Molecular Human Reproduction Vol.9, No.9 pp. 559–567, 2003 DOI: 10.1093/molehr/gag065

Improving clinical preimplantation genetic diagnosis for cystic fibrosis by duplex PCR using two polymorphic markers or one polymorphic marker in combination with the detection of the ΔF508 mutation

V.Goossens[1,3], K.Sermon[1], W.Lissens[1], M.De Rycke[1], B.Saerens[1], A.De Vos[2], P.Henderix[2], H.Van de Velde[2], P.Platteau[2], A.Van Steirteghem[2], P.Devroey[2] and I.Liebaers[1]

[1]Centre for Medical Genetics and [2]Centre for Reproductive Medicine, University Hospital and Medical School, Dutch-speaking Brussels Free University, Laarbeeklaan 101, 1090 Brussels, Belgium

[3]To whom correspondence should be addressed. E-mail: veerle.goossens@az.vub.ac.be

Cystic fibrosis (CF) is an autosomal recessive disease characterized by obstruction and chronic infection of the respiratory tract and pancreatic insufficiency. The first preimplantation genetic diagnosis (PGD) for CF was carried out in 1992. At our centre the first cycle was performed in 1993. However, the number of known CF mutations is >1000, so developing mutation-specific PCR protocols for PGD is unfeasible. This is why a number of marker-based duplex PCRs were developed at the single cell level. A duplex PCR of a mutation and one or two microsatellites is not only a diagnostic tool, but it can also be used as a control for allele drop-out and contamination. During PGD, embryos obtained *in vitro* are analysed for the presence or absence of a particular genetic disease, after which only embryos shown to be free of this disease are returned to the mother. In total, 22 PGD cycles with duplex PCR (IVS8CA/IVS17BTA, ΔF508/IVS8CA, ΔF508/IVS17BTA and D7S486/D7S490) were carried out in 16 couples, which resulted in four ongoing pregnancies and one miscarriage.

Key words: cystic fibrosis/duplex PCR/preimplantation genetic diagnosis

Introduction

Cystic fibrosis (CF) is one of the most common severe, lethal genetic disorders (Zielenski and Tsui, 1995; Welsh, 1995), and the first non-X-linked monogenic disorder for which preimplantation genetic diagnosis (PGD) was performed (Handyside *et al*., 1992). The median life expectancy is now >30 years and it is projected that in newborn infants it will become >40 years. But although the life expectancy has improved beyond all recognition, the quality of life is far from good (Doull, 2001). The carrier frequency in the Caucasian population of this autosomal recessive disorder is 1 in 25; 1 in 2500 newborns is affected (Welsh, 1995). Clinical characteristics are obstruction and chronic infections of the respiratory tract and pancreatic insufficiency. The cystic fibrosis transmembrane conductance regulator (CFTR) gene, which causes CF when mutated, is located on chromosome 7q22-31 (Riordan *et al*., 1989). A phenylalanine deletion at amino acid position 508 is the most common mutation and accounts for ~70% of the CF alleles worldwide. The remaining known CF alleles carry other mutations (>1000 listed, www.genet.sickkids.on.ca/cftr), of which some occur with a frequency of a few per cent, although most are private mutations (occurring only once in the whole population).

Congenital bilateral absence of the vas deferens (CBAVD) is a particular form of CF, which leads to male infertility due to obstructive azoospermia. Sperm can be retrieved by fine needle aspiration (FNA) and used for IVF through ICSI (Van Steirteghem, 1997). CBAVD patients are assumed to be compound heterozygotes for two different CFTR mutations or to have one CFTR mutation in combination with a 5T 'splice' variant in intron 8 (Lissens *et al*., 1996).

PGD can be considered as an early form of prenatal diagnosis which circumvents the problem of therapeutic abortion. The technique has become possible due to the simultaneous development of IVF, micromanipulation of the embryo, the PCR for monogenic disorders, and fluorescence in-situ hybridization (FISH) for chromosomal abnormalities. With PGD, only embryos diagnosed at the preimplantation stage as being unaffected by the disease under consideration are transferred to the uterus. Since genetic analysis is performed on one or two single blastomeres, it has to meet high standards of efficiency, including low allele drop-out (ADO) rates and contamination control (Lissens and Sermon, 1997). We have previously described a number of marker-based protocols for CF, because mutation-specific PCR protocols for every possible CF mutation are not feasible (Goossens *et al*., 2000). For this purpose we used three intragenic polymorphic markers IVS8CA, IVS17BTA and IVS17BCA, and four extragenic polymorphic markers, D7S490, D7S486, D7S480 and D7S523. IVS8CA is a CA-repeat in intron 8 of the CFTR gene with 12–24 repeats and a heterozygosity of 48%. IVS17BCA is a CA-repeat in intron 17B with 11–20 repeats and 39% heterozygosity. The IVS17BTA marker is a TA-repeat in intron 17B and has 7–54 repeats with 87% heterozygosity (Zielenski *et al*., 1991). The extragenic polymorphic markers are also CA-repeats with heterozygosity rates of 80, 77, 87 and 81% respectively (Dreesen *et al*., 2000).

Downloaded from https://academic.oup.com/molehr/article/9/9/559/1110549 by U.S. Department of Justice user on 16 August 2022

Table I. Cystic fibrosis (CF) mutations in 32 couples checked for informativity

Couple	No. of PGD cycles	Male	Female	PCR method
1	1	ΔF508/M265R (CBAVD)	ΔF508/+	IVS17BTA
	3			ΔF508/IVS17BTA
2	2	G551D/+	ΔF508/+	ΔF508/IVS17BTA
3	1	ΔF508/+	W1282X/+	ΔF508/IVS17BTA
4	2	ΔF508/+	?/+	ΔF508/IVS17BTA
5	1	W1282X/+	ΔF508/+	IVS8CA/IVS17BTA
6	1	ΔF508/+	ΔF508/+	ΔF508
7	2	ΔF508/+	W1282X/+	ΔF508
	1			IVS8CA/IVS17BTA
8	4	2183AA→G/+	1717–1G→A/+	1717-1G→A
	2			M470V[a]
9	1	G542X/+	W1282X/+	IVS8CA
10	1	ΔF508/ΔF508 (CF)	R334W/+	D7S486/D7S490
11	3	ΔF508/R117H (CBAVD)	ΔF508/+	M470V[a]
12	0	W1282X/+	W1282X/+	D7S523/D7S486
13	2	ΔF508/+	ΔF508/+	ΔF508
14	1	ΔF508/+	R553X/+	ΔF508/IVS17BTA
15	0	ΔF508/I336K (CBAVD)	G576A/+	D7S486/D7S490
16	0	?	?	IVS17BCA
17	0	ΔF508/+	R117H/+	D7523/D7S486
18	0	L206W/+	D1168G/+	D7S480/M470V
19	3	ΔF508/ΔF508	ΔF508/+	ΔF508/IVS17BTA
20	0	ΔF508/+	ΔF508/+	D7S480/M470V
21	1	ΔF508/+	ΔF508/+	ΔF508/IVS17BTA
22	0	ΔF508/+	W1282X/+	ΔF508/IVS8CA
23	0	G673X/+	ΔF508/+	ΔF508/IVS17BTA
24	2	R117H/+	ΔF508/+	ΔF508/IVS8CA
25	2	ΔF508/+	ΔF508/+	D7S486/D7S490
26	2	ΔF508/+ (CBAVD)	ΔF508/+	ΔF508
	1			ΔF508/IVS17BTA
27	0	G542X/+	ΔF508/+	ΔF508/IVS17BTA
28	1	R1162X/+	3272–26G→A/ΔF508 (CF)	D7S486/D7S490
29	1	R553X/+	ΔF508/+	ΔF508/IVS17BTA
30	0	ΔF508/+	ΔF508/+	ΔF508/IVS17BTA
31	1	ΔF508/+	ΔF508/+	ΔF508/IVS17BTA
32	0	?/+	ΔF508/+	IVS8CA/IVS17BTA

[a]M470V: bi-allelic intragenic polymorphism.
CBAVD = Congenital bilateral absence of the vas deferens; ? = unknown mutation.

Here we describe duplex PCRs that amplify two markers or one marker combined with the ΔF508 mutation.

This approach allows us to offer PGD to couples in whom the mutation(s) is (are) unknown or occur(s) with low frequency. Moreover, a duplex PCR for these markers has to be developed once and can then be applied to all couples who are informative. Informativity means that chromosomes carrying the mutation can be identified. By analysing family members, it is possible to determine which alleles co-segregate with CF. If the mutations are not known, the couple must have an affected child in order to be able to analyse which alleles co-segregate with the mutation.

A duplex PCR of a mutation and a linked marker is not only a diagnostic tool, but it can also be used as a control for ADO and contamination. For example, if, during a PGD, two marker alleles, coupled to the wild-type CF allele of both partners, are observed in an embryo, then only one fragment representing the wild-type allele should be observed. The finding of a carrier genotype with mutant and normal CF alleles would indicate contamination in the PCR carried out for the mutation or recombination between the marker and the mutation. If, on the other hand, analysis of the marker alleles indicates one allele linked to the wild-type allele and one linked to the mutant CF allele, a carrier CF genotype is expected. If, however, only a wild-type CF allele is observed, the conclusion of ADO can be drawn. Without co-amplification of the marker, these embryos could be diagnosed incorrectly, which could lead to the transfer of affected embryos. Undetected ADO of the normal CF allele on the other hand leads to the loss of unaffected embryos (Dreesen *et al*., 2000; Eftedal *et al*., 2001; Moutou *et al*., 2002).

Here we present the development and clinical application of four duplex PCR: a PCR for the marker IVS8CA in combination with IVS17BTA, one for the ΔF508 mutation and the marker IVS8CA, a PCR for the duplex ΔF508 and IVS17BTA and a fourth duplex PCR of D7S486 and D7S490.

Materials and methods

Collection of single lymphoblasts

Lymphoblasts transformed by the Epstein–Barr virus (EBV) were cultured as described by Ventura *et al*. (1988). A few colonies were collected and washed three times in 500 µl phosphate-buffered saline (PBS).

Single lymphoblasts were then collected as described earlier by Sermon *et al*. (1998). Briefly, they were transferred to droplets of Ca^{2+}- and Mg^{2+}-free medium, washed in three different droplets and transferred blindly to a PCR tube containing 2.5 µl alkaline lysis buffer (ALB; 50 mmol/l dithiothreitol, 200 mmol/l KOH or 200 mmol/l NaOH) (Li *et al*., 1988). An aliquot from the last washing droplet was taken to serve as a blank.

Downloaded from https://academic.oup.com/molehr/article/9/9/559/1110549 by U.S. Department of Justice user on 16 August 2022

Table II. PCR procedures

	Alkaline lysis buffer	1st round			2nd round	
		Reaction mix	Program		Reaction mix	Program
IVS8CA/IVS17BTA	KOH	RB1[a] + 2.5% DMSO 0.4 μmol/l CFTR 8.1/8.2 0.4 μmol/l CFTR 1/5	5 min 96°C, 10×(30 s 96°C, 30 s 50°C, 30 s 72°C), 5 min 72°C	IVS8CA	RB2[b] + 2.5% DMSO 0.4 μmol/l CFTR 8.1/8.2	5 min 94°C, 40×(30 s 94°C, 30 s 50°C, 30 s 72°C), 5 min 72°C
				IVS17BTA	RB2[b] + 5% DMSO 0.4 μmol/l CFTR 1/5	
ΔF508/IVS8CA	NaOH	RB3[c] 0.2 μmol/l KSCF1/CF2 0.4 μmol/l CFTR 8.1/8.2	5 min 95°C, 10×(30 s 96°C, 30 s 50°C, 30 s 72°C) 5 min 72°C	ΔF508	RB2[b] 0.4 μmol/l KSCF1/CF2	5 min 94°C, 29× (30 s 94°C, 30 s 55°C, 30 s 72°C), 5 min 72°C
				IVS8CA	RB4[d] + 2.5% DMSO 0.4 μmol/l CFTR 8.1/8.2	5 min 95°C, 40× (30 s 95°C, 30 s 50°C, 30 s 72°C), 5 min 72°C
ΔF508/IVS17BTA	NaOH	RB3[c] + 5% DMSO 0.2 μmol/l KSCF1/CF2 0.4 μmol/l CFTR1/5	5 min 95°C, 10×(30 s 95°C, 30 s 55°C, 30 s 72°C), 5 min 72°C	ΔF508	RB2[b] 0.4μmol/l KSCF1/CF2	5 min 94°C, 29× (30 s 94°C, 30 s 55°C, 30 s 72°C), 5 min 72°C
				IVS17BTA	RB4[d] + 5% DMSO 0.4 μmol/l CFTR 1/5	5 min 95°C, 37× (30 s 95°C, 30 s 50°C, 30 s 72°C), 5 min 72°C
D7S486/D7S490	NaOH	RB3[c] + 5% DMSO 0.2 μmol/l D7S486F/R 0.4 μmol/l D7S490F/R	5 min 95°C, 10×(30 s 95°C, 30 s 55°C, 30 s 72°C), 5 min 72°C	D7S486	RB4[d] + 2.5% DMSO 0.4 μmol/l D7S486F/R	5 min 95°C, 35×(30 s 95°C, 30 s 55°C, 30 s 72°C), 5 min 72°C
				D7S490	RB4[d] + 2.5% DMSO 0.4 μmol/l D7S490F/R	

[a]RB (reaction buffer) 1: 200 μmol/l from each dNTP, 100 μmol/l Tris–HCl (pH 8.3), 2 mmol/l $MgCl_2$, 0.01% gelatine, 1.25 IU Taq polymerase.
[b]RB2: 200 μmol/l from each dNTP, 1×RBII (Perkin Elmer), 2 mmol/l $MgCl_2$, 1.25 IU Taq polymerase.
[c]RB3: 200 μmol/l from each dNTP, 1×Hifi buffer 2 (Roche), 0.875 IU Hifi polymerase.
[d]RB4: 200 μmol/l from each dNTP, 1×Hifi buffer 2, 1.4 IU Hifi polymerase.

Table III. Primer sequences

	Primers	Sequences
ΔF508	KSCF1	5′Cy5-AATTGGAGGCAAGTGAATCC-3′
	CF2	5′-GTTGGCATGCTTTGATGACGCCTC-3′
IVS8CA	CFTR8.1 8.8.18.1	5′-Cy5-ACTAAGATATTTGCCCATTATCAAGT-3′
	CFTR8.2	5′-TCTATCTCATGTTAATGCTG-3′
IVS17BTA	CFTR 1	5′-GCTGCATTCTATAGGTTATC-3′
	CFTR 5	5′Cy5-GACAATCTCGTGTGCATCG-3′
D7S486	D7S486F	5′Cy5-AAAGGCCAATGGTATATCCC-3′
	D7S486R	5′-GCCCAGGTGATTGATTGATAGTGC-3′
D7S490	D7S490F	5′Cy5-CCTTGGGGCCAATAAGGTAAG-3′
	D7S490R	5′-AGCTACTTGCAGTGTAACAGCATTT-3′

Patient work-up

Ethical approval was granted for the study from the institutional ethical committee and all patients gave informed consent. Couples requesting PGD for CF in whom two or more (as in CBAVD and CF patients) different mutations were found or in whom the mutation was not yet known were checked for informativity. A fluorescent PCR, using the polymorphic markers, was therefore carried out on the couples' genomic DNA, extracted from peripheral blood. A couple was considered to be informative if the affected alleles from both partners could be clearly distinguished from the non-affected. This meant that both partners of the couple were heterozygotes for one of the polymorphic markers and that segregation from the affected alleles could be determined. It is not necessary for all polymorphic alleles to be different.

Table I shows the different mutations that were encountered as well as relevant clinical information on the 32 couples checked for informativity of CFTR markers.

Downloaded from https://academic.oup.com/molehr/article/9/9/559/1110549 by U.S. Department of Justice user on 16 August 2022

Table IV. Informativity of 29 informative couples (out of 32 couples investigated)

Patient		IVS17BTA	IVS8CA	IVS17BCA	D7S523	D7S486	D7S480	D7S490
1	A	**248[a]–274**	177–177					
	B	**240[a]–240[a]**	177–177					
	D	**242–248[a]**	177–?					
	E	**254–274**	177–?					
	F	**240[a]–240**	177–?					
	G	**198–240[a]**	177–?					
2	A	**248[a]–252**	**177[a]–191**	136–136				
	B	**200[a]–248**	**177[a]–191**	136–144				
	C	**200[a]–248[a]**	**177[a]–177[a]**	136–144				
3	A	**250[a]–248**	179–193	136–136				
	B	**200[a]–280**	179–179	136–144				
	C	**200[a]–250[a]**	179–193	136–144				
4	A	**200[a]–250**	177–177	136–144				
	B	**200–246[a]**	177–179	136–144				
	C	**200[a]–246[a]**	177–179	136–144				
5	A	**200–248[a]**	**179[a]–191**	**134–144[a]**	234–234	140–140	188–198	96–96
	B	**200[a]–244**	**135[a]–177**	**136[a]–144**	228–234	140–144	196–196	96–106
	C	**200[a]–248[a]**	**135[a]–179[a]**	**136[a]–144[a]**	228–234	140–140	196–198	96–96
6	A	**248–280**	**177–185**	136–136				
	B	**244–248**	**175–189**	136–136				
7	A	**200[a]–246**	**179[a]–189**	136–144				
	B	**248[a]–274**	**177–191**	136–136				
	C	**200[a]–248[a]**	**179[a]–191[a]**	136–144				
9	A	200–200	**177–179[a]**	134–142				
	B	244–256	**177–191[a]**	134–134				
	C	200–256	**179[a]–191[a]**	134–134				
10	A	200–278	177–177	136–144	232–232	**132–142[a]**	190–190	**90–102[a]**
	B	250–282	179–179	136–136	230–240	**140[a]–142[a]**	200–200	**94[a]–94[a]**
	D	200–278	177–177	136–144	232–232	**140–142[a]**	190–190	**88–102[a]**
	E	200–278	177–177	136–144	232–232	**132–140[a]**	190–194	**90–94[a]**
12	A	200–200	179–179	144–144	230–230	**134[a]–136**	194–194	**94–96[a]**
	B	200–200	177–179	144–144	228–234	**140–142[a]**	194–194	**92–94[a]**
	C	200–200	177–179	144–144	228–230	**134[a]–142[a]**	194–194	**94[a]–96[a]**
14	A	**244–296[a]**	177–179	130–134				
	B	**248[a]–248**	177–179	134–134				
	C	**248[a]–296[a]**	179–179	130–134				
15	A	200–248	177–177	136–144	**234–238[a]**	**134[a]–144**	**190–192[a]**	**96[a]–98**
	B	250–250	177–177	136–136	**230[a]–234[a]**	**140[a]–140[a]**	**198[a]–200[a]**	**92[a]–94[a]**
	D	200–248	177–177	136–144	**230–238[a]**	**134[a]–140**	**188–192[a]**	**92–96[a]**
16	A	200–246	177–177	**142[a]–144**	**230[a]–236**	**136[a]–142**	**196–200[a]**	**92[a]–98**
	B	246–246	177–177	**134–142[a]**	**226–230[a]**	**134–136[a]**	**188–200[a]**	**92[a]–96**
	C	246–246	177–177	**142[a]–142[a]**	**230[a]–230[a]**	**136[a]–136[a]**	**200[a]–200[a]**	**92[a]–92[a]**
17	A	250–252	177–177	136–136	**230–234[a]**	140–144	**190–200[a]**	**90–92[a]**
	B	250–252	177–191	136–136	**230–234[a]**	140–140	**190–200[a]**	**94[a]–96**
	C	250–252	177–191	136–136	**234[a]–234[a]**	140–144	**200[a]–200[a]**	**92[a]–94[a]**
18	A	202–202	177–179	144–144	230–234	140–140	**194–196[a]**	90–102
	B	202–250	177–177	136–144	230–234	140–142	**186[a]–200**	90–94
	D	202–256	179–191	136–144	234–234	140–140	**188–196[a]**	90–102
	E	202–248	177–177	136–144	230–230	140–140	**194–200**	90–102
	F	250–252	177–177	136–136	230–234	140–142	**200–200**	90–98
	G	202–202	177–177	144–144	230–230	140–140	**186[a]–194**	90–94
19	A	**248–250[a]**	177–191	136–136				
	B	**202[a]–250[a]**	179–191	136–144				
	D	**230–248**	177–177	136–144				
	E	**202–250[a]**	177–191	136–144				
20	A	248–248	175–189	134–134	228–228	140–140	**186–196[a]**	84–96
	B	248–250	189–189	134–134	230–236	140–142	**186–190[a]**	96–96
	D	244–248	175–175	134–136	228–232	140–144	**186–186**	84–94
	E	246–248	175–189	134–134	228–228	140–140	**182–196[a]**	96–102
	G	246–250	175–189	134–134	234–236	132–140	**186–196**	96–96
21	A	**250[a]–278**	**175–177[a]**	136–136				
	B	**228–252[a]**	**177[a]–187**	136–148				
	C	**250[a]–252[a]**	**177[a]–177[a]**	136–136				
22	A	**200[a]–274**	**175–177[a]**	136–136				
	B	**244–248[a]**	**175–191[a]**	136–136				
	D	**274–280**	**175–175**	134–134				
	E	**200[a]–200**	**175–177[a]**	144–144				
	F	**248[a]–248**	**175–191[a]**	134–134				
	G	**244–246**	**175–175**	134–138				
23	A	**256[a]–278**	177–181	136–136				
	B	**250[a]–254**	191–191	136–136				
	C	**250[a]–256[a]**	177–191	136–136				

Downloaded from https://academic.oup.com/molehr/article/9/9/559/1110549 by U.S. Department of Justice user on 16 August 2022

Table IV. *Continued*

Patient		IVS17BTA	IVS8CA	IVS17BCA	D7S523	D7S486	D7S480	D7S490
24	A	250–250	**177–179[a]**	134–134				
	B	226–248	**177[a]–191**	134–148				
	E	202–250	**179–179[a]**	134–144				
	F	248–252	**177–177[a]**	134–134				
	G	202–226	**177–191**	144–148				
25	A	250–250	177–177	136–138	234–234	**132–142[a]**	**196[a]–200**	**90[a]–102**
	B	250–250	179–191	136–136	230–230	**132[a]–140**	**198[a]–202**	**96–98[a]**
	D	250–250	177–191	136–138	230–234	**132–140**	**200–200**	**92–102**
	E	246–250	177–177	136–136	230–234	**140–142[a]**	**196[a]–200**	**90[a]–90**
	F	202–250	187–191	136–146	230–234	**140–140**	**196–202**	**90–96**
	G	205–250	177–179	136–148	230–230	**132[a]–142**	**198[a]–198**	**92–98[a]**
26	A	**244[a]–248**	175–177	136–136	230–230	**140[a]–144**	**194–196[a]**	**90[a]–102**
	B	**246–248[a]**	175–177	136–136	226–230	**144[a]–144**	**198–200[a]**	**96[a]–98**
	C	**244[a]–248[a]**	175–177	136–136	230–230	**140[a]–144[a]**	**196[a]–200[a]**	**90[a]–96[a]**
27	A	**248–250[a]**	177–177	136–136				
	B	**250[a]–254**	179–189	136–136				
	C1	**248–250[a]**	177–189	136–136				
	C2	**248–250[a]**	177–189	136–136				
28	A	**200[a]–250[a]**	173–175	138–146		**140[a]–146[a]**	**186[a]–196[a]**	**88[a]–102[a]**
	B	**244–248[a]**	173–175	140–140		**140–144[a]**	**186[a]–198**	**94–96[a]**
	D	**200[a]–200[a]**	173–173	146–146		**140[a]–140**	**196[a]–200**	**92–102[a]**
	F	**200–244**	173–175	140–148		**138–140**	**194–198**	**88–94**
	G	**248[a]–272**	173–175	140–140		**142–144[a]**	**186[a]–186**	**88–96[a]**
29	A	**246–250[a]**	175–175	136–136				
	B	**200–256[a]**	175–179	136–144				
	D	**246–280**	175–175	136–136				
	F	**248–256[a]**	175–179	136–136				
30	A	**244[a]–274**	177–189					
	B	**244[a]–252**	189–189					
	C1	**244[a]–244[a]**	189–189					
	C2	**244[a]–274**	**177–189[a]**					
31	A	**248[a]–274**	**175–177[a]**					
	B	**246[a]–286**	**177–189[a]**					
	C1	**246[a]–274**	**175–189[a]**					
	C2	**246[a]–248[a]**	**177[a]–189[a]**					
32	A	**242–246[a]**	**175–189[a]**					
	B	**246[a]–256**	**171[a]–189**					
	C	**246[a]–246[a]**	**171[a]–189[a]**					

A = wife; B = husband; C = child; D = mother of the wife; E = father of the wife; F = mother of the husband G = father of the husband.
Bold type: informative markers.
[a]Alleles that segregate with the mutation.

ICSI procedure

ICSI was performed in all cycles to prevent contamination with sperm adhering to the zona pellucida (ZP) and to ensure fertilization (Liebaers *et al*., 1998). The ICSI procedure was carried out as described by Joris *et al*. (1998).

Briefly, the patients underwent controlled ovarian stimulation by administration of a GnRH agonist or antagonist in association with human urinary menopausal or recombinant gonadotrophins and hCG (Vandervorst *et al*., 1998).

Oocyte retrieval was carried out by vaginal ultrasound-guided puncture of the ovarian follicles, 36 h after hCG was administered. The cells of the cumulus oophorus and corona radiata were removed (Van de Velde *et al*., 1997) and metaphase II oocytes were injected with a single sperm into the ooplasm. Ejaculated sperm or testicular sperm after fine needle aspiration were used (Van Steirteghem, 1997). The injected oocytes were incubated at 37°C in 25 µl of G1/G2 medium in an incubator (37°C, 5% O_2, 5% CO_2, 90% N_2) (Heraeus; Vander Heyden, Belgium). After 16–18 h, fertilization was assessed and further development of the embryos was evaluated on days 2 and 3 prior to biopsy.

Embryo biopsy

A hole was made in the ZP using a non-contact 1.48 µm diode laser system (Fertilase; MTM Medical Technologies, Switzerland) coupled to an inverted microscope in order to deliver two or three laser pulses of 16 ms to the ZP, creating a funnel-shaped hole (Sermon *et al*., 1999).

One or two blastomeres were then gently aspirated through the hole. Embryos in which compaction had already started were incubated for 5–10 min in Ca^{2+}- and Mg^{2+}-free medium (In Vitro Life, Sweden) before biopsy.

Further procedures were then performed using a stereomicroscope. Each blastomere was washed three times in (home-made) Ca^{2+}- and Mg^{2+}-free medium (Sermon *et al*., 1998) and transferred to a 0.2 ml PCR tube containing 2.5 µl ALB. They were kept at –80°C for ⩾30 min (Sermon *et al*., 1998).

Cell lysis

The cells in ALB were further lysed by incubating them at 65°C for 10 min (Sermon *et al*., 1995). After lysis the PCR tubes were immediately put on ice.

The ALB was neutralized afterwards with 2.5 µl neutralization buffer (NB) (0.9 mol/l Tris–HCl; pH 8.3, 0.3 mol/l KCl, 0.2 mol/l HCl) for KOH-containing ALB or with 2.5 µl Tricine (500 mmol/l; pH 4.9) for NaOH-containing ALB (Sermon *et al*., 1998).

PCR procedures

The procedures used to perform the intragenic and extragenic marker-based duplex PCR are summarized in Table II. Two consecutive PCR rounds were performed in all four duplex PCR. Three microlitres from the first PCR round were taken as a template for the second round PCR (Goossens *et al*., 2000). The reason for separating our PCR reactions after 10 cycles is that we started from published primer sequences without any adaptations with regard to annealing

Downloaded from https://academic.oup.com/molehr/article/9/9/559/1110549 by U.S. Department of Justice user on 16 August 2022

Table V. Results on lymphoblasts: comparison between single PCR and duplex PCR for intragenic markers. Results on lymphoblasts: extragenic duplex PCR

	ΔF508	IVS8CA	IVS17BTA	ΔF508/IVS8CA		ΔF508/IVS17BTA		IVS8CA/IVS17BTA		D7S486/D7S490	
				ΔF508	IVS8CA	ΔF508	IVS17BTA	IVS8CA	IVS17BTA	D7S486	D7S490
Amplification	27/30 (90)	27/30 (90)	43/45 (96)	46/50 (92)	47/50 (94)	39/40 (98)	37/40 (93)	29/30 (97)	28/30 (93)	43/50 (95)	42/45 (93)
Allele drop-out rate	0/27 (0)	0/27 (0)	1/43 (2.3)	1/46 (2.2)	1/47 (2.1)	0/39 (0)	1/37 (3)	1/29 (3.5)	1/28 (3.6)	2/43 (4.6)	0/42 (0)
Contamination rate	0/30 (0)	0/30 (0)	3/45 (6.7)	1/50 (2)	1/50 (2)	0/40 (0)	0/40 (0)	1/30 (3.3)	1/30 (3.3)	2/45 (4.4)	2/45 (4.4)

Values in parentheses are percentages.

Table VI. Details of the clinical preimplantation genetic diagnosis cycles with duplex PCR

	Patient	Cycle	COC	ICSI	2PN	Embryos					Transfer	Pregnancy	Baby born
						Biopsied	Affected	Carrier	Unaffected	No diagnosis			
IVS8CA/IVS17BTA	5	1	16	14	10	3	2	1	0	0	1	0	0
	7	1	8	6	4	3	1	1	0	1	1	0	0
ΔF508/IVS8CA	24	1	4	4	4	4	1	3	0	0	2	0	0
		2	5	3	3	3	0	2	1	0	3	1	0
ΔF508/IVS17BTA	1	1	19	16	13	10	4	4	0	2	2	0	0
		2	22	16	11	8	4	2	0	2	2	0	0
		3	22	17	15	7	4	2	0	1	0	0	0
	2	1	6	5	5	5	2	1	2	0	2	0	0
		2	4	4	4	4	1	3	0	0	3	0	0
	3	1	10	8	6	3	1	1	1	0	2	0	0
	4	1	9	6	5	5	1	3	0	1	3	1[a]	0
		2	13	13	8	8	2	2	1	3	2	1	1
	14	1	15	12	8	7	2	4	0	1	2	0	0
	19	1	4	4	4	0	0	0	0	0	0	0	0
		2	6	5	4	3	2	1	0	0	2	0	0
		3	7	6	4	3	2	0	1	0	1	0	0
	21	1	5	5	5	3	1	2	0	0	2	0	0
	26	1	11	10	7	3	1	1	1	0	2	1	0
	29	1	11	10	6	5	0	4	1	0	3	0	0
	31	1	15	12	9	6	2	3	0	1	2	0	0
D7S486/D7S490	10	1	21	18	15	9	2	4	0	3	2	1	1
	25	1	6	4	3	0	0	0	0	0	0	0	0
		2	9	9	8	3	0	0	1	2	1	0	0
	28	1	5	4	3	3	1	2	0	0	2	0	0
Total		24	253	211 (83.4)	164 (77.7)	108 (65.9)	36 (33.3)	46 (42.6)	9 (8.3)	17 (15.7)	42	5 (20.8)	2

Values in parentheses are percentages.
COC = cumulus–oocyte complexes; 2PN = presence of two pronuclei.

temperature or fragment length. Some of the fragments did overlap in length, which meant that we were not able to detect them by using the ALF, since only one fluorescent label is available. In addition we also noticed that the efficiency of the PCR is often much better when the reaction is separated. Primer sequences are given in Table III. The primers were labelled with Indocarbocyanine (CY5) since electrophoresis was performed on an automated laser fluorescence DNA sequencer (Amersham Pharmacia Biotech, The Netherlands).

Results

Informativity

In a first stage, the informativity of all candidate couples was checked for the three intragenic polymorphic markers IVS8CA, IVS17BTA and IVS17BCA and/or for the four extragenic polymorphic markers D7S486, D7S480, D7S490 and D7S523. Therefore, PCR was carried out on 100 ng of the couples' genomic DNA as well as on the DNA of affected children if present or of the patients' parents. Table IV shows the results of the informativity testing.

In total, informativity was analysed in 32 couples. Twenty couples were only tested for the three intragenic polymorphic markers. Seven couples were found to be informative for both IVS17BTA and IVS8CA. Nine other couples were informative for the IVS17BTA polymorphism only while two were informative for IVS8CA. Two couples remained uninformative (not shown in Table IV). Twelve out of the 32 couples were also checked for the extragenic polymorphic markers. One couple was informative for all three intragenic markers but for none of the extragenic markers. Two couples were informative for D7S480 only, two other couples were informative for D7S486 and D7S490. One couple was found to be informative for D7S480, D7S490 and D7S523. One other couple was informative for the three extragenic markers: D7S480, D7S486 and D7S490. One couple was informative for all extragenic markers D7S480, D7S486, D7S490 and D7S523. Furthermore, two couples were informative for IVS17BTA and D7S480, D7S486 and D7S490. One couple was informative for IVS17BCA, D7S480, D7S486, D7S490 and D7S523. For one couple, diagnosis remained impossible using this strategy, since they were uninformative for all seven markers (not shown in Table IV). If one

Downloaded from https://academic.oup.com/molehr/article/9/9/559/1110549 by U.S. Department of Justice user on 16 August 2022

Table VII. PCR characteristics on blastomeres

	ΔF508[a]	IVS8CA[a]	IVS17BTA[a]	D7S486	D7S490
Amplification efficiency	150/158 (95)	21/22 (95)	137/152 (90)	25/27 (93)	25/27 (93)
Allele drop-out	6/75 (8)	1/15 (7)	6/122 (5)	0/19 (0)	0/23 (0)

Values in parentheses are percentages.
[a]Combined results of three different duplexes (ΔF508/IVS8CA, ΔF508/IVS17BTA and IVS8CA/IVS17BTA).

takes into account the patients who were checked for the intragenic markers only and the patients checked for all seven markers, 29 out of 32 couples were informative for at least one polymorphic marker.

Lymphoblasts from heterozygous individuals

To develop a single cell PCR procedure, lymphoblasts from heterozygous individuals were analysed to evaluate the amplification, the allele drop-out (ADO) and the contamination rate.

The results are summarized in Table V. The amplification rate for all four duplex PCR ranges from 92 to 98%, after split of the reaction. The ADO rate in the four PCR ranges from 0 to 4.6%, while 0–4.4% of the blanks were contaminated.

Data from simplex PCR that were developed earlier (Goossens *et al*., 2000) have been included for comparison of amplification, ADO and contamination rates.

Clinical PGD procedures

Eighteen of the informative couples have already undergone PGD in 31 cycles. Twenty-two cycles in which a biopsy could be performed (16 couples) were carried out with a duplex PCR: two cycles with the duplex IVS8CA/IVS17BTA, two cycles with the duplex ΔF508/IVS8CA, fifteen cycles with the duplex ΔF508/IVS17BTA and three cycles with the duplex D7S486/D7S490. Two started cycles (one with the duplex ΔF508/IVS17BTA and one with the duplex D7S486/D7S490) remained without biopsy because no good quality embryos were available.

In total, 253 cumulus–oocyte complexes were obtained and ICSI was performed in 211 metaphase II oocytes (83.4%). A total of 164 injected oocytes (77.7%) showed two pronuclei (2PN), proving normal fertilisation. Of these, 108 (65.8%) cleaved further beyond the 6-cell stage and could be biopsied removing one or two blastomeres. Because of a recently started study that compares the removal of one versus two blastomeres, only one blastomere was removed in four cycles. Thirty-six of all biopsied embryos (33.3%) were affected, 46 embryos were carriers (42.6%) and nine (8.3%) were unaffected. Seventeen embryos remained without diagnosis (15.7%). Forty-two embryos were transferred in 21 transfers (2.05 embryos per transfer). Total pregnancy rates were 23.8% per transfer, 22.7% per PGD cycle with biopsy and 20.8% per started PGD cycle (Table VI).

In the first two cycles (two couples) with the duplex IVS8CA/IVS17BTA, six embryos were analysed, two were carriers, three were affected and one remained without diagnosis. The two carrier embryos were transferred, one in each cycle, but the patients did not achieve pregnancy.

The two cycles (one couple) with the duplex ΔF508/IVS8CA gave the following results. Seven embryos were analysed, five were carriers, one was affected and one was unaffected. In the first cycle, two carrier embryos were transferred. The second transfer cycle with two carrier and one healthy embryo resulted in an ongoing pregnancy.

In the 15 cycles to biopsy (10 couples) with the duplex ΔF508/IVS17BTA, 80 embryos were analysed, of which seven were unaffected, 33 were carriers and 29 were affected. Eleven embryos remained without diagnosis. In total, 30 embryos were transferred in 14 transfer cycles. One cycle ended without transfer due to technical problems: the dish with embryos was mixed up in the IVF laboratory and it was impossible to identify the healthy embryos afterwards. In the other cycles three pregnancies occurred of which one ended in a miscarriage. One twin pregnancy is ongoing. The other pregnancy resulted in the birth of a healthy boy.

There were three cycles to biopsy with the duplex D7S486/D7S490 in three couples. Fifteen embryos were analysed. Six embryos were carriers, three were affected, one embryo was unaffected and five remained without diagnosis. Five embryos were transferred in three transfer cycles. One patient became pregnant and delivered a healthy boy.

When no diagnosis was obtained (in total: 17 out of 108 embryos i.e. 15.7%), it was a result of either ADO in one or two cells or total failure of cell amplification. In this series of blastomeres no discordant results were obtained. Diagnosis is of course only assigned when both cells yield the same result, or when both loci provide concordant results if only one cell result is available (one cell biopsy or amplification failure in one cell). Amplification efficiency and ADO are shown in Table VII.

Discussion

Here we have described the development and clinical application of preimplantation genetic diagnosis for CF using four duplex PCR. In total, 16 couples were treated. In a preclinical setting, the single cell duplex PCR were tested on single lymphoblasts.

Until now we have used the ALF system for fragment analysis. To avoid overlapping fragments while using this single colour system, we would like to switch to a multicolour system such as the ABI prism 3100-Avant Genetic Analyzer (Applied Biosystems). This would make it possible to detect two fragments of the same length.

The use of polymorphisms in duplex PCR (either two different polymorphisms or one polymorphism combined with the ΔF508 detection) can be important on three levels.

Initially, we used polymorphisms when the partners carried different mutations. The diagnosis of compound heterozygote embryos using a duplex for two different mutations can lead to the transfer of affected embryos if ADO of the affected allele occurs in one mutation. This is why in the past we used to analyse only one mutation in two cells and transferred only embryos free from the mutation (Goossens *et al*., 2000). Carrier embryos for the analysed mutation cannot be transferred through this approach because of their unknown status for the second mutation. However, half of these embryos are indeed free of the second mutation, which means that healthy carrier embryos are lost for transfer. By means of polymorphisms, two or more (CBAVD, CF patients) different mutations can be indirectly analysed at once, decreasing the possible loss of healthy embryos for transfer.

A second advantage of these polymorphisms in duplex with a second polymorphism or a specific mutation detection is that ADO and contamination, two well-known problems of single cell PCR, can be detected.

Downloaded from https://academic.oup.com/molehr/article/9/9/559/1110549 by U.S. Department of Justice user on 16 August 2022

A very important third reason is that so far >1000 CF mutations have been identified. It would be very labour-intensive to develop a PCR assay for all PGD patients presenting different mutations. It is much easier to develop a battery of tests for polymorphisms to be used instead of direct mutation analysis. By using four extragenic polymorphic markers, two on both sides of the gene, and three intragenic polymorphic markers, the whole gene can be covered without too large a risk for recombination (2.2–6% between the most 3′ and the most 5′ marker) (Dreesen *et al*., 2000). It must be stressed that if the markers are well chosen in relation to the mutations, recombination will lead to the embryos not being assigned a diagnosis, rather than to transfer of affected embryos.

We would like to emphasize that although the heterozygosity rates of IVS17BTA (87%), IVS8CA (48%), IVS17BCA (39%), D7S523 (81%), D7S486 (77%), D7S480 (87%) and D7S490 (80%) are relatively high and the ΔF508 mutation accounts for 70% of all CF mutations, this does not mean that the couples are also informative. This is often the case in families where there are no affected children and the informativity testing has to rely on the patient-couple and their respective parents. We often see individuals who are heterozygotes, but the same combination of allele sizes is often observed in a family. This leads to a high heterozygosity rate, but a lower informativity rate.

In our 32 patient-couples, there were 27 couples (84.37%) in whom at least one of both partners carried a ΔF508 mutation. In thirteen of the 27 couples (48%) for whom a duplex PCR was developed, the duplex IVS17BTA/ΔF508 could be used. In three out of 27 (11.11%) cases IVS8CA/IVS17BTA could be used and in two out of 27 (7.41%) cases IVS8CA/ΔF508 was used. For the extragenic polymorphic markers in four out of 27 (14.81%) patients, D7S486/D7S490 was used, and for the combinations D7S480/D7S523 and D7S480/M470V (no PGD cycles yet) two out of 27 (7.41%) couples were informative each time. In total, six duplex PCR were developed, useful for 27 couples out of the 32 couples checked for informativity (84.37%). Moutou *et al*. (2002) presume that by using the combinations IVS17BCA/ΔF508 and IVS8CA/ΔF508, 80% of their patient-couples can be helped. Our own numbers are therefore much lower than would be expected in theory. This means that a whole battery of duplex PCR will have to be developed. A more efficient solution would be to develop a multiplex of all extragenic/intragenic markers in combination with the ΔF508 mutation (Dreesen *et al*., 2000).

So far, five pregnancies in four patients have resulted from our 22 PGD with duplex PCR for CF. One patient had a miscarriage but became pregnant a second time. In two patients the PGD results were confirmed by prenatal diagnosis. The two other patients refused prenatal diagnosis because of objections against abortion. Two healthy babies were born.

The amplification and ADO rates obtained during PGD did not differ from the preclinical data obtained with lymphoblast analysis, showing the usefulness of these cells as a model. The relatively low number of homozygous normal embryos (8.3%) is partially due to the presence of two CBAVD patients and two CF patients, who cannot have homozygous normal embryos. If we exclude these patients, there are eight homozygous normal embryos (11.5%), which is still somewhat lower than expected.

Development of duplex PCR with other combinations of markers and the development of multiplex PCR will continue in order to be able to help almost all couples requesting PGD for CF.

Acknowledgements

The authors wish to thank the clinical, paramedical and laboratory staff at the Centres for Medical Genetics and Reproductive Medicine for their help and commitment. Furthermore we are grateful to J.Deconinck of the Language Education Centre at our University. This work was supported by grants from the Belgian Fund for Scientific Research, Flanders (FWO-Vlaanderen). V.G. was funded by the Forton Foundation and is now supported by grants from Fund for Scientific Research Flanders. K.S. and and M.D.R. were supported by grants from the Funds for Scientific Research Flanders. B.S. is employed by the Forton Foundation.

References

Doull, I.J.M. (2001) Recent advances in cystic fibrosis. *Arch. Dis. Child.*, **85**, 62–66.

Dreesen, J.C.F.M., Jacobs, L.J.A.M., Bras, M., Herbergs, J., Dumoulin, J.C.M., Geraedts, J.P.M., Evers, J.L.H. and Smeets, H.J.M. (2000) Multiplex PCR of polymorphic markers flanking the CFTR gene; a general approach for preimplantation genetic diagnosis of cystic fibrosis. *Mol. Hum. Reprod.*, **6**, 391–396.

Eftedal, I., Schwartz, M., Bendtsen, H., Andersen, A. and Zieve, S. (2001) Single intragenic microsatellite preimplantation genetic diagnosis for cystic fibrosis provides positive allele identification of all CFTR genotypes for informative couples. *Mol. Hum. Reprod.*, **7**, 307–312.

Goossens, V., Sermon, K., Lissens, W., Vandervorst, M., Vanderfaeillie, A., De Rijcke, M, De Vos, A., Henderix, P., Van de Velde, H., Van Steirteghem, A. and Liebaers, I. (2000) Clinical application of preimplantation genetic diagnosis for cystic fibrosis. *Prenat. Diagn.*, **20**, 571–581.

Handyside, A.H., Lesko, J.G., Tarin, J.J., Winston, R.M. and Hughes, M.R. (1992) Birth of a normal girl after in vitro fertilization and preimplantation diagnostic testing for cystic fibrosis. *N. Engl. J. Med.*, **327**, 905–909.

Joris, H., Nagy, P., Van de Velde, H., De Vos, A. and Van Steirteghem, A. (1998) Intracytoplasmic sperm injection: laboratory set-up and injection procedure. *Hum. Reprod.*, **13** (Suppl. 1), 107–116.

Li, H., Gyllensten, U.B., Cui, X., Saiki, R.K., Erlich, H.A. and Arnheim, N. (1988) Amplification and analysis of DNA sequences in single human sperm and diploid cells. *Nature*, **335**, 414–417.

Liebaers, I., Sermon, K., Staessen, C., Joris, H., Lissens, W., Van Assche, E., Nagy, P., Bonduelle, M., Vandervorst, M., Devroey, P. and Van Steirteghem, A. (1998) Clinical experience with preimplantation genetic diagnosis and intracytoplasmic sperm injection. *Hum. Reprod.*, **13** (Suppl. 1), 86–95.

Lissens, W. and Sermon, K. (1997) Preimplantation genetic diagnosis: current status and new developments. *Hum. Reprod.*, **12**, 1756–1761.

Lissens, W., Mercier, B., Tournaye, H., Bonduelle, M., Férec, C., Seneca, S., Devroey, P., Silber, S., Van Steirteghem, A. and Liebaers, I. (1996) Cystic fibrosis and infertility caused by congenital bilateral absence of the vas deferens and related clinical entities. *Hum. Reprod.*, **11** (Suppl. 4), 55–78.

Moutou, C., Garde, N. and Viville, S. (2002) Multiplex PCR combining deltaF508 mutation and intragenic microsatellites of the CFTR gene for pre-implantation genetic diagnosis (PGD) of cystic fibrosis. *Eur. J. Hum. Genet.*, **10**, 231–238.

Riordan, J.R., Romens, J.M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J. *et al*. (1989) Identification of the cystic fibrosis gene: cloning and characterisation of complementary DNA. *Science*, **245**, 1066–1072.

Sermon, K., Lissens, W., Devroey, P., Van Steirteghem, A. and Liebaers, I. (1995) Amplification of exon 11 of the gene for the α-chain of β-N-acetylhexosaminidase in single blastomeres. *Fertil. Steril.*, **63**, 407–409.

Sermon, K., De Vos, A., Van de Velde, H., Seneca, S., Lissens, W., Joris, H., Vandervorst, M., Van Steirteghem, A. and Liebaers, I. (1998) Fluorescent PCR and automated fragment analysis for the clinical application of preimplantation genetic diagnosis of myotonic dystrophy (Steinert's disease). *Mol. Hum. Reprod.*, **4**, 791–796.

Sermon, K., Seneca, S., Vanderfaillie, A., Lissens, W., Joris, H., Vandervorst, M., Van Steirteghem, A. and Liebaers, I. (1999) Preimplantation diagnosis for fragile X syndrome based on the detection of the non-expanded paternal and maternal CGG. *Prenat. Diagn.*, **19**, 1223–1230.

Vandervorst, M., Liebaers, I., Sermon, K., Staessen, C., De Vos, A., Van de Velde, H., Van Assche, E., Joris, H., Van Steirteghem, A. and Devroey, P. (1998) Succesful preimplantation genetic diagnosis is related to the number of available cumulus–oocyte complexes. *Hum. Reprod.*, **13**, 3169–3176.

Van deVelde, H., Nagy, Z.P., Joris, H., De Vos, A. and Van Steirteghem, A.C. (1997) Effects of different hyaluronidase concentrations and mechanical

Downloaded from https://academic.oup.com/molehr/article/9/9/559/1110549 by U.S. Department of Justice user on 16 August 2022

procedures for cumulus cell removal on the outcome of intracytoplasmic sperm injection. *Hum. Reprod.*, **12**, 2246–2250.

VanSteirteghem, A. (1997) Intracytoplasmic sperm injection. *Ballière's Clin. Obstet. Gynecol.*, **11**, 725–738.

Ventura, M., Gibaud, A., Le Pendu, J., Hillaire, D., Gerard, G., Vitreac, D. and Oriol, R. (1988) Use of a simple method for the Epstein–Barr virus transformation of lymphocytes from members of large families of Reunion Island. *Hum. Hered.*, **38**, 36–43.

Welsh, M.J., Tsui, L.C., Boat, T.F. and Beaudet, A.L. (1995) In Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. (eds), *The Metabolic and Molecular Bases of Inherited Disease*. McGraw-Hill, New York, pp. 3799–3879.

Zielenski, J., Markiewicz, D., Rininsland, F., Rommens, J. and Tsui, L. (1991) A cluster of highly polymorphic dinucleotide repeats in intron 17b of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. *Am. J. Hum. Genet.*, **49**, 1256–1262.

Zielenski, J. and Tsui, L.C. (1995) Cystic fibrosis: genotypic and phenotypic variations. *Annu. Rev. Genet.*, **29**, 777–807.

Submitted on February 14, 2003; resubmitted on April 24, 2003; accepted on May 2, 2003

Downloaded from https://academic.oup.com/molehr/article/9/9/559/1110549 by U.S. Department of Justice user on 16 August 2022