HLA ALLOIMMUNIZATION IN MULTI-TRANSFUSED PATIENTS WITH B-THALASSEMIA MAJOR IN BASRAH.

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ABSTRACT

Alloimmunization to human leukocyte antigens (HLA) can occur with chronic transfusion therapy and may result in graft rejection during stem cell transplantation. The prevalence and risk factors for HLA alloimmunization in multiply transfused β-thalassemia major (β-TM) patients are unknown. A case control study of HLA alloimmunization among multiply transfusedβ-TM patients aged 3 to 17 years compared to apparently healthy controlindividuals matched with age and gender was performed to detect the antibody to HLA class I and/or class II measured by Panel Reactive Antibody using luminex® technique. One hundred and twenty three individuals (73 with β-TM on regular blood transfusion and 50 healthy individuals) were tested. HLA antibodies were detected in70/73 (95.9%) compared to 10/50 (20%) of the controls,antibodies to HLA class I and II occurred in 52/73 (71.2%), HLA class I (only)antibody occurred in 14/73 (19.2%), which was significantly higher than the controls in which the antibodies detected in 4/50 (8 %) and 3/50 (6.0%) respectively, and class II (only)antibody occurred in 4/73 (5.5%), no significant difference in the antibody to HLA class II (only) between the patients 4/73 (5.5%) and the control 3/50 (6.0%).The current study also showed that the mean levels of the pRBC hematological parameter were low compared to the WHO recommended levels. The current study suggests close association between HLA alloimmunizaton and chronic transfusion therapy.For patients with β-TM on regular transfusion therapyconsideration should be taken for HLA-alloimmunization identification and corresponding Provision of safe, effective and quality blood and blood products for transfusion.

Keywords:HLA alloimmunization, Multiple transfusions, Thalassemia major

No: of Figures: 2 **No: of Tables: 3** **No: of References: 29**

INTRODUCTION

Beta-thalassemias are a group of hereditary blood disorders characterized by partial or complete suppression in the synthesis of beta chains of normal hemoglobin molecules, resulting in variable phenotypes, ranging from severe anemia to clinically asymptomatic individuals**(Giardina et al, 2008)**. It is considered one of the most serious health problems worldwide,accounting for a major number of child deaths per year **(Joly et al. , 2013; Thein et al., 2016).** It was estimated that nearly 3% of the world's populations carry genes for β-thalassemia **(Debaun et al., 2011)**. In Arab countries, thecarrier rates of β-thalassemia ranging from 1–11 % **(Hamamy et al., 2013)**. In Iraq, the reported prevalence rate of β-thalassemia ranges from 3.7% - 6.5% in different provinces and cities**(Jalal et al., 2008)**. In Basra, the overall carrier rate is 4.6% **(Hassan et al., 2003).** Certain cultural and demographic characteristics of most Arab populations influence the incidence and prevalence rate of this genetic disorder, such as their consanguineous marriage, marriage at early age, large family size and first cousin union**(Hamamy et al., 2013; Qari et al., 2013)**.

The majority of infants with B thalassemia major and many with thalassemia intermedia who develop transfusion-dependent β-thalassemia (TDT), present anemia that usually develops during the first few months of life and becomes progressively more severe in thalassemia major and later in life in intermedia **(Srinoun et al., 2009) .** Regular transfusion program is the main stay in the managementrequired to maintains a minimum pre-transfusion Hbconcentration of 9.5 to 10.5 g; growth and development tend to be normal up to 10 to 11 years **(Olivier et al., 2006; Thein et al., 2016)**

Regular blood transfusion, iron chelation therapy, and reasonable use of splenectomy are the main stay for symptomatic treatment of thalassemia**(Olivier NF et al., 2006; Pignatti CB, 2009).** Bone marrow transplantation is the only cure and the best candidates for transplantation are young patients who have not yet developed complications of thalassemia or its treatment **(Pignatti CB, 2009).**

Despite the importance of chronic transfusion therapy in thalassemia, it is consider as a double-edged sword. Finally, the patients may die either from transfusions or from lack of it **(Shah et al., 2010)**.

Many years ago there was growing awareness that leucocytes presentin blood components intended for transfusion could evoke immune response to human leukocyteantigens (HLA),transmit cytomegalovirus (CMV), cause febrile non-hemolytic transfusion reactions (FNTR) and induce immunosuppression **(Bilgin et al., 2011; Chuansumrit et al., 2001)**.

While trying to diminish antigenic barrier in HSCT transplant as this approach represents the only cure for multiply transfused β-TM patients **(Patel et al., 2013)**, therefore, it is crucial to study the baseline prevalence of HLA alloantibodies, and determine the risk factors for their

occurrence to understand their possible contributions to HSCT complications, so the objectives of this study is to detect the prevalence of HLA alloimmunization in transfusion dependent β-thalassemia patients who are subjects to future bone marrow transplantationcompared to apparently healthy control.

MATERIALS AND METHODS

A case control study was carried out in the period between April 2016 and July 2017.A total of 123child and adolescents were enrolled in the study**, 73** patients with β-TM who has been registered at Center for Hereditary Blood Diseases (CHBD) in Basra, Iraq and **50** apparently healthy controls (matched by age and gender) who were attending Primary Health Care (PHC) Centers for minor health problems. The patients were regular recipients of blood transfusion, received at least three lifetime RBC transfusions with age range from 3-17 years, excluding patients with other hemoglobinopathies, platelet transfusion, transplantation, fever, pregnancy hepatitis C and on systemic steroid

An information was obtained from each patient includes age, gender, age of diagnosis, frequency of blood transfusion, transfusion of filtered PRBC, reaction to the blood history of splenectomyand residence, the transfusion records were also reviewed for date of birth, age at first transfusion, frequency of transfusion, transfusion of leukoreduced blood, splenectomy and infection with hepatitis C

Laboratory investigation

A 2 mL of blood sample was collected from each enrolled subject by venipuncture using a sterilesyringe, emptied in gel free plain tube which was left stand for one hour at room temperature allowed to clot and then centrifuged at 3000(rpm) for 5 minutes, the resulted serum drawn by clean pipette and emptied into sterile Eppendorf tube with the identification code of the patient or control individual and kept frozen at -20C◦ until time of examination was performed.

Hemoglobin capillary electrophoresis or HPLC was done for all control groups to exclude hemoglobinopathy

An estimation of some hematological parameters (WBC, Hb and hematocrit) of 56 pRBC units recommended for transfusion in BCHBD blood bank was also done by obtaining one ml blood samples from pRBC unit by closed method,the indices were analyzed nearly 1-2 hours after collection using automated hematology analyzer (Ruby). The 56pRBC units were also weighed by accurate balance and the volume was calculated using the fallowing formula as recommended by the Iraqi National Blood Bank.

$$\text{pRBC volume} = (\text{Wt} - 26.7)/1.08$$

Panel reactive antibodies (PRA) screening tests for detection of ant-HLA class I and class II antibody were performed using Luminex® assay kits LIFECODES Lifescreen Deluxe (Immucor Transplant Diagnostics, Inc. Stamford, CT,

USA),based on immobilization of Soluble HLA molecules to fluorescently labeled polystyrene microbeads used as a target antigen, the percentage of beads that react with antibody in the patient's serum is termed the panel reactive antibody (PRA) activity, the results were interpreted as positive and negative.

The data were analyzed using Statistical Package for Social Science (SPSS) version 24. Chi-square (X2) and Fisher's Exact tests were used to determine the difference between the study groups. Student's t-test was used for comparing the means. Comparisons of proportions were performed by crosstab using the χ2 test to assess the significance of difference between groups. The significance level was set at $p < 0.05$, and the highly significance level was set at $p < 0.001$.

ResultsThe Distribution of case and control according to age groups and gender:Table-1 below showsno significant difference in the age and gender distribution between the two groups, P value > 0.05.

Table -1 Distribution of case and control according to age groups and gender

Variables	Subject		Total	P-value
	Case N=73	Control N=50		
Mean age ± SD				
	9.8 ± 4.4	9.8 ± 4.0	9.8 ± 4.2	.417
Age-group				
< 5	12 16.4%	6 12.0%	18 14.6%	.786
5-10	34 46.6%	24 48.0%	58 47.2%	
> 10	27 37.0%	20 40.0%	47 38.2%	
Gender				
Female	30 41.1%	24 48.0%	54 43.9%	.449
Male	43 58.9%	26 52.0%	69 56.1%	

Selected clinical variables for patients with β-TM

As shown in Table -2, the mean age of patients with β-TM was 9.2 ± 4.2 years and10.4 ± 4.6 years for male and femalerespectively. The mean age of diagnosis as having β-TM was 20.9 ± 23.0 months, with the majority (72.6%) of them were diagnosed in their first year of disease onset. The mean pre-transfusion Hb level was 6.9 ± 1.2 and the mean annual blood transfusion was 15.7 ± 4.9 with a rate of 12 – 14 units per year in 68(93.2%) of them. Nearly half (47.9%) of patients received their blood transfusion without filter, the other half (47.9%) were infrequently transfused with filtered blood according to the availability of the bed side filters in the center, except for 3(4.1%) patients who always transfused with filtered blood products.

All of patients have had developed transfusion reaction, at least once, in the form of febrile 29(39.7%), allergic 29(39.7%) or hemolytic 15(20.5%) transfusion reaction. Their hemoglobin concentrations prior to transfusion were low in majority of patients and no patient in the enrolled subject had a Hb level that reach to 9 g/dl. Less than half 15 (20.5) of enrolled patients were splenectomised and 31 (72.1%) of them live in the rural area.

Table - 2Selected clinical variables for patients with β-TM

Variables	Males	Females	Total: n (%)
Number (%)	43 (58.9)	30 (41.1)	73
Mean age ± SD	9.2 + 4.2	10.4 + 4.6	
Mean age of diagnosis in months	18.8 ± 17.4	22.4 ± 26.3	20.9 ± 23.0*
Age at which thalassemia diagnosed/year			
≤ 1 year	30 (71.4)	23 (43.4)	53 (72.6)
1-3 years	7 (16.7)	5 (16.1)	12 (16.4)
3-5 Years	1 (2.4)	3 (9.7)	4 (5.5)
>5 years	4 (9.5)	0	4 (5.5)

Mean pre-transfusion hemoglobin concentration			
Mean Hb ± SD	**7.0 ± 1.0**	**6.8 ± 1.4**	**6.9 ± 1.2**
Mean annual blood transfusion	**15.3 ± 4.5**	**15.9 ± 5.3**	**15.7 ± 4.9**
Rate of transfusion/ year			
<12	**1 (2.3)**	**0**	**1 (1.4)**
12-24	**42 (97.7)**	**26 (86.7)**	**68 (93.2)**
>24	**0**	**4 (13.3)**	**4 (5.5)**
Transfusion of filtered blood			
Always	**2 (4.7)**	**1 (3.3)**	**3 (4.1)**
Infrequent	**20 (46.5)**	**15 (50.0)**	**35 (47.9)**
Never	**21**	**14 (46.7)**	**35 (47.9)**
Reaction to blood transfusion			
Allergic	**25 (34.2)**	**12(16.4)**	**37 (50.7)**
Febrile	**15 (20.5)**	**13 (17.8)**	**28 (38.3)**
Hemolytic	**3 (4.1)**	**5(6.8)**	**8 (11.0)**
Splenectomy Yes	**6 (14.0)**	**9 (30.0)**	**15 (20.5)**
No	**37 (86.0)**	**21 (70.0)**	**58 (79.5)**
Residency Rural	**31 (72.1)**	**18 (60.0)**	**49 (67.1)**
Urban	**12 (27.9)**	**12 (40.0)**	**24 (32.9)**

Average parameters of blood units

Table -3 shows marked variation in the range of Hb (**23.76 – 65.6**) and hematocrit (**26.8 -82.2**) as compared to the criteria recommended by the WHO and the average WBC (x10⁶/unit) counts is 2300 ± 3800, ranging from 300 – 12600, which is far away from the WHO quality criteria.

Red Blood Cells SAGM	volume (mL) N=56	Hemoglobin (g) N=56	Hematocrit (L/L) N=56	Leukocytes ($x10^6$) N=56
Mean ±2 SD	280 ± 36	47 ± 22	0.51 ± 0.26	2300 ± 3800
Range	238 - 480	23.76 – 65.6	26.8 -82.2	300 – 12600
WHO Quality criteria*	150- 200ml ±10% labelled volume	≥45 g/unit	55-75%	<5x10⁶/unit in all units tested

Table - 3 Hematological parameters of blood units in BCHBD

The correlation between the pre-transfusion hemoglobin level and the rate of transfusion.

The correlation between the pre-transfusion hemoglobin level and the rate of transfusion per year is shown in fig -1.Out of 38/73 (52.1%) patients with Hb level ≤7 g/dl, 34 (46.6%) appear to have their blood transfusion in a rate of 12-24 units per year, one (1.4%) patient with infrequently transfused blood less than 12 units per year and only 3 (4.1%) patients have had their blood transfusion rate more than 24 units per year. The remaining 35/73 (47.9%) patients with pre-transfusion Hb level > 7g/dl, have received their blood transfusion in a rate of 12-24 per year.

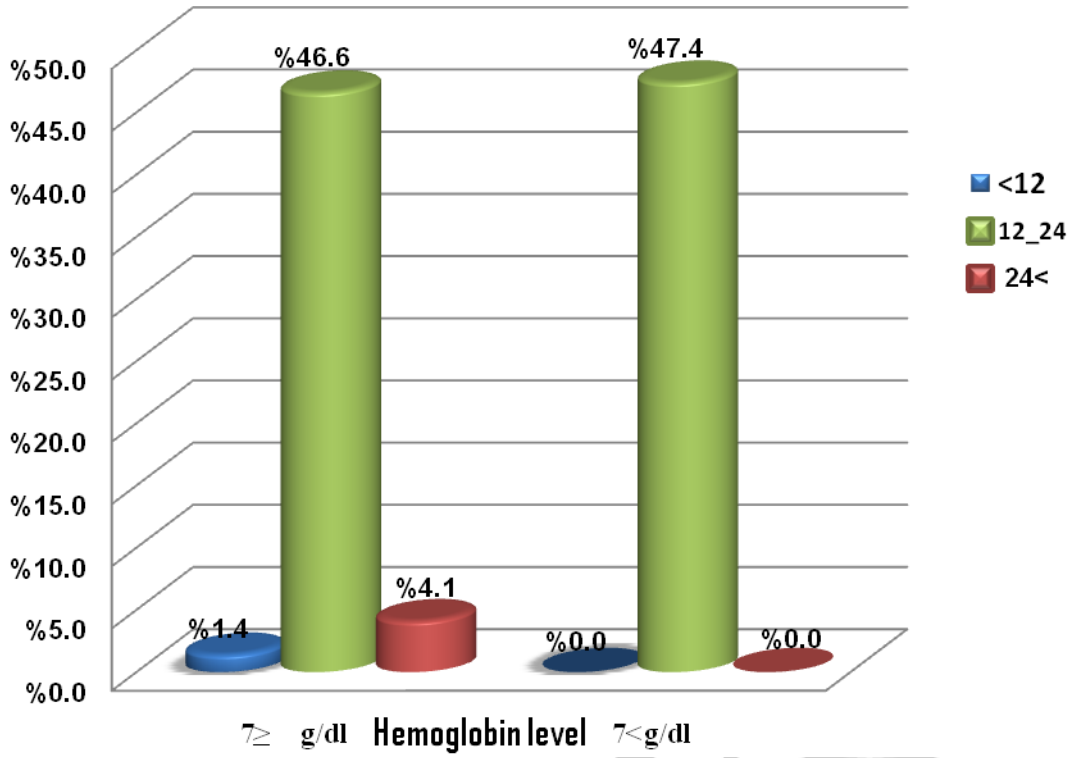

Fig – 1 The correlation between pre- transfusion Hb level and the interval of transfusion in days

The prevalence of HLA antibodies in multiply transfused β-TM patients versus non-transfused healthy control individuals.

As shown in Fig-2. Antibodies to HLA were detected in 70 (95.9%) out of 73 multiply transfused β-TM patients versus 10 (20%) out of 50 non-transfused healthy control individualsand statistically the difference were highly significant (P value < 0.001).Of the 70 patients with positive anti-HLA antibodies, 52 (71.2%) developed antibodies to both classes of HLA molecule (class I & II) and 14 (19.2%) developed antibody to HLA class I only compare to 4(8 %) and 3(6.0%) out of the 10 apparently healthy individuals respectively, and the differences were statistically significant in both. While no statistical difference was found in the percentage of antibody to HLA class II only between the patients 4 (5.5%) and the control 3 (6.0%).

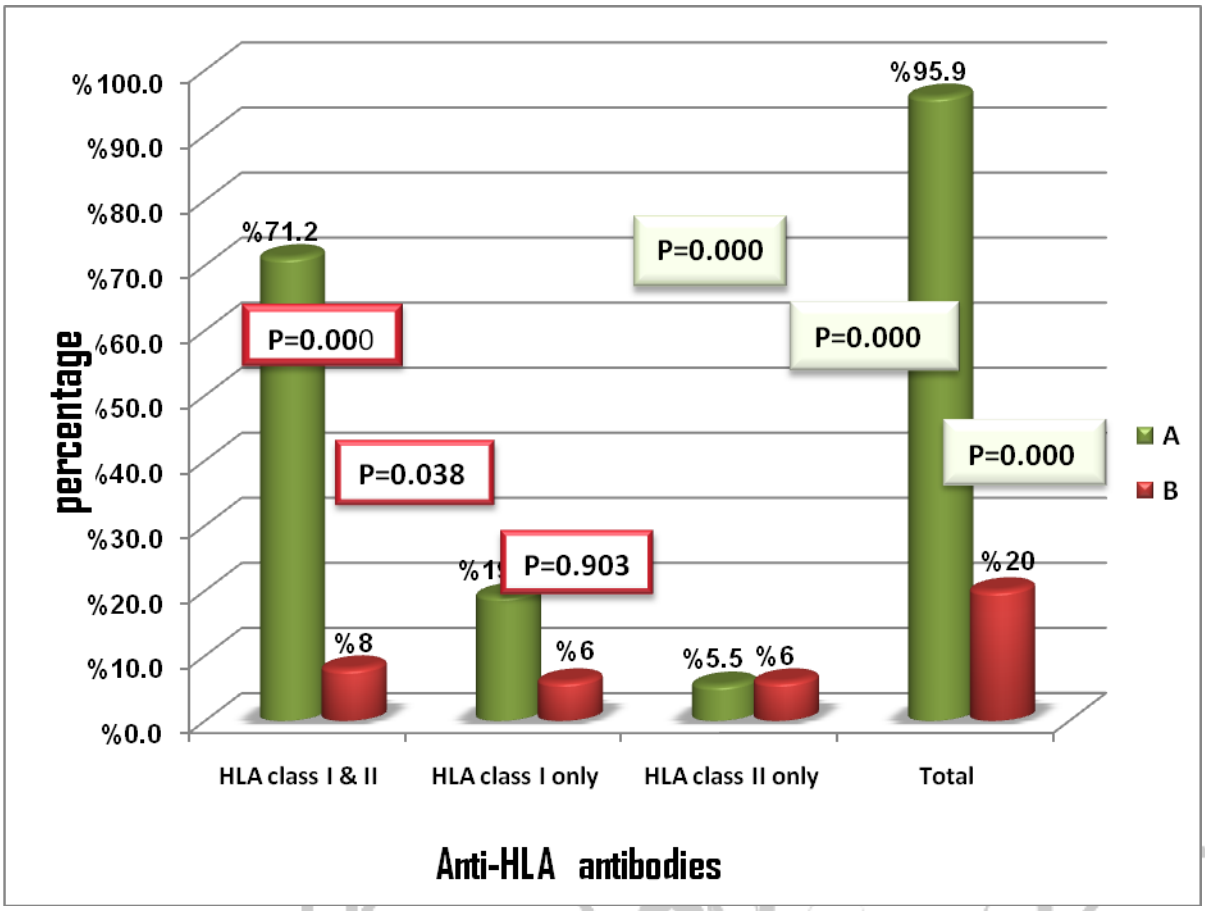

Fig -2 The prevalence of HLA antibody in multiply in transfused β-TM patients vs non-transfused healthy control individuals

DISCUSSION

Up to our knowledge this is the first study that focuses on the prevalence of HLA alloimmunization in multiply transfused β-TM in Iraq, HLA alloimmunization due to blood transfusion is an important phenomenon, while it is well-documented in the field of solid organ transplant, still a matter of concern that is under-recognized in transfusion dependent hemoglobinopathy. Knowledge about this phenomenon in multiply transfused β-TM may help to identify patients who may be at risk that threat their future HSCT.

In the current study the age of the enrolled subject ranged from 3 – 17 years as this is the best age eligible for future HSCT **((Bhatia, 2008;Giardina PJV, 2008. Triulzi, 2009**). Their age of diagnosis as having β-TM was 21.6 ± 24.5 months, which is in accordance with the range of23.11±26.94 months reported by Karamifar 2005. **(Karamifar et al., 2005)**.

It is well documented that HLA-alloimmunization is strongly correlated with the number of residual WBC in the blood units. Leukofiltration, whether pre-storage or bed-side, plays a major role in this context**(Clare, 2009)**. Our study showed that the mean WBC number per unit of pRBC was 2300x10^6 compared to <5 x10^6 in standard leukofiltred units, so RBC packing procedure does not reduce WBC adequately. Furthermore, the blood units show high variation in volume and Hb/Hct, although the mean is within the accepted level but still there are some patients transfused an anemic blood.

The strategy of the BCHBD were arranged to allow all their registered patients to receive the blood product from the center directly without the need for donors, assesses their general wellbeing, regularly supply iron chelating therapy, gives further treatment if needed and follow up to maintain their pre-transfusion Hb level in the range of 9.0 - 9.5 g/dl in order to achieved good quality of life. However, our data showed that the majority of patients had pre-transfusion Hb level ≤ 7g/dl and no one patient had Hb level equal or more than 9 g/dl at time of transfusion**(Thein et al., 2016)**, and the majority of patients, whether their Hb level was above or below 7 g/dl, have a transfusion rate between 12 and 24 units / year. Provided that most of the enrolled patients were living in rural areas far away from the center of the city, reflecting their education level and awareness about disease complication, this finding may not be surprising.Low pre-transfusion Hb level may not be explained by the infrequent transfusion rate only, but also by the quality of blood transfused.

Regular blood transfusion and iron chelation therapy are the recommended treatment for beta thalassemia major from early childhood. In the whole world, despite the advance in the strategy of transfusion and chelation therapy, still there is a limitation in the life expectancy for patients with β-TM related to long term effect of transfused iron on the heart and liver which may lead to organ failure **(Borgna-Pignatti, 2010. Borgna-Pignatti, 2005)**. Thus patients may seek for transplantation as a curative approach to eliminate their suffering.

Antibodies to HLA Class I and II may lead to rejection of HSCT, even with HLA-matched donors **(Brand et al., 2013;Ciurea et al., 2009;Yoshihara et al., 2012;Ciurea et al., 2011)**. Thus screening for HLA alloimmunization is mandatory in patient candidates for transplantation.

The current study showed a very high prevalence (71.2%) of antibody to HLA class I and class II among the multi-transfused β-TM patients. This figure is far from that reported by others. In a study done in Atlanta Georgia in 2010 by the author McPherson et al. the estimated prevalence of HLA alloimmunization was 32.8% among multiply transfused patients with hemoglobinopathy, they also found a significant association between the HLA alloimmunizaton and RBC alloimmunization **(McPherson et al., 2010)**. This is, most likely, related to the policy of pre-storage

leukoreduction of blood units that is strictly applied since 2000 in Atlanta. The same scenario may be suggested with Chuansumrit's study in Thailand. His study was about HLA antibody detection in patients received multitransfusion of red blood cells, comparing different methods of leukoreduction asthe total prevalence of HLA alloimmunization was 34% **(Chuansumrit et al., 2001)**. Our results are inconsistent with the prevalence of HLA alloimmunization (33%) reported by Hyun et al. in 2012 inSeoul, Korea. They studied the effect of particular sensitizing events in parallel (including previous transfusion), using similar assay method (Luminex technology) on patients candidate for solid organ transplant **(Hyun et al., 2012)**. Similar study on sensitizing events done by the authors Lopes et al, in 2015 in Portugal and the reported prevalence of HLA antibody was 22.8% **(Lopes et al., 2015)**.

A previous study done in 1994, demonstrated the effect of leukofiltered blood on the incidence of HLA antibody among multiply transfused patients using complement-dependent microlympho-cytotoxicity test. The reported incidence was (12%) for patients who received prestorage filtered red blood cells and platelets before and during study period and (31%) in those with prior non-leukocyte-reduced blood transfusions, (2.7%) in the control with a negative history for transfusions. **(Novotny, van Doorn, Witvliet, Claas, & Brand, 1995)**. Despite the low figures reported by this study which may be related to the low sensitivity of the assay used it reflects the negative correlation between the leukoreduction and HLA alloimmunization.

Our finding showed that some of the apparently healthy control had antibodies to HLA antigens. This is consistent with others' reports of "natural" antibody to HLA in non- alloimmunized individuals **(Aston et al., 2014; Morales-Buenrostro et al., 2008)**. The production of these antibodies is thought to be provoked by non-allogeneic stimuli, such as immunization **(Alberú et al., 2007; Katerinis et al., 2011; Roddy et al., 2005)** or presence of cross-reactive epitopes from viral or bacterial infections **(Ogasawara, 1986: Raybourne, 1988)**.The non-allogenically stimulated antibodies appear to be unstable and more transient as compared to that triggered by RBC transfusion**(Nickel et al., 2015).** Thus, the detection of auto-reactive HLA anti-bodies may suggest that alloimmunization is not the only cause of HLA antibodies.

In pRBC units HLA class I may cause sensitization in the recipients even with the advanced leukoreduction. The source of which may be from the residual leukocyte after leukoreduction, the residual platelets which express HLA class I on their surface, and the free molecules of HLA that contaminate all RBC unit, in addition to the few adherent HLA antigens to the surface of RBC **(Balasubramaniam et al., 2012; Yabu et al., 2013).** A previous study showed that after a prior contact with allogeneic leukocytes, a memory immune responseis apparently boostered by HLA-class I-bearing blood components **(Novotny, 1995)**.

Class II molecules are expressed only on B lymphocytes, antigen-presenting cells (monocytes, macrophages, and dendritic cells), and activated T lymphocytes.

The current study showed a significant difference ($P < 0.05$) in the prevalence of antibody to HLA class I only among multiply transfused β-TM patients (19.2%) and the non-transfused control individuals (6.0%). Whereas no statistical deference ($P>0.05$) appeared between the prevalence of antibody to HLA class II only in β-TM patients (5.5%) and the control individuals (6.0%). Therefore, alloimmunization for class II HLA is only significant in the setting of common HLA Class I & II alloimmunization. This findings are close to that reported by the authors Lopes et al, in 2015 in Portugal, 18.9% for HLA class I versus 10.0% for control individuals and 11.0% of patients with history of transfusion had antibody to HLA class II vs 5.2% for control group **(Lopes et al., 2015)**.However, higher prevalence reported by the author Thiyagarajan et al. They found that 24.8% of patients have antibody to class I HLA antibodies, and 16.3% with antibody to HLA class II **(Thiyagarajan, Bagul, Frost, Horsburgh, & Nicholson, 2012)**when analyzed the clinical impact of HLA antibodies on transplant recipient using Luminex® technology, Recently Nickel et al. in a study done in Atlanta showed the distribution of HLA class I values for the transfused and never transfused patients where 33%of transfused and 13% of never transfused patients had HLA class I antibodies ($P = 0·046$) while HLA class II antibodies were 7% and 8% respectively. It's worthy to mention that the patients had received exclusively prestorage-leukoreduced blood since the year 2000 **(Nickel et al., 2015)**

Conclusions

1. The current study confirm the close association between the multiple transfusion and HLA alloimmunization. Higher prevalence of HLA antibody was reported in our patients as compared to other countries.
2. The quality of blood units provided to multiply transfused β-TM patients may affect the pre-transfusion Hb level and hence the frequency of blood transfusion.
3. Leukoreduction plays an important role in reduction of HLA alloimmunization.
4. The extreme values for HLA alloimmunization make it difficult to assess the effect of other parameters on the prevalence of theis event.

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