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Adaptation of PCR technique for quantitative estimation of genetic material from different regions of chromosome 21 in cases of trisomy 21✪

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Pre- and postnatal diagnosis of chromosomal aberrations is generally based on conventional cytogenetic analysis. In this paper, we have devised a quantitative polymerase chain reaction (Q-PCR) method to determine gene dose effects and applied it in cases of regular trisomy 21 as a model. The method is based on quantitative assessment of PCR products after using primers amplifying DNA fragments located in the pericentromeric, heterochromatic, euchromatic and telomeric regions of chromosome 21. A gene dose effect on the amount of PCR product in cases of trisomy 21 was confirmed. Moreover, a correlation between the amount of the PCR product of the examined sequences and their location in the chromosome was observed. The obtained results suggest that the Q-PCR technique can be applied in the diagnosis of aneuploidies.

Chromosomal abnormalities are the most frequent ethiopatological event observed in both miscarriages and live births. The majority of chromosomal aberrations are aneuploidies involving chromosomes 21, 18, 13, X and Y (Smith *et al.*, 1999). Up to now, the diagnosis of chromosomal aberrations has been generally based on conventional cytogenetic analysis, which can be performed on dividing cells only. Therefore, these techniques require time-consuming cell culturing. Because of that a rapid and sensitive method

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Abbreviations: Q-PCR, quantitative polymerase chain reaction.

for detection of chromosomal aneuploidy is needed.

Several different approaches of the quantitative polymerase chain reaction (Q-PCR) analysis of chromosomal aberrations have been reported recently (Von Eggeling *et al.*, 1993; Celi *et al.*, 1994; Pertl *et al.*, 1996; Adinolfi *et al.*, 1997; Lee *et al.*, 1997; Mann *et al.*, 2001; Bili *et al.*, 2002; Pont-Kingdon & Lyon, 2003). This technique proved to be especially applicable in diagnosis of the most common aneuploidies of chromosomes 21, 18, 13, X and Y and other unbalanced aberrations such as deletions and duplications. The quantitative PCR methodology offers high sensitivity and significantly early diagnosis. The costs of routine PCR based diagnostics are much lower when compared with the cytogenetic techniques.

The aim of the present work is the development of faster and cheaper methods of chromosomal diagnosis of aneuploidy. We have devised a quantitative PCR (Q-PCR) to determine gene dose effect and applied it also in cases of regular trisomy 21 as a model. The test is based on amplification of a target sequence from chromosome 21 and a target sequence that lies on chromosome 11 or 6 as an internal control. Another aim of the study was to find out whether DNA sequences from different regions of the chromosome are equally suitable for this purpose.

To do so, we carried out quantitative assessment of PCR products after using primers amplifying DNA fragments located in the pericentromeric, heterochromatic, euchromatic and telomeric regions of chromosome 21, D21S16, *APP* (*amyloid precursor protein*), *SOD1* (*Cu/Zn superoxide dismutase*) and *S100,* respectively (Fig. 1).

MATERIALS AND METHODS

Subjects. Fifty Down syndrome patients and 90 normal individuals as controls were examined. Their karyotypes had previously been determined by high resolution trypsin-Giemsa staining.

DNA purification. DNA samples were obtained from amniocytes, skin fibroblasts and peripheral blood lymphocytes of Down syndrome patients and control individuals. Genomic DNA from skin fibroblasts and peripheral blood lymphocytes was isolated essentially as described by Miller *et al.* (1988). DNA was extracted from amniocytes by Erlich's method (1989).

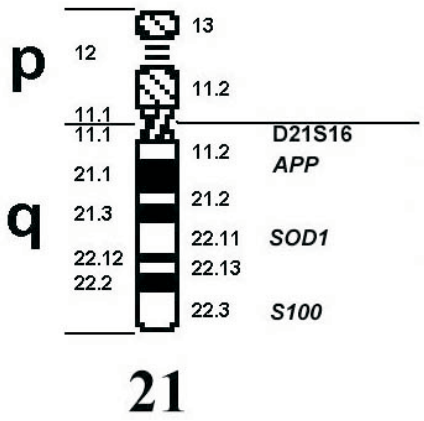

Figure 1. Ideogram of the human chromosome 21.

PCR amplification. PCR amplification was carried out in four separate assays. PCR was performed in a total volume of 50 μl containing genomic DNA, 100 μM dNTPs, 5 μCi [^{32}P]dCTP (Amersham), 40 pmol of each primer, 1 × *Taq* polymerase buffer (50 mM Tris/HCl, pH 8.9, 50 mM KCl, 2 mM $MgCl_2$) and 2U *Taq* polymerase. The primers used for loci on chromosome 21 and for a locus on chromosome 11 (*INS*-insulin) or chromosome 6 and the technical conditions are given in Table 1. PCR was performed in a UNO II thermal cycler (Biometra).

Electrophoresis and quantitative analysis of PCR products. After PCR amplification, 20 μl PCR product was electrophoresed through 8% polyacrylamide gel, stained with ethidium bromide and analyzed by densitometry using Multiscan system and radio-labelling method after cutting bands from the gel (Beckman Scintillator). The ratio of the amount of the analysed sequence to the inter-

nal control was calculated. The results of the Q-PCR analysis were compared with the control group. Statistical analysis was performed using Student's *t*-test ($P \leq 0.05$).

RESULTS

50 regular trisomy 21 cases were examined, an increased amount of PCR products (D21S16, *APP, SOD1* and *S100* fragments) was observed in comparison to the control group, as shown in Table 2 and Fig. 2. The difference between these two groups is significant ($P \leq 0.05$). Radio-labelling analysis showed an increase of the amount of PCR products of D21S16, *APP, SOD1* and *S100* of about 26%, 39%, 40% and 25%, respectively. Densitometry analysis confirmed the increase of the quantity of PCR products of D21S16, *APP, SOD1* and *S100* in comparison to the control group (25%, 38%, 37% and 25%, respectively). Moreover, a correlation between the amount of the PCR product of the examined sequences and their location in the chromosome was observed. The increase of the quantity of PCR products of the pericentromeric (D21S16) and telomeric

Table 1. Primer sets used in quantitative PCR and conditions of amplification

Lp.	Sequence	Sequence of primers	Chromosome location	Annealing temperature (°C)	Internal control	Size of product PCR (bp)	Reference
1	D21S16	5′-cca taa aca caa tct tca agg cc-3′ 5′-ccc aag gac aat agt aca cat tct c-3′	21q11.1	56	D6S1835	190	Genome database
2	*APP*	5′-gga gtt gtc atc ctt tga tgc t-3′ 5′-agg cga cat tcc tcc agt ctt aca a-3′	21q11.2-21.05	55	*INS*	334	Genome database
3	*SOD1*	5′-cag ctg ttt tct ttg ttc ag-3′ 5′-cac ctg ctg tat tat ctc caa-3′	21q22.1	56	*INS*	168	Pramatarova *et al.*, 1995
4	*S100*	5′-gct ggg ccc tcc tgc tga acg-3′ 5′-tgc tct gtg ccc ctt cct cgc-3′	21q22.3	56	D6S1835	216	Von Eggeling *et al.*, 1993
5	*INS*	5′-tgc ctg tct ccc aga tca ct-3′ 5′-ggg tct tgg gtg tgt aga aga a-3′	11q	55/56	–	198	Genome database
6	D6S1835	5′-aac ttt gaa gtc tga ttt tcc tgg-3′ 5′-atg cca cac atc ctc ttt acg-3′	6q22-23	56	–	141	Genome database

Table 2. Quantitative analysis of PCR products in trisomy 21 in comparison to the control group as 100%

Lp.	Sequence	Densitometry analysis (%)	Radio-labelling analysis (%)
1	D21S16	125.4 ± 3.95	126.4 ± 2.848
2	*APP*	138.7 ± 7.03	138.6 ± 5.29
3	*SOD1*	137.9 ± 6.19	140.1 ± 5.56
4	*S100*	125.2 ± 3.83	125.1 ± 4.66

Trisomy 21, 50 cases; Control group, 90 cases

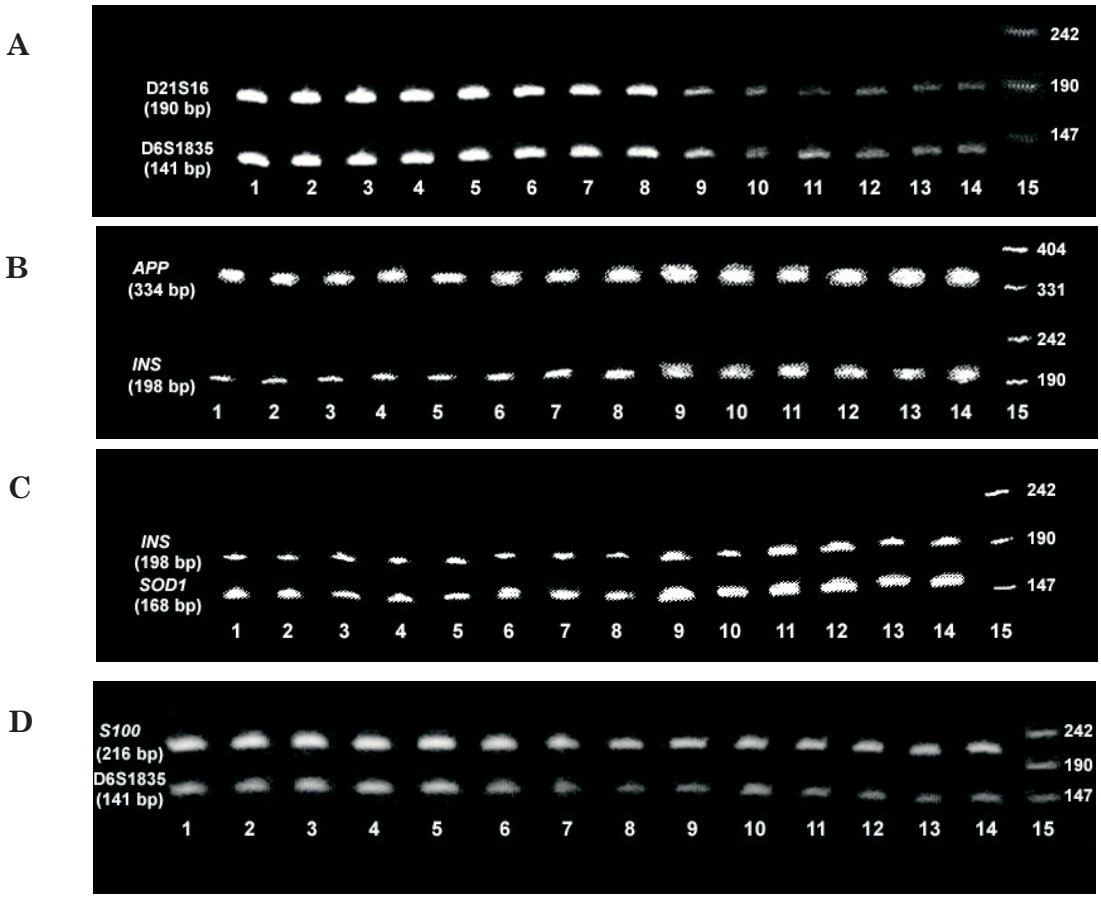

Figure 2. Polyacrylamide gel electrophoresis of PCR products.

A. D21S16 (190 bp) and D6S1835 (141 bp); **B**. APP (334 bp) and INS (198 bp); **C**. SOD1 (168 bp) and INS (198 bp); **D**. S100 (216 bp) and D6S1835 (141 bp). Lanes 1–7 are patients with trisomy 21; lanes 8–14 are the control group; lane 15 represents molecular size standard (pUC19/MspI). Densitometric analysis shows increase quantity of PCR products as compared with the control group.

(*S100*) regions was smaller than for the euchromatic (*SOD1*) and the heterochromatic (*APP)* regions. The difference between these two groups of the regions of chromosome 21 is significant**.**

DISCUSSION

Reports from the recent years confirm the usefulness of the quantitative PCR technique for the analysis of chromosomal aberrations (del Rio *et al.*, 1998; Pertl *et al.*, 1999; Blake *et al.*, 1999; Chen *et al.*, 2000a; 2000b). In this study, a variant of the Q-PCR technique which consists amplification of the studied DNA fragment together with DNA fragment from another site of the same matrix (internal control) on which the studied fragment is multiplied, was applied. This method has certain technical requirements (Raeymaekers, 1998). The two primer pairs used in one reaction should have similar lengths and melting temperatures (t_m). However, the products of the two reactions (the studied and the reference sequence) should have different lengths in order to detect them easily.

The competition resulting from coamplification of the studied and the reference sequence is the main difficulty using internal standards (Mallet, 1999). The differences in the composition of the two sequences may also influence amplification efficiency. The higher the homology of these two sequences

the greater is the chance that both templates will amplify with the same efficiency in various conditions, including the saturation phase of PCR (Raeymaekers, 1998).

In the presented method, the quantitation of the analysed sequence is based on a comparison of its amount after amplification with the amount of the control sequence by means of direct densitometry of the gel as well as by scintillation measurement of radioactively labelled products of PCR.

The usefulness of the presented method was confirmed by performing it for cases of regular trisomy of chromosome 21. The method is a valuable tool allowing quantitative evaluation of genetic material in regions involved in chromosomal aberration. The obtained results indicated the effect of dose for the D21S16, *APP, SOD1*, and *S100* genes in trisomy 21 as well as a correlation between the amount of the PCR product of the studied sequences and their localisation in the chromosome. As it was observed, the amount of the PCR product in the case of sequences from eu- and heterochromatic regions is larger in comparison to the sequences from the pericentromeric and telomeric regions. This could be due to the differences in the structure of the particular region of the chromosome. According to our results sequences from euchromatic and heterochromatic regions are the most effective in gene dose estimation by the Q-PCR method.

The increase in the amount of the PCR product was lower than the theoretically expected 50% for all of the analysed sequences. This could have resulted from the fact that various physical and chemical factors may influence the efficiency of DNA amplification *in vitro* and disturb its quantitative nature. Even small differences in the kinetics and efficiency of particular amplification steps may significantly influence the amount of product accumulated after a certain number of cycles (Reischl & Kochanowski 1999).

The homologous gene quantitative polymerase chain reaction method (HGQ-PCR) to detect regular and translocation trisomy 21 could solve this problem (Lee *et al.*, 1997). It is based on the amplification with one pair of primers of two highly homologous genes: *PFKL-CH21* (human liver-type phosphofructokinase) and *PFKM-CH1* (human muscle-type phosphofructokinase) localised on chromosome 21 and 1, respectively. The estimation of the copy number is carried out by comparison of the PCR products of the analysed and control sequences after direct densitometry of the gel. Still, also in this case, the authors indicate that DNA quality may affect the amplification of homologous genes, thus leading to an increased the ratio of these sequences, especially in the control group. An alternative solution is quantitative fluorescent PCR (QF-PCR) using small tandem repeats (STRs) although it needs an automated DNA sequencer (Adinolfi *et al.*, 1997; Pertl & Adinolfi, 1998; Pertl *et al.*, 1999; Tóth *et al.*, 1998a; 1998b; Verma *et al.*, 1998; Cirigliano *et al.*, 1999; 2001; Samura *et al.*, 2001).

The results of this work confirmed the usefulness of the Q-PCR technique as a valuable tool for quantitative assessment of genetic material in regions involved in chromosomal aberrations. This method is applicable to numerical as well as unbalanced structural aberrations. The Q-PCR technique may be advantageous in the diagnosis of aneuploidies as a screening test. The most important features of this technique are the small amount of DNA needed for the testing and the availability of results in a short time. The cost of this methodology in a routine laboratory is much lower than that of conventional cytogenetic technique. However, the quantitative PCR technique can wholy not replace the cytogenetical analysis.

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