Very early prenatal diagnosis of genetic diseases based on coelomic fluid analysis: a feasibility study

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BACKGROUND: Coelocentesis may represent the ideal technique for very early prenatal diagnosis. Although cell density in coelomic fluid (CF) is very low, the results of analyses on the cellular compartment have been proposed for prenatal diagnosis. METHODS and RESULTS: We aimed to evaluate the amount of total DNA (i.e. cellular and cell-free) in 14 samples (0.4–0.8 ml) of CF, taken from women at 8- to 9-week gestation, who are about to undergo termination of pregnancy, and to assess the feasibility of multiple single-gene analyses using multiplex real-time PCR. We found that the amount of total DNA in the CF was very low and varied widely. Genetic testing using multiplex real-time PCR was successfully achieved in 10 of 14 samples (71%). However, when considering samples that could provide a reliable prenatal diagnosis (i.e. successful PCR analysis and no marked maternal contamination), reliable CF-DNA-based prenatal diagnoses were obtained in only 8 of the 14 (58%) samples. CONCLUSION: The development of highly reliable procedures adapted to pauci-cellular CF is crucially needed before coelocentesis could be proposed for early prenatal diagnosis of genetic diseases before 10 weeks.

Key words: coelocentesis/first trimester/PCR/prenatal diagnosis

Background

Exocoelomic cavity fluid may be the earliest fluid of the gestational sac amenable to prenatal genetic testing, as it can be selectively aspirated under ultrasound guidance using a transvaginal route as of 5 weeks of gestation. As pioneered in 1993 (Jurkovic et al., 1993), coelocentesis might be preferable to chorionic villous sampling (CVS), which is currently the earliest technique available for invasive prenatal diagnosis, because (i) it would shorten the time to diagnosis by at least 1 month, (ii) it would exclude the risk of placental vascular damage and its associated risk of fetal abnormalities, (iii) it would limit discordance resulting from pseudo-mosaicism encountered with classical chorionic preparations and (iv) it would make in utero stem-cell therapy feasible before the fetus becomes immunologically competent.

Two major issues associated with coelocentesis are still a matter of debate. First, procedure-related fetal loss may be approximately 2% but can only be estimated from descriptive studies of women undergoing coelocentesis a couple of weeks before termination of pregnancy (TOP) (Makrydimas et al., 2002). In addition, because of the limited number of ongoing pregnancies following coelocentesis (Makrydimas et al., 2004a), it is currently impossible to conclude as to its potential impact on the subsequent fetal development. These coelocentesis-related risks are likely to be minimized by using a thin needle and withdrawing a small sample volume. The second issue concerns the risk of contamination of the sample by maternal material that may preclude molecular biology assays for genetic analyses. To the best of our knowledge, only the results of analyses on the cellular compartment of the coelomic fluid (CF) have been reported so far. The relatively low number of cells in CF (Jauniaux et al., 2003) and the apparent necessity to retrieve only a small amount of it should encourage analyses of cellular and cell-free DNA in CF.

The aims of our study were to use real-time PCR to evaluate the amount of total (cellular and cell-free) DNA contained in CF and then to examine the feasibility to simultaneously analyse multiple single-base mutations in a single reaction using CF DNA and controlling for the absence of maternal contamination.

Methods

CF sampling and nucleic acid extraction

An experienced operator performed coelocentesis on 14 women with uncomplicated singleton pregnancies at 8–9 weeks of gestation immediately before surgical TOP for psychological reasons. Gestational age was determined from the first day of the last menstrual period and confirmed by ultrasound measurement of the crown–rump length. The local Ethics Committee (CCPRB Aulnay-sous-Bois, France) approved the study, and patients gave their informed written consent.
CF were obtained by transvaginal puncture under ultrasound guidance, as previously described (Jurkovic et al., 1993), using a 20 G needle (Cook, Limerick, Ireland). During the procedure, the tip of the needle was visualized continuously, and care was taken to avoid puncture of the amniotic membrane or the yolk sac. The syringe containing the first 0.2 ml of each fluid was discarded to limit the risk of contamination by maternal blood, and the next 0.4–0.8 ml was retained for analyses. Maternal venous blood was always collected before coelocentesis. At the end of the procedure and before performing suction TOP, we verified that all fetal heart rates were normal.

As soon as the laboratory received the samples (within 8 h after coelocentesis), CF and the corresponding maternal blood were gently homogenized, aliquoted (0.2 ml) and immediately frozen at −30°C until further processing. DNA was then extracted using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Meylan, France), according to the manufacturer’s recommendations, except that DNA was eluted into 50 μl of elution buffer (to increase its concentration) of which 5 μl was used for each analysis.

**Quantification of DNA using duplex real-time PCR**

DNA in CF was quantified using a duplex real-time PCR (Guibert et al., 2003) targeting the SRY (Yq11) and F8 (Xq28) genes. The extracted DNA sample (5 μl) was amplified in a LightCycler® v2.0 (Roche Diagnostics), and PCR was run in a final volume of 20 μl using the Fast DNA Master Hybridization Probes Kit (Roche Diagnostics) containing 0.5 μM of each primer, 0.25 μM of each probe, 1.25 units of uracil DNA glycosylase (UDG; Biolabs, Saint-Quentin-en-Yvelines, France) and 4.5 mM of magnesium chloride. After an initial 1-min incubation at 50°C to allow UDG to cleave putative contaminant PCR products from previous reactions, a first denaturation step, 8 min at 95°C, was followed by amplification as follows: 45 cycles of denaturation (95°C, 10 s, temperature ramping rate 20°C/s), annealing (56°C, 10 s, ramping rate 20°C/s) and extension (72°C, 15 s, ramping rate 2°C/s). Each of the PCR products was simultaneously and specifically detected using hybridization probes, respectively labelled with fluorescent LCRed610 (SRY gene) or LCRed670 (F8 gene). Characteristics and sequences of the primers and probes (Proligo, Paris, France) are summarized in Table I. Each sample of extracted DNA was tested in duplicate, and the elution buffer for DNA extraction served as the negative control.

Using an external standard curve established with serial dilutions of a quantified DNA sample, amounts of specific fetal DNA (for male fetuses) and/or total DNA can be determined.

**Single-base mutation detection using triplex real-time PCR**

As a model for single-base mutation studies, prothrombotic mutations suspected of being involved in recurrent fetal losses (i.e. prothrombin G20210A, factor V G1691A and methylenetetrahydrofolate reductase C677T mutations) were detected in a single-tube multiplex real-time PCR that is rapid, reliable and sensitive and, because it is a closed system, avoids contamination by PCR products.

Maternal blood and CF DNA were tested simultaneously for these mutations as follows. The 5-μl DNA sample was amplified in a LightCycler® v2.0 (Roche Diagnostics) in a final volume of 20 μl, using the LC Multiplex DNA Master Hybridization Probes Kit (Roche Diagnostics), containing 0.5 μM of each primer, 0.25 μM of each probe and 1.25 units of UDG. Cycle conditions were as follows: 1-min incubation at 50°C and denaturation step for one cycle, 95°C for 8 min, amplification of the target DNA for 45 cycles at 95°C for 10 s, 60°C for 10 s and 72°C for 15 s, each with a ramping rate of 20°C/s, melting curve analysis for one cycle at 95 and 45°C for 2 min each and then ramping to 75°C at 0.1°C/s with a continuous fluorescence acquisition. Characteristics and sequences of the primers and probes (Proligo) are reported in Table II. Each of the PCR products was simultaneously and specifically detected using hybridization probes, respectively labelled with fluorescent LCRed610 (MTHFR gene), LCRed640 (F5 gene) or LCRed670 (F2 gene). Each sample extract was tested in duplicate. Each run included a heterozygous DNA control, and elution buffer for DNA extraction served as the negative control.

**Multiplex PCR for short-tandem repeat analysis**

CF contamination by maternal tissue was systematically controlled by studying 10 polymorphic short-tandem repeat (STR) markers located on chromosomes X (HPRT gene), 13 (D13S346, D13S258, D13S794), 18 (D18S51, D18S535, D18S386) or 21 (D21S11, D21S1442, D21S1411). The assay also included SRY gene detection in one multiplex PCR according to a previously described protocol (Brisset et al., 2003) with minor modifications.

PCRs were run in a final volume of 20 μl. The amplification mixture consisted of ×1 PCR buffer, 100 μM of each dNTP, 1.25 mM of MgCl2, 1.25 units of FastStart Taq DNA polymerase (Roche Diagnostics) and sets of primers (Proligo), one of which was fluorescently labelled. After an initial denaturation step, 8 min at 95°C, amplification was performed as follows: 35 cycles of denaturation (95°C, 30 s), annealing (60°C, 30 s) and extension (72°C, 60 s) in a 9700 thermocycler (Applied Biosystems, Courtabœuf, France). The PCR products were then analysed on an ABI310 sequence analyzer using Genescan analysis software (Applied Biosystems). Electrophoreogram profiles of amplified maternal and CF DNA were compared.

**Results**

The pertinent data for all 14 paired maternal–fetal DNA samples are summarized in Table III. The total concentration of DNA amplified using a real-time PCR assay ranged from

<table>
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<tr>
<th>Targeted gene</th>
<th>PCR product</th>
<th>Primers and probes sequences (5' &gt; 3')</th>
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<tr>
<td>SRY</td>
<td>113 bp</td>
<td>GCAACGTCGGATAGAAGTGA&lt;br&gt;GCTGATCTGGATTTCCGATC&lt;br&gt;CCATGAAAGCAATCATCGTGGGCTCT (3'FTC)&lt;br&gt;CGATCAAGGGCAGATGCTCT (5'LCRed640, 3'Ph)&lt;br&gt;TGGGTCAGGGAAGCTGCA&lt;br&gt;TCCCTCAAGCAGCTTAACCTC&lt;br&gt;GAGACGCTTTACCTTGGGCTC (3'FTC)&lt;br&gt;CAAGCAGCTCACGGAGATCTTT (5'LCRed670, 3'Ph)</td>
</tr>
<tr>
<td>F8</td>
<td>133 bp</td>
<td>F8, factor VIII gene; SRY, sex-determining region Y chromosome.</td>
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undetectable to 777.5 ng/ml (median: 17.9 ng/ml). For sample 9, no DNA could be detected despite the use of a sensitive real-time PCR amplification assay. That finding was confirmed in a second independent analysis, starting with the initial material, thereby excluding a technical failure during the DNA-extraction step. For samples 7, 8 and 13, whose DNA concentrations were below or around 1 ng/ml, the conventional protocol currently used in our laboratory for blood or amniotic fluid analysis yielded no further results. In these cases, the sample volume was 0.5, 0.4 and 0.6 ml respectively. Finally, DNA analysis could be performed in 10 of 14 (71%) CF. Interpretation was clear and unambiguous in all cases. No contamination by PCR product carry-over was observed.

Among the 10 samples that could be analysed, very weak maternal DNA contamination was observed in sample 4, but it had no impact on the subsequent mutation analysis. Surprisingly, STR analysis of samples 6 and 11 generated profiles similar to those obtained with the corresponding maternal blood; notably, those samples had been clear but their DNA contents differed widely, at 139.7 and 6.6 ng/ml, respectively. Pertinently, fetal sexing and mutation analyses were feasible for all samples.

### Discussion

According to our results, multiplex real-time PCR of CF DNA was able to successfully diagnose genetic diseases prenatally for 10 of the 14 (71%) samples. However, when considering samples that could provide a reliable prenatal diagnosis (i.e. successful PCR analysis and no marked maternal contamination interfering with interpretation) and applying protocols currently used in our laboratory but not specifically developed for CF, reliable CF–DNA-based prenatal diagnoses were obtained for only 8 of the 14 (58%) samples.

These results might be considered discouraging, if they are compared with those obtained previously (Findlay et al., 1996; Jauniaux et al., 2003). But that would not take into consideration that this investigation, unlike others, was designed as a prospective clinical study, which means that CF samples (<1 ml) were compared with the currently applied real conditions for fetal genetic testing by invasive procedures, i.e., a sufficient quantity of fetal material for successful analysis and a sample free of maternal contamination.

Several explanations can be advanced to explain the notable difference between our success rate and the approximately 90% reported by several teams (Findlay et al., 1996; Makrydimas...
et al., 2004b; Jauniaux et al., 2003). Most of those groups used volumes 5–10 times larger and collected most of the CF, which could have an effect in terms of cell density. Although the CF quantity collected is unimportant in a fundamental research setting, the amount withdrawn is critical in the context of prenatal testing for genetic disorders. Indeed, this latter CF volume must be as small as possible to limit the risk of a miscarriage.

In addition, results can be analysed and viewed differently depending on whether one considers the overall success of PCR assays to study a given fetal gene, the rate of contaminated samples obtained and, finally, the number of samples for which several gene analyses could be obtained at the same time.

This point is clearly illustrated by the findings reported by Findlay et al. (1996), which is one of the most extensive studies published to date. The authors claimed reliable fetal sexing for 96% of their samples. However, that percentage can be interpreted in different ways. Among the 23 samples tested, 20 were compared with placental samples and retained by the authors. Concordant results were obtained for 18 of the 20 samples (90%). Two samples were marred by marked maternal contamination interfering with interpretation of the data. PCR testing for cystic fibrosis is still not as reliable, as only 19 of the 23 samples (83%) were interpretable, among which one sample yielded discordant results with the placenta and another was contaminated by maternal material. Thus, if we express the final result in terms of reliable prenatal diagnoses, the success rate would be only 17 of 23 (74%). Finally, the 11% false-positive (contamination with PCR products) rate reported by Findlay et al. seems unacceptably high for a clinical setting. Furthermore, Jauniaux et al. (2003) too reported an excellent success rate for fetal sexing but with six samples (35%) with marked maternal contamination.

In our opinion, those data justify the development of highly reliable and safe procedures (i.e., real-time PCR-based assays), adapted to the analysis of pauci-cellular CF, like those we have described herein. For these reasons, genetic testing based on real-time PCR assays is particularly well adapted to our needs. But they need to be further improved and refined. The diagnostic results obtained with preimplantation embryos demonstrated that PCR techniques can be adapted to very small amounts of DNA.

It is not unreasonable to think that the sensitivity of the techniques that we applied in this study can be improved, for example, in the preparation of the samples to increase the yield of nucleic acid extraction (cell pellet, concentration). In addition, gene amplification by simple PCR, rather than the multiplex PCR described here, and increasing the number of cycles should achieve higher sensitivity. However, as for embryo sampling, coelocentesis must imperatively satisfy two essential criteria: a sufficient quantity of analysable material and the lowest possible rate of maternal contamination.

Our results demonstrate, for the first time, that not only does the CF-DNA content vary widely among samples but that it was most frequently very low. They indirectly confirm that the CF cell content, if considered the main source of DNA, is poor, severely limiting the possibility of achieving precise and reliable chromosomal analyses using conventional cytogenetic techniques. To the best of our knowledge, no study has yet clearly estimated the cell density in CF. Therefore, we cannot conclude as to whether the variability of the DNA quantities obtained is linked to that of the cell density from one sample to another or simply reflects the variability of cell-free DNA content.

By offering the possibility of prenatal diagnosis of genetic diseases at least 4 weeks earlier, we are of opinion that coelocentesis constitutes a highly attractive advance. From a clinical point of view, it represents a real advantage, as it would allow women to undergo medical TOP at 7–10 weeks of gestation, which is less traumatic than second trimester surgical TOP (Findlay et al.). Moreover, coelocentesis may be a valid alternative to early prenatal diagnosis for Jewish patients, as it would comply with Orthodox Jewish law that allows TOP before 40 days postconception and for whom early CVS, with its associated risk of limb-reduction defects, remains the only option (Firth et al., 1991; Brambatti et al., 1992; Wapner et al., 2002).

Our results and those reported to date do not yet allow us to propose this early prenatal diagnostic procedure to our patients. Nonetheless, we continue to think that coelocentesis remains an attractive technique for early prenatal diagnosis of genetic disorders. We intend to pursue our efforts to develop reliable procedures adapted to pauci-cellular CF to improve our understanding of the mechanisms leading to the presence of genetic material in the coelomic cavity.

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References


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