INTRODUCTION

Haematopoietic stem cell transplantation (HSCT) is widely used as curative treatment for patients suffering from malignant as well as non-malignant haematological diseases. It can be accomplished by either bone marrow transplantation (BMT), or peripheral blood stem cell transplantation or cord blood stem cell infusion. Aplastic anaemia, beta-thalassaemia major, chronic myeloid leukemia and acute leukemia form the bulk of diseases for which BMT is carried out in Pakistan.

Following a BMT, the recipient develops varying degrees of chimerism of the donor and recipient cells. This chimerism can be broadly categorized as complete or mixed depending upon the percentage of donor cells in the recipient's blood or bone marrow. Monitoring the chimerism status is valuable in determining the success of allogeneic stem cell transplant and also following the course of mixed donor chimerism patients.

Chimerism testing involves identifying the genetic profiles of the recipient and of the donor and then evaluating the extent of mixture in the recipient's blood or bone marrow. Analysis of the genetic profile by short tandem repeats (STR) using polymerase chain reaction (PCR) is the preferred method for assessing chimerism. STRs are highly polymorphic regions of extrageneic DNA that consists of a sequence of 2 to 8 bases in length. The sequence is repeated in tandem i.e. one after another like carriages of a train and it is the number of these repeats that vary from individual to individual. The amplified STR markers can either be resolved on capillary electrophoresis by a genetic analyzer, or by gel electrophoresis which can later on be quantified. A pre-transplant genetic profile of the recipient done by STR analysis should be available ideally for comparison with the post-transplant STR profile. However, if it is not available, then a sample of the buccal mucosa of the recipient taken carefully with a swab can be used for extraction of the DNA and it can be treated as pre-transplant STR genetic profile.

Despite its immense potential and utility, studies pertaining to donor chimerism have not been done in Pakistan. Therefore, information regarding this important aspect is lacking in our setting.
parameter in post-BMT patients is deficient. This study describes in detail the materials required and the procedure followed for chimerism analysis best suited for our setup. It also highlights the STR markers which are most informative.

The aim of this study was to determine the frequency of mixed donor chimerism in patients of non-malignant haematological diseases after allogeneic bone marrow transplant.

**METHODOLOGY**

It was cross-sectional study and was carried out in the Department of Haematology at Armed Forces Institute of Pathology, Rawalpindi. A total of 84 patients; 50 with beta-thalassaemia major and 34 with aplastic anaemia were part of the study. Patients of either gender who had a post-transplant duration between 2 months to 2 years were included in the study, whereas patients who were undergoing a second transplant after a graft failure were excluded. Sample size was calculated by WHO sample size calculator; using 95% confidence interval and absolute precision as 10%.

Mixed donor chimerism was defined as a percentage of donor origin DNA between 5 – 95% in the recipient's blood or bone marrow as assessed by STR-PCR analysis. It was further sub-divided into two categories of > 50% mixed donor chimerism and < 50% mixed donor chimerism. Patients with > 95% donor DNA in blood or bone marrow were labeled as complete chimerism (CC), while those with < 5% were regarded as graft failure.

Aseptic technique was used to sample 2.5 – 3 ml venous blood in ethylene diamine tetra-acetic acid (EDTA) and complete blood counts were performed on Sysmex KX - 21 to assess the total leukocyte count (TLC). In individuals with low TLC; bone marrow aspirate collected in EDTA was used as a source of DNA. Most of the requests for donor chimerism analysis were post-transplant patients and in order to determine the patient's pre-transplant STR genotype, sample of the buccal swab was used to extract DNA.

DNA extraction was done using a commercial kit (Gentra, USA); whereas amplification of the extracted DNA for STR markers was done by PCR. The contents included 2 uL of extracted DNA, 1 uL of the specific STR primer (Gene Link Company), 20 uL PCR mix and 0.1 uL Taq DNA polymerase. The detail of STR primers that were used is shown in Table I. Initial denaturation was done for 5 minutes at 94°C followed by 30 cycles of amplification with denaturation at 93°C for 48 seconds, annealing at 60°C for 48 seconds and extension at 72°C for one minute. The PCR amplified products of STR genetic profile/alleles were then resolved by electrophoresis on 8% polyacrylamide gel. The gel was stained with silver nitrate. Quantitative analysis of STR alleles was done by densitometry. The selection of a particular STR marker in a patient was based on its informativeness in that patient.12

Results of the donor chimerism testing were analyzed using Statistical Package for Social Sciences (SPSS) version 13. Mean values and standard deviation were calculated for numerical data like age and duration of transplant. Frequencies of mixed donor chimerism, STR markers and gender of the patient were calculated.

**RESULTS**

Out of the total 84 patients, 52 (62%) were males and 32 (38%) females. Among the 50 patients of beta-thalassaemia major, 20 (40%) were females and 30 (60%) were males; while in aplastic anaemia, 22 (64.7%) were males and 12 (35%) were females. Collectively, the age ranged from 2 to 48 years. The overall mean age of the patients in the study was 10.3 ± 8.72 years; the mean age of beta-thalassaemia patients was 5.6 years and that of aplastic anaemia was 17.3 years. The source of stem cells in all the patients was a sibling.

The mean time of chimerism testing in aplastic anaemia patients was 9.5 ± 7.9 months; while that of beta-thalassaemia major patients was 8.3 ± 5.9 months. The most commonly used STR markers were for the loci, D3S1358, D5S818, D8S1179, D9S305 and TH01. The informativeness of these markers was calculated for our setup. It also highlights the STR markers which are most informative.12

<table>
<thead>
<tr>
<th>STR Locus</th>
<th>Primer sequence</th>
<th>STR Alleles (repeats)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D3S1358</td>
<td>F 5'-ACT GCA GTC CAA TCT GGG T</td>
<td>14,15,16,17,18</td>
</tr>
<tr>
<td></td>
<td>R 5'-ATG AAA ACA GAG GCT TG</td>
<td></td>
</tr>
<tr>
<td>FGA</td>
<td>F 5'-GCC CAT AGG TTT GGA ACCTCA</td>
<td>19,21,22,23,24</td>
</tr>
<tr>
<td></td>
<td>R 5'-TGA GTT GCTG TTA TGG CCT GAC</td>
<td></td>
</tr>
<tr>
<td>D5S818</td>
<td>F 5'-GGG TATT TCT CCT TTT GT</td>
<td>10,11,12,13</td>
</tr>
<tr>
<td></td>
<td>R 5'-TGA TCC AAT CATA GCC ACA</td>
<td></td>
</tr>
<tr>
<td>D7S820</td>
<td>F 5'-GCTA TATT TAGA CAGA AACTACG</td>
<td>7,8,9,10,11</td>
</tr>
<tr>
<td></td>
<td>R 5'-CTGAG TATC AAAAAAC TCA GAG</td>
<td></td>
</tr>
<tr>
<td>D8S1179</td>
<td>F 5'-TTTT TTG ATTT CTT GTG ACATT GGG</td>
<td>10,11,12,13,14,15</td>
</tr>
<tr>
<td></td>
<td>R 5'-CGTAC GCT TAA TTT GCT TAT TCC</td>
<td></td>
</tr>
<tr>
<td>TH01</td>
<td>F 5'-GTG GGT GAG CAG TC GCC GAT TAT</td>
<td>6,7,9,10</td>
</tr>
<tr>
<td></td>
<td>R 5'-ATT CAA AGG GAT TAC TTG GCT TGG</td>
<td></td>
</tr>
<tr>
<td>D21S11</td>
<td>F 5'-ATG AAT GAC ATG AAT GGA TGG</td>
<td>27,28,29,30,31,</td>
</tr>
<tr>
<td></td>
<td>R 5'-AATGT GTG TCT CTT CCG AGG</td>
<td>31,2,32,2,33,3</td>
</tr>
</tbody>
</table>
Mixed donor chimerism after allogeneic bone marrow transplantation

Table II: Disease wise chimerism status of the patients in the study.

<table>
<thead>
<tr>
<th>Disease</th>
<th>CC*</th>
<th>Mixed donor chimerism</th>
<th>Graft failure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>&gt; 50% MC</td>
<td>&lt; 50% MC</td>
</tr>
<tr>
<td>Aplastic anaemia</td>
<td>n = 13 (38.2%)</td>
<td>n = 17 (50.0%)</td>
<td>n = 13 (76.4%)</td>
</tr>
<tr>
<td>Beta-thalassaemia major</td>
<td>n = 13 (26%)</td>
<td>n = 31 (62%)</td>
<td>n = 22 (71.0%)</td>
</tr>
</tbody>
</table>

* Complete chimerism

D3S1358, D5S818 and D8S1170 (Table II). A total of 49 (58.3%) patients developed MC according to the criteria defined; while 25 (29.8%) patients achieved CC and 10 (11.9%) patients had graft failure.

Out of those who achieved MC, 35 (72.9%) patients had > 50% MC, while 13 (27.1%) patients had < 50% MC. The disease wise frequency of MC, CC and graft failure is shown in detail in Table III. The percentage of donor DNA present in the recipient hold prognostic significance; so it was also assessed in the patients that showed MC in the study. Among the patients of aplastic anaemia with > 50% MC, the mean quantity of donor cells was 86.3 ± 9.15% while among patients of beta-thalassaemia major with > 50% MC, it was 90.9 ± 3.18%.

**DISCUSSION**

Evaluating the donor chimerism status in the recipient is crucial for assessing and monitoring engraftment of the transplanted stem cells in the recipient. Mixed donor chimerism occurs when both the donor and recipient origin DNA are detected in the recipient's blood or bone marrow. MC is an ongoing dynamic relation between the donor and recipient's cells and can be further subdivided into stable, increasing, decreasing, or variably fluctuating (also called progressive mixed chimerism).5,16 Patients who achieve stable MC; irrespective of the percentage of donor DNA have excellent survival and no chronic graft versus host disease. Whereas recipients with progressive mixed chimerism have a higher incidence of developing graft rejection.

In the study, donor chimerism was determined using STR analysis, a PCR based technique and the results were resolved on gel electrophoresis. The percentage of donor DNA was measured on densitometer. Although the STR analysis was initially developed for use in forensic criminal identification,13 these markers can be effectively used to distinguish donor DNA from that of the recipient in an post BMT patient.17,18 Results of the study show that D3S1358, D5S818, D8S1170 were the three most frequently used STR markers for assessing chimerism in the study; used in 25, 23 and 18 patients respectively, i.e. a total of 66 patients (78.5%). The utility of these statistics is that it can help in developing an algorithm of informative markers for evaluating chimerism after allogeneic BMT.19,20 If these three STR markers are not informative in a patient, the next three can be checked in the patient donor pair i.e. D7S820, TH01, FGA. Furthermore, the commonly used STR markers can also be set together in a multiplex PCR to yield cost effective results.

The patients in the study were predominantly males (61.9%); the female underrepresentation could be because of gender bias in our society when seeking costly medical attention for different diseases. Secondly, no study shows gender as a prognostic marker for post-transplant outcomes in either aplastic anaemia or beta-thalassaemia. In this study also, the percentage of males and females achieving MC was 59.6% and 56.3%, which is not a major difference.

In this study, the frequency of MC in patients of non-malignant haematological diseases at mean post-transplant duration of 8.8 months was 58.3%. Since this study is the first of its kind in Pakistan; therefore, no local studies were available on the subject for purpose of comparison of these results despite thorough search in the literature. Many foreign studies assessing donor chimerism in the patients of either beta-thalassaemia or aplastic anaemia alone have been done. Majority of these studies follow the patients in the post-transplant period and sequentially monitor donor chimerism to assess the state of haemopoiesis. Additionally, no exact definition for MC was available; which could be one of the reasons for an increase percentage of MC. Most of the present patients had > 50% MC with a mean percentage of donor origin DNA of 86.3% and 90.9% in aplastic anaemia and beta-thalassaemia major respectively.

Lisini et al. showed that out of the 79 patients of beta-thalassaemia major, 51 (64.5%) exhibited MC and 24 (30.3%) developed CC which is comparable to results of this study, where out of the 50 post-transplant patients of beta-thalassaemia, 31 (62%) achieved MC and 13 (26%) developed CC. However, in an Italian study by Andreani et al., the incidence of MC in 93 patients of beta-thalassaemia major who underwent allogeneic BMT was 46.2% (n = 43) while that of CC was 53.7% (n = 50). The difference in results could probably be due to the mean duration of assessing chimerism in study by Andreani et al. was between 20 days to 60 days, whereas in this study, it is 8 months. The study by Andreani et al. also highlighted that the percentage of donor cells may decrease with subsequent follow-up leading to graft rejection, if at initial assessment, the percentage of residual host cells was > 25%.21

In a large study done by Huss et al. on 116 post-BMT patients with aplastic anaemia, who received stem cells from HLA-identical siblings, MC was seen in 63 (54%) patients. This is comparable to that of our study, where MC was seen in 50% of aplastic anaemia patients. In an older study by Hill et al., almost 58.3% of the patients of aplastic anaemia had mixed chimerism after allogeneic
BMT. Here again, the difference may be because the chimerism was assessed between post-transplant days 14 and 100.

Some of the limitations of the study were that the sampling technique was non-probability sampling that could affect the results. Additionally, the factors affecting engraftment and post-transplant chimerism like exposure to blood components, disease severity, stem cell dose, donor and transplant related characteristics e.g. blood group missmatch and gender missmatch were not available.

Local studies pertaining to mixed donor chimerism in patients of allogeneic BMT are virtually non-existent in Pakistan, whereas the number of transplant centre continues to rise in the country. Therefore, a lot of work needs to be done in this regard. In the future prospective studies that follow the patients in a longitudinal manner and assess the degree of mixed donor chimerism at 3 months, 6 months and 12 months need to be carried out. Also the correlation of mixed donor chimerism with the blood counts of the patient at these intervals may provide a clue, as to which haematological parameter of blood counts is best indicative of a higher mixed donor chimerism percentage.

CONCLUSION

Following a BMT for non-malignant haematological diseases majority of the patients achieve mixed donor chimerism. Analyses of D3S1358, D5S818 and D8S1170 STR markers are most informative in these patients. However, an isolated analysis of donor chimerism percentage is not sufficient and serial chimerism analyses over a period of post-transplant duration are required for better interpretation of the donor haematopoiesis in the recipient.

REFERENCES