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Fetal aneuploidy diagnosed at celocentesis for early prenatal diagnosis of congenital hemoglobinopathies

Running title: Early Prenatal diagnosis by celocentesis

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Conflict of Interest statement

There is no conflict of interest in this paper.

Abstract

Introduction: Currently, prenatal diagnosis of genetic disorders requires chorionic villus sampling or amniocentesis carried out after 11 and 16 weeks of gestation, respectively. Celocentesis is a procedure for prenatal diagnosis that could be used from as early as seven weeks. The present investigation evaluated the feasibility of performing diagnosis for monogenic diseases using celomic fluid containing cells of fetal origin. *Material and methods:* Analysis consisted of 489 singleton pregnancies undergoing celocentesis for the prenatal diagnosis of hemoglobinopathies (n=367) or before surgical termination of pregnancy for social indications (n=122). Embryo-fetal cells were isolated from celomic fluid using CD71 antibodies or by micromanipulation. Quantitative fluorescent polymerase chain reaction of short tandem repeat sequences of chromosomes 13, 18, 21, X and Y were used to determine the presence of maternal DNA. *Results*: 357/489 (73%) of celomic fluid samples were contaminated with maternal cells. In two cases, diagnosis was not possible due to the high contamination of celomic fluid Eighty-seven (23.8%) fetuses were affected by hemoglobinopathies, and in five cases, chromosomal aneuploidies were found, including three cases of trisomy 21, one of trisomy 13 and one of triploidy. In all cases, the diagnosis of hemoglobinopathies and chromosomal abnormalities were confirmed by molecular and traditional cytogenetic analysis after amniocentesis, chorionic villus or placental tissue collection following pregnancy termination. *Conclusions*: The findings of this study demonstrate that embryo-fetal cell selection from celomic fluid allows reliable and early prenatal diagnosis of hemoglobinopathies and can give more information on eventual fetal aneuploidy following the control of maternal contamination by quantitative fluorescent-PCR.

Keywords

chromosomal abnormalities; coelocentesis; first trimester; prenatal diagnosis; aneuploidy; thalassemia, celomic cavity.

Abbreviations

CF: celomic fluid

CVS: chorionic villus sampling

QF-PCR: Quantitative fluorescence polymerase chain reaction

STR: short tandem repeat

Key Message

Celocentesis is a procedure for invasive prenatal diagnosis that can be performed as early as the seventh week of gestation. Fetal cells contained in the celomic fluid may be used to identify single gene disorders or chromosome aneuploidies.

Introduction

Prenatal diagnosis of monogenic diseases and fetal chromosomal abnormalities relies on obtaining fetal tissue by chorionic villus sampling (CVS) or amniocentesis performed after 11 and 16 weeks of gestation, respectively. In recent years, some investigators have attempted to make prenatal genetic diagnosis at a very early stage of pregnancy (7-9 weeks) using celomic fluid (CF) extracted from the celomic cavity (1-2).

The celomic or extraembryonic cavity develops during the fourth week of gestation within the extraembryonic mesoderm (3-4). During the first ten weeks of gestation, the celomic cavity is the largest space inside the gestational sac, surrounding both fetus and amniotic cavity, reaching maximum volume at 7-9 weeks, and then subsequently disappearing at approximately 12 weeks. The exocelomic cavity can be clearly identified by ultrasound, and CF can be selectively aspirated from the 7^{th} week of gestation with a success rate of>95% (5-6). Celocentesis involves the transvaginal insertion of a needle within the celomic cavity and aspiration of 1 mL of fluid containing cells of fetal origin to use for prenatal diagnosis (videoclip is available as Supporting Information S1). Initially, the majority of studies previously published on celocentesis focused on the biochemical composition of celomic fluid (7-9). Successively, different studies reported on the prospective clinical feasibility of the safety of this procedure for earlier prenatal diagnosis of sickle cell disease, Marfan syndrome, or β-thalassemia and for fetal sex or paternity tests (10-18). In most of these reports, a large volume of CF, approximately 10-20 mL, was sampled, and this is critical for the safety of the pregnancy.

Differences in success rates (35-90%) obtained by celocentesis reported by several authors highlight the discordant opinions on the use of CF.

Jouannic et al. highlighted some limitations of the CF analysis, demonstrating that the amount of DNA varies considerably among samples and, frequently, is very low. Furthermore, many samples showed a variable grade of maternal contamination, which could compromise the reliability of the diagnosis (19-20).

In recent years, we reported maternal DNA contamination of celomic fluid to occur at highly variable rates (5-95%) that become more significant as sample size decreases to less than 1 mL for clinical diagnostics (21). Furthermore, a laboratory workflow for the performance of prenatal diagnosis of hemoglobinopathies with a high rate of success before the ninth week of gestation was reported (22). One of these steps needed to assess the maternal DNA contamination of the sample to avoid misdiagnosis. A simple and rapid method to detect maternal DNA contamination is quantitative fluorescent polymerase chain reaction (QF-PCR) using a set of fluorescent primers that allows the amplification of specific highly polymorphic short tandem repeat (STR) sequences of chromosomes (up to 15–20 alleles) (23, 24). This methodology has been used successfully for prenatal diagnosis of aneuploidies of chromosomes 13, 18, 21, X and Y, but it can also provide information on maternal contamination of fetal samples (25). The aim of our study was to perform and evaluate the feasibility of prenatal diagnosis of hemoglobinopathies.

Material and methods

The study population consisted of 489 singleton pregnancies undergoing celocentesis either for the prenatal diagnosis of hemoglobinopathies (n=367) or before surgical termination of pregnancy for social indications (n=122). In cases of prenatal diagnosis for hemoglobinopathies, the advice from the ethical committee was that if the results from celocentesis suggested that the fetus was not affected, the diagnosis should be confirmed by CVS at 12 weeks of gestation or amniocentesis at 16 weeks.

Celocentesis was carried out at between 6^{+6} and 9^{+2} weeks. Sampling involved transvaginal sonography and insertion of a 20-G needle through the fornix into the celomic cavity (26). In women undergoing pregnancy termination, the procedure was performed under general anesthesia and 2-4 mL of CF was aspirated; a sample of placental tissue and maternal blood were obtained after the surgical evacuation of the products of conception. In patients undergoing prenatal diagnosis for hemoglobinopathies, no anesthesia was used, and a total of 0.8-1.2 mL of CF was aspirated into three different syringes. Contamination was higher in the first two samples, and although all samples were analysed, the third was considered to be the diagnostic sample. Blood samples were also obtained from both parents. In all cases where the fetus was affected by thalassemia and the parents chose to

have a pregnancy termination, placental tissue obtained at the time of the termination was analysed. In the cases where the fetus was not affected by thalassemia, the diagnosis was confirmed by CVS or amniocentesis, at the request of the Ethical Committee. Molecular analysis and karyotyping were performed via chorionic villus sampling or amniotic fluid in all cases.

Celomic fluid (50 μL) was placed in a 0.5 mL Eppendorf tube, and after centrifugation at 12000 rpm for 10 min, the cell pellet was dissolved in 30 μL of 5% IstaGene Matrix (Bio-Rad Laboratories, Hercules, CA, USA). The sample was incubated at 56 °C for 30 min and subsequently at 95 °C for 10 min, and the supernatant (25 μL) containing the DNA was then removed and transferred into a clean 0.5 mL Eppendorf tube. First, maternal contamination analysis was performed with two multiplex PCR reactions using the QST*R kit containing 16 STR markers for chromosomes 13, 18, 21 and the QST*R v2 kit containing STR for X and Y chromosomes (Elucigen Diagnostics, Manchester, UK) (Supporting Information Table S2). Four different fluorescent dyes (PET, VIC, NED, 6-FAM) were used to label one primer to obtain fluorescent PCR amplicons. Master mix (10 μL) was mixed with 2.5 μL (10-20 ng) of DNA and used for amplification according to the manufacturer's instructions. Fifteen minutes of denaturation at 95 °C were followed by 26 cycles of 95 °C 30'', 59 °C 90'', 72 °C 90'' and a final extension at 72 °C for 30'. PCR products (2 μL) were mixed with 15 μL of HiDi and 0.5 μL of LIZ 500 (Applied Biosystems, Foster City, CA, USA) as an internal size standard. Capillary electrophoresis was performed by an automated ABI Prism 3130 Genetic Analyser, and data analysis was completed with Gene Mapper 4.0 software (Applied Biosystems, Foster City, CA, USA). Genomic DNA was extracted from parental blood and placental tissue using standard protocols (27).

Evaluation of contamination and fetal cell isolation

Celomic fluid samples with no or very low (<5%) maternal contamination were successfully analysed without preliminary treatment. Samples containing more than 5% maternal contamination were purified using two different procedures to isolate embryo-fetal cells, by the positive selection of embryo-fetal erythroid precursors using anti-CD71 MicroBeads (Milteny Biotec, Bergisch Gladbach, Germany) or by picking embryo-fetal cells with a micromanipulator on the basis of their morphology (28).

ß-globin gene analysis (MIM 141900)

After cell selection, maternal cell contamination was re-tested by QF-PCR to be sure that all selected cells were of fetal origin. Samples were analysed for the ß-globin gene (HBB, MIM #141900) by PCR and direct sequencing (22).

Ethical approval

Written informed consent was obtained from all women who participated in the study, which was approved by the Institutional Review Board (Hospital Ethical Committee authorization number 80, 26 January 2005).

Results

Interpretation of QF-PCR electropherograms

Normal chromosome pattern without maternal contamination was characterized by two different alleles, one of paternal origin and one of maternal origin, with a ratio between fluorescent peak areas of 1 to 1 (range 0.8–1.3). In the presence of maternal contamination, the characteristic allele pattern for all chromosomes consisted of skewed bi-allelic or tri-allelic peaks. Trisomy was detected by either a three-peak area ratio close to 1:1:1 or two unbalanced bi-allelic patterns with skewed area ratios of 2:1 or 1:2 (range 1.7–2.4) for at least two different STRs on the same chromosome with a normal Mendelian pattern, while other chromosomes appeared normal. Alleles were considered non-informative when there was a single fetal homozygous allele or two different heterozygous fetal alleles identical to the maternal genotype.

In 33% (161/489) of the CF samples, low or absent maternal contamination (<5%) was observed, but in 67% (328/489), the maternal contamination was higher, from 5-95%. In two (0.5%) of the 367 cases, diagnosis was not possible from CF because the samples were highly contaminated. These celocenteses were performed before the introduction of the micromanipulation procedure.

In 87 (23.8%) of the 365 cases studied for hemoglobinopathies, the foetuses were affected by β-thalassemia major or sickle cell/β-thalassemia. Only three women chose to continue the pregnancy despite the documented presence of an affected fetus. Diagnosis of these affected fetuses were confirmed after birth, while in the other cases (84 pregnancies), in which women chose to terminate the pregnancy, the prenatal diagnosis by celocentesis and karyotyping was confirmed by the molecular analysis of placental tissue. The results obtained via celocentesis in the unaffected fetuses (278) were confirmed in all cases without false negative or positive data by CVS or amniocentesis or after birth (Table 1).

Analysis by QF-PCR of the 489 samples of celomic fluid demonstrated no aneuploidy for chromosomes 13, 18, 21, X or Y in 484 cases and aneuploidy in five, including three cases of trisomy 21, one of trisomy 13 and one of triploidy. The electropherograms for the five cases with chromosomal abnormalities are shown in Figures 1-5.

The first case was a 31-year-old woman. Celocentesis was carried out at 8^{+2} weeks of gestation for the prenatal diagnosis of thalassemia. Analysis of CF revealed approximately 60% maternal cell contamination, and after purification using anti-CD71 MicroBeads, QF-PCR demonstrated normal bi-allelic balanced patterns for chromosomes 13 and 18, but for chromosome 21, there was a bi-allelic pattern with a ratio of 2:1 for markers D21S1435 and D21S11 and two tri-allelic peaks in a 1:1:1 ratio for markers D21S1409 and D21S1437. Trisomy 21 was confirmed by traditional karyotyping and QF-PCR of chorionic villi obtained by CVS and subsequent placental tissue after termination of the pregnancy (Figure 1).

The second case was a 33-year-old woman. Celocentesis was carried out at 8^{+1} weeks of gestation for the prenatal diagnosis of thalassemia. Analysis of CF revealed no maternal cell contamination; QF-PCR demonstrated normal bi-allelic balanced patterns for chromosomes 13 and 18, but for chromosome 21, there was a bi-allelic pattern with a ratio of 2:1 for markers D21S1435, D21S1409, D21S11, D21S1411 and D21S1437. Trisomy 21 was confirmed by traditional karyotyping and QF-PCR of chorionic villi obtained by CVS and subsequent placental tissue after the termination of pregnancy (Figure 2).

The third case was a 38-year-old woman. Celocentesis was carried out at 8^{+2} weeks of gestation prior to pregnancy termination for psychosocial reasons. Analysis of CF revealed approximately 50% maternal cell contamination, and after purification using anti-CD71 MicroBeads, QF-PCR demonstrated normal bi-allelic balanced patterns for chromosomes 13 and 18, but for chromosome 21 (markers D21S1409, D21S11, D21S1411 and D21S1437) there were four skewed 2:1 or 1:2 peak areas of maternal to paternal contribution, indicative of maternally derived trisomy 21.Trisomy 21 was confirmed by traditional karyotyping and QF-PCR of placental tissue after the termination of pregnancy (Figure 3).

The fourth case was a 32-year-old woman. Celocentesis was carried out at 7^{+5} weeks of gestation for the prenatal diagnosis of sickle cell disease. Analysis of CF revealed no maternal cell contamination; QF-PCR demonstrated normal bi-allelic balanced patterns for chromosomes 21 and 18, but for chromosome 13, there was a bi-allelic pattern with a ratio of 2:1 for markers D13S252 and D13S634 and tri-allelic peaks in a 1:1:1 ratio for markers D13S305 and D13S628. At 11 weeks of gestation before planned CVS, ultrasound examination showed no fetal heartbeat, and after the surgical evacuation of remaining products of conception, QF-PCR of the placental tissue demonstrated trisomy 13 (Figure 4).

The fifth case was a 40-year-old woman. Celocentesis was carried out at 8^{+3} weeks of gestation for the prenatal diagnosis of thalassemia. Analysis of CF revealed no maternal cell contamination; QF-PCR demonstrated a tri-allelic pattern for D13S325 and a bi-allelic pattern with a ratio of 2:1 for paternal to maternal DNA contribution (markers D18S978, D21S1409, D21S11,

D18S391 and D21S1437). The presence of two amelogenin peaks (AMEL-XY), one from the X chromosome and one from the Y chromosome, with a skewed allele peak 2:1 ratio of paternal to maternal DNA contribution and the presence of SRY and DXYS218 markers were compatible with a male pregnancy. However, the bi-allelic pattern of DXS6803, DXS1187 and DXS981 was compatible with a female pregnancy. The combination of all these findings was suggestive of paternally derived triploidy. At 10 weeks of gestation, spontaneous abortion occurred, and triploidy was confirmed by traditional karyotyping and QF-PCR on placental tissue (Figure 5).

Analysis by traditional karyotyping of amniotic fluid confirmed the absence of chromosomal anomalies in the other fetalcelomic fluid obtained by celocentesis (Table 2).

The total fetal loss following celocentesis was 2.8% (8 of 283 pregnancies, including 2 fetuses with aneuploidies (manuscript in preparation) (Table 1).

Discussion

This study, assessing a large number of pregnancies by celocentesis, has highlighted that using specific procedures for the analysis of CF samples can allow for accurate prenatal diagnosis during the embryonic stage of intrauterine life. The crucial point was represented by the discovery and selection of embryo-fetal cells contained in the CF. We have previously demonstrated that embryo-fetal cells contained in celomic fluid can be selectively obtained using accurate methodologies (22, 28). The main difficulty in prenatal diagnosis to analyse CF was maternal contamination, whereby the use of microsatellite markers in QF-PCR to determine sequence copy number provides a genotype of the tested sample, minimizing possible misdiagnosis. Our objective was to evaluate the diagnostic feasibility for hemoglobinopathies and the reliability of the results obtained. This objective has been achieved by controlling the maternal cell contamination by QF-PCR and using a micromanipulator to pick up even a few morphologically recognizable fetal cells. We showed that diagnostic feasibility was very high (> 99%) with the introduction of the isolation of fetal cells by a micromanipulator. The reliability of celocentesis in this clinical diagnostic trial was confirmed with subsequent analysis of fetal tissue (amniotic fluid, chorionic villus or placental tissue), and no false positive or negative diagnosis were observed. We carried out this procedure in a large number of continuing pregnancies, and the risk of miscarriage was low, similar to villocentesis (29) and less than the spontaneous miscarriage rate within the first 12 weeks of pregnancy (30, 31). However, it is necessary to extend the casuistry to assess the real risk of abortion associated with the procedure. QF-PCR analysis was performed initially to evaluate maternal contamination using several markers for chromosomes 13, 18, 21, X and Y. Accidentally, this test highlighted the presence of 5 cases of chromosomal aneuploidy, subsequently confirmed on fetal tissues. The incidence of aneuploidies reported in this

study may look very high (1,0%). This could be associated with the increased maternal age and early gestational age (7 weeks) in our study. Indeed, the incidence of chromosomal abnormalities is much higher at 7 weeks than at term due to the high intrauterine demise/miscarriage of fetuses with chromosomal abnormalities.

This is the most extensive clinical study published to date in terms of the number of samples analysed, success of PCR assays, rate of suitable non-contaminated samples obtained and accuracy of the data obtained at the same time. Although standard karyotyping remains undoubtedly more complete in that it allows the evaluation of all chromosomes and the identification of numerical as well as structural alterations, QF-PCR analysis of chromosomes X, Y, 21, 18, and 13 has the advantage of allowing the quick diagnosis of a group of aneuploidies accounting for the vast majority of clinically relevant chromosomal disorders. Due to low number of embryonic erythroid precursors nucleated red blood cells (megaloblasts) obtained from CF and the difficulty in culturing them to obtain metaphase for conventional chromosomal analysis, the traditional cytogenetic test is not possible. In our opinion, the use of QF-PCR remains as a control for maternal contamination, allowing for the collection of some data on aneuploidy of the examined chromosomes. We believe that celocentesis cannot be used alone for the analysis of a few chromosomes on fetal material, even based on the small number of cases we have examined. The use of other techniques that allow the examination of the whole chromosomal structure might be more justified. Nevertheless, in our continuing studies, we aim to apply, at the same time, molecular tests for monogenic diseases and chromosomal microarray analysis based on a comparative genomic hybridization array (CGH-array) or a single nucleotide polymorphism array (SNP-array) to analyse the unbalanced number of copies of genomic sequences with greater resolution than standard chromosomal techniques. These procedures are actually used for preimplantation genetic screening (PGS) to determine the presence of aneuploidy in a developing embryo, whereby the few fetal cells isolated from celomic fluid may be sufficient to obtain reliable results on any aneuploidy of the entire genome (32-33).

Though celocentesis remains an attractive procedure for the early prenatal diagnosis of genetic disorders, in the last few years, this procedure has been abandoned by the pioneering groups that described it for the first time. We hypothesize that this is due to a greater interest in non-invasive prenatal diagnosis techniques (NIPT) rather than the difficulty of developing reliable procedures adapted to low volume of CF-DNA and contaminated CF. Moreover, celocentesis may seem time-consuming and expensive.

Conclusions

We propose celocentesis to our patients for early diagnosis of monogenic diseases, such as hemoglobinopathies. Given the low risk of miscarriage and the high acceptability, we believe that celocentesis remains an attractive technique for early prenatal diagnosis, as demonstrated by the high demand to access to the procedure of celocentesis by many women from other Italian regions and some from countries outside Italy.

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Supporting Information legend.

Videoclip S1. Celocentesis technique.

Table S2. Markers used for QF-PCR.

Legends

Table 1. Results of celomic fluids analysis.

Table 2. Results of positive QF-PCR performed on 485 elegible celomic samples.

Figure 1: Quantitative fluorescent analysis (QF-PCR) of short tandem repeats (STRs) in celomic fluid from the first case of trisomy 21. Panel A provides the genotype profile that demonstrates a 60% maternal contamination; Panel B provides the genotype profile after purification of the coelomic fluid. Panel C provides the genotype profile of maternal DNA.

Figure 2: Quantitative fluorescent analysis (QF-PCR) of short tandem repeats (STRs) in coelomic fluid from the second case of trisomy 21. Panel A provides the genotype profile which demonstrates balanced alleles indicating absence of maternal contamination. A genotype profile of trisomy 21 was assumed by the presence of two peaks with 2:1 ratio in five markers of chromosome 21; Panel B provides the genotype profile of maternal DNA.

Figure 3: Quantitative fluorescent analysis (QF-PCR) of short tandem repeats (STRs) in coelomic fluid from the third case of trisomy 21. Panel A provides the genotype profile that demonstrates a 50% maternal contamination; Panel B provides the genotype profile after purification of the coelomic fluid; Panel C provides the genotype profile of maternal DNA.

Figure 4: Quantitative fluorescent analysis (QF-PCR) of short tandem repeats (STRs) in coelomic fluid from a case of trisomy 13. Panel A provides the balanced genotype profile indicating absence of maternal contamination. Panel B provides the genotype profile of maternal DNA.

Figure 5: Quantitative fluorescent analysis (QF-PCR) of short tandem repeats (STRs) in coelomic fluid from a case of triploidy compared to alleles from maternal blood. Panel A provides the balanced genotype profile indicating absence of maternal contamination. Panel B provides the genotype profile of maternal DNA; Panel C provides the profile of coelomic indicating absence of maternal contamination; Panel D provides the genotype profile of maternal DNA.

Table 1. Results of celomic fuid analysis.

Pregnancies having celocentesis	489
Pregnancies for prenatal diagnosis of hemoglobinopaties	367/489
Pregnancies before TOP	122/489
Elegible samples for analysis	487/489[a]
Results of prenatal diagnosis for hemoglobinopathies	365/367
Fetuses affected by hemoglobinopathies	87/365
Women chose to terminate the pregnancy	84/87
Fetuses affected by aneuploidies	5/487
Analysis of placental tissue after TOP	217/217[b]
Analysis of samples by CVS or amniocentesis or after bird	282/282
Diagnosis errors from celocentesis	0/487
Fetal loss following celocentesis	8/283[c]

[a]Two samples for hemoglobinopathies prenatal diagnosis resulted highly contamined with maternal DNA.

[b]122 placental tissue of women chose to terminate the pregnancy, 84 placental tissue of fetuses affected by hemoglobinopathies, 5 placental tissue of fetuses affected by aneuploidies and 6 fetal loss.

[c]two fetuses were diagnosed having trisomy 13 and paternal triploidy.

CVS, chorionic villus sampling; TOP,termination of pregnancy.

Table 2. Results of positive QF-PCR performed on 485 eligible celomic samples.

Case	Woman Age	Hemoglobin Genotype	Chromosomal Alteration	Fetal Loss before CVS control	Confirmation Analysis	False Negative or Positive Results
1	31	Normal	Trisomy 21	No	Yes, CVS	No
2	33	Heterozygote	Trisomy 21	No	Yes, CVS	No
3	38	Normal	Trisomy 21	No	Yes, CVS	No
4	32	Heterozygote	Trisomy 13	Yes	Yes, Placental Tissue	No
5	40	Heterozygote	Paternal Triploidy	Yes	Yes, Placental Tissue	No

CVS: chorionic villus sampling.

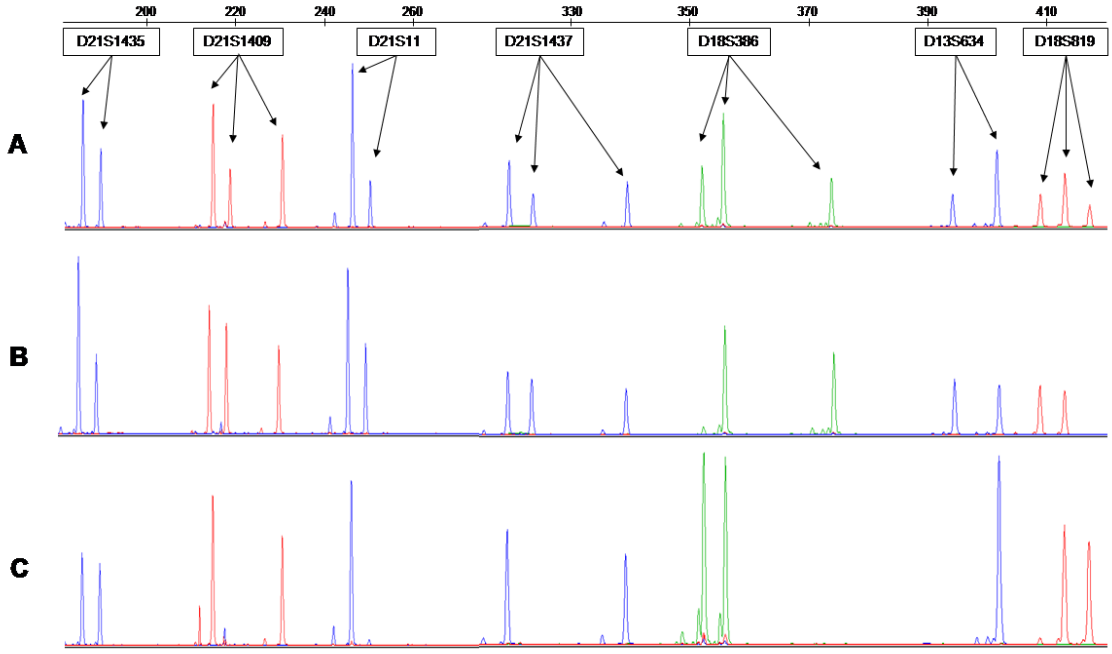

Figure 1

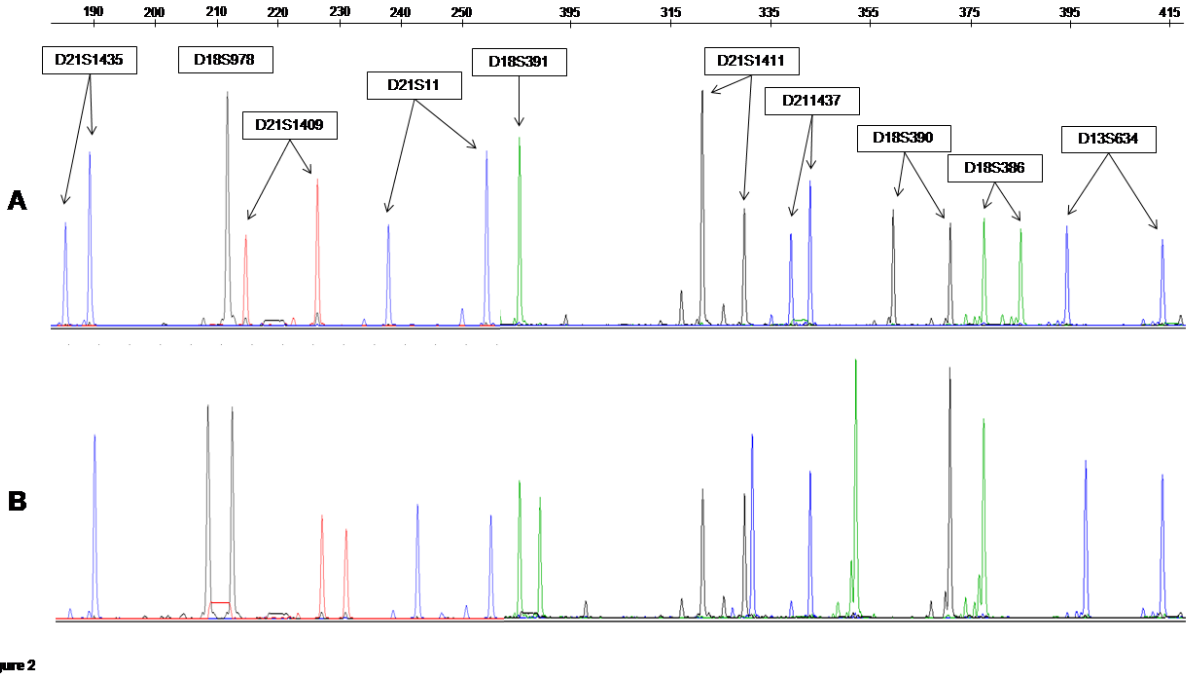

Figure 2

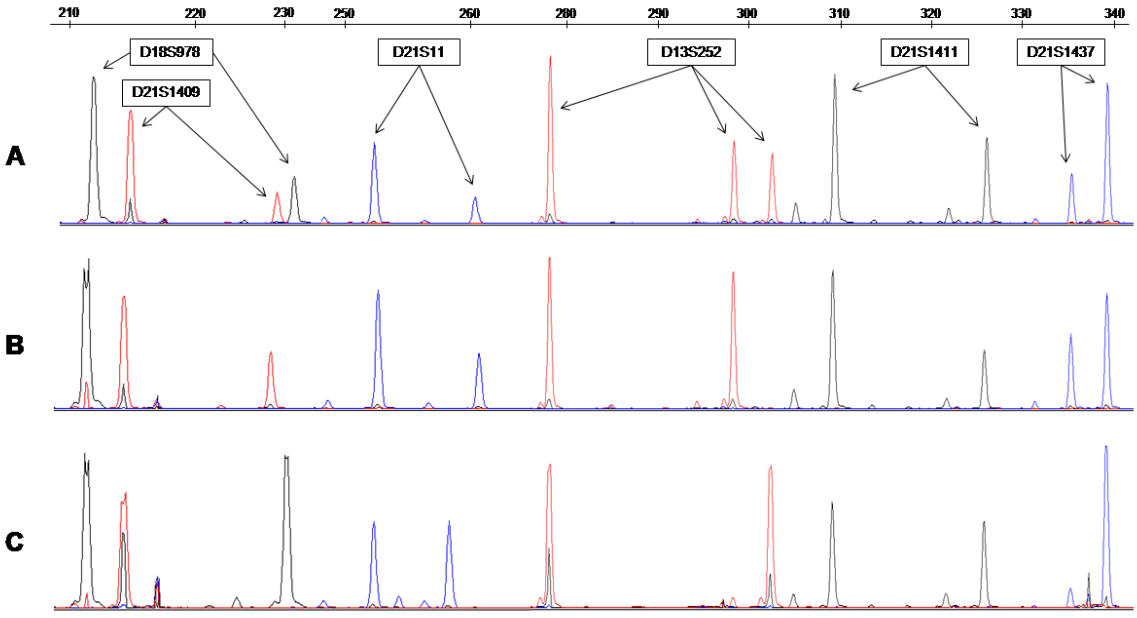

Figure 3

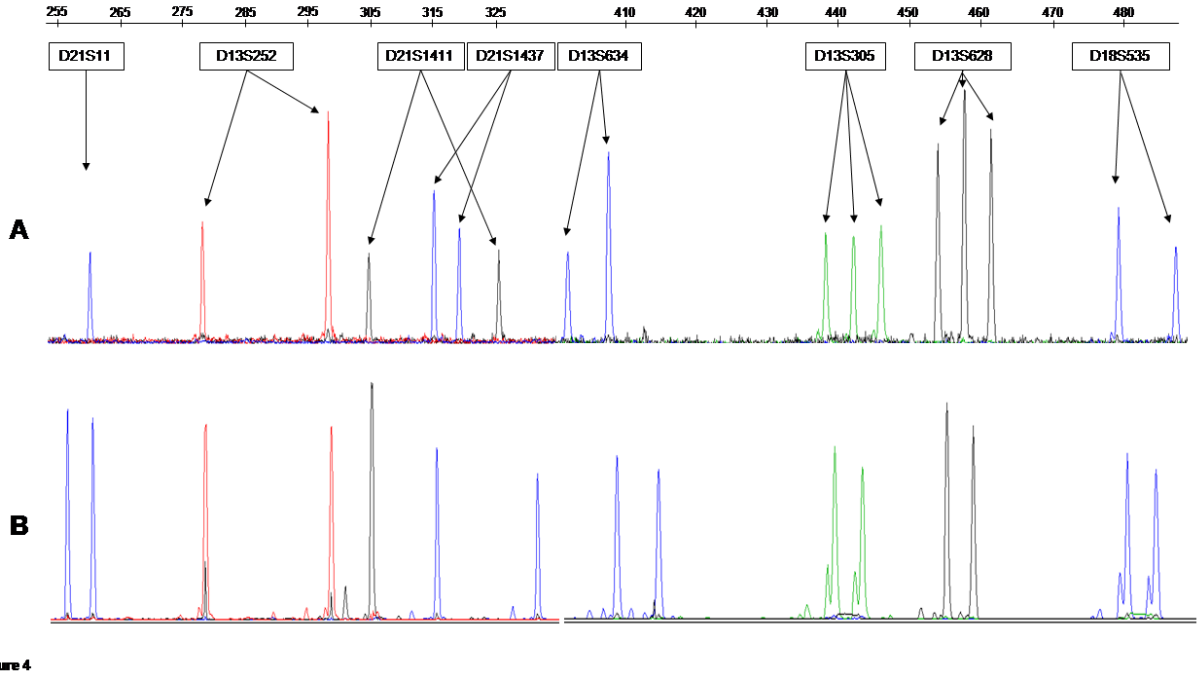

Figure 4