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RHD MPX PCR to support serology and for prenatal typing, five years of experience

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Introduction: Since 1999, we use an RHD MPX PCR, amplifying exons 3 to 7 and 9 of RHD, to define serologically aberrant RhD expression (weak or variant D; n=169) and the fetal RhD status with amniotic fluid (n=107).
Results: In 61 cases, the RHD MPX confirmed the presence of an RhD variant: a DAR (n=28; serologically difficult to define), DVI (n=19), DIII (n=2), DIVa (n=1), DVa (n=3), DFR (n=6) and RoHar (n=2). In 11 cases, a variant D was not confirmed. Subsequent sequence analysis, with genomic DNA and RHD-exon-specific PCRs, showed DVII (n=4), DNB (n=2) and not yet conclusive results in 5 cases. In 75 cases, the RHD MPX PCR was performed to confirm the presence of a normal or weak-D phenotype. Weak D was further proven by specific PCRs. In 10 cases an assumed RhD negativity was confirmed. In case of prenatal screening, in 72 cases RhD positivity was predicted; in 35 cases RhD negativity. In one case, a false positive normal RHD MPX result was noted after birth. Sequence analysis of RHD of the newborn and the father showed that both were carrier of an RHD gene with a deletion of 329T and 330G in exon 2, leading to a premature stopcodon in exon 3. In 17 cases, RHD typing of fetal DNA present in maternal plasma was performed with an RHD-exon 7 specific quantitative.
Conclusion: The RHD MPX is a useful addition for RhD serology. It can be used for prenatal RHD typing, but may be replaced by non-invasive RHD typing with maternal plasma.

Automated red cell and platelet antigen genotyping to provide a completely typed donor population

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Introduction: Blood of all donors is currently typed serologically for ABO, the Rh phenotype and Kell. Only a subset of donors is typed for a larger set of red cell antigens and/or platelet antigens. To increase the direct availability of typed red cells and platelets, we are developing rapid genotyping assays.
Methods: We designed, validated and automated TaqMan-based assays for the clinically relevant Human Platelet Antigen (HPA)-1, -2, -3, -5 and -15 systems. The blood sample is mechanically presented to a DNA isolation robot (MagNa-Pure, Roche). Subsequently, isolated DNA is automatically added to a 96-well plate containing the TaqMan assays. For DNA microarray-based blood group antigen typing a multiplex PCR was designed, amplifying and fluorescent labeling 19 different gene fragments containing the allele-specific SNPs (red cell and HPA antigens). This PCR product can directly be loaded on the DNA array.
Results: The TaqMan-based genotyping assays for HPA-1, -2, -3, -5 and -15 were validated with 90 serologically typed donors. No discrepancies between the phenotype and genotype result were found for HPA-1, 2 and 5. One donor with an additional SNP in the HPA-3b-encoding allele could not be automatically scored with the TaqMan assay. For three HPA-15 typings discrepant results were obtained, suggesting the presence of silent alleles. In a study with two different batches of DNA microarrays, ninety-four HPA-typed samples were genotyped and no discrepancies were found.
Conclusion: TaqMan-based blood group antigen typing can be used for HPA typing. DNA microarray-based blood group antigen will facilitate a fast and reliable antigen typing. However, the method needs to be further developed and automated to obtain the necessary throughput for typing of large donor cohorts.

Non-invasive fetal RHD-genotyping is feasible in all blood group RhD-negative pregnant women

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Introduction: Blood group RhD-negative women receive antenatal anti-D prophylaxis irrespective of the RhD-status of the fetus. In about 40% of these women this gift is given unnecessarily, because the fetus is D-negative. To identify women carrying D-positive fetuses, we have developed a fully automated assay for fetal RHD genotyping with cell-free fetal DNA from maternal plasma.
Methods: One ml of maternal plasma is mechanically presented (Tecan) to a DNA isolation robot (Roche). The DNA eluate is tested (after automated pipetting) in triplicate in a real-time quantitative RHD exon7-PCR (ApplBios). From Oct-Dec 2003, plasma from 2397 (serologically confirmed) D-negative pregnant women, whose blood was sent in for 28-30th week antibody screening, was tested. These women were asked by a questionnaire to send us the cord blood serology results.
Results: 1470 of the plasma's were typed RhD positive and 932 RhD negative. At present, of 1257 newborns the serology is known. In 1245 cases (99.1%) serology and PCR were concordant. In 7 cases the genotype suggests D-positivity while serology reports D-negativity. In 5 cases no RHD-DNA sequences were detected in plasma but cord blood was typed D-positive. We observed that 1.44% (35/2432) of the supposed D-negative pregnant women were in our laboratory serologically found to be D-positive. Therefore, for the discrepant cases it cannot be concluded yet, whether serology or PCR is correct. The true RHD status of these cases will be investigated by DNA tests on buccal swabs of the newborns.
Conclusion: This is the first large-scale study demonstrating the feasibility of screening D-negative women to restrict antenatal administration of anti-D to women carrying D-positive fetuses.

Detection of common deletional determinants of alpha-thalassemia using single labeled self-quenched primers

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Introduction: Alpha-thalassemia is in more than 95 percent of the cases caused by a limited number of deletions of one or both alpha-globin genes on chromosome 16p13.3. Southern Blot analysis was the most commonly used technique, but since the breakpoints of most deletions are known, PCR based detection is possible. For diagnostic purposes we are now developing a closed PCR system.
Methods: To prevent contamination, the amplicons are kept inside the PCR vial and quantified by fluorescence. We have modified the primersets described by Chong et al. (Blood, 2000; 98: 250-251). The primers were labeled with a fluorochrome and extended at the 3' end with 7 nucleotides complementary to the 5' end. Upon incorporation of the primer, the fluorescence increases 8-fold. Fluorescence can be measured by real time PCR, but the method is also applicable for end point detection in a fluorescence plate reader.
Results: We compared the outcome of the PCR method with that of Southern Blotting for selected samples with different deletions and found no discrepancies. For the rare THAI, MED and 20.5 deletions we tested the DNA of only one individual. For the other deletional types we tested at least 3 different samples.
Conclusion: With this method we can recognize the wild type and the seven most common deletions in the alpha-globin region ($\alpha\alpha$; -α3.7; α-4.2; --SEA; --FIL; --THAI; --MED; -α20.5). Because primers can be labeled either with JOE or FAM, it is possible to combine two primer sets in one tube; as a result four PCR vials are enough to screen for the seven most frequently occurring deletions.

Detection of minimal residual disease in acute lymphoblastic leukemia

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Introduction: In the Netherlands about 120 children are diagnosed each year with acute lymphoblastic leukemia (ALL). Treatment intensification brought progress in ALL patient survival, but still 20 to 30 % of these patients suffer from relapse. Identification of low-risk and high-risk patients may further improve outcome. Numerous studies have shown that the early treatment response, as measured by the level of minimal residual disease (MRD) is a good prognostic factor.
Methods: Real-Time quantitative PCR based detection of MRD in ALL relies on the identification of patient-specific immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements. The junctional regions of rearranged Ig and TCR genes can serve as a unique sequence for the detection of leukemic cells. Together with the EUR (group of prof. J.J. van Dongen) we have adapted this technically advanced approach for routine application. In series of 5 patients, for each patient 23 different (multiplex) PCRs are run, amplifying all possible junctional regions in ALL (IgH, Igkappa-, TCRdelta, TCRgamma and TCRbeta loci). The PCR products are analyzed by heteroduplex analysis. Monoclonal targets are sequenced. Next, for each patient 3 patient-specific real-time Taqman based PCRs are developed.
Results: This work has to be completed within 3 months after diagnosis as then the patients will be stratified. This year, a prospective study will be started, in which all children in the Netherlands with ALL will be treated according to MRD-based risk classification.
Conclusion: In conclusion, PCR-based MRD detection shows how important the development of new advanced laboratory techniques can be for treatment-strategy. Furthermore, ALL might serve as a model for the development of new treatment protocols in other diseases.

Selection and implementation of best practice guidelines for reporting molecular genetic diagnostics in the Amphia hospital

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Introduction: The Amphia hospital is the resultant of a recent merge between four hospital locations in the Breda region. Facing four different Laboratory Information Systems (LIS) across the locations it was decided to purchase a new LIS (Molis, Charles Goffin), which, among other goals, should enable tailored reporting of DNA diagnostics.
Methods: Best draft practice guidelines from CMGS/EMQN, SSGS and NCCLS/CAP were surveyed. Criteria considered to be essential for clear, concise, accurate and unambiguous reporting of DNA diagnostics are: complete lab identification; unequivocal patient identification; knowledge of the clinical request/indication; clarification of the disease and gene/mutation tested; elucidation of the methodology used as well as the extent and sensitivity of the method; concise and univocal genotyping according to HUGO standard nomenclature; thoughtful interpretation of the genotype taking into account clinical context, ethnicity and other lab results, and clarification of the posterior genetic risk of having the disease in case of negative testing; suggestions for additional investigations, disclaimers and authorisation of the report by an independent clinical chemist.
Results: Molis is programmed in such a way that DNA diagnostic reports meet the selected criteria above. Per disease, genotype-phenotype specific comments are added. To simplify computerised and customised DNA reporting, manual data entry is minimised and specific comments are programmed into codes and text blocks. Pre- and postanalytical processes are redesigned: an OCR-based request form is introduced which allows to include clinical context and indication, and third line authorisation is foreseen.
Conclusion: Tailored reporting of DNA diagnostics, according to best practice guidelines, has become feasible in our setting with the introduction of a new LIS. It is experienced as a major step forward to GMP/GLP.

Significance of HFE (C282Y, H63D) and FPNI (N144H) gene mutations in the development of cardiovascular disease. Results from the Dutch prospective monitoring project on cardiovascular disease risk factors

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Introduction: Increased iron stores may play a role in the development of cardiovascular disease (CVD) by increasing lipoprotein oxidation. Beyond the major HFE (C282Y) mutation, HFE (H63D) and FPNI (N144H) mutations have been discovered which may cause hereditary hemochromatosis (HH). We investigated whether these mutations were determinants of CVD in the general Dutch population.
Methods: Study subjects (N = 1381) were selected from participants of the Monitoring Project on Cardiovascular Disease Risk Factors in the Netherlands, carried out between 1987 and 1991 in three Dutch towns among >35000 subjects, 20-59 years of age. Mortality follow-up lasted until January 2000. All CVD deaths (N = 302) were genotyped, while a random sample from the cohort provided information about the total cohort (N= 1079) (case-cohort design). Relative risks (RR) for fatal CVD were calculated according to C282Y and H63D genotype, adjusted for age and town of investigation.
Results: All subjects tested negative for the N144H mutation. The frequencies of homozygosity and heterozygosity in the random sample were respectively 0.3% and 13% for the C282Y mutation, and 2.2% and 24% for the H63D mutation. The frequency of compound heterozygosity was 2%. Genotype distributions were in Hardy-Weinberg equilibrium. In this study the C282Y mutation was not associated with fatal CVD. Female homozygous carriers of the H63D mutation had an increased risk for fatal CVD (RR: 5.9; 95% CI = 1.5-22.8), especially when postmenopausal (RR: 20; 95% CI = 3.1-131). In men, no significant association was observed (RR: 1.9; 95% CI = 0.7-5.2).
Conclusion: Our prospective findings suggest a prominent role of the H63D mutation in the development of fatal CVD in the general Dutch population, especially in postmenopausal women.

Alfa-thalassemie: opzetten van een multiplex-PCR voor detectie van 7 meest voorkomende deleties

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Inleiding: Hemoglobinopathieën behoren tot een erfelijke groep afwijkingen, welke gekenmerkt worden door vermindering van de synthese van één of meer globineketens (thalassemieën) of door de synthese van abnormaal hemoglobine. De meerderheid van de alfa-thalassemieën wordt veroorzaakt door deleties op het alfa-globinegen op chromosoom 16. De meest voorkomende deleties zijn: -α3.7, -α4.2 (enkelvoudige gendeleties) en --SEA, --FIL, --THAI, --MED, -α20.5 (dubbele gendeleties).
Methode: Binnen het laboratorium van de Isala klinieken bestaat een protocol voor de diagnostiek van hemoglobinopathieën. Monsters worden eerst gescreend m.b.v. HPLC-techniek. Bij verdenking op alfa-thalassemie vindt vervolgonderzoek plaats in het DNA-lab. Voor de meest voorkomende deleties is een multiplex-PCR opgezet (1). Als controle op de amplificatie worden LIS1-primers meegenomen. In de PCR-reactie wordt gebruik gemaakt van Q-solution.
Resultaat: Aanvankelijk leverde de PCR onvoldoende product. Door gebruik te maken van een andere isolatie en door het verwijderen van de LIS1-primers uit de reactie, verbeteren de resultaten aanzienlijk. Deze extra positieve controle op de amplificatie is niet nodig, want in geval van een deletie ontstaat er ook een product. Door het op elkaar afstemmen van 'primer'-concentraties en juiste 'annealing'-temperatuur is er uiteindelijk een goede multiplex-PCR-methode ontstaan waarmee alle 7 deleties gedetecteerd kunnen worden.
Conclusie: Voor de multiplex-PCR van alfa-thalassemie is optimalisatie van reactieomstandigheden essentieel. Door reactieomstandigheden zoals primerconcentraties, goed op elkaar af te stemmen kon een betrouwbare methode worden ontwikkeld voor de detectie van 7 meest voorkomende deleties op het alfa-globinegen.

Literatuur: 1. Tan ASC, Quah TC, Low PS, Chong SS. A rapid and reliable 7-deletion multiplex polymerase chain reaction assay for alpha-thalassemia, Blood 2001; 98: 250-251.

Molecular evaluation of the human erytrocyte ankyrin gene in hereditary spherocytosis

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Introduction: Hereditary spherocytosis (HS) is a congenital haemolytic anaemia, the severity of which varies from asymptomatic to severe condition, giving rise to symptoms including icterus, anaemia and splenomegaly. One of the erytrocyte membrane proteins is ankyrin-1 (ANK-1), belonging to a family of proteins coordinating interactions between various integral membrane proteins and cytoskeletal elements. In fact, the most common cause (~35 to 65%) of typical, dominant HS is caused by gene mutations in the erytrocyte isoform (also known as band 2.1, Mr = 210 kDa). The ANK-1 gene is composed of 42 exons, and the composite cDNA contains 5636 base pairs (1879 amino acids). A broad variety of mutations in the ankyrine gene are described.
Methods: We started a program to analyse genetic mutations in 24 patients suspected to suffer from HS. We set up two approaches to identify mutants in order to develop a sensitive screening strategy for suspected HS patients: 1) screening of all 42 coding exons plus the 5' untranslated/promoter region; 2) comparison of genomic DNA and cDNA for common ANK1 polymorphisms.
Results: We screened all coding exons and found in some HS patients known polymorphisms and described mutations in several exons. Moreover, a new missense mutation was detected in exon 34 in 2 familial patients (codon 1386, TAC--CAC, Tyr--His). We started a comparison of polymorphisms in exon 26 and 39 of genomic DNA and cDNA to identify reduced expression of mRNA as screening strategy for these patients. Polymorphisms in exon 26 and 39 are frequent in our patient population (80%).

Effectief onderzoek naar thalassemieën en hemoglobinopathieën in Arnhem

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Inleiding: Systematisch onderzoek naar thalassemieën en hemoglobinopathieën wordt in Arnhem sinds lang verricht, onder meer vanwege de vrij aanzienlijke populatie van allochtone herkomst in de stad (met name Turks).
Methode: Beta-thalassemie en hemoglobinopathieën onderzoeken wij sinds meer dan 10 jaar door middel van scheiding van de verschillende hemoglobinevormen met HPLC. Diagnosticering van alfa-thalassemie wordt verricht met DNA-onderzoek. Tot voor kort bestond dit uit aparte PCR-reacties met specifieke primers voor de grote deleties (SEA, MED, 20.5), en restrictie-enzym-kartering met Southern-blot-hybridisatie voor de kleine deleties (-α3.7, 4.2, 5.0) (1). Vanaf 2003 doen we een zogenaamde 'one tube'-multiplex-PCR-methodiek, waarbij met specifieke primers in één (of twee) PCR-reacties de zeven meest voorkomende deleties tegelijk kunnen worden opgespoord (2).
Resultaat: In het HPLC-onderzoek worden bij ons bij 10-15% van de patiënten afwijkingen gevonden duidend op beta-thalassemie of hemoglobinopathie. Bij de ca. 500 patiënten waarvan we gedurende 8 jaar DNA-onderzoek voor alfa-thalassemie verrichtten was de hemoglobineconcentratie in bloed m: 7,4 ± 1,1 en v: 6,7 ± 1,1 mmol/l (referentiewaarden 8,4-10,8 resp. 7,4-9,9 mmol/l); het MCV was 71,2 ± 10,8 resp. 74,0 ± 11,2 fl (ref. 80-100 fl, m/v). In totaal werden bij 24,9 % van de onderzochte patiënten deleties gevonden. Onderverdeeld naar de verschillende mutaties was dat: patiënten met één kleine deletie (-α/αα + -α/ααα) 16,5%, met twee kleine deleties (-α/-α) 5,2%, en met grote deleties (--/αα SEA en MED) 3,2%.
Conclusie: Onze resultaten tonen dat de voorscreening effectief is en dat het bij de bestaande bevolkingsopbouw realistisch is onderzoek naar thalassemieën en hemoglobinopathieën te verrichten.

Literatuur: 1. Schmidt-Hieltjes YMG, Neerbos BR van. Analyse 1995; 50: 124-127. 2. Tan ASC, Quah TC, Low PS, Chong SS. Blood 2001; 98: 250-251.

Rapid parallel detection of the Factor V Leiden mutation and the Factor II G20210A mutation with TaqMan MGB probes

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Introduction: Individuals that carry the Factor V Leiden mutation or the Factor II G20210A mutation have a higher risk to develop venous thrombosis than wild-type individuals. We developed robust and convenient assays for the parallel detection of the Factor V Leiden mutation (1) and the Factor II G20210A mutation, using TaqMan probes conjugated to a minor groove binder (MGB) group. The conjugation of a MGB group allows the design of probes which are over 25% shorter than traditional TaqMan probes, resulting in increased specificity due to increased mismatch discrimination. Primers and probes were designed with Primer Express software of Applied Biosystems, which makes it possible to run PCRs under universal conditions, allowing parallel detection of different single nucleotide polymorphisms (SNPs).
Methods: DNA was isolated from EDTA treated blood with the QIAamp DNA mini kit (Qiagen). PCR was performed under universal conditions in a final reaction volume of 25 µL, containing TaqMan universal PCR master mix (Applied Biosystems), primers and probes and 5 µL DNA solution. Amplification and detection was carried out in optical 96 well PCR plates with an ABI prism 7000 sequence detection system (Applied Biosystems).
Results: For both mutations, 60 clinical samples were analysed retrospectively. Analysis with the allelic discrimination software of the ABI prism 7000 instrument resulted in clear genotype discrimination. The established genotypes were 100% identical to the results obtained previously with PCR followed by restriction fragment analysis.
Conclusion: Parallel detection of SNPs with TaqMan MGB probes is a robust and convenient method that allows an efficient workflow when the number of different SNPs that are detected in the clinical laboratory increases.

Literature: 1. Luderer R, Verheul A, Kortlandt W. Rapid detection of the Factor V Leiden mutation by real-time PCR with TaqMan minor groove binder probes. Clin Chem 2004; 50: 787-788.

Nut van sequentieanalyse van de α1- en α2-globinegenen

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Inleiding: Hemoglobinopathiën zijn een heterogene groep erfelijke aandoeningen, veroorzaakt door mutaties die leiden tot een gereduceerde synthese van de α- of β-globineketens (thalassemiën) of de synthese van globineketens met een structurele verandering (Hb-varianten). α-Thalassemie wordt meestal veroorzaakt door deletie van één (m.n. de -α3.7- en -α4.2-deletie) of beide (m.n. de -α20.5-, --SEA en --MED deletie) α-globinegenen op chromosoom 16. Het ziektebeeld van α-thalassemie kan variëren van een milde microcytaire anemie tot hydrops foetalis, afhankelijk van het aantal ontbrekende α-globinegenen. Bij een aantal patiënten, waarbij de persisterende microcytaire anemie niet verklaard kon worden door een mutatie in het ß-globinegen en/of screening op de aanwezigheid van de bovengenoemde α-globinegendeleties waarmee 85 % van de α-thalassemiën te diagnosticeren zijn, is gekeken of sequentieanalyse van de α-globinegenen tot een diagnose kan leiden.

Methode: Genomisch DNA werd geïsoleerd uit EDTA-volbloed met behulp van Puregene (Gentra Systems). De α1- en α2-globinegenen werden met behulp van genspecifieke primers separaat geamplificeerd. Sequentieanalyse werd uitgevoerd met een ABI Prism 310 genetic analyzer (Applied Biosystems).
Resultaat: Tot nu toe zijn, op basis van de hierboven beschreven criteria, bij 6 personen de α-globinegenen gesequenced. Dit heeft bij 2 personen een mutatie van IVS-1 nt 116 in het α2-globinegen opgeleverd, passend bij een α+-thalassemie. Daarnaast zijn bij 2 personen, bekend met middels capillaire elektroforese geïdentificeerde Hb-varianten, puntmutaties in het α2-globinegen aangetroffen, leidend tot de aanwezigheid van respectievelijk Hb-Stanleyville-II en HbG-Philadelphia.
Conclusie: Sequentieanalyse van de α-globinegenen kan in voorkomende gevallen een verklaring geven voor de aanwezigheid van een persisterende microcytaire anemie of Hb-variant.

A newly identified deletion at the alpha-globin locus removing the promoter region of the alpha-1 gene

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Introduction: The most common causes of alpha-thalassemia are deletions that remove a part, one or both of the functional alpha-globin genes. These deletions cause diminished expression of the alpha-globin protein, which may result in relatively low Hb and/or MCV values. We here report the identification of a new deletion in the alpha-globin gene that affects the alpha-1 regulatory sequences.
Methods: A 25-year-old pregnant woman presented with low hemoglobin level (6.6 mmol/l) and slightly decreased MCV value (76 fl). Iron deficiency was excluded. HPLC analysis indicated the presence of normal HbA2 concentration (excluding ß-thalassemias) and no Hb variants. Genomic DNA was isolated from blood cells.
Results: Alpha-1 and alpha-2 genes were present and the MED, SEA, 20.5, 3.7 and 4.2 deletions were absent. However, the presence of an additional band of unexpected size in one of the PCR's suggested a genomic polymorphism. Sequencing of the PCR product revealed a 970 bp deletion (36599 until 37568; sequence GI:14523048), together with a 3 bp insertion. The deleted region encompasses the entire promoter region of the alpha-1 gene, including the "CAAT" and "ATA" box and 8 putative SP1 sites, and 26 bp encoding the 5' end of the mRNA. The affected alpha-1 globin gene is very unlikely to be expressed. Similar to the –alpha3.7 allele, just one alpha globin gene driven by the alpha-2 promoter can be transcribed and translated into protein.
Conclusion: We have identified an up to now unknown alpha-globin allele (alpha-alphaΔ970) characterized by a 970 bp deletion that, most likely, completely abolishes alpha-1-globin expression. In the patient, carrying the alpha-alpha/alpha-alphaΔ970 genotype, the deletion may well be responsible for the anemia and slightly lowered MCV-value.