Rapid Detection of the Three Celiac Disease Risk Genotypes HLA-DQ2.2, HLA-DQ2.5, and HLA-DQ8 by Multiplex Ligation-Dependent Probe Amplification

Raymon Vijzelaar,¹ Ellen van der Zwan,² Adriaan van Gammeren,³ Rizkat Yilmaz,¹ Alice Verheul,² Ingrid van Hoogstraten,⁴ Ellen de Baar,³ Lianne Schrauwen,³ and Wouter Kortlandt²

Background: Genotyping of HLA-DQ2.2, HLA-DQ2.5, and HLA-DQ8 is important in celiac disease (CD). The absence of these three genotypes has a strong negative predictive value. **Methods:** We designed multiplex ligation-dependent probe amplification (MLPA) for the combined detection of HLA-DQ2.2, HLA-DQ2.5, and HLA-DQ8. The MLPA probe mix was validated against a set of 59 samples characterized by conventional techniques. **Results:** The MLPA assay genotyped all 59 samples correctly when compared to the results obtained by PCR-SSCP/HD or PCR-SSO and PCR-SSP. **Conclusion:** The MLPA assay provides a reliable single-reaction analysis of the CD risk genotypes HLA-DQ2.2, HLA-DQ2.5, and HLA-DQ8 allowing for stratification or exclusion of disease risk.

Introduction

CELIAC DISEASE (CD, #212750; OMIM) is a chronic small-intestinal enteropathy, characterized by an autoimmune response triggered by gluten proteins found in wheat, barley, or rye resulting in small intestine mucosal injury. CD is associated with HLA-DQ2.5, HLA-DQ2.2, and HLA-DQ8 molecules of the human leukocyte antigens (HLA) class II (Mubarak *et al.*, 2013; Barakauskas *et al.*, 2014). The heterodimeric HLA-DQ protein is found on the surface of antigen-presenting cells and consists of the α and β subunits, which are encoded by *HLA-DQA1* and *HLA-DQB1* genes, respectively (Monsuur *et al.*, 2008; Megiorni and Pizzuti, 2012).

More explicitly, HLA genotypes HLA-DQ2.5 (*HLA-DQA1*05* and *HLA-DQB1*02*), HLA-DQ8 (*HLA-DQA1*03* and *HLA-DQB1*03:02*), and more recently, found by Mubarak *et al.* (2013), HLA-DQ2.2 (*HLA-DQA1*02* and *HLA-DQB1*02*) have been highly associated with CD (Table 1). However, ~30–40% of the healthy population carries one of these risk variants, while the worldwide prevalence of CD is about 1%. Consequently, the presence of HLA-DQ risk alleles alone is not the only prerequisite to develop CD.

The development of CD appears to be multifactorial, including potential, additional genetic, and environmental

factors (Megiorni and Pizzuti, 2012; Ludvigsson *et al.*, 2014). Genotyping of the *HLA-DQA1* and *HLA-DQB1* genes is important since genetic HLA-DQ2.2, HLA-DQ2.5, and HLA-DQ8 testing has a very strong negative predictive value (Anderson *et al.*, 2013; Ludvigsson *et al.*, 2014). Testing for relevant alleles is recommended for risk stratification of individuals genetically susceptible to CD, such as in first-degree relatives. It is also recommended in case of autoimmune conditions such as type I diabetes or thyroiditis and in specific genetic disorders such as Down, Turner, or Williams syndrome (Fasano and Catassi, 2012; Barakauskas *et al.*, 2014). Hence, the development of a single assay that can detect all three genotypes is essential to identify or exclude people at risk for CD.

Several studies show that nearly 100% of CD patients contain one or combination of HLA-DQ2.5, HLA-DQ2.2, or HLA-DQ8 (Megiorni *et al.*, 2009; Mubarak *et al.*, 2013; Lund *et al.*, 2015). In ~90% of CD cases, the DQ2.5 haplotype is found. The remaining 10% of CD patients are positive for either DQ2.2 or DQ8 or are homozygous for *HLA-DQB1*02* (Mubarak *et al.*, 2013; Ludvigsson *et al.*, 2014). The European Society for Paediatric Gastroenterology, Hepatology, and Nutrition guideline (ESPGHAN) (Husby *et al.*, 2012) recommends genetic testing on the presence of only HLA-DQ2.5 and HLA-DQ8. However, Mubarak *et al.* (2013)

¹MRC-Holland BV, Amsterdam, The Netherlands.

²Department of Clinical Chemistry, Hematology and Immunology, Diaconessenhuis, Utrecht, The Netherlands.

³Department of Clinical Chemistry and Hematology, Amphia Hospital, Breda, The Netherlands.

⁴Department of Pathology, VU University Medical Center, Amsterdam, The Netherlands.

TABLE 1. PROBES FOR THE DETECTION OF HLA ALLELES ASSOCIATED WITH CELIAC DISEASE

Length	Partial sequence (24 nt adjacent to ligation site)	HLA allele	DQ2.5	DQ2.2	DQ8
200	TAAATCCTTCTC-GGAGAGGTCTCA	DQA1*05	+	–	–
332	TGCTGAGGAGAG-TTATGACTGCAA	DQA1*05	+	–	–
183	AACTATGGGGTA-TGGGGACAAACA	DQB1*02	+	+	–
319	TATAACCGAGAA-GAGATCGTGCGC	DQB1*02	+	+	–
221	AGACTGTCTGGA-AGTTGCCTCTGT	DQA1*02	–	+	–
288	AACCCAGGGCA-33 nt spanning oligo-GCTGGAATCTCA	DQA1*02	–	+	–
177	GGTCCCTCTGGG-CAGTACAGCCAT	DQA1*03	–	–	+
142	ATTTGCACTGAC-35 nt spanning oligo-TGATTAAACGCT	DQA1*03	–	–	+
233	GACCCGGGCGGA-32 nt spanning oligo-TGGAGCTCCGCA	DQB1*03	–	–	+
135	CAGTACTCGGCG-30 nt spanning oligo-TACACCCCCACG	DQB1*03:02 *03:05	–	–	+
148	GGGCCGGGGCCT-40 nt spanning oligo-CCAGTTTAAGGG	DQB1*03:02 *03:03	–	–	+

HLA, human leukocyte antigens.

showed that 9 of 155 CD patients were negative for both HLA-DQ2.5 and HLA-DQ8, but positive for HLA-DQ2.2 demonstrating that HLA-DQ2.2 should be also considered as a susceptible HLA-DQ haplotype in CD, even without the presence of HLA-DQ2.5 or HLA-DQ8 (Mubarak *et al.*, 2013). This was confirmed in a recent study by Lund *et al.* (2015).

Previously, we published a novel multiplex ligation-dependent probe amplification (MLPA) assay to confirm or exclude HLA-DQ2.5 and HLA-DQ8 genotypes (Van Beek *et al.*, 2013). This MLPA assay has been validated by genotyping 155 samples using single-strand conformation polymorphism and heteroduplex analysis (PCR-SSCP/HD) at the VU University Medical Center (Van Beek *et al.*, 2013). We have improved our previously published MLPA assay by including probes for the simultaneous detection of the HLA-DQ2.2 genotype, in addition to HLA-DQ2.5 and HLA-DQ8. Results obtained by this redesigned MLPA assay were 100% consistent with the results of conventional methods such as PCR-SSCP/HD, PCR-SSO, and PCR-SSP.

Materials and Methods

The MLPA probemix P438-D1 (MRC-Holland, Amsterdam, The Netherlands) was developed to simultaneously detect the α and β subunits of the heterodimeric DQ2.2, DQ2.5, and DQ8 molecules. For each allele, that is, *HLA-DQA1*02*, *HLA-DQA1*03*, *HLA-DQA1*05*, and *HLA-DQB1*02*, two probes have been added to the probemix (Table 1 and Fig. 1). In comparison to the previous version, two probes for *HLA-DQA1*05* have been removed from the probemix. It was shown that two probes for each allele are sufficient for detection of the relevant allele. Therefore, the two best performing probes have been selected. Nine reference probes, targeting other chromosomes for internal quality control on the polymerase chain reaction (PCR) process, have also been incorporated. MLPA probe design requires the presence of unique sequences to prevent the amplification of homologous sequences (Schouten *et al.*, 2002). The design of a single-MLPA probe unique for *HLA-DQB1*03:02* (HLA-DQ8) detection is impossible due to the large number of *HLA-DQB1* gene variants. Therefore, a combination of three probes has been included to detect the *HLA-DQB1*03:02* allele of the HLA-DQ8 β -unit.

The MLPA assay was performed according to manufacturers' instructions (www.mlpa.com). In brief, the MLPA

reaction requires 50–100 ng of sample DNA, which is denatured and mixed with the MLPA probemix before an overnight hybridization at 60°C. After the ligation (15' at 54°C) and amplification by PCR (35 cycles), the PCR fragments are analyzed on a capillary electrophoresis. Electrophoresis was carried out on an ABI 3130 sequencer (Life Technologies/Applied Biosystems, Carlsbad, CA) by the Amphia Hospital (Breda, The Netherlands) or a Beckman 8800 sequencer (Beckman Coulter, Woerden, The Netherlands) by the Diaconessenhuis Hospital (Utrecht, The Netherlands).

Reference genotyping was performed by either the VU University Medical Center (Amsterdam, The Netherlands) using a PCR-SSCP/HD-based method or Sanquin (Amsterdam, The Netherlands) using the sequence-specific oligonucleotide-primed PCR (PCR-SSO) and sequence-specific primer-based PCR (PCR-SSP) (Barakauskas *et al.*, 2014). The Diaconessenhuis Hospital evaluated 35 samples and compared results to those obtained by the VU University Medical Center. The Amphia Hospital evaluated 24 samples and compared their results to those of Sanquin. The samples have been selected with a bias for HLA-DQ2.2.

Results and Discussion

The MLPA probemix P438-D1 has been validated by two centers, the Diaconessenhuis Hospital and Amphia Hospital, using 59 samples consisting of different HLA-DQ variants. The sample composition consisted of 19 HLA-DQ2.2 positive (Fig. 1B), 3 HLA-DQ2.5 positive, 6 HLA-DQ8 positive, 4 both HLA-DQ2.2 and HLA-DQ8 positive (Fig. 1C), 11 both HLA-DQ2.2 and HLA-DQ2.5 positive (Fig. 1D), 3 both HLA-DQ2.5 and HLA-DQ8 positive, and 13 HLA DQ2.5, DQ2.2, or DQ8 negative (Fig. 1A). Results obtained by the MLPA assay were 100% consistent with the reference genotyping results (Table 2).

Current guidelines (ESPGHAN) suggest that CD can be excluded in DQ2.5- and DQ8-negative patients (Husby *et al.*, 2012; Mubarak *et al.*, 2013). Recently, it has been demonstrated that DQ2.2, with a prevalence of about 5%, which is as frequent as DQ8, is another risk genotype for CD (Mubarak *et al.*, 2013). Collectively, these three risk genotypes are found in almost all CD patients. We have included *HLA-DQA1*02* allele-specific MLPA probes in this MLPA assay to detect the DQ2.2 genotype.

FIG. 1. Electropherograms of samples harboring different human leukocyte antigen (HLA) alleles detected in this study. (A) Sample negative for HLA-DQ2.2, HLA-DQ2.5, and HLA-DQ8. (B) HLA-DQ2.2-positive sample, showing a heterozygous signal for the HLA-DQB1*02 allele. (C) HLA-DQ2.2- and HLA-DQ8-positive sample. (D) HLA-DQ2.2- and HLA-DQ2.5-positive sample, showing a homozygous signal for the HLA-DQB1*02 allele. Probe identities are described in Table 1 by matching the corresponding length.

Validation of this renewed method by two independent institutes showed that the method with specific probes for all relevant CD risk genotypes is a very reliable and complete assay to exclude or stratify the risk for CD (Van Beek *et al.*, 2013; Barakauskas *et al.*, 2014). In addition, MLPA has been shown to be able to distinguish between heterozygous and homozygous state of the alleles (Van Beek *et al.*, 2013; Barakauskas *et al.*, 2014), which is of importance since ho-

mozygous *HLA-DQB1*02* in the presence or absence of *HLA-DQA1*02* or *HLA-DQA1*05* also predisposes for CD (Megiorni *et al.*, 2009).

In conclusion, the new MLPA probemix is able to correctly detect multiple targets within one reaction, in contrast to other more conventional multistep technologies, and provides a quick, reliable, and complete method to determine all three HLA-DQ genotypes relevant for CD.

TABLE 2. GENOTYPING RESULTS OF SAMPLES RESTED WITH THE IMPROVED MLPA ASSAY (P438-D1) COMPARED WITH REFERENCE METHODS

Genotype combination	Reference method 1: PCR-SSO/SSP	Reference method 2: PCR-SSCP/HD	Results by MLPA	Specificity
HLA-DQ2.2, -DQX	4	15	19	19/19
HLA-DQ2.2, -DQ2.5	4	7	11	11/11
HLA-DQ2.2, -DQ8	0	4	4	4/4
HLA-DQ2.5, -DQX	0	3	3	3/3
HLA-DQ2.5, -DQ8	3	0	3	3/3
HLA-DQ8, -DQX	3	3	6	6/6
HLA-DQX, -DQX	10	3	13	13/13
Total	24	35	59	59/59

DQX, any HLA type other than HLA-DQ2.2, HLA-DQ2.5, and HLA-DQ8; MLPA, multiplex ligation-dependent probe amplification; SSCP/HD, single-strand conformation polymorphism/heteroduplex; SSO/SSP, sequence-specific oligonucleotide/sequence-specific primer based.

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Author Disclosure Statement

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References

- Anderson RP, Henry MJ, Taylor R, *et al.* (2013) A novel serogenetic approach determines the community prevalence of celiac disease and informs improved diagnostic pathways. *BMC Med* 11:188.
- Barakauskas VE, Lam GY, Estey MP (2014) Digesting all the options: laboratory testing for celiac disease. *Crit Rev Clin Lab Sci* 51:358–378.
- Fasano A, Catassi C (2012) Clinical practice. Celiac disease. *N Engl J Med* 367:2419–2426.
- Husby S, Koletzko S, Korponay-Szabó IR, *et al.* (2012) European Society for Pediatric Gastroenterology, Hepatology, and Nutrition guidelines for the diagnosis of coeliac disease. *J Pediatr Gastroenterol Nutr* 54:136–160.
- Ludvigsson JF, Bai JC, Biagi F, *et al.* (2014) Diagnosis and management of adult coeliac disease: guidelines from the British Society of Gastroenterology. *Gut* 63:1210–1228.
- Lund F, Hermansen MN, Pederson MF, *et al.* (2015) Mapping of HLA-DQ haplotypes in a group of Danish patients with celiac disease. *Scand J Clin Lab Invest* 75:519–522.
- Megiorni F, Mora B, Bonamico M, *et al.* (2009) HLA-DQ and risk gradient for celiac disease. *Hum Immunol* 70:55–59.
- Megiorni F, Pizzuti A (2012) HLA-DQA1 and HLA-DQB1 in Celiac disease predisposition: practical implications of the HLA molecular typing. *J Biomed Sci* 19:88.
- Monsuur AJ, de Bakker PI, Zhernakova A, *et al.* (2008) Effective detection of human leukocyte antigen risk alleles in celiac disease using tag single nucleotide polymorphisms. *PLoS One* 3:e2270.
- Mubarak A, Spierings E, Wolters VM, *et al.* (2013) Human leukocyte antigen DQ2.2 and celiac disease. *J Pediatr Gastroenterol Nutr* 56:428–430.
- Schouten JP, McElgunn CJ, Waaijer R, *et al.* (2002) Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res* 30:e57.
- Van Beek EM, Roelandse-Koop EA, Vijzelaar R, *et al.* (2013) A multiplex assay to rapidly exclude HLA-DQ2.5 and HLA-DQ8 expression in patients at risk for celiac disease. *Clin Chem Lab Med* 51:1191–1198.

Address correspondence to:

Wouter Kortlandt, PhD

Department of Clinical Chemistry, Hematology
and Immunology
Diakonessenhuis
Bosboomstraat 1
3582 KE Utrecht
The Netherlands

E-mail: wkortlandt@diakhuis.nl