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.
**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 LCRed640 (SRY gene) or LCRed670 (F8 gene). Characteristics and sequences of the primers and probes (Proligo) are summarized in Table I. Each sample of extracted DNA was tested in duplicate, and the elution buffer used 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, D21S142, D21S141). 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, Courtaboeuf, France). The PCR products were then analysed on an ABI310 sequence analyzer using Genescan analysis software (Applied Biosystems). Electrophoregram 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

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**Table I.** Characteristics and sequences of primers and probes used in the duplex real-time PCR assay for DNA quantification

<table>
<thead>
<tr>
<th>Targeted gene</th>
<th>PCR product</th>
<th>Primers and probes sequences (5’ &gt; 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRY</td>
<td>113 bp</td>
<td>GCAACGTCCAGATAGAGTGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCTGATCTCGAGTTCGACAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCAAGGACATCATCATGTCCCT (3’FITC)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CGATCGAGGCGCAAGATGCCCTC (5’LCRed640, 3’Ph)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGGTGTCAGTTGAACTGTCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCCCTCAAGCAGACTTACATCC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGACATGCTTTTACCTCGTGTCC (3’FITC)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CAGGCAGCTCAAGAGATCCTT (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>
</tr>
</tbody>
</table>
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. 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., 2003).
conclude as to whether the variability of the DNA quantities
techniques. To the best of our knowledge, no study has yet
CF cell content, if considered the main source of DNA, is poor,
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 tech-
iques 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 addi-
tion, gene amplification by simple PCR, rather than the multi-
plex 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 relia-
bile 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 coelo-
centesis 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 alter-
native 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 under-
standing of the mechanisms leading to the presence of genetic
material in the coelomic cavity.

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