplification was performed in a thermal cycler (MJ Research PTC-100 USA) by preliminary denaturation at 94˚C for 5 minutes, followed by 30 cycles of denaturation at 95˚C for 30 secs, cooling at 62˚C for 1 min, extension at 72˚C for 1.5 mins, and a final chain extension at 72˚C for 10 mins. The amplicons were subjected to gel electrophoresis in 2% agarose with ethidium bromide (Seakem, 50002, USA) at 120 volts for 60 minutes. After electrophoresis, the gel was monitored using a system (EC App. Corp. EC135 USA), and then 50-1000 bp markers (Fermentas) were used for the determination of band size (Oral Zeytinli and Köksal 2012).

Table 2. Copy numbers and discriminatory power determined using 12-locus MIRU VNTR method.

Allel	Allel Copy Numbers**											Discriminatory power (h*)
	0	1	2	3	4	5	6	7	8	9	10	
MIRU 2			70									0,00
MIRU 4				70								0,00
MIRU 10			40	1		7	19	3				**0,58**
MIRU 16				41	29							0,48
MIRU 20			70									0,00
MIRU 23					70							0,00
MIRU 24			70									0,00
MIRU 26						10	5	38	17			**0,60**
MIRU 27					70							0,00
MIRU 31			29	19		22						**0,65**
MIRU 39			70									0,00
MIRU 40		5	58	7								0,29

*h=1-∑ xi² [n/(n-1)]
**xi: repeats of the allele locus

Results

In the study, in which 12 loci MIRU-VNTR was used to investigate the molecular epidemiological characteristics of 70 cattle isolates that were isolated and identified using classical and molecular methods, it was found that the repeat numbers for MIRU10, MIRU16, MIRU26, MIRU31, and MIRU40 loci varied between strains and these loci had a discriminatory power (0.25≤h) [MIRU-VNTRPlus, www.miru-vntrplus.org].

It was also found that the repeat numbers for MIRU2, MIRU4, MIRU20, MIRU23, MIRU24, MIRU27, and MIRU39 loci showed no variation between the strains and had no discriminatory power. It was determined from the genotyping analysis that 29 subgroups with 1-14 isolates were formed; that subgroup 16 comprised the largest gene group, with 13 members, followed by subgroup 18 with eight members and subgroup 17 with five members, while one subgroup had four members, three subgroups had three members, eight subgroups had two members and 14 subgroups had one member.

Table 3. VNTR profiles formed as a result of the MIRU-VNTR analysis of cattle isolates.

MIRU-VNTR Profiles	Strain number
232324274222	13
236424274522	8
235424274522	5
232324254323	4
236324284522	3
236424284522	3
237424274322	3
236324284322	2
232324254221	2
232324274221	2
232324254322	2
232324274223	2
232324284322	2
232324284522	2
232324284222	2
232324264222	1
232324254222	1
232324284222	1
232324264522	1
232424274322	1
232424254322	1
232424274222	1
232424274223	1
232424264222	1
233324264322	1
235324284222	1
235424284322	1
236324274221	1
236424264322	1
236424274322	1
	N=70

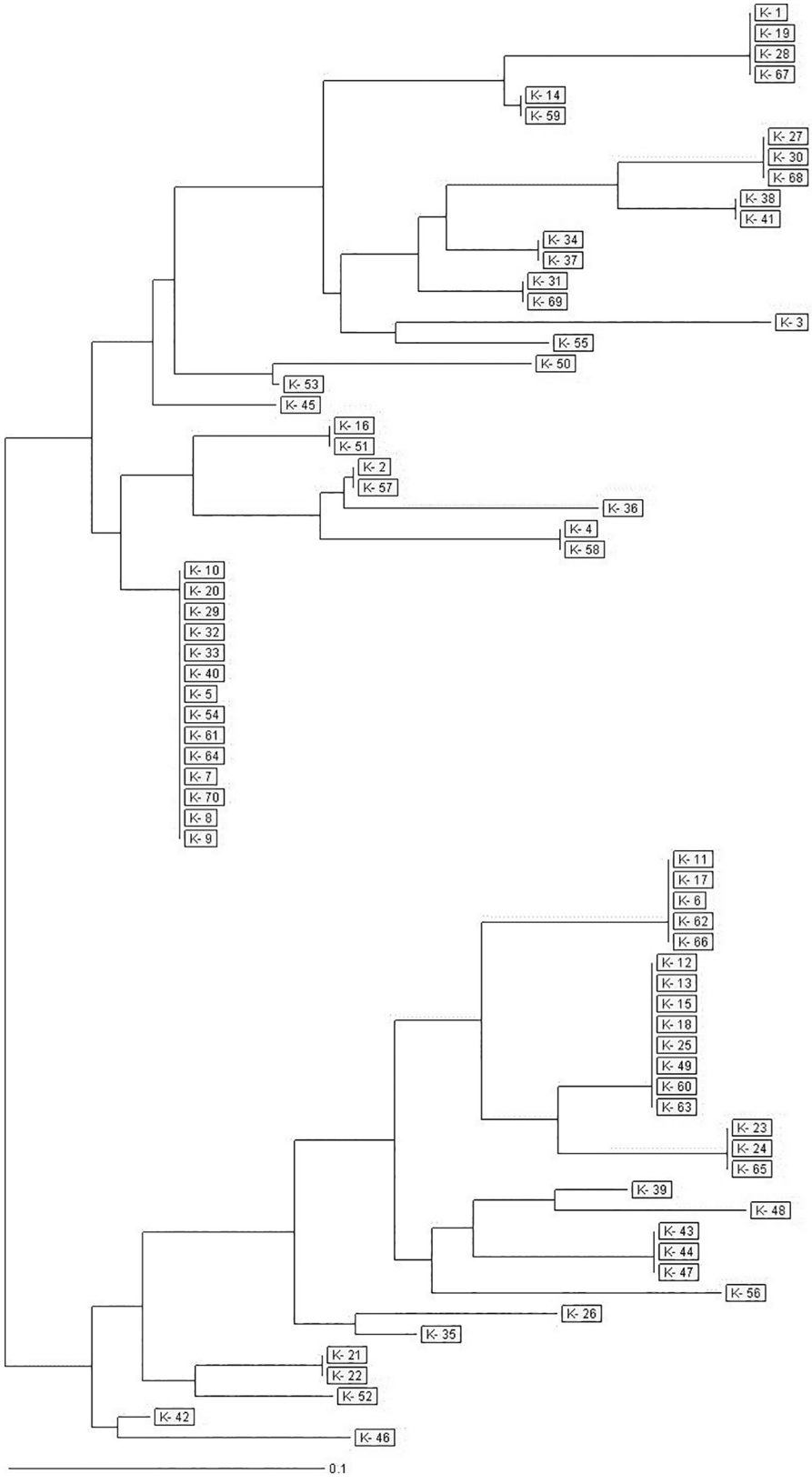

Figure 1. 12 MIRU-VNTR dendrogram of 70 cattle isolates.

Discussion

Genotyping studies of *M. bovis* strains are considered a valuable tool for the identification of sources of infection and transmission routes, and for the epidemiological monitoring of bovine tuberculosis (Boniotti et al. 2009). The lack of information or epidemiological assessments are the main reasons for the failure to isolate the agent in many tuberculosis cases (Frothingham and Meeker-O'Connell 1998). Thanks to the epidemiological studies that are increasing in number with the development of molecular methods, the distinction between reinfection and reactivation, in particular, can be made, with the source and dynamics of outbreaks can be determined, and iatrogenic infections and laboratory contaminations can be detected (Esen 2003). Genotyping studies are not only effective for regional backwards tracing, but also for the global comparison of tuberculosis isolates (Yang et al. 2015). The IS*6110* REA (Restriction Enzyme Analysis) and/or PGRS (Polymorphic GC Rich Sequence) methods used in these studies have recently been replaced by such methods as spoligotyping and MIRU-VNTR (Esen 2003).

For the typing of *M. bovis* isolates, the importance of MIRU-VNTR (a PCR-based method that allows the locus analysis of 12 independent loci after amplification) in genotyping has been proven in previous studies, while there is as yet no internationally accepted MIRU-VNTR panel for *M. bovis*. The fact that each locus shows allelic diversity between countries makes the detection and use of a standardized locus panel important for future comparisons between laboratories (Bolado-Martínez et al. 2015). The most significant advantages of MIRU-VNTR are its high discriminatory power and repeatability, its suitability for multi-center studies, its lack of a requirement for large amounts of DNA, and its ability to be performed easily and in vitro (Esen 2003; Oral Zeytinli and Köksal 2012). The method also forms more discriminatory profiles than IS*6110* RFLP and spoligotyping (Esen 2003; Peterson 2009). In addition to being sensitive, specific, cheaper, and faster for the complex isolates of *M. tuberculosis*, the sufficiency of its resolution in many cases has led the MIRU-VNTR to become the standard first step in the epidemiology of *M. bovis* (Esen 2003; Oral Zeytinli and Köksal 2012; Yang et al. 2015).

In the present study, 70 *M. bovis* isolates from cattle in Konya province were analyzed using the 12 loci MIRU-VNTR method using the primers given in Table 1. According to our results, MIRU31, MIRU26, and MIRU10 loci (h= 0.65, 0.6, and 0.58 respectively) had high discriminatory power, while the MIRU16 and MIRU40 loci (h= 0.48 and 0.29 respectively) had a lower discriminatory power. On the other hand, MIRU2, MIRU4, MIRU20, MIRU23, MIRU24, MIRU27, and MIRU39 were found to have no discriminatory power. The finding that MIRU31 had the highest discriminatory power was in line with those of Jeon et al. (2008), Besirovic et al. (2012), and Bolado-Martínez et al. (2015), while Supply et al. (2006) found the discriminatory power of this locus to be medium and El Sayed (2019) reported this locus to have the lowest discriminatory power. MIRU26 was reported to have the second highest discriminatory power, which was in line with the findings of several researchers (Hilty et al. 2005; Allix et al. 2006, Supply et al. 2006; Boniotti et al. 2009; Parreiras et al. 2012; Laniado-Laborín et al. 2014; Bolado-Martínez et al. 2015; Vargas et al. 2016), who reported the same result. Another locus with discriminatory power was MIRU10, in line with the findings of Besirovic et al. (2012), and Elsayed (2019) while Bolado-Martínez et al. (2015) and Vargas et al. (2016) reported that this locus had only medium discriminatory power. On the other hand, the lack of discriminatory power of the MIRU 02, MIRU 20, MIRU 23, MIRU 24, and MIRU 39 loci in our study was in concurrence with studies carried out in Ireland and Brazil (Roring et al. 2004; Figueiredo et al. 2011; Parreiras et al. 2012).

Many studies (Hilty et al. 2005; Allix et al. 2006; Supply et al. 2006; Boniotti et al. 2009; Parreiras et al. 2012; Laniado-Laborín et al. 2014, Bolado-Martínez et al. 2015; Vargas et al. 2016) have been carried out in Europe and in other parts of the world aiming to establish a reference method for the high-resolution genotyping study of mycobacterial strains that can serve as a tool for epidemiological and phylogenetic research. As a result of these researches differences in the discriminatory powers of VNTR loci have been identified in different countries and regions. In the study by Laniado-Laborín et al. (2014), the locus with the highest discriminatory power among the *M. bovis* strains was MIRU26, while the MIRU4 and MIRU31 loci were reported to make no contribution to the discrimination of strains. Bolado-Martinez et al. (2015) and Vargas et al. (2016) from the same country reported MIRU26 and MIRU31 to have the highest discriminatory power, while MIRU4 had only medium discriminatory power. Also, Parreiras et al. (2012) found MIRU26 and MIRU16 to have high discriminatory power in Brazil, while Carvalho et al. (2016) reported MIRU16 to have medium discriminatory power and MIRU26 to have a very low dis-

criminatory power. The different findings reported in the same or different geographical locations can be attributed to the genetic variations between the causative agents of the disease.

Researchers (Carvalho et al. 2016) have concluded that the movement of animals between different farms or regions can lead to the transmission of *M. bovis* strains between herds. An analysis of the results of the present study revealed variations in the loci with high discriminatory power. Due to the difference between the discriminatory powers of genetic markers between regions and countries, forming an ideal MIRU-VNTR panel will only be possible with access to the data obtained from studies be carried out in different regions or countries of the world (Roring et al. 2004; Hilty et al. 2005). The development of a standard set of loci and a database at an international level will enable *M. bovis* strains to be monitored and compared globally with the genotyping data that will be obtained in the near future, and the sharing of this data. For the accurate genotyping of *M. bovis* strains circulating in the field, it is necessary to establish an international standard model that includes MIRU loci with high discriminatory power, requiring more studies to contribute to the epidemiological resolution. The obtained data will contribute to the easy assessment of interrelated disease foci, with an epidemiological focus view in disease control studies, and will help clarify the transmission dynamics of the disease agents as well as cluster growth, while also being valuable for monitoring infections and determining risk factors.

Through this study, which is a first for Turkey in this field, preliminary data was used to develop a minimal panel that can be used in later genotyping studies of *M. bovis*. We consider that the use of MIRU31, MIRU26, and MIRU10, as loci with high discriminatory powers in the 12 loci MIRU-VNTR panel, as markers for the genotyping of *M. bovis* isolates can contribute to the results.

The use of common pastures by different herds in our region, contact with wildlife, and the high intensity of animal movements due to local trade were found to cause *M. bovis* strains to vary between herds. The fact that the cattle that were the source of isolates in our study came from different herds led to variations in the discriminatory power of MIRU loci.

Ethics statement: This research was approved by the Ethics Committee of the Experimental Medicine Research and Application Center of Selcuk University in Konya, Turkey (Protocol Number: 2009/057).

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