



FEASIBILITY OF DNA DIAGNOSIS OF HAEMOGLOBINOPATHIES ON COELOCENTESIS

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Key Words:	THALASSAEMIA, HAEMOGLOBINOPATHIES, PRENATAL DIAGNOSIS, celocentesis, celomic fluid

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FEASIBILITY OF DNA DIAGNOSIS OF HAEMOGLOBINOPATHIES ON COELOCENTESIS

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SUMMARY

At 5-12 weeks of gestation the amniotic sac is surrounded by celomic fluid which contains cells of fetal origin. This fluid can be sampled by celocentesis, which involves the ultrasound-guided insertion of a needle through the vagina. The aim of this study was to examine the feasibility of prenatal diagnosis of hemoglobinopathies from the celomic fluid using a specific protocol. Celocentesis was performed at 7-9 weeks' gestation in 26 singleton pregnancies at risk for hemoglobinopathies. In 25 cases more than 30 fetal cells were recovered from the celomic fluid and in all these cases molecular analysis for hemoglobinopathies was possible and the results were confirmed by subsequent chorionic villus sampling or amniocentesis. The results of this study suggest that reliable diagnosis of thalassemia syndromes can be performed from 7 weeks' gestation by celocentesis. **Further work is necessary to demonstrate the safety of celocentesis before widespread use.**

Key words: Haemoglobinopathies, Thalassemia, Prenatal diagnosis, Celocentesis, celomic fluid.

INTRODUCTION

Hemoglobinopathies are a major public health problem worldwide (Weatherall, 2010). Actually, β -thalassemia and hemoglobin disorders account for 3.4% of deaths in children less than 5 years of age (Modell & Darlison, 2008). Prevention of thalassemia major can be achieved by population screening in combination with prenatal diagnosis (Rosatelli *et al*, 1986; Hartevelde *et al*, 2009).

Prenatal diagnosis of chromosomal disorders and genetic abnormalities necessitates the analysis of fetal material obtained by chorionic villus sampling (CVS) at 11-13 weeks' gestation or amniocentesis from 16 weeks onwards. An alternative technique for earlier diagnosis is celocentesis (Jurcovic *et al*, 1993; Makrydimas *et al*, 1997; Makrydimas *et al*, 2002; Makrydimas *et al*, 2004). At 5-12 weeks' gestation the amniotic sac is surrounded by celomic fluid (CF) which contains cells of fetal origin (Jurcovic *et al*, 1993; Renda *et al*, 2010). This fluid can be sampled by a technique which involves the ultrasound-guided insertion of a needle through the vagina from as early as 6 weeks. The desirability of such technique is not only based on the feasibility of achieving prenatal diagnosis several weeks earlier than with CVS but also on the potential for in-utero stem cell transplantation before the fetus becomes immunologically competent. However, studies investigating the feasibility of prenatal diagnosis from fetal DNA extracted from cells in the CF reported failure rates of more than 40% which was attributed to the small number of celomic cells, the high degree of maternal contamination and the unknown nature of the maternal and fetal cells in CF (Jouannic *et al*, 2006; Jouannic *et al*, 2008).

In this study we present a CF-specific protocol that maximises the success rate of prenatal genetic testing using this fluid.

MATERIALS AND METHODS

The institutional review board approved the study which complied with the declaration of Helsinki and the participating couples provided written informed consent (Hospital Ethical Committee authorization on date 01.26.2005, N° 80). The patients included in this study are part of an ongoing investigation examining the safety of celocentesis.

Sampling

Celocentesis was carried out at 7-9 weeks' gestation in 26 singleton uncomplicated pregnancies at risk for hemoglobinopathies, including 15 for β -thalassemia, 9 for Sickle-Cell/beta-thalassemia and 2 for β -thalassemia and Hb Lepore Boston (Table I). **The procedure was offered to all women**

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seeking prenatal diagnosis of haemoglobinopathy at our center. The procedure was performed by the same operator (GM), essentially involves transvaginal sonography and the insertion of a 20 G needle through the fornix into the celomic cavity (Figure 1; Makrydimas *et al* 2002). Samples of CF were aspirated into three different syringes (0.2 mL, 0.2 mL and 0.6 mL). The first two samples were discarded to reduce the risk of contamination by maternal blood and the third sample was used for analysis.

Microscopical observation

The CF (50 µL) was put in a petri disk and observed by optical microscopy to assess the number and composition of cells as previously reported (Renda *et al*, 2010).

Purification

The CF samples where the percentage of maternal cells were more than 5% of the total cell content were purified using anti-CD45 and anti-CD105 MicroBeads for negative selection. The sample of CF was put in an Eppendorf tube and centrifuged at 2.000 rpm for 10 min, the pellet was dissolved in 50 µL of PBS buffer and MicroBeads of anti-CD 45 (2.5 µL) and anti-CD 105 (2.5 µL) (Miltenyi Biotec, Bergisch Gladbach, Germany) were then added and the tube was incubated at 4 °C for 15 min. Subsequently, PBS (500 µL) was added to the tube and centrifuged at 2.000 rpm for 10 min and the pellet was dissolved in PBS (550 µL). The sample was then applied to MiniMACS separation column (Miltenyi Biotec, Bergisch Gladbach, Germany) and all the liquid containing fetal cells (negative selection) was eluted in an Eppendorf tube. The pellet of was collected by centrifugation at 12,000 rpm for 10 min, dissolved in 50 µL of 5% IstaGene Matrix (Bio-Rad Laboratories, Hercules, CA, USA) and incubated at 56 °C for 30 min and at 95 °C for 10 min. The supernatant (45 µL) containing DNA was removed and transferred to an 0.5 mL Eppendorf tube.

Molecular analysis of β-globin gene

To analyse β-globin gene (HBB MIM#141900) PCRs were performed in 30 µl reactions using 5 µl of extracted DNA, 200 µM of each dNTP (Roche Diagnostics GmmH, Mannheim, Germany), 1X PCR buffer (Invitrogen, Valley Blvd, San Diego, CA, USA), 1.5 mM MgCl₂, 10 µM of each primer and 1.5units of Taq Polymerase (Invitrogen , San Giuliano Milanese, Milano, Italy). The reaction mix was denatured at 96°C for 5'. The reaction was performed for 35 cycles of 94 °C 30'', 55 °C 30'' and 72 °C for 45'' followed by 10' at 72 °C. Specific primers were used to amplify one or two fragments of β-globin gene from -130 nucleotides 5' to the CAP site to + 150 nucleotides 3' to the Poly-A. Amplified fragments were successively analyzed by direct sequencing method using

BigDye Terminator 3.1 Cycle Sequencing Kit (Applied BioSystems, Foster City, CA, USA) and run on ABI PRISM 3130 DNA Analyzer (Applied BioSystems, Foster City, CA, USA). β -globin gene polymorphisms (Cd 2 C→T, IVSII nt 16 C→G, IVS II nt 26 T→G, IVS II nt 74 G→T, IVSII nt 81C→T and IVSII nt 666 T→C) presented in both fragments were also analysed to check contamination with maternal DNA and for linkage analysis.

Maternal contamination control

Maternal contamination of the CF sample was evaluated before and after negative selection of maternal cells using a very sensitive multiplex fluorescence PCR (QF-PCR), containing primers for highly variable microsatellites of many chromosomal markers (STR) 13, 18, 21, X and Y (D13S631, D13S634, D13S258, D18S535, D18S386, D21S1435, D21S1411, D21S1414, AMXY, X22, HPRT, DXS6803, DXS6809). Products were denatured and fragments of different sizes were run on ABI PRISM 3130 DNA Analyzer (Applied BioSystems, Foster City, CA, USA). Data were analysed by GeneScan software (Applied BioSystems, Foster City, CA, USA) (Figure 2).

RESULTS

Sampling. Aspiration of celomic fluid was possible in all cases and this was not associated with a change in fetal heart rate. The volume of the CF sample used for diagnosis was 450-710 μ L (Table I). On the basis of previous investigations (data not shown), only samples containing more than 30 fetal cells were considered adequate for molecular analysis and this was achieved in 25 of our 26 cases. One sample contained only 10 cells and this was considered inadequate for further analysis.

Microscopical observation. We observed the presence of megaloblastic and other cells as previously reported (Figure 3; Renda *et al*, 2010). Surface antigens were characterized as CD71+ (fetal cells), CD45+ (maternal white cells) and CD105+ (mesenchymal / endothelial cells). The number of the cells, estimated manually in a Burker chamber, was 10-6400 (Table 1).

Purification. The CF samples were considered appropriate for molecular prenatal β -globin gene without preventive purification in 7 cases. The other 18 CF showed reduction of contamination to less than 1%, after negative selection of maternal cells.

Molecular analysis. This was conducted in 25 of the 26 samples. Polymerase Chain reaction (PCR) amplification of DNAs was proportional to the number of observed embryo- fetal erythroid cells (Renda *et al*, 2010). In nine cases the fetus was affected from thalassemia major and sickle-cell/beta-thalassemia and the parents decided to terminate the pregnancies. Ten and six were

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carriers and normal, respectively. Molecular analysis of prenatal diagnosis was confirmed by CVS or amniocentesis at 12 or 16 weeks' gestation. Moreover, molecular diagnosis was also confirmed on placental tissue following pregnancy termination in those cases with affected fetuses.

DISCUSSION

This study has established a specific protocol for assessment and analysis of CF samples which has made it possible to achieve accurate prenatal diagnosis during the embryonic stage of intrauterine life.

The critical components of the protocol were firstly, expertise in obtaining CF samples and discarding the first samples with high maternal contamination, secondly, microscopical examination of the CF sample immediately after celocentesis to estimate the total number of fetal cells, and thirdly, purification of the sample to minimize the number of maternal cells. The findings of the study confirm our previous results that CF contains embryo-fetal erythroid precursors, megaloblasts (CD 71+/Cd 45-) and maternal white cells (CD 45+) (Renda *et al*, 2010). All CF samples showing maternal contamination exceeding 5% were purified successfully using anti-CD45 and anti-CD105 for negative selection of maternal white blood cells and endothelial meenchymal cells. Accurate prenatal diagnosis of hemoglobinopathies was achieved in all CF samples containing more that 30 fetal cells. In all 25 cases with adequate number of fetal cells, the diagnosis was correct as confirmed by CVS, amniocentesis or placental tissue. The low success rate in diagnosis reported in previous studies is the inevitable consequence of failure to firstly, identify and distinguish between foetal and maternal cells and secondly, develop an effective method for removal of the maternal cells (Jouannic *et al*, 2006; Jouannic *et al*, 2008).

The CF contains embryo-fetal erythroid precursors or megaloblasts (Migliaccio *et al*, 1986; Renda *et al*, 2010). Hematopoiesis in the embryo starts in the 2nd or 3rd week of gestation yolk sac blood islands develop clones of primitive nucleated erythroblasts or megaloblasts (Zambidis *et al*, 2009; Valtieri *et al*, 1989; Stamatoyannopoulos, 2005). Detailed analysis of these embryonic cells revealed a prevalent megaloblasts pattern with CD71+/CD45- membrane expression, consistent with embryonic erythroid precursors (Migliaccio *et al*, 1986). The presence of such precursors in CF may infer that there is cellular trafficking between the embryo and the celomic cavity. In theory, the possibility of an early access to the celomic cavity in combination with the cell trafficking between the embryonal compartments could also allow a novel strategy for in utero cell transplantation (Noia *et al*, 2004).

This study has shown that reliable molecular diagnosis is possible from CF provided the sample contains at least 30 fetal cells. Future studies will determine the feasibility of prenatal diagnosis in CF samples containing less fetal cells by employing specific selection of such cells directly under the microscope and the application of techniques similar to those used in pre-implantation diagnosis. Ultimately, widespread clinical application of celocentesis will need to await the results of ongoing studies on the safety of the technique.

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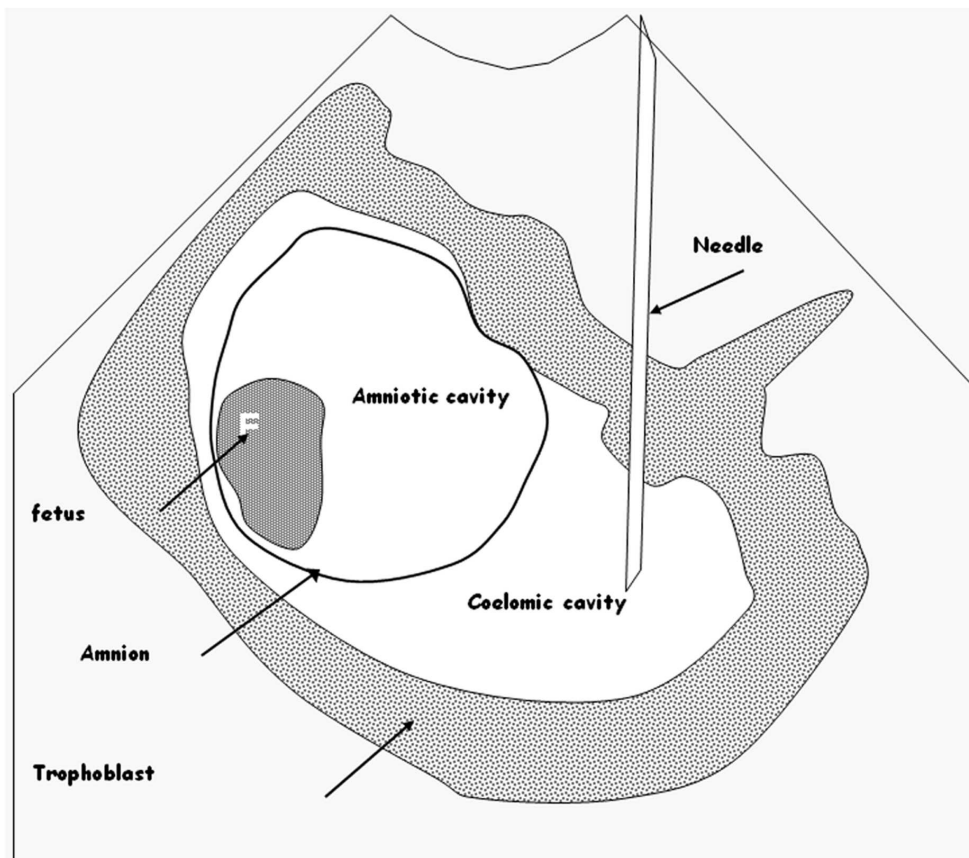
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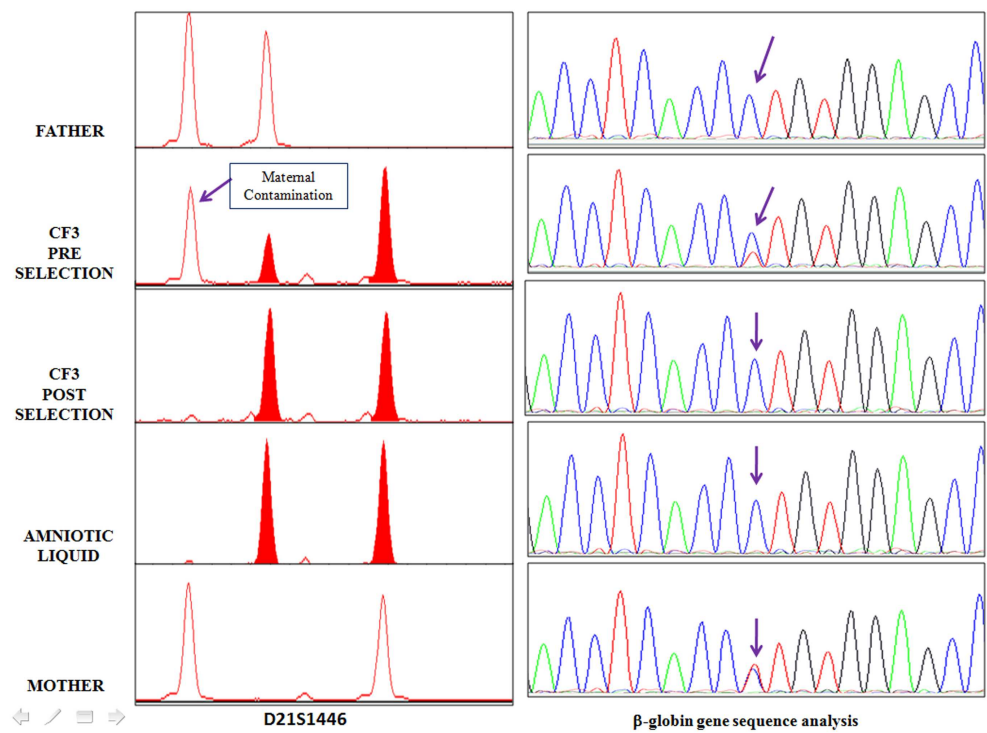
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Case	Parental mutation	Gestation (w)	Sample (μL)	Total Fetal cells	Maternal cells contamination before purification (%)	Fetal Genotype
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11						
12	IVS II-745/β ^S	8+5	600	380	25	IVS II-745 / β ^S
13						
14	Codon 39 / Codon 39	8+6	630	530	10	Codon 39 / Normal
15						
16	IVS I- 110 / β ^S	8+5	550	610	20	Normal / Normal
17						
18	IVS I- 110 / Codon 39	7+1	540	370	65	Codon 39 / Normal
19						
20	IVS II-745 / IVS-I-6	8+2	590	410	15	Normal / Normal
21						
22	β ^S / Codon 6 (-A)	7+2	610	720	5	β ^S / Codon 6 (-A)
23						
24	IVS I-110 / IVS I-110	7+1	560	130	60	IVS I-110 / IVS I-110
25						
26	β ^S / β ^S	8+6	480	210	40	Normal / Normal
27						
28	β ^S / IVS I-110	8+3	450	500	15	β ^S / IVS I-110
29						
30	β ^S / Codon 39	8+4	650	250	20	Codon 39 / Normal
31						
32	IVS I-1 / β ^S	8+6	650	700	10	IVS I-1 / Normal
33						
34	Codon 39 / IVS I-110	8+5	500	300	15	Codon 39 / IVS I-110
35						
36	Codon 39 / IVS I-110	8+2	600	480	15	IVS I-110 / Normal
37						
38	Hb Lepore / Codon 39	8+1	600	520	5	Hb Lepore / Normal
39						
40	Codon 39 / IVS II-1	8+0	520	4000	5	Normal / Normal
41						
42	Codon 39 / IVS II-745	8+4	500	2000	5	Codon 39 / Normal
43						
44	β ^S / IVS I-6	9+2	670	160	40	β ^S / IVS I-6
45						
46	-101 / IVS II-745	8+3	710	6400	5	Normal / Normal
47						
48	IVS I-110 / Hb Lepore	8+4	640	180	25	Normal / Normal
49						
50	IVS I-6 / IVS I-110	8+4	620	10	///	CF inadequate for diagnosis
51						
52	IVS I-110 / Codon 39	7+4	600	4800	5	IVS I-110 / Codon 39
53						
54	IVS I-110 / Codon 39	8+5	680	120	25	IVS I-110 / Normal
55						
56	IVS II-745 / Cod 39	8+5	700	3600	5	IVS II-745/ Codon 39
57						
58	IVS I-110 / Codon 39	8+4	650	140	30	IVS I-110 / Codon 39
59						
60	IVS I-6 / IVS I-110	8+3	540	200	30	IVS I-6 / Normal
25						
26	IVS I-6 / β ^S	7+4	610	190	30	IVS I-6 / Normal

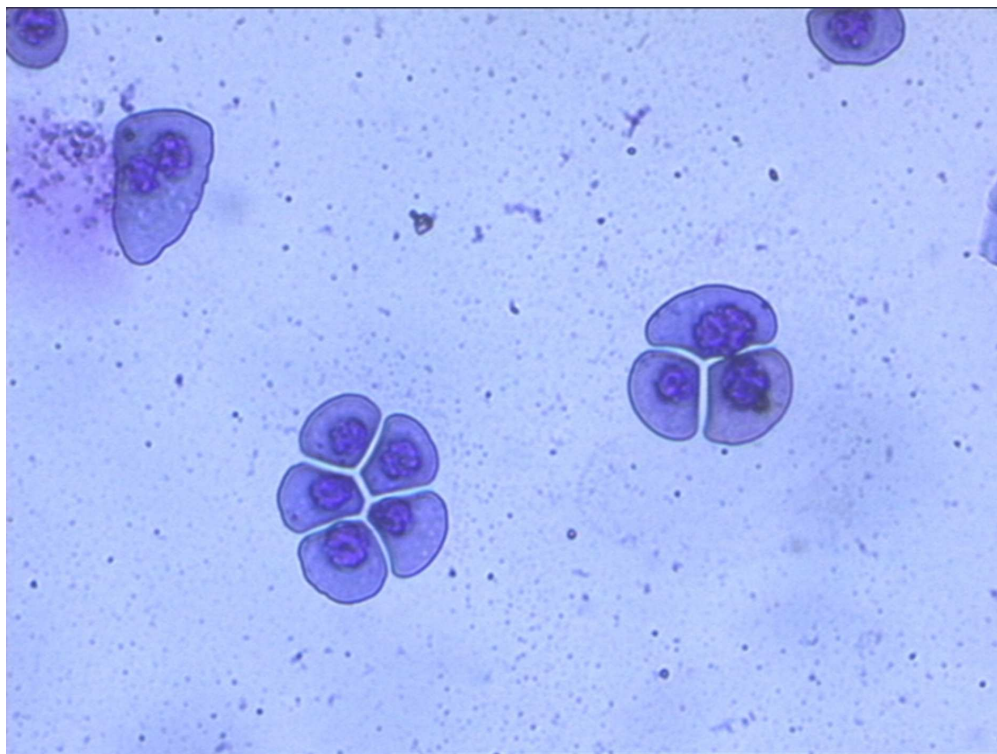


Schematic representation of celocentesis.

80x69mm (600 x 600 DPI)



DNA fingerprinting by QF-PCR and mutation detection by sequencing analysis of coelomic fluid before and after purification are showed.
117x87mm (600 x 600 DPI)



May Grunwald-Giemsa staining showing erythroid megaloblasts contained in the coelomic fluid
80x59mm (600 x 600 DPI)