A comparison of fluorescent *in situ* hybridization and multiplex short tandem repeat polymerase chain reaction for quantifying chimerism after stem cell transplantation

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Background and Objectives. Despite the great utility of chimerism analysis after allogeneic stem cell transplantation, a gold standard method for its quantification has not yet been defined. The objective of the present investigation was to compare the sensitivity (detection limit) and the quantification accuracy of fluorescent *in situ* hybridization with specific probes for the sex chromosomes (XY-FISH) and multiplex short tandem repeat polymerase chain reaction (STR-PCR) revealed by capillary electrophoresis for the quantification of chimerism after stem cell transplantation.

Design and Methods. A first experiment was performed on two sets of artificial cell mixtures from two sex-mismatched healthy donors mixed in different proportions (% male: 100, 75, 50, 25, 10, 5, 3, 1, 0.1, 0). In a second experiment, 58 samples obtained from 10 selected patients with different clinical courses and chimerism evolution after sex-mismatched stem cell transplantation, which had been studied by XY-FISH, were retrospectively analyzed by STR-PCR. In a third experiment, 60 unselected prospective samples belonging to 15 patients (5 of whom had also been included in the retrospective study) were analyzed by both XY-FISH and STR-PCR.

Results. Both techniques showed high quantification accuracy and were highly reproducible. The sensitivity of both approaches reached 1% under standard conditions. Moreover, the use of long injection times for the capillary electrophoresis (30 and 50s vs. the standard 10s) resulted in an increase of sensitivity of the STR-PCR assay up to 0.1%, which has interesting clinical implications.

Interpretation and Conclusions. Considering the high sensitivity and quantification accuracy of multiplex STR-PCR and the fact that this assay is sex-independent and can be applied to virtually all patients, STR-PCR could be considered as the method of choice for chimerism quantification after stem cell transplantation when high sensitivity is not a requirement.

Key words: stem cell transplantation, chimerism quantification, FISH, STR-PCR.

Haematologica 2005; 90:1373-1379

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Allogeneic hematopoietic stem cell transplantation is nowadays the therapy of choice for a number of malignant and non-malignant hematologic diseases such as severe aplastic anemia, severe combined immunodeficiency, acute and chronic leukemia and lymphoma.[1] The quantification of chimerism after allogeneic stem cell transplantation is of great utility because it allows prediction or early detection of engraftment, graft rejection and disease relapse.[2-6] Several methodological approaches have been used for chimerism analysis.[7-9] Probably the most widespread technique is the polymerase chain reaction for variable number of tandem repeats/short tandem repeats (VNTR/STR-PCR) revealed by conventional agarose-acrylamide gel electrophoresis. This method has a moderate sensitivity (3-5% depending on the marker used)[3], and quantification of donor and recipient cells is cumbersome because it is performed by densitometry of gel bands. On the other hand, if a sufficient number of VNTR/STR markers are used, the technique can be applied for the follow-up of virtually all patients.

Chimerism in sex-mismatched transplantations can be analyzed by using interphase fluorescent *in situ* hybridization (FISH) with specific probes for the sex chromosomes (XY-FISH).[10] XY-FISH is a fully quantitative technique and offers 1% sensitivity when 500 cells are scored.[5]

More recently, a growing number of laboratories are using multiplex STR-PCR with fluorescently-labeled primers, revealed by capillary electrophoresis in a DNA sequencer. This allows direct quantification of donor and recipient hematopoiesis with a sensitivity of 1-3%.[11-14] However, a gold

Table 1. Characteristics of patients, transplants performed and evolution post-SCT.

UPN	Age	Diag.	Type of donor	Type of allo-SCT	Cond. regimen	a/c GVHD	Chimerism evolution	Clinical evolution	DLI/day (T cells/kg)	Status at last F-U	Study
34	49	CML	IFD	conv./CD34$^+$	Bu/Cy/ATG	I/E	CC-MC-CC	Rel	**	Alive RC/+2670	R,P
41	52	CML	IFD	conv./CD34$^+$	Bu/Cy/ATG	II/E	CC-MC	CR	–	Dead RC/+1547	R
121	52	CLL	IFD	conv.	TBI/Cy	II/E	CC	CR	–	Dead RC/+1792	P
126	36	AML	NIFD	conv.	Bu/Cy	-/-	CC-MC-CC	Rej	–	Dead RC/+163	R
129	33	AML	IFD	conv./CD34$^+$	Flu/Mel/Thio/ATG	I/-	MC-CC	CR	–	Dead RC/+171	R
139	48	MCL	IFD	conv./CD34$^+$	Bu/Cy/ATG	I/E	MC-CC	Rej	1×10^7/+106	Alive RC/+1602	R
146	49	AML	IFD	RIC	Flu/Mel	II/L	CC-MC	Rel	1×10^7/+198	Dead Rel/+294	P
149	31	CML	IFD	conv.	Bu/Cy	III/L	CC	CR	–	Alive RC/+1571	P
157	2	RAEBt	IFD	conv.	Bu/Cy	-/-	MC	Prog	–	Dead Prog/+36	R
181	39	AML	NIFD	conv./CD34$^+$	Flu/Mel/Thio/ATG	III/L	CC	CR	–	Alive RC/+1128	P
183	24	ALL	MUD	conv.	TBI/Mel/ATG	II/L	MC-CC	CR	–	Alive RC/+309	P
209	41	ALL	IFD	conv.	TBI/Cy	I/E	MC-CC	CR	–	Alive RC/+770	R,P
214	44	AML	IFD	RIC	Flu/Mel	II/L	MC-CC	Rel	1×10^8/+384	Dead Rel/+269	R,P
218	14	BL	IFD	RIC	Flu/Mel	-/-	CC	CR	–	Alive RC/+716	P
227	34	AML	NIFD	conv./CD34$^+$	Flu/Mel/ATG	-/-	CC-MC	Rel	–	Dead Rel/+360	R,P
228	60	AML	IFD	RIC	Flu/Mel/ATG	-/L	MC-CC	Rej	1×10^7/+35	Dead RC/+249	R,P
232	35	BL	NIFD	conv./CD34$^+$	Flu/Mel/Thio/ATG	-/-	MC*-CC	CR	–	Dead RC/+30	P
246	48	NHL	IFD	RIC	Flu/Mel	II/E	CC	CR	–	Dead RC/+355	P
249	42	MDS	IFD	conv.	Bu/Cy	III/E	MC-CC	CR	–	Alive RC/+401	P
283	56	AML	IFD	RIC	Flu/Mel	-/-	MC-CC	CR	–	Alive RC/+80	P

*UPN: unique patient number. Diag: diagnosis. Allo-SCT: allogeneic stem cell transplantation. Cond. regimen: conditioning regimen. a/c GVHD: acute/chronic graft versus host disease. Chimerism evolution: evolution of chimerism during the study period. DLI: Donor leukocyte infusion. F-U: follow-up. CML: chronic myeloid leukemia. CLL: chronic lymphocytic leukemia; AML: acute myeloid leukemia; MCL: mantle cell lymphoma. RAEBt: refractory anemia with excess of blasts in transformation; ALL: acute lymphoid leukemia; BL: Burkitt's lymphoma; NHL: non-Hodgkin's lymphoma; MDS: myelodysplastic sindrome; IFD: HLA-identical family donor. NIFD: Non-HLA-identical family donor. CD34+: positive selection of CD34 cells. RIC: reduced intensity conditioning. Bu: busulphan. Cy: cyclofosfamide. ATG: antithymocyte globulin. Flu: fludarabine. Mel: melphalan; Thio: thiotepa; TBI: total body irradiation; E: extensive. L: limited. CC: complete chimerism. MC: mixed chimerism. Rel: relapse. Rej: rejection. CR: complete remission. Prog: progression. R, included in the retrospective study. P, included in the prospective study. *MC detected in the first sample post-SCT only by STR-PCR. **1×10^7/+1186; 1×10^7/+1229; 5×10^7/+1277; 5×10^7/+1319; 1×10^8/+1461. Study: study in which the patient was included. R: retrospective; P: prospective.*

standard method for quantifying chimerism has not been clearly defined yet. Therefore, the purpose of the present investigation was to compare the efficacy of multiplex STR-PCR with that of a well-established method for chimerism quantification, namely XY-FISH, which shows high sensitivity and quantification accuracy.

Design and Methods

Sample preparation

A total of 138 peripheral blood and bone marrow samples were analyzed with both techniques, XY-FISH and multiplex STR-PCR. In a first experiment two independent groups of artificial mixtures of male and female peripheral blood cells in different proportions (% male: 100, 75, 50, 25, 10, 5, 3, 1, 0.1, 0) were prepared. In this experiment, male individuals were considered as the *recipient* while females were the *donor* of the fictitious stem cell transplant. Each artificial mixture contained a total of 20×10^6 cells, from which one half was used to prepare routine smears for XY-FISH analysis, and the other was processed to purify genomic DNA (QIAamp DNA blood kit, Qiagen) for chimerism analysis by multiplex STR-PCR. Additionally, the first group of mixtures was independently analyzed twice with both techniques to determine the reproducibility of the two techniques. Finally, in order to test whether greater amounts of DNA in the capillary electrophoresis would result in an increase in sensitivity (detection limit) of the STR-PCR technique, samples containing 5, 3, 1, 0.1 and 0% of male (*recipient*) DNA were analyzed using injection times of 10s (standard), 30s and 50s. In a second experiment, chimerism was retrospectively quantified by multiplex STR-PCR in 58 samples (40 peripheral blood and 18 bone marrow) obtained after allogeneic stem cell transplantation from 10 patients; these samples have been previously studied by XY-FISH. Patients showing different degrees of chimerism and clinical courses following transplantation were selected for this study (Table 1).

In a third experiment, 60 unselected prospective samples (39 peripheral blood and 21 bone marrow) belonging to 15 patients (5 of whom had also been included in the retrospective study; Table 1) were analyzed by both XY-FISH and STR-PCR.

Finally, samples identified as showing complete chimerism with both techniques were analyzed again, using DNA injection times of 10s, 30s and 50s in the capillary electrophoresis. The present research project was approved by local (in-house ethics and research committees) and national (Ministry of Health) institutions, and procedures were conducted in compliance with the Helsinki Declaration.

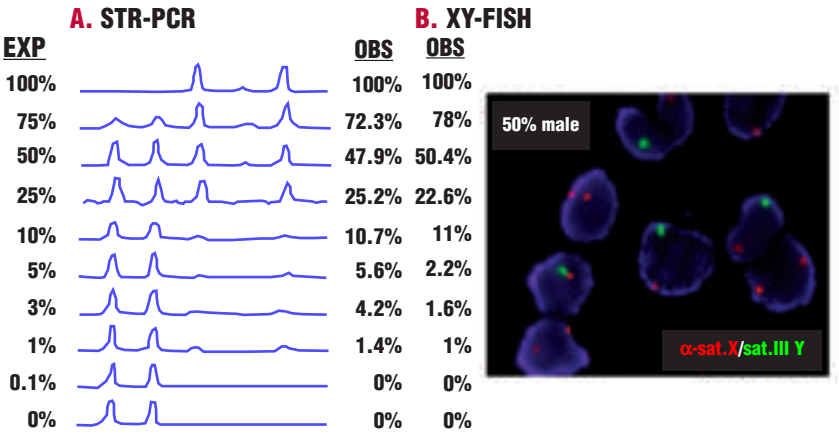

Figure 1. Results of quantifying chimerism by STR-PCR (A; peak constellation for the informative marker D3S1358 is shown as an example) and XY-FISH (B; photomicrograph of the 50% male/50% female peripheral blood smear is shown as an example) in the first set of artificial mixtures (first experiment) with known proportions of male and female cells. EXP. Percentage of male (*recipient*) cells in the artificial mixtures. OBS. Percentage of male (*recipient*) cells observed with each method.

XY-FISH

XY-FISH was performed on routine smears using a dual-color CEP XY probe (Figure 1; Vysis Inc.) and following the protocol suggested by the manufacturer with slight modifications.[5] In all cases, 500 nuclei were scored per slide to reach a 1% sensitivity.[5] Moreover, slides from the first experiment were examined under the microscope in a blind fashion to eliminate observer bias.

Multiplex STR-PCR

Multiplex STR-PCR was performed on 2 ng of genomic DNA using the AmpFlSTR SGM Plus kit (Applied Biosystems), which contains 10 STR loci plus the X-Y homologous gene amelogenin labeled in three different colors (blue 5-FAM, green JOE and yellow NED). Amplified PCR products were subjected to capillary electrophoresis in an ABI Prism 3100 (Applied Biosystems; Figure 1) automated DNA sequencer using the conditions recommended by the manufacturer, as previously described.[12]

In order to increase specificity, only markers in which recipient-specific peaks showed no residual fluorescence (stutter peaks, peaks resulting from spectral overlapping, etc.) in the donor sample (Figure 1), and no other peak in any other color, were considered as informative to distinguish donor and recipient cells. Although this strategy could theoretically reduce the number of patients suitable for STR-PCR analysis, this was not a problem in our group of patients, in whom a mean number of three markers (range 2-5) were selected for chimerism quantifiction.

Chimerism was quantified as suggested by Thiede *et al.*[12] using Genotyper 3.7 software (Applied Biosystems) and peak areas to perform calculations.

Statistical analysis

Although frequently used to this end, the correlation coefficient is not a good indicator of agreement between two methods of measurement.[15] Therefore, an alternative approach proposed by Bland and Altman[15] was used in the present investigation. This method is based on graphical representation of the difference between the measurements of both methods against their mean (Figures 2, 3 and 4) and calculation of the limits of agreement mean d – 2SD and mean d + 2SD, where mean d is the mean difference of the measurements and SD the standard deviation of the differences. The two methods are considered to agree when measurement differences are not statistically significant because they are included in the 95% confidence interval calculated from the t distribution with n-1 degrees of freedom ($x_i \pm 2.03$).[15] The reproducibility coefficient was calculated following the definition of the British Standards Institution,[16] which expects 95% of differences to be less than 2SD. The standard deviation of the differences is $(\sum d^2/n)^{1/2}$, where d is the difference between repetitions.

Results

First experiment: sensitivity of XY-FISH and STR-PCR

We had previously established that the sensitivity (detection limit) of XY-FISH in our hands was lower than 1% when 500 cells are scored.[5] Therefore, samples containing 1% or more recipient cells are considered as mixed chimeras (Figure 1). All informative markers identified by multiplex STR-PCR (five in the first male-female pair and four in the second) were able to detect male (*recipient*) DNA in mixtures containing 3% or more male DNA. Moreover, at least one of the markers was able to detect male *(recipient)* DNA in mixtures containing 1% male DNA (Figure 1, Table 2). When long injection times (30s and 50s versus the standard 10s) were used for the capillary electrophoresis, male (*recipient*) DNA was also detected in the sample containing 0.1% male DNA (Table 3). Therefore, this approach results in an increase in sensitivity of one order of magnitude.

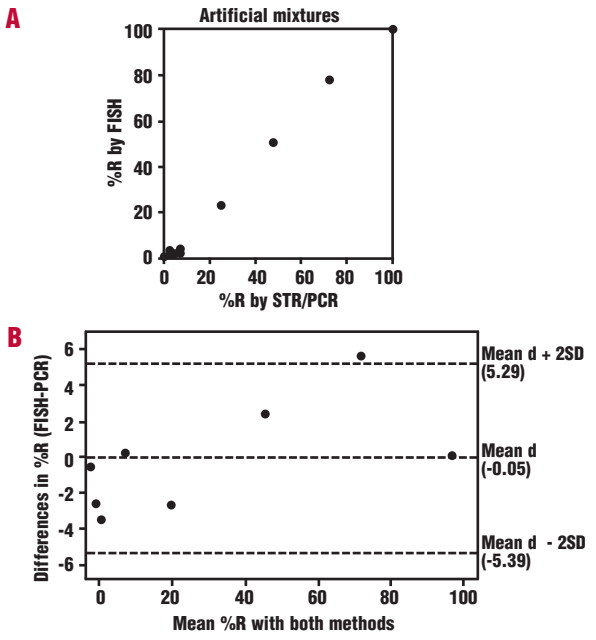

Figure 2. Comparison of the results obtained from chimerism quantification with STR-PCR and XY-FISH in the first set of 10 artificial mixtures (first experiment) using the method proposed by Bland and Altman.[15] (A) Plot of the results obtained by STR-PCR against those of XY-FISH. (B) Plot of the difference between the results of both methods against their mean.

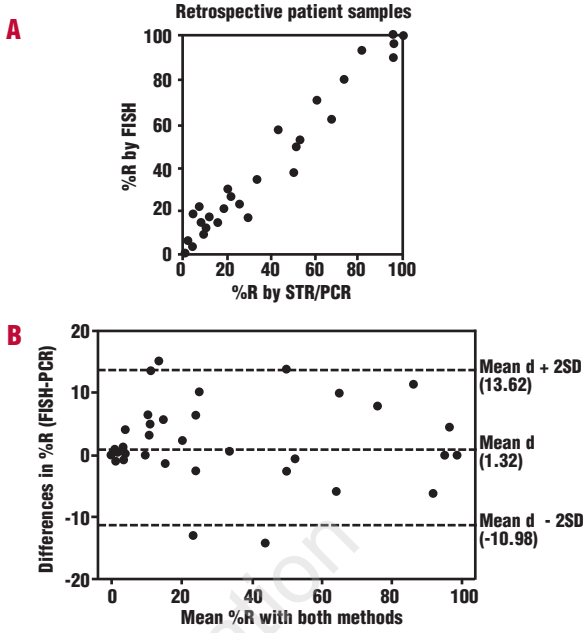

Figure 3. Comparison of the results obtained from chimerism quantification with STR-PCR and XY-FISH in 58 retrospective samples from patients (second experiment) using the method proposed by Bland and Altman.[15] (A) Plot of the results obtained by STR-PCR against those of XY-FISH. (B) Plot of the difference between the results of both methods against their mean.

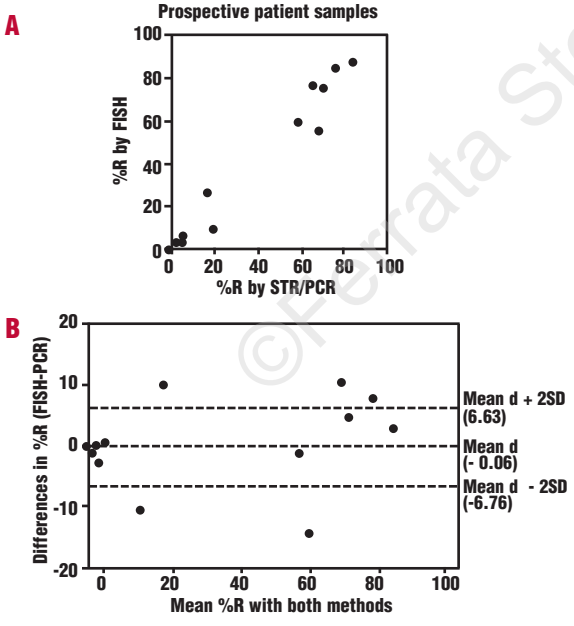

Figure 4. Comparison of the results obtained from chimerism quantification with STR-PCR and XY-FISH in 60 prospective samples from patients (third experiment) using the method proposed by Bland and Altman.[15] (A) Plot of the results obtained by STR-PCR against those of XY-FISH. (B) Plot of the difference between the results of both methods against their mean.

First experiment: quantification accuracy

The results obtained in the first experiment on the quantification of male (*recipient*) and female (*donor*) cells by XY-FISH and multiplex STR-PCR (Figure 1) on artificial mixtures showed a good agreement since most differences lay between mean d - 2SD and mean d + 2SD (Figure 2).[15] Although the sample size in this experiment was small, differences between measurements of both approaches were not statistically significant because they were included within the 95% confidence interval calculated from the t distribution with n-1 degrees of freedom ($x_i \pm 2.03$).[15] Moreover, both techniques were highly accurate since results obtained were close to those expected from the percentages of male and female DNA in the artificial samples (Figures 1 and 2). On the other hand, the accuracy of quantification decreased when long injection times were used for the capillary electrophoresis (Table 3). Longer injection times tended to overestimate DNA fragments which were underrepresented in the samples analyzed.

First experiment: reproducibility of XY-FISH and STR-PCR

Reproducibility of the results obtained from two independent analyses of the first set of artificial mixtures with both techniques was high, with reproducibility coefficients of 3.3% for XY-FISH and 1.3% for STR-PCR.

Table 2. Sensitivity of the different informative STR markers capable of detecting male (*recipient*) DNA in artificial cell mixtures (first experiment).

STR marker	*Sensitivity**
First male/female pair	
D3S1358	1
VWA	3
Amelogenin	1
D21S11	1
TH01	1
Second male/female pair	
VWA	1
D2S1338	3
Amelogenin	3
FGA	3

**percentage of male ("recipient") cells.*

Table 3. Mean area of recipient specific peaks and chimerism estimated in artificial cell mixtures containing 5, 3, 1, 0.1 and 0% of male (*recipient*) cells when different injection times (10s [standard], 30s and 50s) were used for capillary electrophoresis.

% male expected	*Injection time 10s*	*30s*	*50s*
Mean area of recipient specific peaks			
5	800.13	2139.50	2429.25
3	1036.12	5528.12	7278.12
1	284.12	2228.87	5338.62
0.1	0	182.87	294.87
0	0	0	0
Chimerism (%R estimated)			
5	4.95	4.8	4.75
3	3.5	7.81	11.32
1	1.35	3.88	5.98
0.1	0	0.7	0.63
0	0	0	0

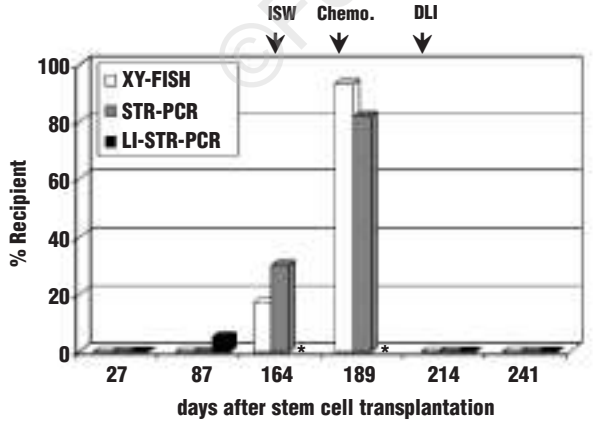

Figure 5. Follow-up of chimerism in UPN 214 by XY-FISH, STR-PCR with a standard injection time for the capillary electrophoresis (10s) and STR-PCR with long injection (LI) times (30s and 50s). ISW: withdrawal of immunosuppression; Chemo.: chemotherapy (arsenic trioxide, idarubicin); DLI: donor leukocyte infusion; SCT: stem cell transplant; *: not done.

Second and third experiments: samples from patients

Results obtained from the quantification of chimerism in retrospective (Figure 3) and prospective (Figure 4) patients' samples with both techniques also showed a good agreement. Differences between measurements of both approaches were not statistically significant because they were included within the 95% confidence interval calculated from the t distribution with n-1 degrees of freedom ($x_i \pm 1.92$ for retrospective and $x_i \pm 0.86$ for prospective samples).[15] In the second experiment, all samples previously identified as mixed chimeras by XY-FISH were also defined as mixed chimeras by multiplex STR-PCR. Likewise samples with complete chimerism according to XY-FISH were also defined as such with multiplex STR-PCR. Therefore, acquisition of complete chimerism or detection of reappearing recipient cells was identified in the same sample (data-point) with both techniques in the follow-up of all patients. Among the 60 samples included in the third experiment, mixed chimerism was observed in 14 by STR-PCR and in 13 samples by XY-FISH. The first bone marrow sample after stem cell transplantation from UPN 232 (Table 1) showed 1.2% recipient DNA by STR-PCR while it was designated as showing complete chimerism by XY-FISH (3 cells of recipient origin out of 500 cells scored, 0.6%, which falls within the intrinsic error of the technique).

All samples with complete chimerism, as determined by XY-FISH and STR-PCR, were analyzed by STR-PCR using long injection times (30s and 50s vs. the standard 10s). Mixed chimerism was detected in 5 of the 73 samples analyzed. Two of these samples were the first peripheral blood samples obtained after stem cell transplantation in UPN 246, who has maintained complete chimerism since, and another one was the first sample with complete chimerism (day +97) in a patient previously showing mixed chimerism (UPN 209; Table 1). However, the two remaining samples preceded the diagnosis of mixed chimerism (UPN 214 and 227; Table 1). The increase in sensitivity obtained using long injection times allowed the detection of reappearing recipient cells 77 days in advance in UPN 214, in the context of a disease relapse (Figure 5), and 7 days earlier than the standard approach in UPN 227, in the context of a graft rejection.

Discussion

Quantitative monitoring of chimerism after allogeneic stem cell transplantation can predict engraftment/graft failure, graft rejection or relapse.[2-6] Within this scenario, an appropriate quantitative method is

needed for the follow up of the transplanted patients. One of the most widespread methods for chimerism analysis is VNTR-/STR-PCR revealed by conventional agarose/acrylamide gel electrophoresis. This approach can be used in virtually all transplanted patients if enough markers are tested, but is of moderate sensitivity (3-5% depending on the marker) and, even more troublesome, allows only semi-quantitative estimations based on densitometry of gel bands.[3] XY-FISH shows a slightly better sensitivity (1% when 500 cells are scored) and it is fully quantitative.[10] However, it is only applicable after sex-mismatched transplantation (approximately 50% of cases). More recently, a quantitative method based on multiplex STR-PCR revealed by capillary electrophoresis has been developed.[11,13] In this technique, the use of fluorescently labeled PCR primers makes direct quantification of donor and recipient DNA possible on an automated DNA sequencer.[12]

The aim of the present investigation was to compare the efficacy, focusing on the sensitivity and the quantification accuracy, of XY-FISH and multiplex STR-PCR for chimerism quantification after allogeneic stem cell transplantation. To this end, a first experiment was performed in which two sets of artificial mixtures of male and female cells in different proportions (% male: 100, 75, 50, 25, 10, 5, 3, 1, 0.1, 0) were prepared. These artificial samples were used to test the quantification accuracy of both techniques as well as the reproducibility of the techniques.

Results obtained with both techniques from the quantification of *donor* and *recipient* cells in artificial mixtures showed a good agreement (Figure 2) and were highly reproducible (the reproducibility coefficient was 3.3% for XY-FISH and 1.3% for STR-PCR). Interestingly, this was true throughout the quantification range (0-100%). Direct quantification of fluorescence with the DNA sequences proved easier and more accurate than previous estimations based on densitometry of agarose/acrylamide gel bands. Moreover, standard PCR-based methods evaluate the quantity of the PCR product once the amplification reaction has reached the plateau phase, whose level depends on a large number of variables.[17] However, the kit used here is optimized (excess of PCR reagents, amplification of very low template DNA amounts) to remain in the exponential phase of amplification throughout the whole PCR reaction (28 thermal cycles). In this way, the so-called *end-point* quantification, otherwise inaccurate, is directly proportional to the initial amount of the target DNA sequence.

Finally, it must be considered that differences in quantification of chimerism between two methods in particular samples may be considerable (Figures 3, 4). Therefore, every patient should be followed-up with a single technique, irrespectively of the approach used. In concordance with previous observations,[12,13] a 1% sensitivity was consistently obtained with multiplex STR-PCR when standard injection times were used. This sensitivity is similar to that of XY-FISH, namely 1% when 500 nuclei are scored.[5] In fact, among the 138 samples analyzed, 73 showed complete chimerism and 64 mixed chimerism with both approaches. Only one sample, with a percentage of recipient cells around the level of sensitivity of these techniques, was identified as showing mixed chimerism by STR-PCR but not by XY-FISH. The use of long injection times increases the sensitivity of the assay to 0.1%, which may have interesting clinical applications. In two patients, this approach allowed the detection of mixed chimerism 7 and 77 days earlier than STR-PCR with standard injection times or XY-FISH (Figure 5). Long injection times favor the introduction in the capillary of DNA fragments that are underrepresented in the sample. In this way, the relative increase in area of recipient-specific peaks is greater than that of donor peaks (see results of chimerism quantification in Table 3). This results in an increase in the sensitivity of the assay, which, on the other hand, becomes quantitatively less accurate and, therefore, must not be used for quantification purposes when such conditions are applied. Considering that informative markers are carefully selected, avoiding the presence of confounding peaks (stutter peaks, peaks resulting from spectral overlapping, etc), false positivity can be virtually discarded (see results for the sample with 0% male DNA in Table 3). Some patients retain low levels of residual host hematopoiesis for long periods of time,[18,19] and this has been claimed to avoid the need for high sensitivity assays in the detection of recipient cells.[11] Nevertheless, the highest sensitivity should be available for the follow-up of patients with complete chimerism in order to ensure early detection of the reappearance of recipient hematopoiesis and enable the appropriate clinical decisions to be taken promptly (closer follow-up, withdrawal of immunosuppression, donor leukocyte infusion, etc.).[4-6] For this purpose, optimal assay conditions, especially high quality DNA, must be met in the sequential chimerism studies. Furthermore, approaches for chimerism quantification based on real-time quantitative PCR[17,20,21] have recently been developed. Such methods show increased sensitivity (0.01%) but are difficult to standardize and lose quantification accuracy as the percentage of recipient cells increases.[8,20] As suggested by Thiede *et al.*[8] such methods should therefore be used in combination with multiplex STR-PCR, and in certain cases with XY-FISH, in order to provide the best methodological approach in every clinical situation. If real time quantitative PCR is not

available, we recommend the use of long injection times for the qualitative analysis of patients' samples expected to show complete chimerism.

The results reported here demonstrate that STR-PCR has advantages over XY-FISH for quantifying chimerism after stem cell transplantation. However, in particular patients with neoplastic genetic markers undetectable by PCR (such as aneusomies), sequential FISH approaches could be especially useful to evaluate minimal residual disease (probing the appropriate DNA sequence) focusing the study only on recipient cells previously identified by XY-FISH.[22] In summary, considering the high sensitivity and quantification accuracy of multiplex STR-PCR, the fact that it is a sex-independent assay and can be applied to virtually all patients, this approach could be considered the method of choice for quantification of chimerism after stem cell transplantation when high sensitivity is not a requirement.

Conception and design: IB and JLD-M; analysis and interpretation of data: all authors; article drafting: JLD-M and IB; revision of the article: all authors; final approval: all authors. The first and last authors take primary responsibility for the work reported here in terms of laboratory procedures (IB) and care of patients (JLD-M). The order of the remaining authors reflects their degree of participation in the study. The authors declare that they have no potential conflict of interest.

The authors wish to thank JM Bellón for his valuable assistance with statistics. This work has been partially supported by grants FIS02-1177 and FIS01-1079 from the Ministerio de Sanidad y Consumo and GR/SAL/0228/2004 from Comunidad de Madrid (Spain).

Manuscript received August 3, 2004. Accepted August 8, 2005.

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