# DETECTION OF HLA GENOTYPE

## TECHNICAL FIELD

The present invention relates to a rapid genetic method for detection of HLA genotype based on the loop-mediated isothermal amplification (LAMP) principles. In particular, the present invention relates to a method or a kit for detection of HLA-B*1502 allele based on LAMP principles.

## BACKGROUND OF INVENTION

Human leukocyte antigens (HLA) are cell surface molecules that participate critically in the presentation of processed antigenic peptides for T cell activation. Encoded by a gene complex on the short arm of chromosome 6, it constitutes one of the most important immunogenetic systems of our body. The HLA gene complex is not only polygenic but also polyallelic, so that a high diversity of different HLA proteins could be produced among different individuals. It has been demonstrated that the polymorphism in this machinery contributes to individual variations in susceptibility to immune mediated/controlled diseases and plays an important role in drug induced adverse reactions (1, 2).

Historically, HLA typing was performed by serological detection, which was very imprecise. Latest technology makes use of polymerase chain reaction (PCR)(3) and nucleotide sequencing(4) to decode the HLA genotype on the DNA samples. Although sequence based typing (SBT) yields very precise and high resolution data, the high labor and reagent costs incurred make it an insurmountable barrier for clinical and even research application. Detection of specific HLA genotypes by sequence specific primers (SSP)-PCR may be less labor intensive and cheaper. However, some genotypes could not be differentiated due to presence of cross reactions.

Several recent studies have reported strong genetic associations between HLA alleles and susceptibility to drug hypersensitivity. The genetic associations can be drug specific, such as HLA-B*1502 being associated with carbamazepine-specific severe cutaneous reactions (Stevens Johnson syndrome and toxic epidermal necrolysis) and other forms of hypersensitivity, HLA-B*5701 with abacavir hypersensitivity and HLA-B*5801

with allopurinol-induced severe cutaneous adverse reactions.

In most HLA genetic association studies, limited by financial resources, a statistically adequate sample size could not be achieved. As a result, many studies suffer from a lack of statistical power to detect and confirm associations involved with low frequency alleles. Importantly, although increasing evidence has revealed the importance of HLA testing prior to the prescription of certain drugs so that adverse and potentially fatal drug reactions could be avoided in patients carrying susceptible HLA genotypes(2, 11), the high costs and a relatively long test turnaround time (TAT) (>1 day) required for obtaining the HLA test results pose a major hurdle to the effective practice of even guidelines advised by US Food and Drug Administration (FDA) (i.e. testing for B*1502 prior to prescription of carbamazepine (CBZ) in Asians to prevent the drug induced Steven Johnson Syndrome/Toxic Epidermal Necrolysis (SJS/TEN)(7). Thus, there is an immense need for the development of a simple, accurate and rapid HLA test at low costs.

HLA genotyping is an expensive test available only in specialized centers. It has also a long test turnaround time (TAT) that normally takes over 1 day. Rapid diagnosis of HLA status to guide drug prescription has not been developed and validated.

A novel nucleic acid amplification method, termed loop-mediated isothermal amplification (LAMP) (5, 6), is capable of amplifying DNA under isothermal conditions with high specificity, efficiency, and speed. The most significant advantage of LAMP is the ability to amplify specific sequences of DNA under isothermal conditions between 63 and 65°C. This ability allows the method to be performed with only simple and cost-effective reaction equipment amenable to use in hospital laboratories.

LAMP has been developed for detection of the severe acute respiratory syndrome coronavirus （SARS-CoV） replicase gene (24), the untranslated region in the genome of hepatitis A virus (HAV) (25), Human Influenza A Viruses DNA (26) etc. However, LAMP has not been developed for human HLA genotyping.

## SUMMARY OF THE INVENTION

In these directions, we have developed and validated a new testing approach for HLA genotyping based on the LAMP principles.

One aspect disclosed herein is directed to a method for detection of an HLA genotype in an individual, comprising

providing sets A and B of primers, which target different regions specific to the HLA genotype, such as two exons of the genotype, wherein both of sets A and B include a forward inner primer (FIP), a backward inner primer (BIP), two outer primers (a forward primer and a backward primer), and one or two loop primers (a loop forward primer and a loop backward primer);

preparing reaction mixture A comprising set A of primers, reaction buffer, DNA polymerase and a whole blood or a DNA purified template obtained from the individual, and reaction mixture B comprising set B of primers, reaction buffer, DNA polymerase and a whole blood or a DNA purified template obtained from the individual;

incubating reaction mixtures A and B, preferably at 63-65 °C for 15-50 min, respectively, and

detecting products in the reaction mixtures,

wherein the presences of the products in both reaction mixtures A and B indicate that the individual has the HLA genotype.

Another aspect disclosed herein is directed to a method for detection of an HLA genotype associated with susceptibility to drug hypersensitivity in an individual comprising

providing a blood sample obtained from the individual or a purified DNA template thereof,

providing sets A and B of primers, which target different regions specific to the HLA genotype such as two exons of the genotype, wherein both of sets A and B include a forward inner primer (FIP), a backward inner primer (BIP), two outer primers (a forward primer and a backward primer), and one or two loop primers (loop forward primer and loop backward primer);

preparing reaction mixture A comprising set A of primers, reaction buffer, DNA polymerase and a whole blood or a DNA purified template obtained from the individual, and reaction mixture B comprising set B of primers, reaction buffer, DNA polymerase and whole blood or a DNA purified template obtained from the individual;

incubating reaction mixtures A and B, preferably at 63-65 °C for 15-50 min,

respectively, and

detecting products in the reaction mixtures,

wherein presences of the products in both reaction mixtures A and B indicate that the individual is at increased risk for a hypersensitivity reaction to the drug or experiences a hypersensitivity reaction to the drug.

In some embodiments of the invention disclosed herein, products in the reaction mixtures can be detected by: (i) visual inspection of turbidity formed in the reaction mixture due to the formation of magnesium pyrophosphate as a by-product of the reaction, (ii) inspection of color change by the addition of a staining agent such as SYBR Green I to the reaction mixture, which binds to double-stranded DNA formed in the reaction to emit a fluorescence; (iii) inspection of color change by the addition of calcein and manganese chloride in the reaction mixture, in which pyrophosphate ions will remove manganese ions from calcein, such that calcein can combine with magnesium ion to emit green fluorescence, and the change of color is inspected visually.

In one embodiment of the invention disclosed herein, blood samples in the reaction mixture are first prepared by heat-treatment. In another embodiment, the whole blood is diluted 1:10 with water and is heated at >95°C for 3-5 minutes.

In one embodiment of the invention, the reaction mixture A comprising two loop primers such as forward loop primer and backward loop primer in the set A of primers is incubated at 63°C for 15-25 min. In another embodiment, the reaction mixture B comprising one or two loop primers such as forward loop primer and/or backward loop primer in the set B of primers is incubated at 63°C for 40-50 min.

In some embodiments of the invention disclosed herein, the HLA genotype is selected from the group consisting of HLA-B*1502 being associated with carbamazepine-specific severe cutaneous reactions and other forms of hypersensitivity, HLA-B*5701 being associated with abacavir hypersensitivity, HLA-B*5801 being associated with allopurinol-induced severe cutaneous adverse reactions, HLA-A29，-B12，-DR7 being associated with sulfonamide-SJS, HLA-A2，B12 being associated with oxicam-SJS，HLA- B59 being associated with methazolamide-SJS，HLA-Aw33，B17/Bw58 being associated with allopurinol- drug eruption，HLA-B27 being associated

with levamisole-agranulocytosis, HLA-DR4 being associated with hydralazine-SLE, HLA-DR3 being associated with penicillamine toxicity, HLA-B38, DR4, DQw3 being associated with clozapine-agranulocytosis, HLA-A24, B7, DQw1 being associated with dipyrone-agranulocytosis. Preferably, the HLA genotype is selected from the group consisting of HLA-B*1502 being associated with carbamazepine-specific severe cutaneous reactions and other forms of hypersensitivity, HLA-B*5701 with abacavir hypersensitivity and HLA-B*5801 with allopurinol-induced severe cutaneous adverse reactions, and preferably being HLA-B*1502.

In one embodiment of the invention, the HLA genotype is HLA-B*1502 gene (GenBank assession number: L42145), the primer set A comprises 4 oligonucleotides (SEQ ID NOs. 1-4) which correspond to 6 distinct regions (SEQ ID NOs. 9-14) of HLA-B*1502 exon 2 sequence, and 2 oligonucleotides (SEQ ID NOs. 21-22) which correspond to sequence between SEQ ID NOs. 9 and 10 or 13 and 14, while primer set B comprises 4 oligonucleotides (SEQ ID NOs. 5-8) which correspond to 6 distinct regions (SEQ ID NOs. 15-20) of HLA-B*1502 exon 3 sequence, and 1 oligonucleotide (SEQ. ID NO. 23) which corresponds to sequence between SEQ ID NOs. 15 and 16.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows LAMP-HB primers for detection of HLA-B*1502. Sequences of primer sets A and B used in the LAMP-HB assay are shown in the tables of Figure 1. In primer set A, SEQ ID NOS:1-4 and SEQ ID NOS: 21-22 are primer; and SEQ ID NOS:9-14 are primer binding site. In primer set B, SEQ ID NOS:5-8 and SEQ ID NO: 23 are primers; and SEQ ID NOS:15-20 are primer binding sites. Locations of primer-binding sites in the exon 2 and 3 of B*1502 sequence are indicated. Examples of other HLA-B allele sequences are aligned with B*1502, where only the mismatched nucleotides that differ from B*1502 are shown (matched nucleotides are represented by "-"). Due to cross-reactions of the primers with other HLA-B alleles, 2 sets of primers are needed to confirm the presence of B*1502 allele in the sample.

Figure 2 shows detection of HLA-B*1502 allele by LAMP-HB. As shown in Figure 2, the colours of the LAMP-HB. Reactions were inspected visually after the addition

of Sybr Green I to reaction mixture. Green colour denoted a positive reaction, while orange colour a negative reaction. Sample #1 revealed green colour in both tubes A and B, indicating a positive status for HLA-B*1502 allele whereas sample #2 remained orange in colour in tubes A and B, indicating a negative status for B*1502 allele. A, tube A; B, tube B; +, positive reaction; -, negative reaction.

## DETAILED DESCRIPTION OF THE INVENTION

Definitions

The term"allele" is intended to mean an alternative version of a gene encoding the same functional protein but containing differences in nucleotide sequence relative to another version of the same gene.

The term "genotyping" as used herein is intended to mean a process of determining the allelic patterns or genotypes of a human individual.

As used herein, the term "primer" refers to a single-stranded oligonucleotide that is extended by covalent bonding of nucleotide monomers during amplification or polymerization of a nucleic acid molecule.

As used herein, the term "template" refers to a double-stranded or single-stranded nucleic acid molecule which is to be amplified, synthesized or sequenced. In the case of a double-stranded DNA molecule, denaturation of its strands to form a first and the second strand is performed before these molecules may be amplified, synthesized or sequenced. A primer, complementary to a portion of a template is hybridized under appropriate conditions and a polymerase then synthesizes a molecule complementary to the template or a portion thereof.

As used herein, the term "amplification" refers to any in vitro method for increasing the number of copies of a nucleotide sequence with the use of a DNA polymerase. Nucleic acid amplification results in the incorporation of nucleotides into a DNA molecule or primer, thereby forming a new DNA molecule complementary to a DNA template. The formed DNA molecule and its template can be used as templates to synthesize additional DNA molecules. As used herein, one amplification reaction may consist of many rounds of DNA replication.

As used herein, the term "oligonucleotide" refers to a synthetic or natural molecule

comprising a covalently linked sequence of nucleotides which are joined by a phosphodiester bond between the 3' position of the pentose of one nucleotide and the 5' position of the pentose of the adjacent nucleotide.

As used herein, a drug "hypersensitivity reaction" refers to the development of an immune-like response to a drug molecule or a metabolite of the drug.

This response is typically characterized by multiple symptoms and is consistent with the clinical descriptions of such syndromes (Knowles et al., Lancet. 356: 1587 (2000); Carr et al. , Lancet. 356: 1423, (2000) ). The immunologic reaction shares features of, but is not necessarily identical to, the types present in the Gell and Coombs system (See Sullivan TJ: Drug allergy, In Middleton et al. (eds): Allergy : Principles and Practice, 4th Ed. , St. Louis, Mosby, 1993, p. 1730.)

Based on publicly released genomic sequence of HLA allele, we have developed loop-mediated isothermal amplification (LAMP) assays specifically targeting two different regions of the HLA allele. These assays are able to detect HLA genotype targets in both purified DNA samples and heat-treated blood samples within 1 hour under isothermal conditions. Primers, samples, reaction conditions, methods and interpretations of results are provided as follows.

*Primer design*

The primers for LAMP assay are designed to target specific regions in an HLA allele selected from the group consisting of HLA-B*1502 being associated with carbamazepine-specific, HLA-B*5701 being associated with abacavir hypersensitivity, HLA-B*5801 being associated with allopurinol-induced severe cutaneous adverse reactions, HLA-A29，-B12，-DR7 being associated with sulfonamide-SJS, HLA-A2，B12 being associated with oxicam-SJS，HLA- B59 being associated with methazolamide-SJS，HLA-Aw33，B17/Bw58 being associated with allopurinol- drug eruption，HLA-B27 being associated with levamisole-agranulocytosis ，HLA-DR4 being associated with hydralazine-SLE，HLA-DR3 being associated with penicillamine toxicity，HLA-B38，DR4，DQw3 being associated with clozapine-agranulocytosis，HLA-A24，B7，DQw1 being associated with dipyrone-agranulocytosis. For example, the primers are designed to target

exon 2 to exon 3 of the HLA-B*1502 gene (GenBank assession number: L42145).

Firstly, the sequence is aligned with other HLA-B, DR and DQ allele sequences using the "Sequence Alignment Tool" in the IMGT/ HLA database (http://www.ebi.ac.uk/imgt/hla/align.html) to acquire the specific regions of the HLA to be detected that differ from the other alleles. Primer Explorer V4 software is then used to design the candidate primers that specifically bind to these regions (Eiken Genome, Japan, http://primerexplorer.jp/e/). For optimal amplification in LAMP reaction within a 200-base pair region, cross-reactions with other HLA-B, DR and DQ alleles are unavoidable. Therefore, to enhance specificity in interpretation, 2 sets of primers are designed so that both can amplify the HLA genotype sequence to be detected but not to the other alleles. Two sets of primers (sets A and B), each of which includes a forward inner primer (FIP), a backward inner primer (BIP), two outer primers (F3 and B3), and one or two loop primers (LF and LB), are used for the LAMP assay.

As an illustrated example, in order to detect HLA-B*1502 allele, the primers for LAMP assay are designed to target exon 2 to exon 3 regions of the HLA-B*1502 gene (GenBank assession number: L42145). Firstly, The HLA-B*1502 sequence is aligned with other HLA-B allele sequences using the "Sequence Alignment Tool" in the IMGT/ HLA database (http://www.ebi.ac.uk/imgt/hla/align.html) to acquire the specific regions of B*1502 that differ from the other alleles. Primer Explorer V4 software is then used to design the candidate primers that specifically bind to these regions (Eiken Genome, Japan, http://primerexplorer.jp/e/). For optimal amplification in LAMP reaction within a 200-base pair region, cross-reactions with other HLA-B alleles are unavoidable. Therefore, to enhance specificity in interpretation, 2 sets of primers are designed so that both can amplify B*1502 sequence but not to the other alleles.

The primers directed to HLA--B*1502 allele used for LAMP reaction is schematically depicted in enclosure of Figure 1. Forward Inner Primer (FIP) comprises F2 and the complementary sequence of F1c, and Backward Inner Primer (BIP) comprises B2 and the complementary sequence of B1c when each of sequences (F1, F2, B1c, and B2c) is defined on the template sequence as shown in Figure 1. The two outer primers (a forward primer and a backward primer) are F3 and B3. The loop primers comprise loop forward

primer LF, and loop backward primer LB. In some references such as Notomi et al. (5), a spacer of few thymidines was inserted between F1c or B1c and F2 or B2 in the inner primer (FIP or BIP) so that one and two thymidine spacers are inserted in FIP and BIP, respectively. However, the spacer is not used in this study because the LAMP reaction can progress with the use of inner primers without the spacer.

These oligonucleotides or primers may be synthesized chemically (e.g. solid phase phosphoramidite triester method) and then purified (e.g. desalting or HPLC).

*Samples:*

Samples suitable for analysis using the present technology may be taken from any sources of a human individual. Preferred samples are peripheral blood (for heat treatment) or samples containing genomic DNA of that individual (for DNA extraction). Heat treatment of blood is performed by diluting the blood (e.g. 1/10 dilution) with water and treated at >95°C for 3-5 minutes whereas DNA extraction may be performed by methods well known in the art, e.g. phenol/ chloroform extraction and ethanol precipitation.

*Reaction:*

The LAMP reaction is carried out using the reaction mixture as disclosed herein or according to Notomi et al (2000, 2008). For example, the reaction mixture may contain 12.5ul of 2X reaction buffer, 40 pmol each of FIP and BIP, 20 pmol of loop primer (LF or each of LF and LB) for acceleration of the reaction, 5 pmol each of F3 and B3, 2 ul of heat-treated blood or DNA template, and 1 ul of 8U Bst DNA polymerase. The reaction mixture is then incubated at 60-65°C for 30 to 60 minutes.

*Interpretation of results:*

The LAMP product can be detected by: (i) visual inspection of turbidity formed in the reaction tube due to the formation of magnesium pyrophosphate as a by-product of the reaction, (ii) inspection of color change from orange to green in daylight (or presence of green fluorescence under UV light) by the addition of SYBR Green I to the reaction mixture, which binds to double-stranded DNA formed in the reaction to emit green

fluorescence; (iii) inspection of color change from orange to green in daylight (or presence of green fluorescence under UV light) by the addition of calcein and manganese chloride in the reaction mix, in which pyrophosphate ions will remove manganese ions from calcein, such that calcein can combine with magnesium ion to emit green fluorescence. For results scoring, only the presence of white precipitate or green color in both tubes A and B was considered to be positive for HLA-B*1502 allele, as the positive signal may occur in any one of the tubes due to cross-reaction to other HLA alleles.

Comparison with existing or previous product, if applicable

The detection of an HLA genotype such as HLA-B*1502 can be done by conventional HLA-typing technologies, e.g. sequence-specific primers polymerase chain reaction (SSP-PCR), sequence-specific oligonucleotide probes (SSOP), and sequence-based typing (SBT). These methods rely on expensive equipment (e.g. thermocycler, sequencer) and expertise, and the experimental process is time-consuming and tedious which needs more than a day. Besides, highly-purified and intact DNA is essential to these methods, as they are all PCR-based techniques.

While the LAMP technology requires no sophisticated equipments as the whole amplification process takes place under isothermal conditions. Purified DNA template is not necessary such that the cost and time required for DNA extraction can be waived. Besides, the whole amplification process can be completed within one hour, and the results can be visualized almost immediately, without the need to detect the reaction product as by other techniques (e.g. gel electrophoresis for SSP-PCR, enzymatic reaction for SSOP, and sequence analysis for SBT).

Market potential (anticipated clientele, usage, etc)

Since carbamazepine is a prescription-only medication in most countries, the target user groups will be physicians who treat patients with these diseases. As the diseases are common, both specialists and primary care doctors will be users. In most healthcare settings, specialists who look after patients with epilepsy include neurologists (adult and pediatric), general physicians, general pediatricians, neurosurgeons, geriatricians, and psychiatrists.

Specialists treating neuropathic pain include pain specialists, neurologists, diabetologists, neurosurgeons, and orthopedic surgeons. Patients with bipolar affective disorder are usually under the care of psychiatrists.

The following examples are intended to illustrate embodiments of the invention, and should not be construed as limitations on the scope of the invention.

## EXAMPLES

Materials and Methods:

*Study design and samples*

To investigate the feasibility and accuracy of detection of specific HLA genotypes by the new testing approach using B*1502 as an example, in phase I of the study, 50 DNA (25 positive and 25 negative for B*1502) and 200 frozen blood (30 positive and 170 negative for B*1502) samples from healthy blood donors were recruited and anonymized. All these 250 samples had been typed by high resolution SBT as the controls in a previous HLA genetic association study and thus with known HLA-B genotypes. SBT was performed according to the IHWG Technical Manual(8) and the resulting sequences were analyzed using SBTengine (Genome Diagnostics, the Netherlands). Whereas the DNA samples were directly tested for the carrier status of B*1502 by the new LAMP approach, the frozen blood samples were tested after heat treatment with no DNA extraction. To further validate the application of this new testing approach in clinic setting, in phase II of the study, 200 anonymized fresh patient blood samples were randomly recruited from the routine haematology laboratory. All these 200 samples were prospectively and concurrently typed for B*1502 carrier status by both the new LAMP based method on heated blood (LAMP-HB) and SSP-PCR as previously described (9). Results obtained by the new testing approach were compared with data by the standard SBT or SSP-PCR for evaluation of the accuracies in terms of sensitivities and specificities. The mean test TAT and $TAT_{90}$ (in 90% of samples) were also calculated in the phase II study.

*LAMP-HB (Loop Mediated Isothermal Amplification on Heated Blood)*

*Primer design*

The primers for LAMP assay were designed to target exon 2 to exon 3 of the HLA-B*1502 gene (GenBank assession number: L42145). First, the sequence was aligned with other HLA-B allele sequences using the "Sequence Alignment Tool" in the IMGT/ HLA database (http://www.ebi.ac.uk/imgt/hla/align.html) to acquire the specific regions of B*1502 that differ from the other alleles. Primer Explorer V4 software was then used to design the candidate primers that specifically bind to these regions (Eiken Genome, Japan, http://primerexplorer.jp/e/). For optimal amplification in LAMP reaction within a 200-base pair region, cross-reactions with other HLA-B alleles were unavoidable. Therefore, to enhance specificity in interpretation, 2 sets of primers were designed so that both could amplify B*1502 sequence but not to the other alleles. Two sets of primers (tubes A and B) including, and one or two loop primers (LF and LB), were used for the LAMP assay. Two sets of primers (sets A and B) each consist of 4 oligonucleotides (a forward inner primer (FIP), a backward inner primer (BIP), two outer primers (F3 and B3)) as represented by SEQ ID NOs. 1-8, which recognize 6 distinct regions (SEQ ID NOs. 9-20) of HLA-B*1502 sequence, and 1 to 2 oligonucleotides (loop primers, SEQ ID NOs. 21-23) complementary to the single stranded loop region formed during the assay to accelerate the LAMP reaction.

Figure 1 listed the primers used in this study, and examples of other HLA-B alleles were aligned with B*1502 sequence to demonstrate the specificity of the primers. Most of the cross reacting alleles are extremely rare alleles causing only very little compromise on the specificities (10). In cross-reactivity of the primer sets, rare alleles reported in Asian populations are as follows:

Set A: 1515, 1531,

Set B: 1503, 1506, 1513, 1518, 1521, 1525;

rare alleles not reported in Asian populations are as follows:

Set A: 1555,

Set B: 1516, 1523, 1529, 1536, 1539, 1540, 1567; and

alleles almost non-existent with no reported allelic frequencies in world populations are as follows:

Set A: 1588, 9512, 9521, 9544

Set B: 1544, 1561, 1562, 1564, 1569, 1574, 1580, 1589, 1593, 1595, 1598, 4608,

9503, 9506, 9508, 9512, 9515, 9519, 9521, 9527, 9532, 9538, 9539.

Accordingly, cross-reactivity of the primer sets are as follows:

Set A - B*1502, 1515, 1531, 1555, 1588, 9512, 9521, and 9544;

Set B - B*1502, B*1503, B*1506, B*1513, B*1516, B*1518, B*1521, B*1523, B*1525, B*1529, B*1536, B*1539, B*1540, B*1544, B*1561, B*1562, B*1564, B*1567, B*1569, B*1574, B*1580, B*1589, B*1593, B*1595, B*1598, B*4608, B*9503, B*9506, B*9508, B*9512, B*9515, B*9519, B*9521, B*9527, B*9532, B*9538, and B*9539.

Although B*1502, 9512 and 9521 will react with both primer sets, B*9512 and 9521 are very rare alleles found in only a few individuals.

*LAMP reaction*

The blood samples for LAMP reactions were first prepared by heat-treatment. Ten ul of whole blood collected in EDTA bottle was diluted 1:10 with water, and was heated at 98°C for 3 minutes. The LAMP reaction was carried out using the reaction mixture according to Notomi et al (5, 6). In brief, the reaction mixture A in tube A contained 12.5ul of 2X reaction buffer, 40 pmol each of FIP and BIP (SEQ ID NOs. 3-4), 20 pmol of loop primer (each of LF and LB, SEQ ID NOs. 21-22) for acceleration of the reaction, 5 pmol each of F3 and B3 (SEQ ID NOs. 1-2) of HLA-B*1502 exon 2 sequence, 2ul of heat-treated blood or purified DNA template, and 1ul of 8U Bst DNA polymerase (New England Biolabs, Ipswich, MA); and the reaction mixture B in tube B contained 12.5ul of 2X reaction buffer, 40 pmol each of FIP and BIP (SEQ ID NOs. 7-8), 20 pmol of loop primer (LF, SEQ ID NO.23) for acceleration of the reaction, 5 pmol each of F3 and B3 (SEQ ID NOs.5-6) of HLA-B*1502 exon 3 sequence, 2ul of heat-treated blood or purified DNA template, and 1ul of 8U Bst DNA polymerase (New England Biolabs, Ipswich, MA).

The reaction mixtures were incubated at 63°C for 15-25 min for tube A, and 63°C for 40-50 min for tube B.

*Interpretation of LAMP results*

The LAMP product in the reaction mixture was detected by adding 1ul of 1:10 diluted Sybr Green I (Invitrogen, Calsbad, CA) to the reaction mixture, and the change of

color was inspected visually. A positive reaction was indicated by a green color and a negative reaction by an orange color. For quality control, a positive control blood sample with B*1502 (heterozygote) and a negative control sample (B*1502 negative DNA) were performed with each batch of specimens. The tested specimen was considered positive for B*1502 when the two reaction tubes (both A and B) turned green, while it was non-B*1502 when one or both of the two tubes were orange in color.

Results:

Phase I study:

Results from all 50 DNA samples (25 positive and 25 negative for B*1502) by LAMP showed complete concordance with that obtained by previous SBT. Complete concordance was also observed in the 200 frozen blood samples (30 positive and 170 negative for B*1502) between results from LAMP-HB and previous SBT. Among all the 165 samples tested negative for B*1502, 166 showed negative reactions in both A and B tubes. However, a positive reaction indicated by a green color was found in 1 sample in tube A (1/170) and in 3 other samples in tube B (3/170), suggesting the presence of expected cross reactions and highlighting the need to use 2 sets of specific primers to enhance specificity. Hence, false positive tests as a result of cross positive reactions in both tubes were not observed in the current study. Examination of the SBT data revealed that samples showing cross positive reactions in tube A were actually B*1515 carriers and in tube B were carriers of B*1525.

Phase II study:

In 200 prospectively and randomly recruited fresh blood samples, the B*1502 status indicated by LAMP-HB was identical to that obtained by SSP-PCR performed concurrently. B*1502 was found positive in 35 samples and negative in 165 samples by both methods. The mean TAT for the 200 fresh blood samples was 33.1±10.8 minutes and $TAT_{90}$ was 45 minutes.

In summary, our findings indicate that results on DNA, frozen or fresh blood samples by the new LAMP based HLA testing showed 100% concordance with results

obtained by SBT or SSP-PCR, confirming that LAMP-HB detection of specific HLA genotype, B*1502 in this case, is an accurate method. There were no critical differences in the quality of experimental results between blood or DNA samples and between fresh or frozen blood samples by the LAMP based approach. No false positive or false negative results were encountered. All positive or negative reactions were easily visualized by a clear change of color. None of the samples were found to have undetermined status. Since no DNA extraction is required by combining heat treatment on blood samples prior to LAMP, the TAT and costs are both reduced. All results were quickly available, all within 1 hour.

Discussion:

In this post genome research era, it has been demonstrated that most human diseases have a genetic link in aetiology or pathogenesis. Genetic testing becomes increasingly important in daily clinical practice, which moves more and more towards personalized therapy. However, limited by the need of quality assured sophisticated systems and expertise for accuracy, genetic testing is still rather restricted in general application, being confined to a few specialized centers in most clinical communities. Thus, delayed by referral logistics and a long analytical process, test results may not be available timely enough to best facilitate the clinical management scenarios. This is particularly so with HLA testing. Although detection of specific HLA genotypes provides valuable information in advising disease risk and drug prescription(2, 11), the high costs and expertise required and the long test TAT constitute a main barrier for its effective application.

Drug induced hypersensitivity is a common clinical problem. Useful drugs like allopurinol for the treatment of hyperuricaemia, abacavir for HIV infection and carbamazepine (CBZ) for epilepsy have all been associated with severe drug reactions (2, 11). Recent studies have revealed significantly higher risks of developing allopurinol induced severe cutaneous reaction and abacavir hypersensitivity respectively in carriers of HLA-B*5801 and B*5701(12, 13). Therefore, these HLA alleles may serve as specific genetic risk markers to identify susceptible individuals. A very important example has also been clearly demonstrated by us and others that carriers of HLA-B*1502, a high prevalence allele in Asians including Hong Kong Chinese(14), have a dramatically elevated risk for

developing the potentially fatal CBZ induced Stevens Johnson syndrome / toxic epidermal necrolysis (C-SJS/TEN)(9, 15, 16). SJS/TEN are severe adverse reactions characterized by a rapidly developing blistering exanthema of macules and target-like lesions accompanied by extensive and severe mucosal detachments with potential life threatening consequences(17, 18). Morbidities are significant and fatality has been reported in 10-30% of the cases(19). The high positive (93.6%) and negative (100%) predictive values of B*1502 carrier status for C-SJS/TEN reported by Taiwan's study confirmed the value of B*1502 as a good predictive genetic marker for C-SJS/TEN(15). Since CBZ is a first line drug for epilepsy, in view of these, the US FDA issued an alert in December 2007, which advises testing for HLA-B*1502 in patients with ancestry from areas in which the allele is present, and avoiding the drug if the patient is found to have the allele (7).

However, this advice poses difficulty for clinical implementation for three main reasons. First, present technique (SSP-PCR) to test for HLA-B*1502 relies on multiple PCR of DNA extracted from the patient's blood. Expensive equipment and experimental processing in a laboratory for over a day is needed. The method is costly and available only in specialized centers. A delay in obtaining the test result leads to a delay in starting CBZ and thus in controlling the disease. For in-patients, this may imply delay in hospital discharge and thus higher healthcare costs. Second, patients with epilepsy, neuropathic pain or bipolar disorder that can be treated with CBZ are usually seen in the outpatient clinics. The usual clinical practice is to provide prescription to the patient after medical consultation. It is impractical to ask the patient to wait for the test result and return a few days later to obtain prescription. Third, testing for HLA-B*1502 is most relevant to patients in Asia, but many Asian countries have poor healthcare resources and for which the conventional SSP-PCR test is not affordable (Table 1).

To overcome these practical hurdles, there is a need to develop a more rapid and cost-effective test that ideally can be used at the doctor's clinic. The test developed would have a large market in Asian populations in which HLA-B*1502 is common. It would be applicable to patients with epilepsy, neuropathic pain and bipolar disorder. Together, these diseases are estimated to approximately 10% of the general populations (20, 22).

In this perspective, we looked for alternative approaches of HLA testing and found

LAMP assay the most attractive candidate. LAMP assay is a very new but simple technique developed for detection of specific genes based on the principle of isothermal amplification of nucleic acid. Four highly specific primers recognizing 6 distinct regions are specifically designed on the target gene region, with one set of primers anneal to the target DNA one after the other on the same strand and the primer which anneals later displaces the strand formed by the previous primer through the strand displacement activity of Bst DNA polymerase. The reaction takes place in both strands and primers are designed such that loops are formed under isothermal conditions to produce a series of stem-loop DNAs with various lengths. White precipitate from magnesium pyrophosphate is formed simultaneously during the amplification, which allows the assessment of turbidity simply by visual inspection (5, 6). To ensure high specificities, two sets of specific primers are designed to recognize 6 specific regions on the target gene by each set. As a result, chances of cross reactions are substantially reduced as compared to SSP-PCR approach, where a single region is targeted in each PCR reaction. With this new testing approach, it is expected that it could be applied for the detection of all specific HLA genotypes.

Applied only in recent years, mainly in the field of microbiology and food hygiene industry, LAMP offers great promise in genetic testing (6). It does not require any sophisticated equipment such as thermocycler. As the reaction takes place in an isothermal condition, only a water bath or a heating block is needed. Besides, the results can be visualized within 1 hour as the reaction provides high amplification efficiency, in which DNA is amplified $10^9$-$10^{10}$ times in 15-60 minutes in the positive tubes. By combining amplification of nucleic acid and detection of positive end point by one single reaction, it offers rapid diagnosis with high sensitivity and specificity. This makes it the most suitable genetic test at the bedside or in the clinic.

To improve the visual scoring of results, we added Sybr Green I to the reaction tubes at the end of experiments. Sybr Green I is an asymmetrical cyanine dye that binds to double-stranded DNA to emit green light. It imparts an orange color under visual light and turns green in the presence of significantly increased amount of double-stranded DNA. So a positive reaction is clearly indicated by a green color and a negative reaction by an orange color (Figure 2). To enhance simplicity in operation for application outside laboratory and

further reduce the cost and TAT, we also investigated and confirmed the feasibility of directly applying heat-treated blood samples to the LAMP reaction mixtures without prior conventional DNA extraction.

In this study, we first developed, optimized and confirmed LAMP-HB for fast and accurate detection of B*1502 using DNA and frozen blood samples with known B*1502 carrier status typed previously by SBT. To validate the application of the assay for rapid diagnosis, we went on to recruit prospectively anonymized fresh patient blood samples for parallel HLA testing by LAMP-HB and SSP-PCR. Again, determination of B*1502 status by LAMP-HB was in complete agreement with that by SSP-PCR, indicating that LAMP-HB can replace SSP-PCR for clinical application. Moreover, the TAT (from specimen reception to report of result) was greatly shortened in LAMP-HB (mean TAT= 33.1±10.8 minutes and $TAT_{90}$=45 minutes) as compared to SSP-PCR (TAT=1-2 day). To further demonstrate that this test is simple and easy to follow, we have also prepared a simple instruction manual for use by inexperienced personnel. A previously uninformed junior research assistant with no prior experience in genetic testing was asked to read and follow the instruction to carry out the LAMB-HB testing on 20 fresh blood samples. All the 20 results reported accurately identified the B*1502 carrier status. These findings have validated the cost-effective application of LAMP-HB in the clinical settings where most of the target patients are seen and managed. Importantly, unlike PCR, LAMP does not require multiple steps in temperature control. Thus our approach has the potential to be developed into a "kit" for use at the doctor's clinic. This will greatly enhance acceptability by doctors working in a wide range of practice settings including underdeveloped communities in some geographical regions of Asia.

The new testing approach in effect helps overcome the barrier in effective implementation of FDA guidelines. By avoiding the severe drug induced skin reaction like SJS/TEN, the patient safety and quality of care are remarkably enhanced. Whereas implications in these aspects are tremendous but difficult to quantify, a careful cost benefit analysis highlights the significant impacts on health care and social resource allocation in different clinical scenarios (Table). Huge costs would be saved from the cheaper test, faster results, shorter hospital stays, earlier disease control and no more need of managing

C-SJS/TEN (Table 1) (15 23).

Although false positive detection of B*1502 was not found in our current study, it could be estimated that in extremely rare subjects (2x1/340x3/340=0.005%) carrying combination of both B*1515 and B*1525, they will be tested false positive for B*1502. The clinical implication will be that this extremely rare individual if by chance develops epilepsy or other diseases that may be indicated for CBZ will be denied to the drug when tested by this new LAMP approach. Thus, an alternative drug may be used with minimal clinical consequences. In fact, the estimated false positive rates range from 0.005% (Hong Kong) to 0.058% (Northern China), figures calculated based on the reported allelic frequencies of the rare alleles that cross react with B*1502 in both tubes A and B for regions including Taiwan, Hong Kong, Singapore and China (10). Although there are quite a number of cross reacting alleles listed particularly for tube B, these alleles are all rare alleles that are almost non-existent in most populations and no frequency figures have been reported in our target regions. In conclusion, the new LAMP-HB HLA testing achieves 100% sensitivity and specificity in our study. However, the predicted specificities may be <100% but >99.9%.

More importantly, our study on B*1502 may serve as a prototype in the approach on HLA genotyping. The provision of simple and cheap HLA testing may also imply that a statistically powerfully large sample size could be achieved in HLA association research, particularly in the validation study of rare target HLA allele. The success of this approach as proved in this study will certainly revolutionize HLA testing in both clinical and research arenas.

All patents and publications mentioned in this Specification are incorporated herein by reference in their entireties.

Many modifications and variations of the embodiments described herein may be made without departing from the scope of the invention, as is apparent to those skilled in the art. Changes therein and other use will occur to those skilled in the art which are encompassed within the spirit of the invention and defined by the scope of the appended claims.

Table 1: Cost benefit analysis by introduction of HLA testing and rapid diagnosis by LAMP-HB in C-SJS/TEN

| *Non-compliance with FDA guidelines* | | *Compliance with FDA guidelines* | | |
|---|---|---|---|---|
| *Untested* | | | *Tested by SSP-PCR* | *Tested by LAMP-HB* |
| Chance of developing Steven Johnson Syndrome (SJS) | 18.7% (= 20%x93.6%)<br>B*1502 carrier rate in Hong Kong = 20%<br>PPV=93.6% (Taiwan's study) | Chance of developing SJS | Not developed if B*1502 negative<br>NPV= 100%<br>Avoid CBZ if B*1502 positive (20% carrier rate in Hong Kong) | Not developed if B*1502 negative<br>NPV = 100%<br>Avoid CBZ if B*1502 positive (20% carrier rate in Hong Kong) |
| Mortality from SJS | Up to 30% patients may die | Reagent cost<br>Labor cost<br>Test cost | HK$400<br>HK$600 (trained staff x 4hrs)<br>HK$1,000 | HK$30<br>HK$HK$70 (general staff x 1hr)<br>HK$100 |
| Morbidity from SJS | Pain, severe skin damage, residual scaring, eye complications, chronic xerostomia and pulmonary complications | Turnaround time (TAT) | • 1-2 days<br>• May take longer time with referral from remote areas | • 1 hour<br>• Could be adapted for application in bed side or clinic |
| *Costs of SJS management | Hospital stay: 4-7 days<br>Immunoglobulin, antibiotics | In-patients: Hospital stay | • + 1-2 days<br>• Delay in prescription and controlling the epilepsy | • Early treatment and discharge |
| | | Out-patients: Extra visit to clinic | • Leave<br>• Loss of work production<br>• Traveling to and from clinic<br>• Time and cost for doctor's consultation<br>• Delay in prescription and controlling the epilepsy | • Single visit<br>• Early treatment |

PPV: Positive predictive value; NPV; Negative predictive value; Taiwan's study (Chung et al 2004)
*Estimated total costs of hospital treatment US$4416 (based on average national Medicare reimbursement in USA in 2002 [Kagan et al, 2007]).

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What is claimed is:

1. A method for detection of an HLA genotype in an individual, comprising

providing sets A and B of primers, which target different specific regions in the HLA genotype, such as two exons of the genotype, wherein both of sets A and B include a forward inner primer (FIP), a backward inner primer (BIP), two outer primers (a forward primer and a backward primer), and one or two loop primers (a loop forward primer and a loop backward primer);

preparing reaction mixture A comprising set A of primers, a reaction buffer, a DNA polymerase and a whole blood or a DNA purified template obtained from the individual, and reaction mixture B comprising set B of primers, a reaction buffer, a DNA polymerase and a whole blood or a DNA purified template obtained from the individual;

incubating reaction mixtures A and B, respectively, and

detecting products in the reaction mixtures,

wherein the presence of the products in both reaction mixtures A and B indicates that the individual has the HLA genotype.

2. A method for detection of an HLA genotype associated with susceptibility to drug hypersensitivity in an individual comprising

providing a blood sample obtained from the individual or a purified DNA template thereof,

providing sets A and B of primers, which target different regions specific to the HLA genotype such as two exons of the genotype, wherein both of sets A and B include a forward inner primer (FIP), a backward inner primer (BIP), two outer primers (a forward primer and a backward primer), and one or two loop primers (loop forward primer and loop backward primer);

preparing reaction mixture A comprising set A of primers, a reaction buffer, a DNA polymerase and a whole blood or a DNA purified template obtained from the individual, and reaction mixture B comprising set B of primers, a reaction buffer, a DNA polymerase and

whole blood or a DNA purified template obtained from the individual;

incubating reaction mixtures A and B, respectively; and

detecting products in the reaction mixtures,

wherein the presence of the products in both reaction mixtures A and B indicates that the individual is at increased risk for a hypersensitivity reaction to the drug or experiences a hypersensitivity reaction to the drug.

3. The method according to Claim 1 or 2, wherein the whole blood sample in the reaction mixture is first prepared by heat-treatment.

4. The method according to Claim 1 or 2, wherein whole blood is diluted 1:10 with water and is heated at 98°C for 3 minutes.

5. The method according to Claim 1 or 2, wherein the reaction mixture A comprising two loop primers such as a forward loop primer and a backward loop primer is incubated at about 63-65°C for 15-25 min.

6. The method according to Claim 1 or 2, wherein the reaction mixture B comprising one or two loop primers such as a forward loop primer and/or a backward loop primer is incubated at about 63-65°C for 40-50 min.

7. The method according to Claim 1 or 2, wherein detecting the products in the reaction mixtures comprises: (i) visual inspection of turbidity formed in the reaction tube due to the formation of magnesium pyrophosphate as a by-product of the reaction, (ii) inspection of color change by the addition of a staining agent such as SYBR Green I to the reaction mixture, which binds to double-stranded DNA formed in the reaction to emit a fluorescence; or (iii) inspection of color change by the addition of calcein and manganese chloride in the reaction mixture, in which pyrophosphate ions will remove manganese ions from calcein, such that calcein can combine with magnesium ions to emit green fluorescence, and the change of color is inspected visually.

8. The method according to any one of Claims 1-7, wherein the HLA genotype is selected from the group consisting of HLA-B*1502 being associated with carbamazepine-specific severe cutaneous reactions and other forms of hypersensitivity, HLA-B*5701 being associated with abacavir hypersensitivity; HLA-B*5801 being associated with allopurinol-induced severe cutaneous adverse reactions; HLA-A29, -B12 and -DR7 being associated with sulfonamide-SJS; HLA-A2 and B12 being associated with oxicam-SJS; HLA- B59 being associated with methazolamide-SJS; HLA-Aw33 and B17/Bw58 being associated with allopurinol- drug eruption; HLA-B27 being associated with levamisole-agranulocytosis; HLA-DR4 being associated with hydralazine-SLE; HLA-DR3 being associated with penicillamine toxicity; HLA-B38, DR4 and DQw3 being associated with clozapine-agranulocytosis; and HLA-A24, B7 and DQw1 being associated with dipyrone-agranulocytosis.

9. The method according to Claim 8, wherein the HLA genotype is HLA-B*1502 gene (GenBank assession number: L42145).

10. The method according to Claim 9, wherein the primer set A comprises 4 oligonucleotides (SEQ ID NOs. 1-4) which correspond to 6 distinct regions (SEQ ID NOs. 9-14) of HLA-B*1502 exon 2 sequence, and 2 oligonucleotides (SEQ ID NOs. 21-22) which correspond to sequence between SEQ ID NOs. 9 and 10 or 13 and 14 of HLA-B*1502 exon 2 sequence.

11. The method according to Claim 9, wherein primer set B comprises 4 oligonucleotides (SEQ ID NOs. 5-8) which correspond to 6 distinct regions (SEQ ID NOs. 15-20) of HLA-B*1502 exon 3 sequence, and 1 oligonucleotide (SEQ. ID NO. 23) which corresponds to sequence between SEQ ID NOs. 15 and 16 of HLA-B*1502 exon 3 sequence.

12. A kit for detection of an HLA genotype in an individual, comprising

sets A and B of primers, which target different specific regions in the HLA

genotype, such as two exons of the genotype, wherein both of sets A and B include a forward inner primer (FIP), a backward inner primer (BIP), two outer primers (a forward primer and a backward primer), and one or two loop primers (a loop forward primer and a loop backward primer);

a reaction buffer,

a DNA polymerase and

optionally comprising a whole blood or a DNA purified template as a positive control.

13. A kit for detection of an HLA genotype associated with susceptibility to drug hypersensitivity in an individual comprising

sets A and B of primers, which target different specific regions in the HLA genotype, such as two exons of the genotype, wherein both of sets A and B include a forward inner primer (FIP), a backward inner primer (BIP), two outer primers (a forward primer and a backward primer), and one or two loop primers (a loop forward primer and a loop backward primer);

a reaction buffer,

a DNA polymerase and

optionally comprising a whole blood or a DNA purified template as a positive control.

14. The kit according to Claim 12 or 13, wherein the set A of primers comprises two loop primers such as a forward loop primer and a backward loop primer.

15. The kit according to Claim 12 or 13, wherein the set B of primers comprises one or two loop primers such as forward loop primer and/or backward loop primer.

16. The kit according to Claim 12 or 13, further comprising a staining agent such as SYBR Green I, which binds to double-stranded DNA formed in the reaction to emit a fluorescence; or calcein and manganese chloride.

17. The kit according to any one of Claims 12-16, wherein the HLA genotype is selected from the group consisting of HLA-B*1502 being associated with carbamazepine-specific severe cutaneous reactions and other forms of hypersensitivity, HLA-B*5701 being associated with abacavir hypersensitivity; HLA-B*5801 being associated with allopurinol-induced severe cutaneous adverse reactions; HLA-A29, -B12 and -DR7 being associated with sulfonamide-SJS; HLA-A2 and B12 being associated with oxicam-SJS; HLA- B59 being associated with methazolamide-SJS; HLA-Aw33 and B17/Bw58 being associated with allopurinol- drug eruption; HLA-B27 being associated with levamisole-agranulocytosis; HLA-DR4 being associated with hydralazine-SLE; HLA-DR3 being associated with penicillamine toxicity; HLA-B38, DR4 and DQw3 being associated with clozapine-agranulocytosis; and HLA-A24, B7 and DQw1 being associated with dipyrone-agranulocytosis.

18. The kit according to Claim 8, wherein the HLA genotype is HLA-B*1502 gene (GenBank assession number: L42145).

19. The kit according to Claim 9, wherein the primer set A comprises 4 oligonucleotides (SEQ ID NOS. 1-4) which correspond to 6 distinct regions (SEQ ID NOs. 9-14) of HLA-B*1502 exon 2 sequence, and 2 oligonucleotides (SEQ ID NOs. 21-22) which correspond to sequence between SEQ ID NOs. 9 and 10 or 13 and 14.

20. The kit according to Claim 9, wherein primer set B comprises 4 oligonucleotides (SEQ ID NOS. 5-8) which correspond to 6 distinct regions (SEQ ID NOS. 15-20) of HLA-B*1502 exon 3 sequence, and 1 oligonucleotide (SEQ. ID NO. 23) which corresponds to sequence between SEQ ID NOs. 15 and 16.