Assessment of complement activation in human disease
Wouters, D.

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Chapter 6

High throughput analysis of the C4 polymorphism
by a combination of
MLPA and isotype-specific ELISA’s

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Dennis Schooneman, Taco W Kuijpers, Lucien A Aarden, Dörte Hamann

Submitted
Abstract
Complement factor C4 exists as two main isotypes, C4A and C4B, with different functional properties and encoded by two separate genes. In addition, C4A and C4B genes can occur in multiple copies and may carry a retroviral HERV-K(C4) insertion in intron 9. To study association of C4 polymorphism with disease, accurate genotyping and phenotyping is important. However, current techniques are very laborious and not suitable to study large patient groups. Therefore, we aimed to develop novel assays for C4 geno- and phenotyping, to make high throughput possible. To study C4 gene copy number variation, a novel Multiplex Ligation-dependent Probe Amplification (MLPA) assay was set up with three synthetic probe parts. C4A and C4B protein levels were measured by isotype-specific ELISA’s. The relationship between C4 genotype with C4A and C4B serum concentrations was examined in 104 healthy lab workers and 66 children with meningococcal disease. As expected, a strong positive correlation was found between C4A and C4B gene copy number and serum levels of total C4, C4A and C4B. In the healthy controls, 95.3% of C4A genes and 53.7% of C4B genes carried the HERV-K(C4) insertion. Presence of HERV-K(C4) resulted in less C4B protein expression, while there was no effect on total C4 levels. In the meningitis patients, no increased incidence of hetero- or homozygous deficiency of either C4A or C4B was found.
In conclusion, the combination of MLPA and ELISA is very suitable to study the geno- and phenotype of complement C4 in large patient groups.
Introduction

The fourth complement component (C4) is encoded by two closely linked, highly polymorphic genes, located within the MHC class III region on chromosome 6. These genes encode the isotypic proteins C4A and C4B, each comprising multiple allotypes (1-3). The C4 gene copy number in a diploid genome varies from two to six, due to deletion or duplication of C4 genes; most common is a C4 gene dosage of four. In addition to gene copy number variation, C4A and C4B genes can be long (21 kb) or short (14.6 kb), depending on the presence of a retroviral HERV-K(C4) insertion in intron 9 (4, 5). It has been reported that C4A genes are usually long, whereas C4B genes can be either long or short (6). The C4 gene copy number variation and dichotomous gene size variation result in a wide spectrum of serum C4 protein concentrations. Null alleles are defined by the absence of C4 protein in plasma, which may be due to either large gene deletions or nonexpression of genes. A well known mutation that leads to C4 null alleles is a 2 bp (CT) insertion in exon 29, resulting in a premature stop codon (7). The silencing CT insertion is most often found in C4A genes, and has been described once in C4B (7, 8).

Upon activation of C4, a highly reactive thio-ester is exposed, which can be hydrolysed by surrounding water molecules or react with free hydroxyl or amino groups to form ester or amide bonds, respectively. Despite high sequence homology, the two isotypic variants C4A and C4B exhibit differential chemical reactivities towards hydroxyl- and amino-group-containing targets. After proteolytic cleavage, C4A binds more efficiently to targets expressing free amino groups, whereas C4B has a higher affinity to hydroxyl groups (9, 10). Furthermore, C4B is hemolytically more active towards sensitized sheep erythrocytes. These differences infer that C4B is more involved in propagating the classical and lectin activation pathways leading to the formation of the membrane attack complex to eventually lyse invading organisms. On the other hand, C4A may have evolved for better opsonization and clearance of immune complexes (11).

The difference in C4A- versus C4B-binding affinities is determined by only four amino acids within the C4d region of the alpha chain (12). The C4d region also carries the antigenic blood group determinants Rodgers (Rg) and Chido (Ch) that are strongly associated with C4 isotypes. Usually, C4A proteins carry the Rg blood group antigen, while C4B proteins carry the Ch antigen. However, rare C4 allotypes have been recognized that express reversed antigenicity: C4A1 usually carries Ch1 instead of Rg1 (13) and some C4B5 allotypes carry Rg1 but lack Ch determinants (14).

Complete C4 deficiency is very rare and is strongly associated with severe immune complex disease, such as systemic lupus erythematosus (SLE). However, partial C4 deficiency is very common in humans, due to gene deletions, conversions or the presence of null alleles. Increased
frequencies of heterozygous or homozygous C4A and C4B deficiency are associated with autoimmune disease and susceptibility for bacterial infections, respectively. Many studies have shown C4A null alleles to be a risk factor for SLE (15, 16). It has been postulated that low C4A levels, as found in patients with C4A deficiency, result in impaired clearance of immune complexes (11). Complete or partial C4B deficiencies are related to vulnerability and severity of microbial infections (17-19). Recently, Yang et al reported that C4 genotyping and phenotyping is relevant in diagnosing human immune complex disease such as SLE. According to that study, low C4A gene copy number is a risk factor for SLE susceptibility (16). In general, knowledge on C4A and C4B gene copy number variation, gene size and protein polymorphisms may be relevant for determining susceptibility for microbial infections or development of autoimmune disease.

In the present study, we report an extensive investigation of C4 genotype and C4A and C4B protein serum levels in a healthy study population. Since standard techniques such as RFLP and allotyping are very laborious and sometimes difficult to interpret, we aimed to develop alternative techniques that render it possible to study large patient groups. We describe a novel Multiplex Ligation-dependent Probe Amplification (MLPA) assay for C4 genotyping. MLPA was originally developed as alternative for RFLP and Southern blot analysis. The assay is very suitable to study gene copy number variation, but can be easily adapted to detect polymorphisms as well in the same assay. For MLPA only small amounts of DNA are required and high throughput is possible. We have used a synthetic probe set approach. Probes consisting of three probe parts were used instead of the usual half probes. This way the number of different probes could be extended from 10 to 20 in one reaction with probe lengths up to 180 bp, without the need to have the probe parts purified or to use cloned probes. The C4 MLPA assay enabled us to determine the copy number of C4A and C4B genes and the coding sequences for Rg and Ch epitopes. Moreover, in the same multiplex assay the retroviral HERV-K(C4) insertion in intron 9 was detected to distinguish between long and short genes as well as the presence of a 2 bp (CT)-insertion in exon 29.

To render high throughput analysis of the C4 polymorphism in large patient cohorts possible, we investigated whether a combination of genotyping by MLPA and phenotyping by isotype-specific ELISA’s is suitable to study the C4 polymorphism. To this end, both assays were performed in 104 healthy lab workers and geno- and phenotypic results were combined. Furthermore, since C4B deficiency has been reported to be a risk factor for bacterial meningitis, C4 analysis was performed in a patient group of sixty-six children that survived bacterial meningitis.
Materials and methods

Serum and DNA

Informed consent was obtained from all individuals included in this study. For validation of the MLPA assay, we used DNA that was obtained from 8 individuals that were geno- and phenotyped with standard techniques as part of another study as described before (20).

Serum and DNA were collected from 104 healthy Dutch lab workers. Peripheral blood leukocytes were isolated from EDTA blood, and DNA was purified by means of the Blood and Cell culture DNA midi kit of Qiagen (Hilden, Germany). Sixty-six Caucasian children with meningococcal disease were also included in this study. The characteristics of these patients have been described elsewhere (21). Serum was collected at least twelve months after recovery and DNA was available from 38 of the patients.

Antibodies

Anti-C4-4 was obtained by immunizing a mouse with purified human C4. The use of this mAb in immunoassays has been described previously (22, 23). Anti-C4-4 does not react with serum deficient for the Rg antigen or with genetically C4A-deficient serum, indicating that this mAb is specific for the Rg epitope and does not recognize Ch. Anti-Ch (clone 1228) was obtained from Biotest (Dreieich, Germany). The use of this mAb in ELISA was described before (24, 25).

Multiplex Ligation-dependent Probe Amplification

MLPA reagents were obtained from MRC Holland (Amsterdam, The Netherlands) except for the synthetic probes, which were designed by us and purchased from Biolegio (Malden, The Netherlands). Our MLPA assay contained 20 probes: 10 two-half probes (90-120 bp) and 10 probes that consist of three parts (135-180 bp). MLPA probes were designed for each C4A or C4B gene and each Rg or Ch allele. We designed one probe for the silencing CT insertion in exon 29, one probe for the retroviral HERV-K(C4) insertion in intron 9, one probe in intron 9 without HERV-K(C4) insertion, 4 probes on exons 2, 3, 20 and 30 for total gene count, and 9 control probes. The probe mix contained competitors for the probes on exons 2, 20 and 30 to reduce the signal strengths of these probes. For probe sequences see Table I. DNA samples of eight previously genotyped individuals were used for validation of the assay. MLPA was performed according to the first description by Schouten et al. (26).

In brief, 5 μl of DNA (20 ng/μl) was denatured at 95°C for 5 min in a thermal cycler with heated lid. 3.5 μl of probe mix and 1.5 μl of MLPA buffer were added to each sample and incubated for 1 min at 95°C followed by 16 h at 60°C. Then, 32 μl ligase-65 mix were added at 54°C, followed
<table>
<thead>
<tr>
<th>Probe</th>
<th>Location</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4A L</td>
<td>exon 26</td>
<td>C4A</td>
</tr>
<tr>
<td>C4A R</td>
<td>exon 26</td>
<td>C4A</td>
</tr>
<tr>
<td>C4B L</td>
<td>exon 26</td>
<td>C4B</td>
</tr>
<tr>
<td>C4R</td>
<td>exon 26</td>
<td>See C4A R</td>
</tr>
<tr>
<td>Rodgers L</td>
<td>exon 28</td>
<td>Rodgers L</td>
</tr>
<tr>
<td>Rodgers R</td>
<td>exon 28</td>
<td>Rodgers R</td>
</tr>
<tr>
<td>Chido L</td>
<td>exon 28</td>
<td>Chido L</td>
</tr>
<tr>
<td>Chido R</td>
<td>exon 28</td>
<td>See Chido R</td>
</tr>
<tr>
<td>C4 short L</td>
<td>intron 9</td>
<td>C4 short L</td>
</tr>
<tr>
<td>C4 long L</td>
<td>intron 9</td>
<td>C4 long L</td>
</tr>
<tr>
<td>CT ins L</td>
<td>exon 29</td>
<td>CT ins L</td>
</tr>
<tr>
<td>CT ins M</td>
<td>exon 29</td>
<td>CT ins M</td>
</tr>
<tr>
<td>CT ins R</td>
<td>exon 29</td>
<td>CT ins R</td>
</tr>
<tr>
<td>C4 ex 2 L</td>
<td>exon 2</td>
<td>C4 ex 2 L</td>
</tr>
<tr>
<td>C4 ex 2 M</td>
<td>exon 2</td>
<td>C4 ex 2 M</td>
</tr>
<tr>
<td>C4 ex 3 L</td>
<td>exon 3</td>
<td>C4 ex 3 L</td>
</tr>
<tr>
<td>C4 ex 3 M</td>
<td>exon 3</td>
<td>C4 ex 3 M</td>
</tr>
<tr>
<td>C4 ex 3 R</td>
<td>exon 3</td>
<td>C4 ex 3 R</td>
</tr>
<tr>
<td>C4 ex 20 L</td>
<td>exon 20</td>
<td>C4 ex 20 L</td>
</tr>
<tr>
<td>C4 ex 20 M</td>
<td>exon 20</td>
<td>C4 ex 20 M</td>
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<tr>
<td>C4 ex 30 M</td>
<td>exon 30</td>
<td>C4 ex 30 M</td>
</tr>
<tr>
<td>C4 ex 30 R</td>
<td>exon 30</td>
<td>C4 ex 30 R</td>
</tr>
</tbody>
</table>

**Table I.** MLPA probe sequences
Novel assays to study C4 polymorphism

by an incubation of 15 min at 54°C and 5 min at 98°C. This ligation mixture was diluted 4 times. Subsequently, 40 µl of PCR mix containing one single primer pair was added to 10 µl of the previous reaction mixture, PCR conditions were 38 cycles of 30 s at 95°C, 30 s at 60°C and 60 s at 72°C, followed by 20 min at 72°C. After the PCR reaction, 1 µl of the PCR product was mixed with 9.5 µl of HiDi Formamide and 0.5 µl of CXR 60-400 Promega internal size standard. The mixture was incubated for 10 min at 90°C. PCR products were then separated by capillary electrophoresis on a sequence platform (3130xl Genetic Analyzer, Applied Biosystems; Foster City, CA, USA). The programme Genemarker (Softgenetics) was used to analyze the data.

**Long-range PCR for localization of retroviral insertion**

To test the association between C4A or C4B genes and the retroviral HERV-K(C4) insertion in intron 9, a set of primers (Table II) was designed to amplify the long or the short form of the two isotypes C4A and C4B in a long-range PCR. In short, 50 ng of DNA was added to 12.5 µl of Multiplex PCR mix (Qiagen), 10 µM concentration of each primer and 5 µl of DepC. A first denaturation step at 95°C for 10 min was followed by 42 cycles of 30 s at 95°C, 30 s at 60°C and 5 min at 72°C, followed by 10 min at 72°C. PCR products were separated by electrophoresis on 1% agarose gel.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Location</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4 long F</td>
<td>Intron 9 with HERV-K(C4) insert</td>
<td>GTGCCCTTCTGCTGGCCTCAGCATGTACAG</td>
</tr>
<tr>
<td>C4 short F</td>
<td>Intron 9 without HERV-K(C4) insert</td>
<td>GCCACCCCCAAAAAGTCCTTTTCCCCACACCAG</td>
</tr>
<tr>
<td>C4A R</td>
<td>C4 exon 26 C4A</td>
<td>ATGCTCTGTCTACACTGGAC</td>
</tr>
<tr>
<td>C4B R</td>
<td>C4 exon 26 C4B</td>
<td>ATGCTCTATGTATGTAGGAG</td>
</tr>
</tbody>
</table>

**Table II.** Primer sequences for localization of retroviral insertion

**Sequencing for CT insertion and Rg/Ch crossingover**

To test whether the CT insertion was present in C4A or C4B and whether C4A or C4B were linked with Rodgers or Chido, a PCR and subsequent sequencing was done with a C4A- or C4B-specific forward primer and a reverse primer downstream of the CT insertion (Table III).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Location</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4A F</td>
<td>C4 exon 26 C4A</td>
<td>TAGTTCCAGGACCGCTGTGCACTGTAGAG</td>
</tr>
<tr>
<td>C4B F</td>
<td>C4 exon 26 C4B</td>
<td>GCGTCCAGGACCTCTCCTCCAGTGATACAT</td>
</tr>
<tr>
<td>CT R</td>
<td>C4 exon 29 downstream of CT insertion</td>
<td>GTGCTCTTCTGCTCCATCTCTGCTTTGCCCCTCGTG</td>
</tr>
</tbody>
</table>

**Table III.** Primer sequences for localization CT insertion
Chapter 6

C4 protein levels

Total C4 levels in serum were measured by nephelometry, with rabbit polyclonal anti-human C4 (Dade Behring, Marburg, Germany). For detection of C4A in ELISA, mAb C4-4 (Rg-specific) (5 μg/ml in 0.11 M sodium acetate buffer, pH 5.5) was coated onto microtiter plates (Nunc Maxisorp; NalgeNunc Int., Roskilde, Denmark) overnight at room temperature (RT). Sera, diluted in high-performance ELISA buffer [HPE; Business Unit Immune Reagents (BUR), Sanquin, Amsterdam, The Netherlands], were incubated for 1 h at RT. Plates were washed five times with PBS/0.02% Tween and incubated with biotinylated polyclonal sheep anti-human C4c (BUR, Sanquin), diluted in HPE, for 1 h at RT followed by another five washes with PBS/0.02% Tween. Subsequently, plates were incubated with 100 μl of streptavidin-peroxidase (0.1%, v/v, in HPE; Amersham/Pharmacia, Uppsala, Sweden) for 30 min at RT. Development of the assay was with 100 μg/ml tetramethylbenzidine in 0.11 M sodium acetate (pH 5.5) containing 0.003%, v/v, H₂O₂. Substrate conversion was stopped by addition of 2M H₂SO₄. Absorbance was measured at 450 nm with a Titertek multiscan. Serum samples were diluted 1:20 and subsequent three-fold dilutions. C4A concentration was calculated on a titration curve of a serum pool of two healthy volunteers with homozygous C4B deficiency and known C4A concentration (Fig. 1A).

![Figure 1](image_url)

Figure 1. Serial dilutions of serum pools of homozygous C4B-deficient individuals (closed symbols) and homozygous C4A-deficient individuals (open symbols) in (A) C4A ELISA and (B) C4B ELISA. C4 concentration of the serum pools was measured by nephelometry.
C4B levels were assessed with an ELISA similar to that of C4A, using a Chido-specific mAb (clone 1228) as catching antibody (5 µg/ml in 0.11 M sodium acetate buffer, pH 5.5). For optimal results, polymerized streptavidin-peroxidase (BUR, Sanquin) was used, (0.01%, v/v, in HPE). Serum samples were diluted 1:20 and subsequent three-fold dilutions. C4B concentration was calculated on a titration curve of a serum pool of four C4A deficient healthy volunteers with known C4B concentration (Fig. 1B). There was a highly significant correlation between total C4 as measured by nephelometry and the sum of C4A and C4B measured by ELISA (R = 0.89, P < 0.0001), which indicates that all C4 was detected in our assays.

Statistical analysis
Data were analyzed with GraphPad Prism (4.0). Comparisons between different genotypic groups were made with the Mann-Whitney or the unpaired t-test, depending on whether the values were normally distributed. Correlation between total C4 as measured by nephelometry and the sum of C4A and C4B was determined by Spearman’s rank correlation coefficient. P values (2-tailed) less than 0.05 were considered statistically significant.

Results
C4 genotyping
C4 genotyping results obtained by MLPA are summarized in Table IV. In a population of 104 healthy lab workers, the majority (59.6%) had four copies of the C4 gene. The frequencies of C4 gene copy number of two, three and five C4 genes were 5.7%, 26% and 8.7%, respectively. No individuals with six C4 genes were found.
Of the 386 C4 genes present in our study population, 211 genes (54.7%) encoded for the C4A isotype and 175 genes (45.3%) for the C4B isotype. The retroviral HERV-K(C4) insertion in intron 9 was present in 295 out of 386 C4 genes (76.4%). The association between HERV-K(C4) insertion and C4 isotypes was studied by combining long-range PCR results with MLPA data. As expected, almost all C4A genes were long (95.3%) whereas short and long C4B genes were present in similar frequency (46.3% and 53.7%, respectively). Five subjects (4.8%) carried a 2-bp (CT) insertion in exon 29. Sequencing revealed that this silencing mutation was always present in C4A genes.
Total absence of C4A genes (homozygous C4A deficiency) was observed in six of the 104 (5.8%) individuals. In addition, one subject with a single C4A gene (partial C4A deficiency) had a silencing CT insertion in exon 29 of the C4A gene, which resulted in complete absence of C4A protein in serum. In total, seven individuals (6.7%) were completely deficient for C4A in serum.
Sixteen subjects (15.4%) were partially C4A deficient, having only a single copy of the C4A gene (heterozygous C4A deficiency). Three subjects with two C4A genes were bearing a CT insertion in exon 29, silencing one of the C4A genes present. Taking the presence of this silencing mutation into account, nineteen of the 104 subjects (18.3%) were partially C4A deficient, having only one functional C4A gene. Together, 25% of our study population were either partially or completely C4A deficient. High C4A gene copy numbers (more than two C4A genes) were observed in 26.9% of the subjects. Twenty-four subjects (23.1%) had three copies of the C4A gene and 4 subjects (3.8%) had four copies. However, one of the subjects with four C4A genes carried a silencing CT insertion, so in total 25 subjects (24%) had three functional C4A genes and 3 subjects (2.9%) had four functional C4A genes.

<table>
<thead>
<tr>
<th>N= 104</th>
<th>Total (%)</th>
<th>C4A (%)</th>
<th>C4B (%)</th>
</tr>
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<tbody>
<tr>
<td>Gene frequency</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>7 (6.7)</td>
<td>5 (4.8)</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>19 (18.3)</td>
<td>30 (28.8)</td>
</tr>
<tr>
<td>2</td>
<td>6 (5.7)</td>
<td>50 (48)</td>
<td>62 (59.6)</td>
</tr>
<tr>
<td>3</td>
<td>27 (26)</td>
<td>25 (24)</td>
<td>7 (6.7)</td>
</tr>
<tr>
<td>4</td>
<td>62 (59.6)</td>
<td>3 (2.9)</td>
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</tr>
<tr>
<td>5</td>
<td>9 (8.7)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Genes</td>
<td>386 (100)</td>
<td>211 (54.7)</td>
<td>175 (45.3)</td>
</tr>
<tr>
<td>HERV-K(C4) insertion</td>
<td>295 (76.4)</td>
<td>201 (95.3)</td>
<td>94 (53.7)</td>
</tr>
<tr>
<td>CT insertion</td>
<td>5 (4.8)</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

* Table IV. MLPA results in 104 healthy individuals

* Data are corrected for the presence of silencing CT insertion

C4B genes were absent in five subjects (4.8%) (homozygous C4B deficiency). No CT insertion was found in C4B genes. Thirty (28.8%) individuals were partially C4B deficient, having only one C4B gene (heterozygous C4B deficiency). Together, 33.7% of the individuals were either partially or completely deficient for C4B. High C4B gene copy numbers (more than two C4B genes) were less common than high C4A gene copy numbers. Seven subjects (6.7%) had three
copies of the $C4B$ gene. Partial or complete deficiency of $C4A$ or $C4B$ was observed in 58.7% of the individuals studied. $C4A$ and $C4B$ gene copy numbers tend to be inversely correlated (Fig. 2). The inverse correlation between $C4A$ and $C4B$ gene copy numbers was also reflected by serum concentrations (data not shown).

![Graphs showing the distribution of $C4A$ and $C4B$ gene copy numbers](image)

**Figure 2.** Inverse correlation between $C4A$ and $C4B$ gene copy number. Distribution of $C4A$ and $C4B$ genes is shown in a cohort of 104 healthy individuals.

**Correlation between C4 gene copy number and C4 serum concentration**

Total C4 serum levels were positively correlated with $C4$ gene copy number; although a wide variation in C4 concentration between genotypic groups was observed. The median serum concentration of the most common genotypic group, i.e. four $C4$ genes, was 216 µg/ml (range 61-372). Subjects with three or two $C4$ genes, being hetero- or homozygous deficient for one of the C4 isotypes, had significantly ($P < 0.01$) lower C4 serum concentrations than subjects with
four $C4$ genes. No significant difference between hetero- and homozygous $C4$ deficiency was observed (median 175 μg/ml, range 142-209 and 194 μg/ml, range 61-274, respectively). Subjects with high $C4$ gene copy number (having five copies of the $C4$ gene) had significantly ($P < 0.01$) higher $C4$ serum levels (median 289 μg/ml, range 215-362) than all other genotypic groups.

**Correlation between $C4A$ gene copy number and $C4A$ serum concentration**

To study the influence of $C4A$ gene copy number on total $C4$ serum concentration, the population was divided into three groups: low (0 or 1 $C4A$ gene), medium (2 $C4A$ genes) and high (3 or 4 $C4A$ genes). Five subjects were shown to harbour a silencing CT insertion in one of their $C4A$ genes. Since these $C4A$ pseudogenes produce no protein, they were counted as $C4A$ negative. In figure 3, the number of functional, protein expressing $C4A$ genes is shown. There was no correlation between the number of $C4A$ genes and total $C4$ serum concentration (Fig. 3A).

![Figure 3](image)

**Figure 3.** Correlation between $C4A$ gene copy number and total $C4$ serum concentration measured by nephelometry (A) and $C4A$ serum concentration measured by Rg-specific ELISA (B). The number of $C4A$ genes is corrected for the presence of a silencing CT insertion. Horizontal lines indicate the median values. **$P < 0.01$, ***$P < 0.001$.

$C4A$ serum levels of individuals with different $C4A$ gene copy number were quantified by ELISA, with a Rg-specific mAb. $C4A$ serum levels were strongly associated with $C4A$ gene copy number; although a wide overlap was observed between the genotypic groups (Fig. 3B). With a single exception, no $C4A$ was detected in serum of individuals with 0 $C4A$ genes.
Correlation between C4B gene copy number and C4B serum concentration

To study the relationship between C4B gene copy number and total C4 serum concentration, the population was divided in three groups: low (0 or 1 C4B gene), medium (2 C4B genes) and high (3 C4B genes). There was no significant difference in total C4 serum concentration between the low and medium C4B gene copy number group (Fig. 4A). However, total C4 serum concentration was significantly \( (P < 0.05) \) higher in individuals with high C4B gene copy number.

Figure 4. Correlation between C4B gene copy number and total C4 serum concentration measured by nephelometry (A) and C4B serum concentration measured by Ch-specific ELISA (B). Horizontal lines indicate the median values. *\( P < 0.05 \), ***\( P < 0.001 \)
C4B serum levels of individuals with different \( C4B \) gene copy number were measured by Ch-specific ELISA. C4B serum levels were strongly associated with \( C4B \) gene copy number, although the data of different genotypic groups were overlapping (Fig. 4B). No C4B was detected in serum of individuals with 0 \( C4B \) genes (homozygous C4B deficiency). The median C4B concentration in subjects with 1 \( C4B \) gene (heterozygous C4B deficiency) was 70 \( \mu g/ml \) (range 22-168), which was significantly (\( P < 0.001 \)) lower than the C4B serum concentration in subjects with 2 \( C4B \) genes (median 144 \( \mu g/ml \), range 28-292). Seven subjects had a high \( C4B \) gene copy number (3 \( C4B \) genes), and these had the highest C4B serum concentration (median 180 \( \mu g/ml \), range 131-338) (\( P < 0.05 \)).

One subject who was homozygous deficient for C4B had detectable serum protein levels (62 \( \mu g/ml \)) (Fig. 4B). This individual showed reversed antigenicity, having no \( C4B \) genes, but one Ch allele, which was associated with C4A (genotype: 4\text{C4A}/0\text{C4B}; 3 \text{Rg}/1\text{Ch}). Ch epitopes on C4A protein present in this serum were detected with the Ch-specific ELISA. When ELISA results of the whole study population were combined with MLPA data, three subjects (2.9%) showed reversed antigenicity. Thus, the Ch- and Rg-specific ELISAs showed 97.1% agreement with genotyping of the C4 isotypes and 100% agreement with genotyping of Ch and Rg alleles.

**Influence of retroviral HERV-K(C4) insertion on C4 protein expression**

Since virtually all (95.3%) \( C4A \) genes and 53.7% of \( C4B \) genes harbour the retroviral HERV-K(C4) insertion in intron 9, we only focused on \( C4B \) genes to study the influence of this retroviral insertion on C4 protein expression levels. We focused on the most common \( C4B \) gene dosage group, i.e. two \( C4B \) genes. These individuals may have the following distribution of long and short \( C4B \) genes: Long-Long, Short-Long or Short-Short. The presence of long \( C4B \) genes had no effect on total C4 serum concentration (Fig. 5A). However, the presence of HERV-K(C4) insertion in \( C4B \) genes had a significant negative effect on C4B protein expression. Individuals with the Long-Long distribution had lowest C4B serum concentrations (Fig. 5B). The presence of one short gene resulted in significantly (\( P < 0.01 \)) higher C4B serum concentration. No difference was observed in C4B serum concentration between subjects with one or two short genes.

**C4 polymorphism in children with meningococcal disease**

The C4 polymorphism was examined in sixty-six children that survived bacterial meningitis. MLPA was performed in 38 out of 66 patients. C4 genotyping in the patients was not significantly different from healthy controls; 73.7% had four copies of the \( C4 \) gene. Frequencies
of $C4$ gene copy number of two, three and five $C4$ genes were 5.3%, 13.2% and 5.3%, respectively. One patient with six $C4$ genes was found. 74.8% of the genes carried a HERV-K(C4) insertion and four CT insertions (10.5%) were observed. Two patients (5.3%) were homozygous $C4A$ deficient and eight (21%) were heterozygous $C4A$ deficient. None of the patients was homozygous $C4B$ deficient and nine (23.7%) were heterozygous $C4B$ deficient.

In the patient group, $C4A$ levels (median 98 µg/ml, range 0-298) were slightly lower than in the healthy controls (median 116 µg/ml, range 0-365). No difference was observed in $C4B$ levels between the meningitis patients (median 123 µg/ml, range 0-338) and healthy controls (median 126 µg/ml, range 3-346).

**Discussion**

The present study describes an MLPA assay to study $C4$ genetic variation. Results obtained by this assay in a healthy study population were combined with isotype-specific ELISAs to quantify $C4A$ and $C4B$ serum levels.

Determination of $C4A$ and $C4B$ gene copy numbers is generally carried out by RFLP in combination with Southern blot (27). However, this is a laborious technique and relatively large quantities of DNA are required. More recently, PCR-based methods were developed to
determine C4A and C4B gene copy numbers (28-30). However, the mere presence of C4 genes does not ensure C4 protein expression. C4 null alleles can only be detected at the protein level, since PCR assays are not suitable for identification of non-functional C4 genes. The present study describes a novel MLPA assay to study C4 genetic variation. For MLPA, only small amounts of DNA are required, and within one multiplex assay, not only C4A and C4B gene copy number can be determined, but also Ch and Rg alleles can be distinguished. Moreover, the assay detects the HERV-K(C4) insertion in intron 9 and a silencing CT insertion in exon 29. For the C4 MLPA assay synthetic probes were designed in our laboratory. Currently, a C4 MLPA kit (MRC Holland) is available with cloned probes that yield similar results as the synthetic probes, as determined by analyzing DNA of twenty samples in both assays (data not shown).

C4 genetic variation was studied in 104 healthy lab workers. Observed frequencies of C4 gene copy number as well as the presence of HERV-K(C4) were in accordance with the outcome of previous studies (2). Five donors had a silencing CT insertion in exon 29 and sequencing revealed that it was always present in the C4A gene, as was expected (7).

Reported frequencies of homozygous C4A and C4B deficiency vary. In our study group they were higher than those previously published by Blanchong et al. (2). This may in part be explained by the fact that we considered the presence of a CT insertion in exon 29 as a null allele. Our results were comparable to those of a Swedish population: Nityanand et al. observed frequencies of homozygous C4A and C4B deficiencies of 5% and 5.6%, respectively (31).

Standard technology for C4 allotyping is high-voltage agarose electrophoresis with subsequent immunofixation (32, 33). Null alleles are determined by visual scoring of the relative density of C4A and C4B bands on C4 allotyping gels. However, this method is semi-quantitative; an overdose of one isotype (caused by gene duplication) may be misinterpreted as partial deficiency of the other isotype. In the present study we used Rg- and Ch-specific ELISAs to quantify C4A and C4B levels in serum, as described before (24, 34, 35). However, the correspondence between C4 isotypes and these blood group antigens is not absolute. Rare alleles with reversed antigenicity have been recognized before; C4A1 usually carries Ch1 instead of Rg1 (13) and some C4B5 allotypes carry Rg1 but lack Ch determinants (14), so clearly the Ch- and Rg-specific ELISAs cannot be used for typing individuals with A1 or B5 alleles (36). It has been described that these alleles are very rare, and should therefore not give significant problems in Ch- and Rg-specific ELISAs (37). However, in our study group, three subjects (2.9%) were misinterpreted by Ch- and Rg-specific ELISAs because of reversed antigenicity. To our knowledge no C4A and C4B specific mAbs are currently available to circumvent this problem. Interestingly, two of the three individuals with reversed antigenicity were completely deficient.
for either C4A or C4B. This might indicate that gene deletion and crossing-over are linked events.

Several reports describe that C4 serum concentration is mainly determined by gene copy number (38, 39). It has been reported that the presence of null alleles can be predicted by the total C4 serum concentration, by setting reference ranges for different genotypic groups (34, 38, 40). The present study confirms that total serum C4 concentration is closely related to C4 gene copy number. However, because there is considerable overlap between the different genotypic groups, it is impossible to determine the presence of single null alleles from total serum C4. When measuring C4A and C4B serum levels, there was a strong correlation between gene copy number and serum concentration of C4A and C4B. Only when ELISA results are completely negative, homozygous deficiency of one of the isotypes can be predicted. However, the observed extensive overlap between genotypic groups in our study renders it impossible to predict heterozygous deficiency of C4 isotypes. Moreover, in patients with active disease, C4 phenotyping is hampered by low C4 serum concentration due to consumption. Because of the limitations of studying mere genetics or proteins, phenotyping should always be accompanied by genotyping for accurate determination of C4A or C4B polymorphisms.

76.4% of all C4 genes in our study population harbour a HERV-K(C4) insertion. Yang et al. found a strong correlation between the presence of HERV-K(C4) and C4A genes (39). Indeed, in our study population 95.3% of the C4A genes were long. Still, about half of the C4B genes (53.7%) also harbour this insertion. It has been described that the presence of the HERV-K(C4) insertion has a negative effect on C4 expression, resulting in lower protein levels and less hemolytic activity (39). Since nearly all C4A genes were long, we focused on C4B genes and observed that C4B protein expression was negatively influenced by the presence of HERV-K(C4) in intron 9. Furthermore, when comparing serum protein concentration, two C4A genes result in lower protein expression than two C4B genes, which is in accordance with the higher prevalence of the HERV-K(C4) insertion in C4A genes as compared to C4B genes.

We observed an inverse correlation between C4A and C4B levels. Therefore, individuals with similar total C4 levels can differ substantially in C4A and C4B levels. Associations with disease have been mainly reported with deficiencies in either C4A or C4B, because the isotypes differ functionally. Measuring C4A and C4B levels in addition to total C4 is therefore essential for finding disease associations and for patient analysis. Moreover, selective C4A or C4B consumption can be studied during autoimmune disease or infections, respectively.

C4B deficiency has been described to be a risk factor for bacterial meningitis (17, 18) although others could not confirm this association (41). In the present study, sixty-six patients who
Chapter 6

suffered from bacterial meningitis were analyzed by C4 isotype-specific ELISAs. DNA was available from 38 of these patients and MLPA was performed for C4 genetic analysis. None of the patients was homozygous C4B deficient, and the frequency of heterozygous C4B deficiency was not different from that of the healthy controls. In addition, no difference was found in the frequency of heterozygous or homozygous C4A deficiency between patients and healthy controls. Overall, no relation was found between C4 isotypic deficiencies and meningitis, which is in agreement with the results of Cates et al. (41).

In conclusion, we set up a novel MLPA assay to identify gene copy numbers of C4 and the isotypic variants C4A and C4B. The combination of MLPA and ELISA is an accurate approach to study C4 polymorphism and is suitable for high-throughput analysis of patient cohorts.

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Novel assays to study C4 polymorphism

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