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Application of PCR-SSP method for *HLA-B*27* identification as an auxiliary tool for diagnosis of ankylosing spondylitis

*Aplicação da metodologia de PCR-SSP na identificação de HLA-B*27 como auxílio ao diagnóstico de espondilite anquilosante*

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ABSTRACT

Introduction: Human leukocyte antigens (HLA) are molecules that present antigen to the immune system; their presence or absence have been described as an influential factor in some diseases. *HLA-B*27* is an HLA polymorphism that has been associated with increased susceptibility to ankylosing spondylitis (AS) and other spondyloarthropathy. The detection of *HLA-B*27* has been used as diagnostic and prognostic tool in these cases, as well as in the differential diagnosis of other diseases. **Objective:** Standardize the single specific primer-polymerase chain reaction (PCR-SSP) methodology for use in the Immunogenetics Laboratory of the Universidade Estadual de Maringá (UEM), considering its specificity and cost-effectiveness. **Material and methods:** A total of 30 individuals without AS positive for *HLA-B*27* allele and 10 AS negative individuals, were previously tested by PCR-sequence specific oligonucleotide (PCR-SSO) and, in this study, by PCR-SSP. **Results:** One hundred percent of the patients tested confirmed their results, even with different subtypes. **Conclusion:** Considering the high reproducibility and the broad spectrum of subtypes covering, it was concluded that the PCR-SSP identifying method for *HLA-B*27* can be used as a routine diagnostic tool for spondyloarthropathies.

Key words: *HLA-B*27* antigen; ankylosing spondylitis; molecular biology.

INTRODUCTION

The major histocompatibility complex is a set of mapped genes on the short arm of chromosome 6, which encodes molecules presenting antigens to the immune system, called human leukocyte antigen (HLA)⁽¹⁾. HLA molecules are divided into classes: in class I region are loci A, B and C⁽²⁾, that generates products with high degree of allelic variation at each locus, resulting in a large variability in individuals⁽³⁾. The presence or absence of some of these antigens has been related to some diseases⁽¹⁾. By August 2015, 3,977 *HLA-B* alleles were identified in different populations⁽⁴⁾.

The *HLA-B*27* is a polymorphism of the *HLA-B*, described in 1969⁽⁵⁾. The presence of this allele was associated with ankylosing spondylitis (AS) and other spondyloarthropathies⁽⁶⁻⁸⁾, a group

of chronic inflammatory diseases with clinical and radiological manifestations in common⁽⁹⁾. About 184 subtypes of this *HLA*⁽¹⁰⁾ are known, and *HLA-B*27:05* is the most widely distributed and probably is the allele from which others have developed⁽¹¹⁻¹⁴⁾.

The *HLA-B*27* connection to the AS is well established; 90%-95% of patients with the disease have this allele⁽¹⁵⁻¹⁸⁾, suggesting a tendency to family association⁽¹⁹⁻²¹⁾. The prevalence of AS accompanies the frequency of *HLA-B*27* allele in the population⁽²²⁾, however studies indicate that only 2% of the individuals *HLA-B*27* positive develop the disease⁽²³⁾, indicating the involvement of other genetic and/or environmental factors in the onset of it^(24, 25). The exact trigger mechanism has not been identified⁽²⁶⁾.

Moreover, some studies indicate that certain subtypes have a greater association with AS than others, suggesting clinical

specificity⁽²⁷⁻³²⁾. The *HLA-B*27:05* and the *HLA-B*27:02* are the most common subtypes, 90% and 5%-10%, respectively, in Caucasians⁽³³⁾; the *HLA-B*27:04* is the predominant subtype among Chinese and Japanese⁽³⁴⁾ and the *HLA-B*27:04*, the *HLA-B*27:06* and the *HLA-B*27:07* are found only in Asia^(35,36). This allele is rare in black African populations, as well as the frequency of AS in this ethnic group. In Brazil, the disease is found in mulattos, since they have the Caucasian genetic influence⁽³⁷⁾.

The *HLA-B*27* has been used as a diagnostic marker for AS and other spondyloarthropathies^(38,39). The presence of the allele is not linked to the development of AS, but its presence may be useful in the diagnosis and also to auxiliate in the differential diagnosis of other diseases⁽²⁾. Furthermore, it can be used as indicator of patient prognosis; individuals with AS and *HLA-B*27* tend to have more prolonged and severe symptoms⁽⁴⁰⁾.

The identification of *HLA-B*27* and its subtypes is commonly performed by polymerase chain reaction-sequence specific oligonucleotide (PCR-SSO) and polymerase chain reaction-sequence specific primer (PCR-SSP) methodologies. This is a method considered fast, efficient and relatively low cost⁽⁴¹⁾. The advantage is the differentiation of several alleles⁽⁴²⁾, this method allows to detect a single different base in the the deoxyribonucleic acid (DNA) sequence between two alleles, though it can not detect a new undefined allele, unless the change happen in the location detected by the primer⁽⁴³⁾. The PCR-SSO, when compared to the PCR-SSP, may present more ambiguities because the probes used are able to bind to the DNA of the sample in a single region per test; the PCR-SSP can bind to the DNA of the sample in two regions per test⁽⁴⁴⁾.

Due to the benefits of PCR-SSP methodology and clinical applicability of *HLA-B*27*, the standardization of this method for use in routine and research in the Immunogenetics Laboratory of the Universidade Estadual de Maringá (UEM) is justified.

MATERIAL AND METHODS

Case series

We included 30 patients without AS previously tested by PCR-SSO (One-Lambda, low-medium resolution) and proven positive for *HLA-B*27* allele from the National Register of Bone Marrow Donor (Registro Nacional de Doadores de Medula Óssea [REDOME]) of the city of Maringá and surrounding region. The control-group consisted of 10 individuals from the same

database, but negative for *HLA-B*27* allele, evidenced by the same methodology.

The selection of individuals in the database considered the heterogeneity of the *HLA-B*27* alleles to identify the greatest number of subtypes. These have been identified, including cross-reactions, by the National Marrow Donor Program (NMDP) code (<http://www.marrow-donor.org/cgi-bin/DNA/dnatyp.pl>).

Table 1 describes the *HLA-B*27* subtypes tested and confirmed in this study.

TABLE 1 – Positive results for *HLA-B*27* by PCR-SSO and PCR-SSP methods

Patients	NMDP code of subtypes identified by PCR-SSO and PCR-SSP	
	Possible alleles that the subtypes represent	
H1	B*27:AETG	27:03/27:05/27:13/27:14/27:17
H2	B*27:ADDM	27:03/27:05/27:13/27:17/27:19
H3	B*27:09	-
H4	B*27:BWHH	27:03/27:05/27:07/27:11/27:13/27:17/27:32
H5	B*27:TGG	27:02/27:30
H6	B*27:02	-
H7	B*27:BXPE	27:03/27:05/27:13/27:17/27:32
H8	B*27:CXME	27:03/27:05/27:13/27:17/27:38
H9	B*27:CWNS	27:03/27:05/27:13/27:17/27:32/27:37
H10	B*27:08	-
H11	B*27:BRRD	27:03/27:05/27:13/27:17/27:19/27:28
H12	B*27:AS	27:07/27:11
H13	B*27:CFBR	27:03/27:04/27:05/27:10/27:13/27:14/27:15/27:17
H14	B*27:CNCS	27:07/27:11/27:34
H15	B*27:XME	40:01/40:10/40:22N/40:43
H16	B*27:ADDM	27:03/27:05/27:13/27:17/27:19
H17	B*27:BWHH	**
H18	B*27:BXPE	**
H19	B*27:AETG	**
H20	B*27:CXME	**
H21	B*27:TGG	**
H22	B*27:BRRD	**
H23	B*27:CWNS	**
H24	B*27:CXME	**
H25	B*27:BXPE	**
H26	B*27:ADDM	**
H27	B*27:BWHH	**
H28	B*27:BRRD	**
H29	B*27:CWXB	27:03/27:05/27:13/27:17/27:19/27:38
H30	B*27:PEN	27:03/27:05/27:13/27:17

HLA: human leukocyte antigens; *PCR-SSP*: polymerase chain reaction-single specific primer; *PCR-SSO*: polymerase chain reaction-sequence specific oligonucleotide; *NMDP*: National Marrow Donor Program; **: possible alleles mentioned above.

DNA extraction

The DNA extraction was performed with the BIOPUR extraction kit (Kit de Extração Mini Spin Plus 250, Biometrix), using from 200 µl of whole blood collected with ethylenediaminetetraacetic acid (EDTA). After extraction, the DNA was quantified in Nanodrop (NanoDrop 2000 Spectrophotometer, Thermo Scientific-Uniscience).

PCR-SSP

The primers specific for *HLA-B*27* were selected from sequences of the International ImMunoGeneTics Database (IMGT)/HLA (<http://www.ebi.ac.uk/ipd/imgt/hla/probe.html>) and described by Kulkarni *et al.* ⁽⁴⁵⁾; the sequences are shown in **Table 2**. The Amplicon flanked by these primers was designed to identify the *HLA-B*27* subtypes from 27:01 to 27:73 and expand the identification, an additional primer was included⁽⁴⁶⁾. The *HgH* gene was used as an internal control of the reaction.

TABLE 2 – Specific primers for *HLA-B*27*

Sense	Sequence
Sense	1-5' -GCTACGTGACGACACGCT-3'
Antisense	1-5' -CTCGGTCAGTCTGTGCCTT-3'
Antisense	2-5' -TCTCGGTAAGTCTGT GCCTT-3'
<i>HgH</i> sense	-5'-TGCTTCCCAACCAATCCCTTA-3'
<i>HgH</i> antisense	5'-CCACTCAGGATTTC TGTGTGTTTG-3'

HLA: human leukocyte antigens.

The conditions of the reaction used were described by Parasannanavar *et al.* (2013)⁽⁴⁷⁾ with modifications. The tests were initiated at the following conditions: 1 µl buffer, 0.5 µl magnesium chloride (MgCl₂), 0.5 µl deoxyribonucleotide phosphates (dNTP), 2.5 µl primers and 0.5 µl Taq, this volume was used for five samples. The amplifications were performed in a thermocycler (Veriti 96 Well Thermal Cycler, Applied Biosystems) at settings: initial denaturation at 94°C for 5 minutes, 30 cycles at 94°C for 1 minute, at 65°C for 2 minutes, at 72°C for 1 minute and final extension at 72°C for 10 minutes. The amplification products were subjected to electrophoresis (Electrophoresis Power Supply LPS – 300 V, Locus Biotecnologia) in 2% agarose gel with 0.5 µg/ml Syber safe and bromophenol blue in 0.5× buffer of tris-borate-EDTA (TBE) at 80 V for 30 minutes. The identification of bands was performed using a transilluminator (Transiluminador UVB ITB 20 × 20 STV, Locus Biotecnologia); the band corresponding to the *HgH* gene has 434 pb and the positive band for *HLA B*27*, 149 pb.

RESULTS

To standardize the PCR-SSP, tests were performed with several amounts of each compound to improve the visibility of the bands in the gel testes. In the first mix tested, the reaction was not satisfactory, the control bands were visible, but the bands of the *HLA-B*27* were poorly visible. Modifications and sequential test were conducted by modifying a test variable to reach the best reaction condition, which is composed of: 12.5 µl buffer, 4 µl MgCl₂, 2.5 µl dNTP, 1.5 µl primers, 1 µl primers of *HgH* gene and 1.25 µl Taq. Moreover, cycling conditions were also changed to improve the visibility of the bands in the agarose gel, reaching the optimum file of: initial denaturation at 94°C for 5 minutes, 30 cycles at 94°C for 40 seconds, 64°C for 1 minute, 72°C for 1 minute and final extension at 72°C for 10 minutes. The **Figure** shows the pattern of bands obtained using the final mix, standardized.

From the standardization, the *HLA-B*27* alleles were tested and all subtypes were amplified by PCR-SSP. **Table 3** shows the results. One hundred percent of sensitivity and specificity were obtained, there was no false positive or negative result.

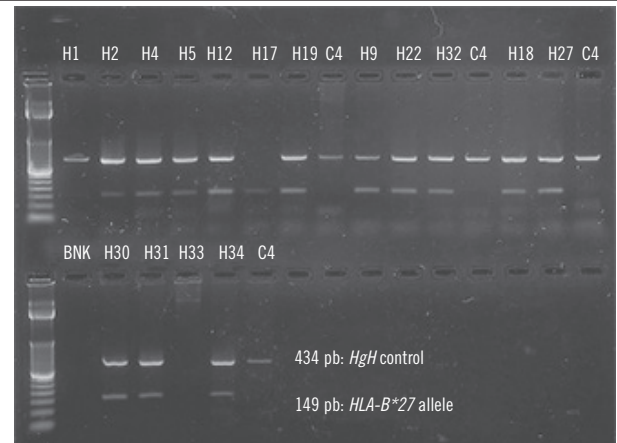


FIGURE – Band pattern of positive and negative samples for *HLA-B*27* by PCR-SSP in agarose gel with standardized mix

PCR-SSP: polymerase chain reaction-single specific primer; HLA: human leukocyte antigens; H: *HLA-B*27* positive; C: *HLA-B*27* negative; BNK: blank. Step ladder: 50 pb.

TABLE 3 – Positive and negative results for PCR-SSO and PCR-SSP

		<i>HLA-B*27</i> (PCR-SSO)	
		Present	Absent
PCR-SSP testing	Positive	30	0
	Negative	0	10

PCR-SSO: polymerase chain reaction-sequence specific oligonucleotide; PCR-SSP: polymerase chain reaction-single specific primer; HLA: human leukocyte antigens.

DISCUSSION

Due to the association of the presence of the *HLA-B*27* allele with spondyloarthropathies, the molecular detection requires this allele, which is of utmost importance for both the study and the diagnosis of these diseases. Furthermore, this detection in asymptomatic patients and the research on the risk of patients relatives developing the disease is also essential, that's because epidemiological studies have shown that 10%-20% of first-degree relatives of patients with AS have higher risk of developing the disease⁽⁴⁸⁾.

According to Parasannanavar *et al.* (2013)⁽⁴⁷⁾, the methods used for identification of *HLA-B*27* (microlymphocytotoxicity and flow cytometry) are difficult because of the lack of a specific antiserum and the possibility of false results due to the change on epitopes. Molecular techniques are more sensitive and specific. The authors tested 90 samples from healthy controls by PCR-SSP and PCR-SSO and found no discrepancy in results between them, data compatible with this study, in which the efficiency of PCR-SSP and

the applicability of the primers used was proven, since all results were consistent with those obtained by PCR-SSO, reaching 100% of specificity and sensitivity.

The difficulty encountered was the standardization of the DNA concentration to be used, Parasannanavar *et al.* (2013)⁽⁴⁷⁾ suggest the use of 80-100 ng, and this value is not always obtained. Alternative for DNA extraction methods may be used, such as salting out method⁽⁴⁹⁾, which expects a higher performance, but requires more time and the use of larger amounts of blood or buffy-coat.

Other authors, such as Frankengerger *et al.* (1997)⁽⁵⁰⁾, also found that identification of *HLA-B*27* by PCR is reliable and reproducible, and therefore recommended for the routine, since it overcomes the weaknesses of serology, such as cross-reactivity and ambiguous results.

It was concluded that the PCR-SSP identification method can be used as a routine diagnostic aid for spondyloarthropathies. It is a relatively simple, quick, low costly, high sensitivity and specificity technique.

RESUMO

Introdução: Os antígenos leucocitários humanos (HLA) são moléculas que apresentam antígenos ao sistema imune; a presença ou a ausência deles é descrita como fator influente em algumas doenças. O *HLA-B*27* é um polimorfismo do HLA que tem sido associado à maior predisposição à espondilite anquilosante (EA) e a outras espondiloartropatias. A detecção do *HLA-B*27* é utilizada como ferramenta diagnóstica e prognóstica nesses casos, assim como no diagnóstico diferencial de outras doenças. **Objetivo:** Padronizar a metodologia de reação em cadeia da polimerase-iniciador específico (PCR-SSP) para utilização no Laboratório de Imunogenética da Universidade Estadual de Maringá (UEM), considerando sua especificidade e seu custo-benefício. **Material e métodos:** Foram utilizados 30 indivíduos comprovadamente sem EA positivos para o alelo *HLA-B*27* e 10 indivíduos negativos, testados previamente por PCR-oligonucleotídeo específico (PCR-SSO) e, neste estudo, por PCR-SSP. **Resultados:** Cem por cento dos pacientes testados tiveram seus resultados confirmados, mesmo com diferentes subtipos. **Conclusão:** Tendo em vista a alta reprodutibilidade e o amplo espectro de subtipos que abrange, concluiu-se que a técnica de identificação de PCR-SSP para o *HLA-B*27* pode ser utilizada como auxílio diagnóstico de rotina para espondiloartropatias.

Unitermos: antígeno *HLA-B*27*; espondilite anquilosante; biologia molecular.

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