

Comparison of Variable Number Tandem Repeat and Short Tandem Repeat Genetic Markers for Qualitative and Quantitative Chimerism Analysis Post Allogeneic Stem Cell Transplantation

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ABSTRACT

Background: Analysis of donor chimerism has become a routine procedure for the documentation of engraftment after allogeneic hematopoietic stem cell transplantation. Quantitative analysis of chimerism kinetics has been shown to predict graft failure or relapse. In this study, we compared the use of variable number tandem repeats (VNTR) and short tandem repeats (STR) as polymorphic genetic markers in chimerism analysis. This study included qualitative and quantitative assessment of both techniques to assess informative yield and sensitivity.

Patients and Methods: We analyzed 206 samples representing 40 transplant recipients and their HLA-identical sibling donors. A panel of six VNTR loci, 15 STR loci and 1 sex chromosome locus was used. Amplified VNTR products were visualized in an ethidium bromide-stained gel. STR loci were amplified using fluorescent primers, and the products were analyzed by capillary electrophoresis.

Results: VNTR and STR analysis gave comparable qualitative results in the majority of cases. The incidence of mixed chimerism (MC) by STR analysis was 45% compared to 32% in cases evaluated by VNTR analysis. STR markers were more informative; several informative loci could be identified in all patients. Unique alleles for both patient and donor could be identified in all patients by STR versus 32/40 by VNTR analysis. The STR markers were also more sensitive in the detection of chimerism. The size of VNTR alleles and differences between the size of donor and recipient VNTR alleles affected the sensitivity of detection. With both techniques, quantitative assessment of chimerism showed some discrepancies between the estimated and the calculated percentage of donor DNA. Discordance between the two estimates was observed in 8/19 patients with MC. However, sequential monitoring of the relative band intensity of VNTR alleles offered some insight into the direction of change in engraftment over time.

Conclusion: The higher yield of informative loci with STR and the automated measurement of amplified STR

products offered the advantages of more rapid and accurate quantitative assessment of chimerism. The choice between these two techniques depends on the need for quantitative or qualitative information, the availability of equipment, and the cost.

Key Words: Chimerism analysis - Variable number tandem repeat (VNTR) - Short tandem repeat (STR) - Mixed chimerism (MC).

INTRODUCTION

Analysis of donor chimerism has become a routine procedure for the evaluation of engraftment after allogeneic stem cell transplantation (ASCT) [1]. Monitoring the dynamics of chimerism has recently gained considerable significance, since several groups have concluded that detection of an increasing population of recipient cells in patients monitored serially is more predictive than a single determination in assessing the risk of relapse [2,3,4]. Serial monitoring allows early therapeutic intervention by rapid tapering of immunosuppressive medications or donor lymphocyte infusion (DLI), especially when a nonmyeloablative conditioning regimen is used before transplantation [5,6,7].

Genetic markers of human identity have been used for chimerism analysis. Tandemly repeated DNA sequences are abundant throughout the human genome. These sequences provide a rich source of polymorphism resulting from variation in the number of copies of a tandemly repeated motif at each locus. These repeats differ in length to form variable number tandem repeat (VNTR) loci when the repeated core sequence is 8-50bp (also referred to as minisat-

ellite loci) or short tandem repeat (STR) loci when the repeated core sequence is 2 to 7bp (also referred to as microsatellite loci) [8,9]. At present, VNTR and STR analysis is the approach most likely to give reproducible and informative data regarding chimerism after allogeneic transplantation [10].

With information from testing these loci, the state of chimerism can be qualitatively assessed after ASCT as being "complete," where only donor cells are detected, or "mixed," where both recipient and donor cells are present. Quantitative estimation of mixed chimerism (MC) is possible by visual inspection of the relative band intensity in ethidium bromide stained gel after amplification of VNTR loci or by determination of the peak area or peak height of fluorescently labeled recipient or donor-specific PCR products after amplification of STR loci.

In this study, we compared the use of VNTR and STR loci for qualitative and quantitative assessment of chimerism after ASCT. Our goals were to assess the frequency of informative results and the sensitivity of testing by amplification of 6 VNTR loci with visualization in an ethidium bromide-stained gel as compared to testing by multiplex amplification of 15 STR loci with automated fluorescence quantitation by capillary electrophoresis using the genetic analyzer.

PATIENTS AND METHODS

This study was performed on 206 samples representing 40 patients undergoing allogeneic stem cell transplantation at the National Cancer Institute, Cairo University and at Nasser Institute, Cairo, Egypt as well, their HLA identical siblings and 126 post transplant samples. A nonmyeloablative pretransplant conditioning regimen was used for 35 patients, while a myeloablative conditioning regimen was used for 5 patients. For chimerism analysis, 20ml heparinized peripheral blood was collected before and after transplantation at scheduled intervals. DNA was extracted using a salting out technique [11]. The concentration of DNA was estimated by UV spectrophotometry and adjusted to 5ng/uL and 0.8ng/uL for VNTR and STR analysis, respectively.

VNTR analysis was performed at the Bone Marrow transplantation Laboratory Unit, Na-

tional Cancer Institute, Cairo University. A panel of 6 VNTR loci (Apo-B, D1S80, YNZ-22, 33.1, 33.4 and 33.6) was used. Amplification of these loci was performed as previously described [12-14]. All reactions were performed in a volume of 50uL containing 50mmol/l KCL, 10mmol/L Tris-HCL (pH 8.3), 1.5mmol/L MgCl₂, 12.5pmol each primer (Pharmacia), 0.2mmol/L each dNTPs (Pharmacia) and 2.5 units Taq polymerase (Promega) followed by visualization in an ethidium bromide-stained gel. STR analysis was performed retrospectively at the Clinical Immunogenetics Laboratory, Seattle Cancer Care Alliance, Seattle, USA. The PowerPlex 16 System (Promega) used for these assays included 15 STR loci (Penta E, D18S51, D21S11, TH01, D3S1358, FGA, TPOX, D8S1179, vWA, Penta D, CSF1PO, D16S539, D7S820, D13S317 and D5S818) and the sex marker Amelogenin. For each locus, one specific primer was fluorescently labeled with fluorescein, JOE or TMR, and all 16 loci were amplified simultaneously in a single tube (multiplex PCR). Alleles of overlapping loci were distinguished by locus-specific primers with different color dyes. Amplification was performed in 25uL reactions with the thermocycler Perkin Elmer 9600, primers, buffer and Taq polymerase used according to the manufacturer's instructions. The amplified fragments were analyzed in a single capillary injection using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems). An internal lane standard 600 (ILS) (Promega) labeled with CXR was injected with each sample for accurate sizing, and an allelic ladder was included in one capillary for allele assignment. The fluorescence intensity patterns, measured as relative fluorescence unit (r.f.u.) of each STR locus were translated into an electropherogram. The GeneScan software (3.1) (Applied Biosystems) was used for size determination and quantitation of peak areas. Data were exported to Genotyper (3.7) to assign allele numbers from known base pair sizes. The PowerTyper 16 Macro (Promega) was used to facilitate analysis of the data. The ratio of the donor and recipient were determined by calculation of the proportion of the peak areas corresponding to donor signals as compared to the sum peak areas of the donor and recipient for each informative STR marker. The proportion of donor cells was calculated as $D = \frac{AD_1 + AD_2}{AD_1 + AD_2 + AR_1 + AR_2} \times 100$, where A is peak area, D donor allele(s) and R recipient allele(s).

Shared alleles were not included in the analysis. The median value of the percentage peak areas of all informative alleles was used as the proportion of donor cells for chimerism analysis. The values were given as percentage donor signal.

To identify an informative locus, pretransplant samples were screened for informative VNTR and STR loci. An informative locus is one for which at least one recipient allele has a different number of repeats than the donor allele(s). Patients were then tested several times after transplantation, using the informative VNTR and STR loci. VNTR analysis provided a qualitative determination of the type of chimerism. Complete chimerism (CC) was reported if only donor cells were detected, whereas mixed chimerism (MC) was reported if at least 1 recipient-specific allele could be detected in addition to donor allele(s). In cases where both the patient and donor had unique alleles, semi-quantitative evaluation of the percent donor cells was estimated by visual inspection of the relative band intensity in the ethidium bromide-stained gel. STR analysis provided quantitative estimation of the percent donor cells. Mixed chimerism was defined as the detection of 5-95% donor cells. STR results for patient samples were compared with VNTR results.

RESULTS

With the panel of 6 VNTR loci, at least one recipient-informative locus could be identified in 39/40 cases. A recipient-informative locus could be identified after one PCR amplification in 16/40 cases (40%). Unique alleles for both patient and donor could be identified in 32/40 patients. With the panel of 15 STR loci and the sex marker, several informative loci could be identified in all patients. The number of informative loci ranged from 3 to 10 (median 7). Unique alleles for both patient and donor could be identified in all patients. The discriminative power among the various VNTR loci could not be evaluated from this study, since we stopped screening when an informative locus was identified.

Table (1) shows the discriminative power among the various STR loci. Some loci, although technically informative, were not optimal for the determination of MC because of inter-

ference from stutter peaks, an artifact reflecting a partial amplification product. In our series, seven STR loci were excluded from consideration on at least one occasion because of stutter interference. Stutter peaks were most commonly encountered with the STR locus VWA (6 patients) and D8S1179 and D5S818 (5 patients each).

Comparison between the qualitative VNTR results and the quantitative STR results showed some discrepancies. In some cases, MC was detected only with STR. The incidence of MC by STR analysis was 45% (57/126 post-transplant samples) representing 27/40 patients. After excluding 16 samples in 6 patients that did not have an informative donor or patient-specific allele, the incidence of MC by VNTR analysis was 32% (35/110). After excluding 9 samples for 4 patients with MC by STR due to the absence of an informative donor or patient-specific VNTR allele, detection of MC by VNTR was missed in 13/48 evaluated samples which were falsely interpreted as CC (8 samples) or AR (5 samples). The involved loci were YNZ-22, 33.1, Apo-B and D1S80, and the range of undetected donor or recipient cells was 8-30% as detected by parallel STR testing (Table 2). Fig. (1) shows chimerism analysis using VNTR and STR markers in unique patient number (UPN) 25. In this case, STR testing showed 17% donor cells, while VNTR testing showed autologous recovery (AR) of recipient cells with no donor cells detected on day +28.

Table (3) compares the threshold of detection by VNTR markers in ethidium bromide stained gel compared to STR analysis using fluorescently labeled primers. The sensitivity of certain VNTR loci reached 3-5%. Fig. (2) shows the difference in sensitivity of detection according to the allele size. In UPN 26, 16% donor cells could be identified on day +28 by VNTR, while 22% patient cells could not be identified on day +56 using the same VNTR locus. The inability to detect recipient cells by VNTR testing was caused by the larger size of the informative allele in the patient.

We compared the semiquantitative assessment of MC by VNTR with the quantitative assessment by STR. Results were considered discrepant when differences were larger than plus or minus 10%. Concordance between the two estimates was observed in 11/19 patients

with MC. Discordance was reported with YNZ-22, 33.4 and 33.1 loci, and differences in the estimated donor chimerism ranged from 20-45% (Table 4). In Fig. (3), for example, the estimated percent donor cells on days 56 and

90 by VNTR was 50% and 30%, respectively, compared to 85% and 67% by STR analysis. It should be noted however, that the increasing percentage of recipient cells was detected by both VNTR and STR analysis for day 56 and 90 samples.

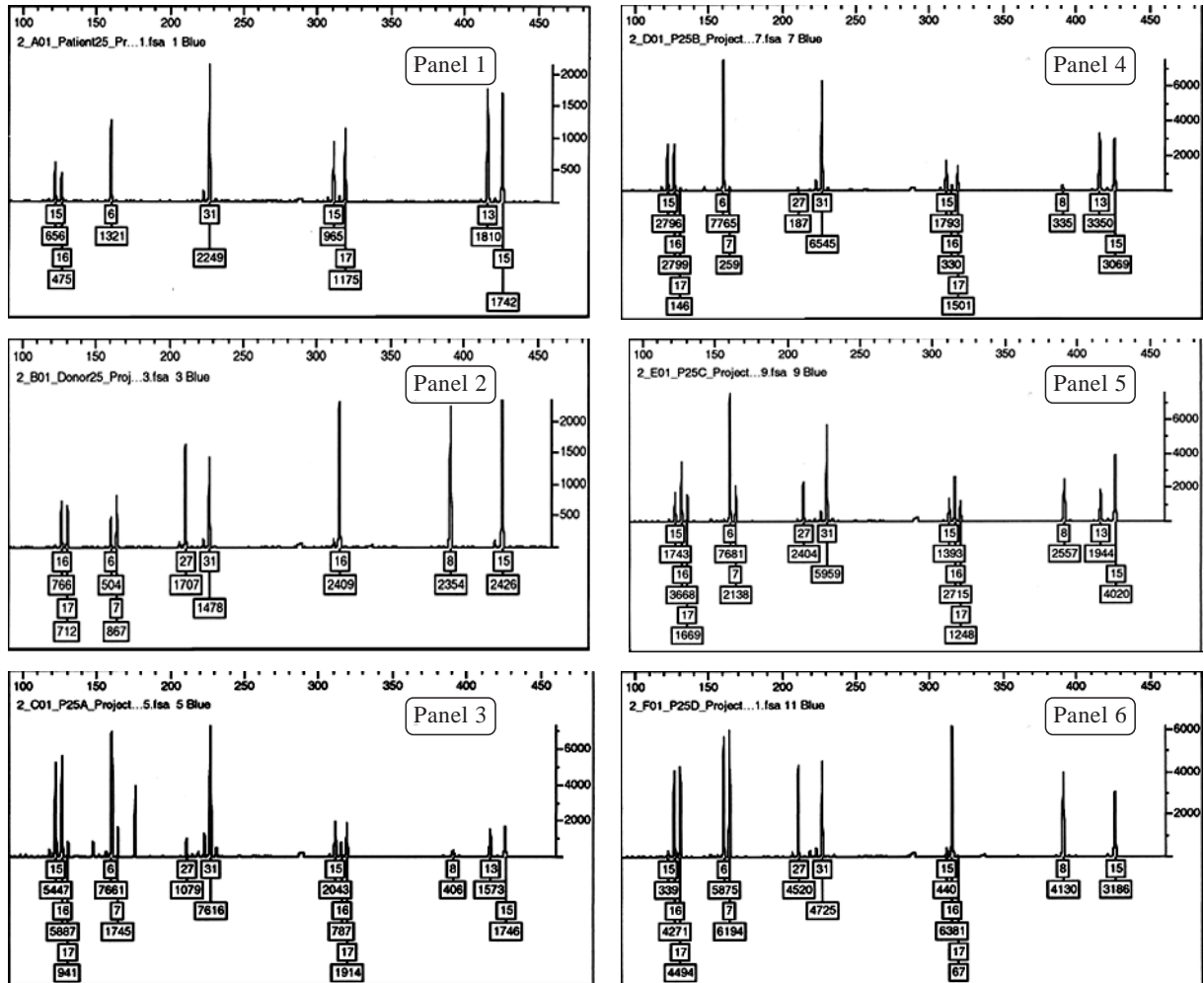


Fig. (1-B): Electropherogram of the patient UPN 25 showing STR loci (**D3S1358**, THO1, D21S11, **D18S51** and **Penta E**) labeled with fluorescein. Panels 1 and 2 represent patient and donor alleles. Panels 3, 4, 5 and 6 represent the post transplant samples on days +28, +56, the first DLI and the second DLI showing 17%, 5%, 50% and 99% donor cells, respectively. Loci in bold are informative for patient and donor and were used in analysis.

1 2 3 4 5 6 7

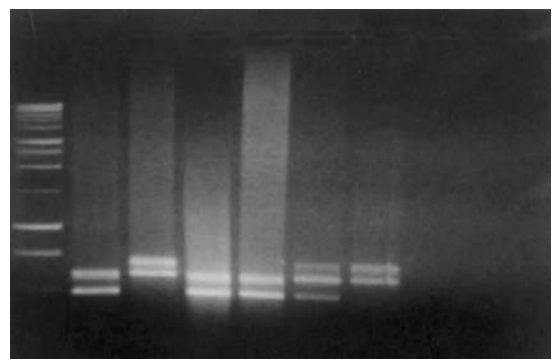


Fig. (1-A): Follow up of chimerism in patient UPN 25 post-nonmyeloablative transplant by amplification of the VNTR locus D1S80. Lane 1: MW marker (1kb); Lane 2: Pre transplant pattern of the patient; Lane 3: Donor pattern; Lanes 4, 5: The patient on day +28 and +56 post transplantation showing autologous recovery (AR) ; Lane 6: Analysis of chimerism after the first DLI showing conversion to mixed chimerism (MC); Lane 7: Analysis of chimerism after second DLI showing conversion to complete chimerism (CC). UPN: unique patient number, MW: Molecular weight, DLI: donor lymphocyte infusion.

Fig. (2-A): Follow up of chimerism in patient UPN 26 post-nonmyeloablative transplant by amplification of the VNTR locus YNZ-22. Lane 1: MW marker (1kb); Lane 2: Pre transplant pattern of the patient; Lane 3: Donor pattern; Lanes 4: The patient on day +28 post transplantation showing mixed chimerism (MC); Lane 5: Day+56 showing conversion to complete chimerism (CC). UPN: Unique patient number, MW: Molecular weight, DLI: donor lymphocyte infusion.

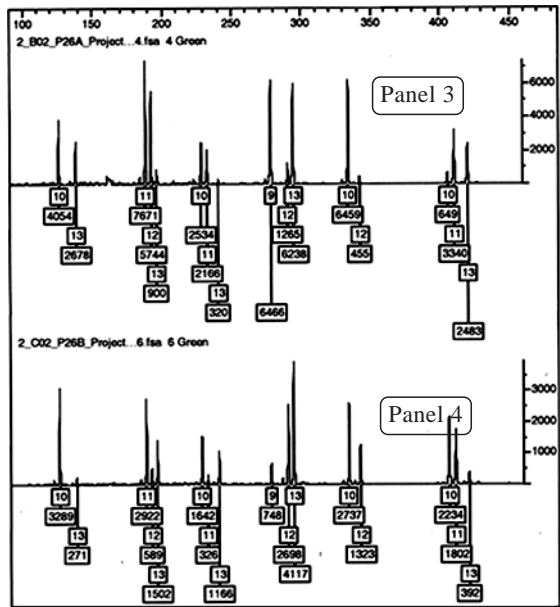
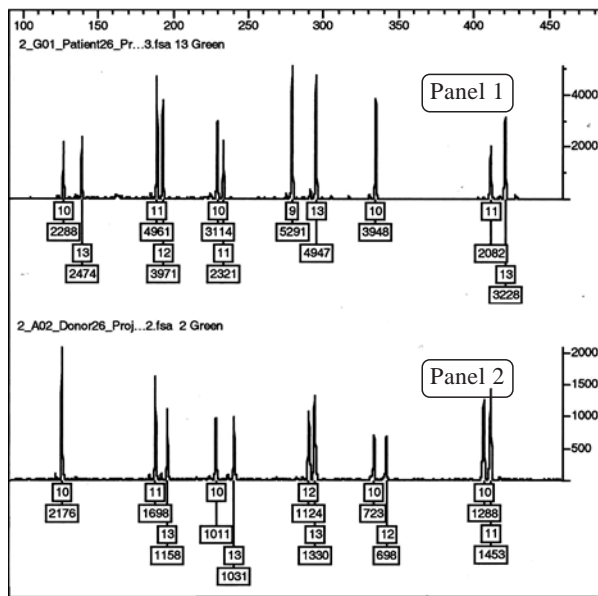
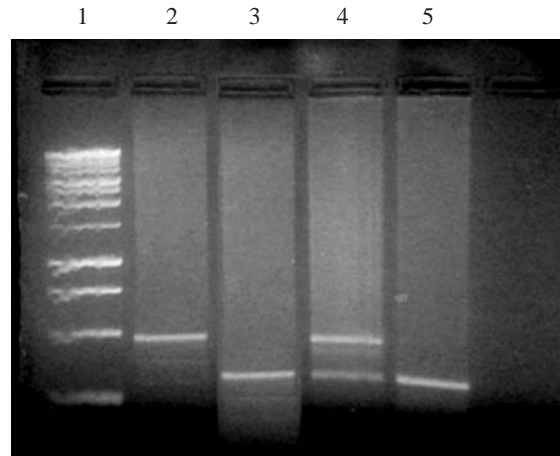


Fig. (2-B): Electropherogram of the patient UPN 26 showing STR loci (**D13S317**, **D7S820**, **D16S539**, CSF1PO and **Penta D**) labeled with JOE. Panel 1 and 2 represent patient and donor alleles. Panel 3 represents day +28 showing 16% donor cells whereas panel 4 represents day +56 showing 78% donor cells and 22% patient cells. Loci in bold are informative for the patient and donor and were used in analysis.

Fig. (3-A): Follow up of chimerism in patient UPN 40 post-nonmyeloablative transplant by amplification of the VNTR locus YNZ-22. Lane 1: MW marker (1kb); Lane 2: Pre transplant pattern of the patient; Lane 3: donor pattern; Lanes 4: The patient on day +28 showing complete chimerism (CC) ; Lanes 5,6: represent analysis of chimerism on days +56 and +90 showing conversion to mixed chimerism (MC); Lane 7: Represents analysis of chimerism at day +120 showing autologous recovery . UPN: Unique patient number, MW: Molecular weight.

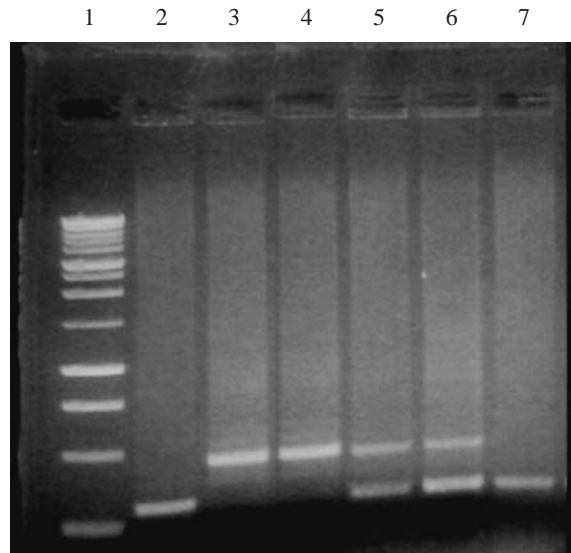


Fig. (3-B): Electropherogram of the patient UPN 40 showing STR loci (**D3S1358**, THO1, D21S11, **D18S51** and Penta E) labeled with fluorescein. Panel 1 and 2 represent the patient and donor alleles. Panels 3, 4, 5 and 6 represent days +28, +56, +90 and +120 showing 99%, 85%, 67% and 0% donor cells respectively. Loci in bold are informative for the patient and donor and were used in analysis.

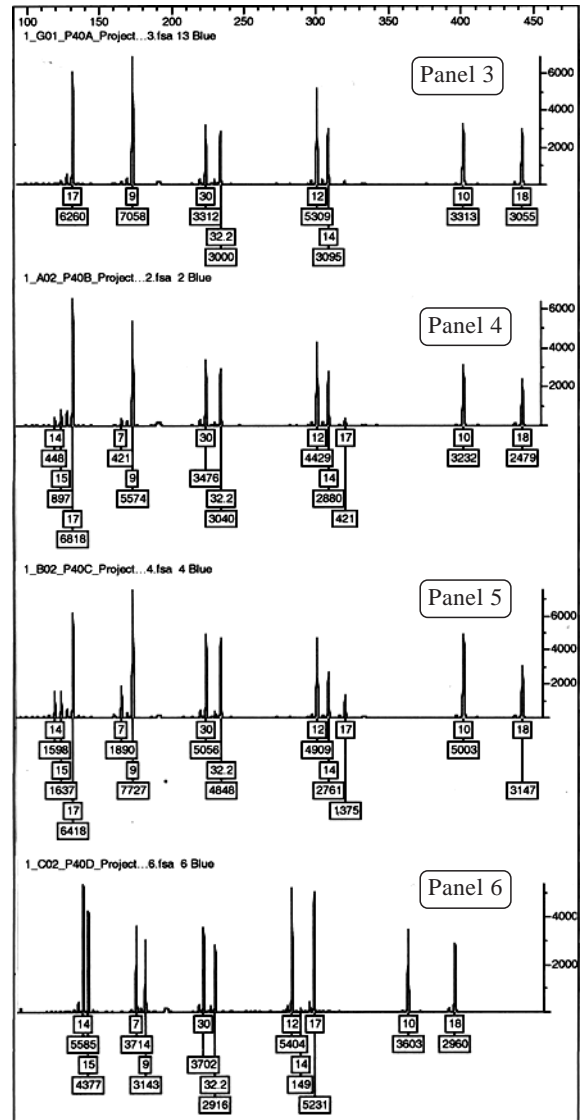
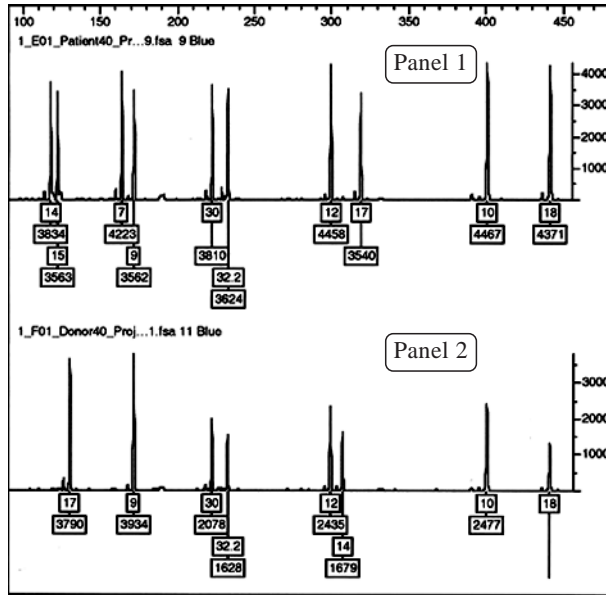


Table (1): Discriminative power among the various STR loci used for the detection of chimerism.

STR Locus	Power of Discrimination
D18S51	80%
Penta E	78%
D5S818	70%
D21S11	65%
D13S317	58%
D8S1179	55%
FGA	55%
D3S1358	53%
D7S820	53%
Penta D	53%
vWA	53%
THO1	45%
CSF1PO	45%
D16S539	38%
TPOX	38%

Table (2): Discrepancies between qualitative VNTR results and quantitative STR results.

UPN	State of chimerism using VNTR		Donor percentages by STR
	Locus	State of chimerism	
32	YNZ-22	CC	70
30	33.1	CC	72, 92
23	Apo-B	CC	75, 80
26	YNZ-22	CC	78
28	D1S80	CC	79, 86
24	Apo-B	AR	24
16	Apo-B	AR	22, 21
25	D1S80	AR	17
36	D1S80	AR	10

UPN : Unique patient number.
 CC : Complete chimerism.
 AR : Autologous recovery.

Table (3): Sensitivity of VNTR in the detection of mixed chimerism.

UPN	State of chimerism using VNTR		Donor or recipient percentage by STR
	Locus	State of chimerism	
35	YNZ-22	MC	3
7	D1S80	MC	5
4	D1S80	MC	9
4	D1S80	MC	14
6	YNZ-22	MC	14
40	YNZ-22	MC	15
9	D1S80	MC	16
26	YNZ-22	MC	16
38	Apo-B	MC	16

UPN : Unique patient number.
MC : Mixed chimerism.

Table (4): Discrepancies between VNTR and STR in quantitation of MC after ASCT.

UPN	Estimated donor percentage by VNTR		Median donor percentage by STR
	Locus	Donor percentage	
11	YNZ-22	30	68
12 (x3)		33.4	60
15 (x5)		33.1	65-80
26	YNZ-22	40	16
31		33.4	75
35	YNZ-22	70	43
40 (x2)	YNZ-22	50,30	70
			85,67

UPN: Unique patient number.

DISCUSSION

Selecting an informative locus is a key step in chimerism analysis, and VNTR/STR markers offer the highest probability of finding an informative marker [15]. The commercial kit PowerPlex 16 enabled rapid identification of more than one (median 7) informative loci in every patient/donor pair after a single multiplex PCR reaction, while VNTR analysis necessitated the use of 1 (in 42% of patients) to 6 PCR reactions to identify an informative locus. STR markers were informative in one patient for whom a recipient specific VNTR allele could not be identified. Moreover, in 5 patients, the absence of a donor specific VNTR allele made it impossible to distinguish between MC and failure of engraftment. The discriminative power among the 6 VNTR loci used, as estimated from our previous study [16], was in the range of 46-58%, and the most informative loci were YNZ-22 and Apo-B. The discriminative power among

the 15 STR loci tested in our present study ranged from 38-80%. The most informative loci were D18S51 and Penta E, as previously reported by Thiede et al. [1]. The combination of D18S51, Penta E and D5S18 gave 100% discrimination.

Selection criteria for STR loci require not only the presence of at least one unique recipient allele, but also the absence of stutter peaks for that allele [1]. A stutter peak is one repeat smaller than the major allele peak. This artifact results from slipped-strand mispairing during amplification and typically represents 2-10% of the corresponding allele [1,17]. A stutter peak also affects the accuracy of detection for small proportions of donor or recipient cells in the mixture [1]. Stutter peaks are more prominent for some STR loci than others. Stutter peaks were more obvious with the STR locus vWA, as previously reported by Walsh et al. [17]. In our series, 7 markers were excluded because of stutter interference. Stutter interference occurs less frequently for pentameric repeats [18]. The PowerPlex 16 kit amplifies two loci with pentameric repeats. Stutter interference did not occur with VNTR amplification, because the repeat unit is much larger than for STR loci [19].

Comparison between VNTR and STR markers showed concordance in the chimerism category for most cases. However, the incidence of MC was higher with STR than with VNTR (45% versus 32%), as also reported by Gonzalez et al. [20]. The discrepancy in the percentage of patients with MC was due to the higher sensitivity of the STR approach [20]. As mentioned by Hancock et al., Lion and Bryant et al. [21-23], the sensitivity of detection depends on the allele size, the difference in size between donor and recipient alleles, the efficiency of the PCR reaction, the quality and quantity of DNA and the method of detection. In our series, discordant results were obtained in 13 samples. The percentage of host or donor DNA found in these 13 cases by STR that could not be detected by VNTR analysis ranged from 8-30%. This could be attributed to the large size of the alleles at certain loci (e.g., 33.1), which hampers the sensitivity of the test, as mentioned by Chandon [24]. Since donor and recipient VNTR alleles can differ substantially in size, preferential amplification of shorter alleles may occur [25,26], resulting in reduced amplification of the larger allele [27].

PCR amplification of some VNTR loci can be as sensitive as STR loci, as previously reported [28]. In our cases, the sensitivity reached 3% and 5% with certain VNTR alleles. This result could reflect preferential amplification of short fragments, allowing minor recipient or donor populations to be detected with greater sensitivity [22]. This phenomenon occurred in UPN 26, due to difference in the size of alleles between the patient and the donor at the VNTR locus YNZ-22. The donor allele was detected at 16% on day 28, but the larger patient allele present at 22% could not be identified on day 56 by using the same VNTR locus.

The sensitivity of detection is usually lower for VNTR loci as compared to STR loci. Using STR, the sensitivity threshold was set at 5% for informative loci with both donor and recipient unique alleles [18,29,30]. Preferential amplification of shorter alleles is less obvious due to overall smaller size differences of STR amplicons [28]. Some investigators have stressed that the amplification efficiency could differ significantly with the two assays [22,24,25]. The quantity of DNA needed for efficient PCR amplification is much lower with the STR reagents employed in these studies as compared to the VNTR reagents used here. The fluorescent signal obtained in the STR analysis is proportional to the number of cells [31], thereby improving the sensitivity of the assay compared to ethidium bromide staining.

Quantitative analysis of chimerism kinetics after ASCT has been shown to predict graft failure or relapse. Increasing levels of MC in patients monitored serially is associated with increased risk of relapse [4,7,32-34]. Measurement of chimerism is a prerequisite for manipulating engraftment by withdrawal of immunosuppressive medications or administration of DLI [35-37]. In general, most PCR-based methods are semiquantitative, although the accuracy of quantitation can be increased by using multiplex PCR [6,29]. Both patient and donor unique alleles are needed for quantitation with VNTR. Such informative markers were available in 32 of the 40 cases we studied. In STR analysis, the use of unshared alleles to calculate chimerism allows a detection limit of 5%, compared to 10% when using one shared and another unshared allele [18]. In our series, multiple STR loci provided both patient and donor unique alleles in all patients.

Preferential amplification due to differences in allele sizes is more obvious with VNTR loci than with STR loci and may result in a nonlinear relationship between the allelic band intensity and the amount of recipient versus donor DNA present in the sample [31]. In our series, preferential amplification was obvious from comparing the quantitative assessment of MC between the estimated relative band intensity of the ethidium bromide-stained gel and the median of the percentage peak areas of the informative STR loci. Difference $\pm 10\%$ were considered discordant, and the 8 discordant cases (8/19) could be all attributed to preferential amplification of small VNTR alleles and underestimation of larger ones. This problem was more obvious with the VNTR locus YNZ-22 (4/8) than with other loci. In order to achieve a reliable relationship between band intensity and the percentage of donor or recipient DNA in the sample, it would be necessary to produce standard curves of specific samples mixtures [32]. As shown in patient UPN 24, however, an increasing or decreasing band intensity might indicate a corresponding change in the relative percentages of patient and donor-derived cells.

The need to use several informative loci emerges from differences in the efficiency of amplification of the alleles at each locus. With STR loci, the accuracy of quantitative chimerism analysis can be increased by testing more than one marker [22] and by taking a mean or median of all informative loci to compensate for random variability in amplification [6]. This allows for a more accurate comparison between sequential samples after transplantation [21,38]. After taking the average percent donor chimerism at several STR loci and the presence of stutter peaks into account, the accuracy of quantitation is best set at $\pm 5\%$ [33,38,39].

Besides enabling rapid identification of more than one informative locus, the use of multiplex STR PCR improves the reproducibility and the quantitative accuracy of chimerism measurements, since a mean or median of several results can be used to estimate donor chimerism. The use of more than one marker is also needed for consecutive donor chimerism evaluation, since loss of specific chromosomal regions during clonogenic evolution has occurred in several haematological malignancies [18,40,41]. However, these advantages are counter-balanced by the

high cost and the lower sensitivity compared to a monoplex assay [22].

To conclude, VNTR and STR analysis gave comparable qualitative results in most cases. However, the chance of discrimination was higher with STR. To increase the sensitivity of VNTR, loci with higher sensitivity are used preferentially; these are usually loci of small size alleles. Some VNTR loci had good sensitivity, but were not optimal for quantitation because of large differences in allele size. This was noted especially for YNZ-22. With these loci, the estimated relative band intensity cannot be used for quantitation without the use of a standard curve constructed from dilutions of recipient DNA in donor DNA. However, sequential monitoring of the relative band intensity may offer some insight into the direction of change in engraftment over time.

Compared to VNTR, STR analysis is more sensitive and therefore does not need as much DNA. The yield of informative alleles is higher with STR loci, and the assay is more rapid and gives more accurate and reproducible results. On the other hand, costs of STR testing are higher, and the analysis of amplified samples requires expensive equipment. Costs could be reduced by selecting a smaller number of highly informative STR loci and by designing a monoplex PCR that also improves the sensitivity of detection. The choice of either technique should be made according to the specific needs (i.e., qualitative or quantitative), the lab facilities and the cost.

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