Use of Single- and Multi-Locus and Polymerase Chain Reaction Systems for Zygosity Determination - Clinical Application in Twins with Clefts of the Lip and Palate

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Abstract. Precision of zygosity determination in twins can be improved by the use of modern methods of DNA analysis. The clinical application of 4 single- (SLS) and 2 multi-locus (MLS), and 6 PCR (polymerase chain reaction) systems for zygosity determination in 12 twin pairs with oral clefts was compared with regard to the quality and quantity of sample material required and the probability of error in monozygosity determination. PCR systems proved to be superior to SLS or MLS, as DNA sampling is much more convenient, while its level of accuracy still fulfills clinical requirements. For this reason, PCR systems should be considered a basic method in modern clinical twin research.

Key words: Cleft lip and palate, Zygosity determination, DNA fingerprint, Restriction fragment length polymorphism, Polymerase chain reaction.

INTRODUCTION

The accuracy of zygosity determination plays a dominant role in twin studies. Alongside placental monochorionicity, which is a problematic criterion for retrospective analysis, the determination of multiple antigens or HLA subtypes has been the preferred method of zygosity determination for many years [19]. However, large sample quantities were required, and the procedures were costly [11]. Monozygosity determined by the more readily available but unreliable methods used in many past clinical twin studies must therefore be regarded as at best "probably acceptable" and at worst "not acceptable" [15]. These deficiencies have been overcome by the use of modern methods of DNA...
analysis, e.g. single- (SLS) and multi-locus systems (MLS), the latter also being called "DNA-fingerprinting".

DNA-fingerprinting for the determination of twin zygosity at birth can be carried out with a small sample of chord blood [10, 15] and has proved to be reliable and authoritative [3, 4]. In a clinical study, we reported on a series of 13 twin pairs with clefts of the lip and palate out of our own collective of 1045 cleft patients between 1973 and 1991. EDTA-blood was collected in 10 pairs of the same sex, and 4 of these were concordant with respect to cleft, although the level of expression varied within the pairs. Monozygosity was determined in 5 pairs with a probability of error of less than $10^{-9}$ using 2 MLS and 4 SLS. 2 out of 5 pairs were concordant in both the MZ and dizygotic (DZ) group [8].

After completion of this study, another 2 twin pairs of the same sex were evaluated using 6 PCR (polymerase chain reaction) systems for zygosity determination, as first described a few years ago [1]. Our paper aims to investigate this modern method of DNA-analysis for clinical twin studies and to compare it to DNA-fingerprinting.

**MATERIALS AND METHODS**

Both sets of twins, a pair of 6-month-old boys and a pair of 12-year-old girls, were discordant for bilateral cleft lip and palate and left-sided cleft lip, respectively. EDTA-blood was collected from both girls and from the affected male twin during cleft surgery. In the unaffected twin brother, a cotton-pad swab from the oral mucosa was taken.

The samples were kept at 4°C until further preparation, according to routine methods described elsewhere [2, 6, 16, 17, 20]. The amplification was carried out with DNA samples of 1 ng with 1 U Taq polymerase (Promega, Heidelberg, Germany), 0.3 μM of the primer for each system (Table) and 100 μM of each nucleotide in a thermocycler (Triothermoblock, Biometra Göttingen, Germany). Electrophoretical separation of the amplified fragments was carried out in polyacrylamide gels. Bands were visualized using a silver staining method. Alleles determination in each polymorphism was carried out by a side-to-side comparison with an allele cocktail, which contained most known alleles of the polymorphism. The family history was evaluated in both pairs.

<table>
<thead>
<tr>
<th>PCR system</th>
<th>chromosome location</th>
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<tr>
<td>MCT118</td>
<td>D1S80 (chromosome 1)</td>
</tr>
<tr>
<td>YNZ22</td>
<td>D17S30 (chromosome 17)</td>
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<tr>
<td>ApoB</td>
<td>2p24-p23</td>
</tr>
<tr>
<td>TC11</td>
<td>11p15-15.5</td>
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<tr>
<td>VWA</td>
<td>12p12-pter</td>
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<tr>
<td>SE33</td>
<td>ACTBP2 (chromosome 6)</td>
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Fig. 1. Family pedigree and PCR bands (SE 33) of the female twins. Squares symbolize male, circles female family members - black symbols indicate a patient affected by a cleft. The electrophoretic lanes are inserted into the pedigrees, and comparison between the 2 central lanes of the twin pair and the 2 marginal lanes of the allele cocktail is possible. The starting point of electrophoresis is at the bottom of the figure. The PCR bands show identity (scale 1:2). A cousin (III8) of the twins and a son of another cousin (IV2) were reported to have cleft lip and palate.

Fig. 2. Family pedigree and PCR bands (YNZ 22) of the male twins. The bands are identical.
RESULTS

Monozygosity was demonstrated for both pairs, with identical bands in all PCR systems evaluated (Figs. 1 and 2). The family history was positive for cleft lip and palate in the female pair (Fig. 1).

DISCUSSION AND CONCLUSIONS

DNA-fingerprinting is based on many dispersed tandem-repetitive and non-coding DNA-sequences, was first described in the mid-1980s [13], and subsequently was used widely in forensic medicine [4, 5, 9, 13]. Its individual specificity is the result of allelic variation in the repeat copy number of these sequences. After DNA-digestion with restriction endonucleases and electrophoretic separation of single-stranded fragments, the repetitive sequences are hybridized with labelled DNA probes. The use of MLS results in a complex pattern of 15-50 bands, and is called a genetic fingerprint, because the probes hybridise with a varying number of repetitive sequences spread over the genome with unknown locations. Although this pattern is highly individual and specific, the lack of defined locations on the genome leads to deficiencies in defining a formal biostatistical genetic model. For forensic applications, autosomal, co-dominant, polymorphic systems which can be tested for deviation from Hardy-Weinberg-equilibrium and for linkage of loci are required. Failure to fulfil these requirements, and low re-test reliability due to the diversity of probe combinations used in the different laboratories, have led to MLS not being recommended for use in forensic case-work, with the exception of paternity-testing [5, 7].

The use of SLS, where the probes hybridise with repetitive sequences on single defined loci, yields 2 bands per sample and system if the individual is a heterozygote. Therefore the use of several SLS in combination is required for a statistically significant evaluation [4, 18]. However, although some of the deficiencies mentioned above for MLS are excluded, the diversity of probe combinations used in each laboratory, relatively high mutation rates which differ from locus to locus, and the continuous allele distribution with resulting measurement of error, remain unsolved problems. Therefore the use of SLS in forensic medicine is also now restricted [5].

Although this criticism of the method is of no relevance for the use of MLS and SLS in zygosity determination (with an extremely low probability of error of less than $10^{-9}$ using 2 MLS and 4 SLS, for example), the clinician must be prepared to cooperate with an institute where a suitable method is available.

Alongside the abandonment of MLS and SLS for forensic testing, the use of PCR has increased. PCR systems are based on the detection and pairing of DNA-sequences at defined loci by special signal-sequences, called primers. The marked sequences are amplified by a heat-resistant enzyme (polymerase), thus multiplying the material for further separation and visualization [6, 16, 17, 20]. Short tandem repeats (STR) with 3-6 base pairs (bp) and amplifiable fragment length polymorphisms (AmpFLP) with 7-80 bp are differentiated according to their repeat lengths, the latter including the PCR systems MCT 118, YNZ22 and ApoB used in this study. These systems are still an object of ongoing research, but they seem to fulfil the forensic requirements mentioned above
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[17, 20]. Furthermore, due to amplification and the short length of repeat sequences, only very small samples are required, which may also be degraded [20] e.g. by bacterial nucleases. Therefore a cotton-pad swab from the oral mucosa delivers sufficient sample material in contrast to at least 0.5-1.0 ml EDTA-blood for genetic fingerprinting [10]: MLS and SLS require 500 ng DNA sample material but for PCR systems only 0.1 ng are needed [5].

However, statistical evidence is limited by the fact that only 2 alleles resulting in 2 bands are found in a heterozygous individual. The number of alleles described for the different systems varies from 6 (TC11) to 26 (SE33) with some alleles showing a higher frequency. In consequence, the probability that siblings or DZ twins inherit identical alleles is at least 0.25 for each PCR system and 0.25^6 (1/4096) for 6 systems, and this is decreased by the probability of a homozygous parent or parents showing identical alleles. However, the statistical evidence of 4 systems is equivalent to that of approximately 20 conventional blood group systems [17].

This comparison demonstrates the important advantages of the PCR systems in relation to conventional methods or MLS and SLS: the reduction of both quality and quantity of the sample material goes hand in hand with a reduction of costs compared to conventional methods over MLS/SLS to PCR systems considering the required statistical evidence of 99.9% for all methods. In clinical twin studies or genetic counselling of twins of the same sex with discordance for an inheritable disease or malformation, PCR systems should be the first choice of method for zygosity determination, where a mucosal swab is a practicable method of obtaining samples, even in the newborn.

In our own series of 10 twin pairs, we demonstrated the use of MLS (genetic fingerprinting) and SLS for zygosity determination [8], but and after completion of that primary study we have followed the developments in forensic medicine by investigating another 2 pairs with PCR systems.

Acknowledgments: We express our thanks to Prof. Dr Dr U. Joos and Prof. Dr Dr A. Hemprich, University Hospital of Oral & Maxillofacial Surgery, Münster, for referring the female twins for evaluation.

REFERENCES


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