

AMBIGUOUS ALLELE COMBINATIONS AT GROUP LEVEL OF HLA-A, -C, AND -B GENES IN MACEDONIAN POPULATION USING REVERSE LINE STRIP TYPING METHOD

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Abstract: The aim of this study was to investigate the occurrence of ambiguous allele combinations at the allele group level of HLA-A, -C and -B loci in the Macedonian population. The DNA samples of 214 healthy unrelated Macedonian volunteers were obtained from our DNA Bank. HLA typing was performed using the IHWG-RLS method (Reverse Line Strip, Roche Molecular Systems, USA) consisting of PCR amplification of exon 2 and 3 of HLA-A, -B and -C genes, followed by hybridization. The statistical analysis of the observed ambiguity frequency was performed by using the Arleqin Software. At the HLA-A locus only one ambiguous allele combination at the allele group level in 214 samples was observed with a frequency of 0.467% ($1/214 = 0.467\%$). A total of 6 different HLA-C ambiguous allele combinations at the allele group level in twelve samples with a frequency of 5.607% ($12/214 = 5.607\%$) and 11 different for HLA-B locus in nineteen samples with a frequency of 8.879% ($19/214 = 8.879\%$) were observed in 214 samples. In conclusion we can say that analysis of the frequency of allele ambiguities revealed that the ambiguities involved some of the most common alleles in our population, obviating the need to introduce ambiguity resolution technique(s)/strategies in the HLA laboratory.

Key words: Allele ambiguity combination; HLA-A, -C, and -B polymorphisms; Macedonian population; Reverse Line Strip.

Introduction

The Major Histocompatibility Complex (MHC) is a region of multiple genes encoding products that play a central role in the development of both

humoral and cell-mediated immune responses. Among the expressed loci, the MHC has the greatest degree of polymorphism in the human genome. This is at such a degree that it is theoretically possible for each human to possess a different set of MHC alleles [1]. To a large extent the polymorphism within the MHC system consists of single nucleotide polymorphisms, many of them silent and having no effect on protein expression levels [2]. However, other studies have demonstrated the central role of substitution polymorphism in shaping HLA diversity. Analyses show that in the antigen recognition sites, nonsynonymous differences between alleles are proportionally more common than synonymous ones. In the rest of the molecule, there is an excess of synonymous changes, as is normally observed in protein coding sequences. In addition, many HLA polymorphisms are whole codon replacements that cannot be explained by a single-nucleotide replacement model [3, 4].

Although the DNA techniques for HLA typing have proved to be powerful in resolving many serological ambiguities, in detecting single nucleotide polymorphisms and actually defining a whole spectrum of new MHC alleles, still, not a single one of these techniques offers ambiguity-free MHC typing. Since the prevalence of ambiguities in HLA typing relates to the nature of polymorphisms in HLA Class I and II genes, defining strategies to resolve ambiguities created by HLA DNA typing remains a focus of interest for HLA DNA typing professionals.

The patterns of allelic sequence diversity for both the class I and class II HLA loci are highly unusual; some alleles differ in the second and third exons by as much as 15%, and the sequence variation is distributed as a patchwork of localized polymorphic sequence motifs. One consequence of this pattern of patchwork polymorphism is that, in PCR-based HLA typing, a large number of different alleles can be distinguished by using a relatively small number of oligonucleotide primers or probes complementary to these discrete sequence motifs. Another more problematic consequence is that a given pattern of sequence motifs, detected either with probes or primers or by sequencing, may be consistent with more than a single genotype because the observed sequence motifs can be combined into more than one unique pair of alleles [5].

Three different kinds of ambiguities are recognized: i. the polymorphic sites distinguishing the alleles in question are located outside the amplified or probed regions, ii. multiple allele pairs display identical heterozygous sequences, so that the phase of polymorphisms cannot be determined and iii. part of the sequence of an allele is not known [6, 7].

The minimum requirements for submission of new sequences into reference databases of HLA sequences are the sequencing of exon 2 and exon 3 for Class I and exon 2 for Class II. This approach has been the standard due to the functional relevance of this region which defines the peptide groove of Class I and Class II molecules, respectively. However, some Class I alleles have iden-

tical sequences across exons 2 and 3. To resolve these alleles it is necessary to analyse the gene at the region where they differ. Numerous ambiguities arise due to an incomplete sequence in exon 4. As DNA sequencing has become easier and more widely applied to defining HLA alleles, additional polymorphisms have been found in other exons, and also in the introns [8].

The precise identification of HLA Class I alleles is crucial for clinical purposes, and particularly essential for bone marrow transplantation from unrelated donors, investigation of genetic inheritance or disease association and most recently in developing cancer treatment strategies and designing vaccines. The extensive diversity of the HLA genes makes the identification of matched donors challenging. As histocompatibility and the transplant survival are inversely correlated with the number of HLA mismatches, the search continues for more efficient HLA typing methods that could unequivocally define HLA alleles, thereby increasing the chances of finding a matching donor and reducing the risk of adverse transplantation outcome due to ambiguous HLA typing [5]. However, ambiguous typing results do not necessarily correspond to clinically relevant mismatches.

Currently, commonly used HLA molecular typing methods include Reverse Line Strip (RLS), Sequence Specific Oligonucleotide Probes (SSOP), Polymerase Chain Reaction (PCR) using Sequence Specific Primers (SSP) and Sequence Based Typing (SBT). In recent years a significant advance has been made in resolution, automation, throughput and data analysis in the non-sequencing based HLA DNA typing techniques such as RLS and SSOP, but still the ambiguities remain as a considerable problem related to these techniques [5]. Sequencing is also not ambiguity free [6]. Due to the nature of HLA polymorphism, there will be ambiguity as long as we are forced to consider the polymorphism of both alleles at the same time. Even with sequencing we are faced with an inability to set phase. The only way to get around this is to consider each allele independently.

The aim of the study was to examine the ambiguous allele combination at the allele group level (cross-serogroup ambiguity) for HLA-A, -C and -B genes in the Macedonian population using a DNA typing method. For example, an ambiguity at the allele group level is found in HLA-A locus for allele combination "A*03011/013/03N/04xA*2501 or A*3204x A*6601", whereas an unambiguous result is obtained for "A*0101/04N/05NxA*2601". In this study we have observed ambiguous allele combinations for HLA-A, -C and -B genes based on the exon 2 and exon 3 sequences.

Material and Methods

Population samples

Two hundred and fourteen unrelated random healthy Macedonian volunteers of Macedonian origin and nationality, Christian Orthodox religion

and residents of different regions of the Republic of Macedonia were included in this study. Their ages ranged between 20 and 59 years. Peripheral blood was drawn after signing of the informed consent. Genomic DNA was extracted from the peripheral blood leukocytes using the standard phenol/chloroform procedure previously described [9], and stored in the anthropology project field of our DNA Bank (hDNAMKD) until processing [10].

PCR amplification

Duplex PCR reactions were carried out in 60 µl volume for HLA-A and -C and 100 µl volume for HLA-B locus, using biotinylated primers for both exons 2 and 3 of HLA-A, -C and -B genes contained in the PCR master mixes provided by the manufacturer. The PCR cycling conditions for all loci were 35 cycles of 95°C for 15s; 65°C for 45s and 72°C for 15s using a MJ Research PTC-100 thermal cycler. PCR products were visualized on a 2% agarose gel prior to hybridization to the RLS strips [11].

HLA-A, -C, and -B genotyping

HLA DNA typing of HLA-A, -C and -B genes was performed by using the International Histocompatibility Working Group's reverse hybridization method (Reverse Line Strip – Roche Molecular System, USA). The labelled PCR products were first denaturated and then hybridized in a single reaction to an array of SSO probes immobilized on nylon membrane. 57 different probes for high resolution HLA-A, 82 probes for high resolution HLA-B, and 36 probes for high resolution HLA-C typing were used with corresponding motifs. The presence of biotinylated PCR product bound to a specific probe was detected using streptavidin-horseradish peroxidase (HRP) and a chromogenic, soluble substrate to produce a blue "line" at the position of the positive probe. Genotyping software (HLA Genotyping Program Roche Molecular System, Inc.) can interpret the probe reactivity pattern as a genotype and indicates potential ambiguities [11].

Statistical analysis

The statistical analysis of the observed ambiguous allele combinations for HLA-A, -C and -B loci was performed by using the Arlequin Software version 2.000 [12].

Results

Reverse Line Strip typing for HLA-A, -C and -B loci was performed on a population of 214 samples. In this study we are reporting the ambiguous allele combinations at the allele group level for HLA-A, -C and -B loci, presented in Tables 1–3.

At HLA-A locus the only observed ambiguous allele combination was "A*03011/013/03N/04xA*2501 or A*3204x A*6601", with a frequency of 0.467% (1/214 = 0.467%) (Table 1).

Table 1 – Табела 1

*Allele ambiguity encountered with RLS for HLA-A locus
Двојбени алели добиени со РЛС во ХЛА-А локусу*

HLA-A No.	Allele ambiguity observed at group level	Number of samples where ambiguity was found	Ambiguity frequency % (total 214)	Oligonucleotide probes
1.	A*03011/013/03N/04	1	0.467	2, 6, 14, 16 , 21, / 23, 31, 33, 37, 39, 46, 48, 54, 57
	A*2501			1, 9, 12, 13, 20 , 21, / 27, 35, 37, 41, 43, 50, 51, 57
	or			
	A*3204			2, 6, 13, 20 , 21, / 23, 31, 33, 37, 39, 46, 48, 54, 57
	A*6601			1, 9, 12, 14, 16 , 21, / 27, 35, 37, 41, 43, 50, 51, 57

At HLA-C locus we found 6 different ambiguous allele combinations observed in twelve samples with a total frequency of 5.607% (12/214 = 5.607%) (Table 2).

Table 2 – Табела 2

*Allele ambiguity encountered with RLS for HLA-C locus
Двојбени алели добиени со РЛС во ХЛА-Ц локусу*

HLA-C No.	Allele ambiguity observed at group level	Number of samples where ambiguity was found	Ambiguity frequency % (total 214)	Oligonucleotide probes
1.	C*1203/06	4	1.869	1, 7, 8, 12, 13 , 16, 17, / 20, 25, 33, 34, 36
	C*15021/022			2, 7, 8, 11, 14 , 16, 17, / 18, 28, 30, 34, 36
	or			
	C*12042			1, 7, 8, 12, 14 , 16, 17, / 20, 25, 33, 34, 36
	C*1507			2, 7, 8, 11, 13 , 16, 17, / 18, 28, 30, 34, 36
	or			
	C*16041			1, 7, 8, 11, 13 , 16, 17, / 20, 25, 33, 34, 36
	C*1503			2, 7, 8, 12, 14 , 16, 17, / 18, 28, 30, 34, 36
2.	C*1203/06			1, 7, 8, 12, 13 , 16, 17, / 20, 25, 33, 34, 36
	C*14021/022			5, 6, 8, 11, 13 , 16, 17, / 23, 25, 31, 34, 36
	or			

	C*1207	2	0.935	1, 7, 8, 12 , 16, 17, / 20, 25, 33, 34, 36
	C*14021/022			5, 6, 8, 11 , 13 , 16, 17, / 23, 25, 31, 34, 36
	or			
	C*16041			1, 7, 8, 11 , 13 , 16, 17, / 20, 25, 33, 34, 36
	C*1404			5, 6, 8, 12 , 16, 17, / 23, 25, 31, 34, 36
3.	C*0501/03	2	0.935	1, 7, 8, 11 , 14 , 16, 17, / 18, 27, 31, 34 , 36
	C*1203/06			1, 7, 8, 12 , 13 , 16, 17, / 20, 25, 33, 34 , 36
	or			
	C*0502			1, 7, 8, 11 , 14 , 16, 17, / 18, 27, 31, 36
	C*1203/06			1, 7, 8, 12 , 13 , 16, 17, / 20, 25, 33, 34 , 36
	or			
	C*0802/07			1, 7, 8, 11 , 13 , 16, 17, / 18, 27, 31, 34 , 36
	C*12042			1, 7, 8, 12 , 14 , 16, 17, / 20, 25, 33, 34 , 36
	or			
	C*0805			1, 7, 8, 12 , 13 , 16, 17, / 18, 27, 31, 34 , 36
	C*1205			1, 7, 8, 11 , 14 , 16, 17, / 20, 25, 33, 34 , 36
4.	C*0501/03	2	0.935	1, 7, 8, 11, 14 , 16, 17, / 18 , 27 , 31, 34 , 36
	C*07011/012/06			3, 6, 8, 12, 13 , 16, 17, / 18 , 25 , 30, 34 , 36
	or			
	C*0501/03			1, 7, 8, 11, 14 , 16, 17, / 18 , 27 , 31, 34 , 36
	C*0705			3, 6, 8, 12, 13 , 16, 17, / 25 , 30, 34 , 36
	or			
	C*0502			1, 7, 8, 11, 14 , 16, 17, / 18 , 27 , 31, 36
	C*07011/012/06			3, 6, 8, 12, 13 , 16, 17, / 18 , 25 , 30, 34 , 36
	or			
	C*0502			1, 7, 8, 11, 14 , 16, 17, / 18 , 27 , 31, 36
	C*0705			3, 6, 8, 12, 13 , 16, 17, / 25 , 30, 34 , 36
	or			
	C*0802/07			1, 7, 8, 11, 13 , 16, 17, / 18 , 27 , 31, 34 , 36
C*0707/09	3, 6, 8, 12, 14 , 16, 17, / 18 , 25 , 30, 34 , 36			
5.	C*0501/03	1	0.467	1, 7, 8, 11, 14 , 16, 17, / 18, 27, 31 , 34 , 36
	C*1507			2, 7, 8, 11, 13 , 16, 17, / 18, 28, 30 , 34 , 36
	or			
	C*0502			1, 7, 8, 11, 14 , 16, 17, / 18, 27, 31 , 36
	C*1507			2, 7, 8, 11, 13 , 16, 17, / 18, 28, 30 , 34 , 36
	or			
	C*0801/03/04			1, 7, 8, 11, 13 , 16, 17, / 18, 27, 30 , 34 , 36
	C*1508			2, 7, 8, 11, 14 , 16, 17, / 18, 28, 31 , 34 , 36
	or			
	C*0802/07			1, 7, 8, 11, 13 , 16, 17, / 18, 27, 31 , 34 , 36
	C*15021/022			2, 7, 8, 11, 14 , 16, 17, / 18, 28, 30 , 34 , 36
or				
C*0806	1, 7, 8, 11, 13 , 16, 17, / 18, 27, 30 , 36			
C*1508	2, 7, 8, 11, 14 , 16, 17, / 18, 28, 31 , 34 , 36			
6.	C*1205	1	0.467	1, 7, 8, 11, 14 , 16, 17, / 20, 25, 33, 34, 36
	C*1507			2, 7, 8, 11, 13 , 16, 17, / 18, 28, 30, 34, 36
	or			
	C*16041			1, 7, 8, 11, 13 , 16, 17, / 20, 25, 33, 34, 36
	C*15021/022			2, 7, 8, 11, 14 , 16, 17, / 18, 28, 30, 34, 36

For HLA-B locus we identified 11 different ambiguous allele combinations that were observed in nineteen different samples with a total frequency of 8.879% (19/214 = 8.879%) (Table 3).

Table 3 – Табела 3

*Allele ambiguity encountered with RLS for HLA-B locus
Двојбени алели добиени со РЛС во ХЛА-А локусу*

HLA-B No.	Allele ambiguity observed at group level	Number of samples where ambiguity was found	Ambiguity frequency % (total 214)	Oligonucleotide probes
1.	B*0801	3	1.402	5, 9, 11, 15, 17, 23, 25, 34, 41 , 42, / 45, 53, 56, 64 , 68, 72, 75, 77 , 80, 82
	B*51011/09			6, 8, 12, 15, 18, 23, 25, 33, 40 , 42, / 46, 53, 56, 64 , 67, 74, 75, 78 , 79, 82
	or			
	B*0801			5, 9, 11, 15, 17, 23, 25, 34, 41 , 42, / 45, 53, 56, 64 , 68, 72, 75, 77 , 80, 82
	B*5103			6, 8, 12, 15, 18, 23, 25, 33, 40 , 42, / 46, 53, 56, 64 , 67, 74, 78 , 79, 82
	or			
	B*0801			5, 9, 11, 15, 17, 23, 25, 34, 41 , 42, / 45, 53, 56, 64 , 68, 72, 75, 77 , 80, 82
	B*5114			6, 8, 12, 15, 18, 23, 25, 33, 40 , 42, / 46, 53, 64 , 67, 74, 75, 78 , 79, 82
	or			
	B*0803			5, 9, 11, 15, 17, 23, 25, 33, 40 , 42, / 45, 53, 56, 64 , 68, 72, 75, 77 , 80, 82
	B*7801/022			6, 8, 12, 15, 18, 23, 25, 34, 41 , 42, / 46, 53, 56, 64 , 67, 74, 75, 78 , 79, 82
	or			
	B*0805			5, 9, 11, 15, 17, 23, 34, 40 , 42, / 45, 53, 56, 64 , 68, 72, 75, 77 , 80, 82
	B*51011/09			6, 8, 12, 15, 18, 23, 25, 33, 40 , 42, / 46, 53, 56, 64 , 67, 74, 75, 78 , 79, 82
	or			
	B*0805			5, 9, 11, 15, 17, 23, 34, 40 , 42, / 45, 53, 56, 64 , 68, 72, 75, 77 , 80, 82
	B*5103			6, 8, 12, 15, 18, 23, 25, 33, 40 , 42, / 46, 53, 56, 64 , 67, 74, 78 , 79, 82
	or			
	B*0805			5, 9, 11, 15, 17, 23, 34, 40 , 42, / 45, 53, 56, 64 , 68, 72, 75, 77 , 80, 82
B*5114	6, 8, 12, 15, 18, 23, 25, 33, 40 , 42, / 46, 53, 64 , 67, 74, 75, 78 , 79, 82			

2.	B*4002	3	1.402	3, 10, 14, 16, 20, 22, 27, 34, 41, 42, / 45, 53, 56, 64, 67, 73, 75, 77, 79, 82
	B*51011/09			6, 8, 12, 15, 18, 23, 25, 33, 40, 42, / 46, 53, 56, 64, 67, 74, 75, 78, 79, 82
	or			
	B*4002			3, 10, 14, 16, 20, 22, 27, 34, 41, 42, / 45, 53, 56, 64, 67, 73, 75, 77, 79, 82
	B*5103			6, 8, 12, 15, 18, 23, 25, 33, 40, 42, / 46, 53, 56, 64, 67, 74, 78, 79, 82
	or			
	B*4002			3, 10, 14, 16, 20, 22, 27, 34, 41, 42, / 45, 53, 56, 64, 67, 73, 75, 77, 79, 82
	B*5114			6, 8, 12, 15, 18, 23, 25, 33, 40, 42, / 46, 53, 64, 67, 74, 75, 78, 79, 82
	or			
	B*4008			3, 10, 14, 16, 20, 23, 25, 34, 41, 42, / 45, 53, 56, 64, 67, 73, 75, 77, 79, 82
	B*51011/09			6, 8, 12, 15, 18, 23, 25, 33, 40, 42, / 46, 53, 56, 64, 67, 74, 75, 78, 79, 82
	or			
	B*4009			3, 10, 14, 16, 20, 22, 27, 34, 41, 42, / 45, 53, 64, 67, 73, 75, 77, 79, 82
	B*51011/09			6, 8, 12, 15, 18, 23, 25, 33, 40, 42, / 46, 53, 56, 64, 67, 74, 75, 78, 79, 82
	or			
	B*4009			3, 10, 14, 16, 20, 22, 27, 34, 41, 42, / 45, 53, 64, 67, 73, 75, 77, 79, 82
B*5103	6, 8, 12, 15, 18, 23, 25, 33, 40, 42, / 46, 53, 56, 64, 67, 74, 78, 79, 82			
or				
B*4019	3, 10, 14, 16, 20, 22, 27, 33, 40, 42, / 45, 53, 56, 64, 67, 73, 75, 77, 79, 82			
B*7801/022	6, 8, 12, 15, 18, 23, 25, 34, 41, 42, / 46, 53, 56, 64, 67, 74, 75, 78, 79, 82			
3.	B*3501/07/11/23	3	1.402	6, 7, 12, 15, 18, 23, 25, 34, 41, 42, / 43, 54, 59, 64, 67, 74, 75, 77, 79, 82
	B*51011/09			6, 8, 12, 15, 18, 23, 25, 33, 40, 42, / 46, 53, 56, 64, 67, 74, 75, 78, 79, 82
	or			
	B*3501/07/11/23			6, 7, 12, 15, 18, 23, 25, 34, 41, 42, / 43, 54, 59, 64, 67, 74, 75, 77, 79, 82
	B*5103			6, 8, 12, 15, 18, 23, 25, 33, 40, 42, / 46, 53, 56, 64, 67, 74, 78, 79, 82
	or			
	B*3521/24			6, 7, 12, 15, 18, 23, 25, 34, 41, 42, / 43, 54, 59, 64, 67, 74, 75, 78, 79, 82
	B*51021			6, 8, 12, 15, 18, 23, 25, 33, 40, 42, / 46, 53, 56, 64, 67, 74, 75, 77, 79, 82
or				
B*5301	6, 7, 12, 15, 18, 23, 25, 33, 40, 42, / 43, 54, 59, 64, 67, 74, 75, 77, 79, 82			

	B*7801/022			6, 8, 12, 15, 18, 23, 25, 34, 41 , 42, / 46, 53, 56, 64, 67, 74, 75, 78 , 79, 82
4.	B*3801	2	0.935	1, 9, 11, 15, 17, 23, 26, 33, 40 , 42, / 44 , 53, 61, 64, 71, 72, 75, 77 , 79, 82
	B*7801/022			6, 8, 12, 15, 18, 23, 25, 34, 41 , 42, / 46 , 53, 56, 64, 67, 74, 75, 78 , 79, 82
	or			
	B*39011/013/05			1, 9, 11, 15, 17, 23, 26, 34, 41 , 42, / 44 , 53, 61, 64, 71, 72, 75, 77 , 79, 82
	B*51011/09			6, 8, 12, 15, 18, 23, 25, 33, 40 , 42, / 46 , 53, 56, 64, 67, 74, 75, 78 , 79, 82
	or			
	B*39011/013/05			1, 9, 11, 15, 17, 23, 26, 34, 41 , 42, / 44 , 53, 61, 64, 71, 72, 75, 77 , 79, 82
	B*5103			6, 8, 12, 15, 18, 23, 25, 33, 40 , 42, / 46 , 53, 56, 64, 67, 74, 78 , 79, 82
	or			
B*39061/062	1, 9, 11, 15, 17, 23, 26, 34, 41 , 42, / 46 , 53, 61, 64, 71, 72, 75, 77 , 79, 82			
B*5106	6, 8, 12, 15, 18, 23, 25, 33, 40 , 42, / 44 , 53, 56, 64, 67, 74, 75, 78 , 79, 82			
5.	B*3801	2	0.935	1, 9, 11, 15, 17, 23, 26, 33, 40 , 42, / 44, 53, 61, 64, 71, 72, 75, 77, 79, 82
	B*4002			3, 10, 14, 16, 20, 22, 27, 34, 41 , 42, / 45, 53, 56, 64, 67, 73, 75, 77, 79, 82
	or			
	B*39011/013/05			1, 9, 11, 15, 17, 23, 26, 34, 41 , 42, / 44, 53, 61, 64, 71, 72, 75, 77, 79, 82
	B*4019			3, 10, 14, 16, 20, 22, 27, 33, 40 , 42, / 45, 53, 56, 64, 67, 73, 75, 77, 79, 82
6.	B*3801	1	0.467	1, 9, 11, 15, 17, 23, 26, 33, 40 , 42, / 44, 53, 61, 64, 71, 72, 75, 77, 79, 82
	B*5001			4, 10, 14, 16, 20, 22, 27, 34, 41 , 42, / 51, 54, 63, 64, 67, 74, 75, 77, 79, 82
	Or			
	B*39011/013/05			1, 9, 11, 15, 17, 23, 26, 34, 41 , 42, / 44, 53, 61, 64, 71, 72, 75, 77, 79, 82
	B*4901			4, 10, 14, 16, 20, 22, 27, 33, 40 , 42, / 51, 54, 63, 64, 67, 74, 75, 77, 79, 82
7.	B*15011/26N/33	1	0.467	6, 7, 12, 15, 19, 22, 27 , 34, 41, 42, / 44, 53, 59, 64, 70, 74, 75, 77 , 79, 82
	B*5107			6, 8, 12, 15, 18, 23, 27 , 33, 40, 42, / 46, 53, 56, 64, 67, 74, 75, 78 , 79, 82
	Or			
	B*1511			6, 7, 12, 15, 19, 23 , 34, 41, 42, / 44, 53, 59, 64, 70, 74, 75, 77 , 79, 82
	B*52012			6, 8, 12, 15, 18, 22, 27 , 33, 40, 42, / 46, 53, 56, 64, 67, 74, 75, 78 , 79, 82
	Or			
	B*1512/19			6, 7, 12, 15, 19, 22, 27 , 34, 41, 42, / 44, 53, 59, 64, 70, 74, 77 , 79, 82

	B*5107			6, 8, 12, 15, 18, 23, 27 , 33, 40, 42, / 46, 53, 56, 64, 67, 74, 75, 78 , 79, 82
	Or			
	B*1515			6, 7, 12, 15, 19, 23, 27 , 34, 41, 42, / 44, 53, 59, 64, 70, 74, 75, 77 , 79, 82
	B*52012			6, 8, 12, 15, 18, 22, 27 , 33, 40, 42, / 46, 53, 56, 64, 67, 74, 75, 78 , 79, 82
8.	B*15011/26N/33			6, 7, 12, 15, 19, 22, 27, 34, 41 , 42, / 44 , 53, 59, 64, 70, 74, 75, 77 , 79, 82
	B*51011/09			6, 8, 12, 15, 18, 23, 25, 33, 40 , 42, / 46 , 53, 56, 64, 67, 74, 75, 78 , 79, 82
	Or			
	B*15011/26N/33			6, 7, 12, 15, 19, 22, 27, 34, 41 , 42, / 44 , 53, 59, 64, 70, 74, 75, 77 , 79, 82
	B*5103			6, 8, 12, 15, 18, 23, 25, 33, 40 , 42, / 46 , 53, 56, 64, 67, 74, 78 , 79, 82
	Or			
	B*1504			6, 7, 12, 15, 19, 22, 27, 34, 41 , 42, / 46 , 53, 59, 64, 70, 74, 75, 77 , 79, 82
	B*5106			6, 8, 12, 15, 18, 23, 25, 33, 40 , 42, / 44 , 53, 56, 64, 67, 74, 75, 78 , 79, 82
	Or			
	B*1508	1	0.467	6, 7, 12, 15, 19, 23, 25, 34, 41 , 42, / 44 , 53, 59, 64, 70, 74, 75, 77 , 79, 82
	B*52012			6, 8, 12, 15, 18, 22, 27, 33, 40 , 42, / 46 , 53, 56, 64, 67, 74, 75, 78 , 79, 82
	Or			
	B*1512/19			6, 7, 12, 15, 19, 22, 27, 34, 41 , 42, / 44 , 53, 59, 64, 70, 74, 77, 79, 82
	B*51011/09			6, 8, 12, 15, 18, 23, 25, 33, 40 , 42, / 46 , 53, 56, 64, 67, 74, 75, 78 , 79, 82
	Or			
	B*1524			6, 7, 12, 15, 19, 22, 27, 33, 40 , 42, / 44 , 53, 59, 64, 70, 74, 75, 77 , 79, 82
	B*7801/022			6, 8, 12, 15, 18, 23, 25, 34, 41 , 42, / 46 , 53, 56, 64, 67, 74, 75, 78 , 79, 82
	Or			
	B*1538			6, 7, 12, 15, 19, 22, 27, 34, 41 , 42, / 44 , 53, 59, 64, 70, 74, 75, 78 , 79, 82
	B*51021			6, 8, 12, 15, 18, 23, 25, 33, 40 , 42, / 46 , 53, 56, 64, 67, 74, 75, 77 , 79, 82
9.	B*3501/07/11/23			6, 7, 12, 15, 18, 23, 25, 34, 41 , 42, / 43, 54, 59, 64, 67, 74, 75, 77, 79, 82
	B*4901			4, 10, 14, 16, 20, 22, 27, 33, 40 , 42, / 51, 54, 63, 64, 67, 74, 75, 77, 79, 82
	Or			
	B*5301	1	0.467	6, 7, 12, 15, 18, 23, 25, 33, 40 , 42, / 43, 54, 59, 64, 67, 74, 75, 77, 79, 82
	B*5001			4, 10, 14, 16, 20, 22, 27, 34, 41 , 42, / 51, 54, 63, 64, 67, 74, 75, 77, 79, 82

10.	B*3501/07/11/23	1	0.467	6, 7, 12, 15, 18, 23, 25, 34, 41 , 42, / 43, 54, 59, 64, 67, 74, 75, 77, 79, 82
	B*4019			3, 10, 14, 16, 20, 22, 27, 33, 40 , 42, / 45, 53, 56, 64, 67, 73, 75, 77, 79, 82
	Or			
	B*5301			6, 7, 12, 15, 18, 23, 25, 33, 40 , 42, / 43, 54, 59, 64, 67, 74, 75, 77, 79, 82
	B*4002			3, 10, 14, 16, 20, 22, 27, 34, 41 , 42, / 45, 53, 56, 64, 67, 73, 75, 77, 79, 82
11.	B*0801	1	0.467	5, 9, 11, 15, 17, 23, 25, 34, 41 , 42, / 45, 53, 56, 64, 68, 72, 75, 77, 80, 82
	B*4901			4, 10, 14, 16, 20, 22, 27, 33, 40 , 42, / 51, 54, 63, 64, 67, 74, 75, 77, 79, 82
	Or			
	B*0803			5, 9, 11, 15, 17, 23, 25, 33, 40 , 42, / 45, 53, 56, 64, 68, 72, 75, 77, 80, 82
	B*5001			4, 10, 14, 16, 20, 22, 27, 34, 41 , 42, / 51, 54, 63, 64, 67, 74, 75, 77, 79, 82

A different combination of specific probes in exon 2 and exon 3 in the HLA -A, -C and -B loci presents ambiguous allele combinations at the allele group level.

The only one ambiguous result for HLA-A locus, "A*03011/013/03-N/04 × A*2501 or A*3204 × A*6601", was found due to different combinations of probes 12, 13, 14, 16 and 20 in exon 2 (Table 1).

Some of the ambiguous results for HLA-C locus were due to different combinations of probes in exon 2, while others were due to different combinations in both exons (exon 2 and exon 3). The ambiguous HLA-C combination "Cw*1205 × Cw*1507 or Cw*16041 × Cw*15021/022" was due to a different combinations of probes 13 and 14 in exon 2, "Cw*1203/06 × Cw*15021/022 or Cw*12042 × Cw*1507 or Cw*16041 × Cw*1503" was due to different combination of probes 11, 12, 13 and 14 in exon 2 and "C*1203/06 × C*14021/022 or C*1207 × C*14021/022 or C*1604 × C*1404" was due to different combinations of probes 11, 12 and 13 in exon 2. Ambiguous results for the remaining HLA-C ambiguities were due to the different probe combinations in exons 2 and 3 (Table 2).

Ambiguous results for the HLA-B locus were also due to different combinations of specific probes in exon 2 and exon 3 (Table 3).

Since, in a total of 214 samples examined, ambiguous allele combinations were found in only one sample for HLA-A locus, the observed resolution of RLS for HLA -A locus at the allele group level was 99.533%. The observed resolution for HLA-C locus, with ambiguous combinations found in 12 samples of the 214 examined, was 94.392%, and was 91.122% for HLA-B locus, with ambiguous combinations in 19 of the 214 examined samples (Table 4).

Table 4 – Табела 4

Overall performance characteristics of the RLS tests
Особини на целокујнајиа изводливосќи на РЛС шесќиовијие

Test	Accuracy (%)	Observed resolution (%)
HLA-A	100	99.533 (at group level)
HLA-C	100	94.393 (at group level)
HLA-B	100	91.121 (at group level)

Discussion

We found ambiguous allele combinations on an allele group level with the frequency of 0.5% for HLA-A locus, 5.6% for HLA-C locus and 8.9% for HLA-B locus, resulting in resolutions of 99.5%, 94.4%, and 91.6% for the HLA-A, -C, and -B loci, respectively. The observed resolution of the INNO-LiPA HLA Update tests (which is similar to the RLS method used in this paper) is 99.4% for HLA-A Update and 92.4% for HLA-B Update at the allele group level [13]. Analysis of the frequency of allele ambiguities revealed that the found ambiguities involved some of the most common allele groups in our population HLA-C*07, C*12, C*15, B*08, B*35, B*38, B*40 and B*51, obviating the need to introduce ambiguity resolution technique(s)/strategies in our lab.

Our results indicate that the RLS (a probe hybridization-based method) has a significant limitation in the precise identification of alleles (allele-level resolution). The results showed that ambiguous allele combinations in all three loci have resulted from combinations of probes for those polymorphisms that are shared between large numbers of alleles in multiple combinations, so that the phase of the polymorphisms is difficult to establish. For HLA-A locus these probes were 12, 13, 14, 16 and 20 in exon 2. For HLA-C locus the most frequent probes found in different combinations were 11, 12, 13 and 14 in exon 2 and 18, 25, 27, 30 31, and 34 in exon 3. For HLA-B locus, ambiguous allele combinations were due to specific probes 22, 23, 25, 27, 33, 34, 40, and 41 in exon 2 and 44, 46, 56, 64, 75, 77 and 78 in exon 3. Since RLS is a probe-based method, the amelioration of the high resolution result (i.e. unambiguous assignments at the four-digit (peptide) level) may require an increase in the number of probes as well as an increase in the number of polymorphisms detected by a single probe for the HLA-A, -C and -B loci, improvement of the minimum requirements for HLA Class I typing and probing outside of exons 2 and 3 (i.e. probing in exon 4). However, such improvements must be balanced against space-constraints and ease of use considerations (i.e. maximizing the number of

probes that can be put on a strip while permitting the strip to be read by a human), as well as the requirement that all probes perform correctly under the same hybridization conditions. If the ambiguity is due to identical probe patterns in different heterozygous genotypes, one of the possible options to overcome ambiguous allele combinations could be the use of primers for separate amplification of each allele in the PCR followed by hybridization.

There are several techniques used to resolve ambiguities in HLA genotyping. The most comprehensive results are published by Rosemuller [6] using SBT resolution. He reported the number of alleles, sequences, genotypes, ambiguous combinations and percentage of all ambiguous combinations for HLA-A, -C and -B loci at a group level. He published 1.19% for HLA-A, 1.69% for HLA-C and 1.68% for HLA-B ambiguous combinations at a group level. However, if ambiguities are analyzed with SBT at the allelic level such as in the papers of Adams *et al.* [8] their results showed 41% of HLA-A alleles and 24% of HLA-B alleles with ambiguities at the allelic level. Although informative and relatively fast for small numbers of samples, the SSP approach requires many separate PCRs to achieve intermediate or high level typing and, in its current format, is not well suited for rapid throughput of a large number of samples. Allele-specific amplification can be used in conjunction with SSO probe typing for high-resolution typing by allowing the separate amplification of the two alleles in a heterozygote [8]. In addition, Pyrosequencing has been applied for the study of gene expression and could be a useful complement to the high throughput single nucleotide polymorphism identification system as a substitute to SBT [14].

In conclusion, using the RLS method we have identified a high frequency of ambiguous allele combinations, particularly for the HLA-C and -B loci, that could perhaps be reduced somewhat with the addition of new probes targeting polymorphisms that are shared between large numbers of alleles in multiple combinations. The only way to eliminate ambiguity is to amplify each allele separately and examine the polymorphism in multiple exons by either probes or sequencing. The elimination of ambiguity, while a desirable goal, may be significantly more costly than the benefits that it could potentially provide.

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Резиме

**ДВОЈБЕНИ АЛЕЛСКИ КОМБИНАЦИИ НА ГРУПНО НИВО
ОД ХЛА-А, -Ц И -Б ГЕНИТЕ ВО МАКЕДОНСКАТА ПОПУЛАЦИЈА
СО МЕТОД ЗА РЕВЕРЗНА ХИБРИДИЗАЦИЈА НА ЛЕНТИ**

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Извадок: Целта на овој труд беше да се испита појавата на двојбени алелски комбинации на алелско групно ниво за ХЛА-А, -Ц и -Б локусите во македонската популација. Беа употребени ДНК примероци од 214 здрави несродни македонски доброволци од нашата банка за хумана ДНК. ХЛА типизирањето беше изведено со употреба на методот за реверзна хибридација на ленти (Reverse Line Strip, Roche Molecular Systems, USA) со користење полимеразно верижна реакција за егзоните 2 и 3 од ХЛА-А, -Б, и -Ц гените, со последователна хибридација на ленти. Статистичката анализа на добиените двојбени фреквенции беше изведена со употреба на Arlequin Software. Во ХЛА-А локусот од 214 примероци беше најдена само една двојбена алелска комбинација на алелско групно ниво со фреквенција од 0,467% (1/214). Во испитуваните 214 примероци беа најдени вкупно 6 различни ХЛА-Ц двојбени алелски комбинации на алелско групно ниво во 12 примероци со фреквенција од 5,607% (12/214) и 11 различни ХЛА-Б локуси во 19 примероци со фреквенција од 8,879% (19/214). Како заклучок можеме да кажеме дека анализата на фреквенциите од двојбените алелски комбинации покажа дека во двојбеностите се вклучени некои од најчестите алели во нашата популација укажувајќи на потребата да се воведат високо разделни методи за разрешување на двојбеностите во лабораториите за ХЛА.

Клучни зборови: ХЛА алелски двојбени комбинации; полиморфизми на ХЛА-А, -Ц и -Б; македонска популација; реверзна хибридација на ленти.

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