

Detection of Common β -Globin Gene Mutation in Eastern Nepal by Multiplex Amplification Refractory Mutation System Polymerase Chain Reaction System

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ABSTRACT

β -thalassemia is the most common autosomal recessive haemoglobin disorder worldwide. Although more than 200 β -thalassemia mutations have been reported, few ethnic group-specific alleles account for 90-93% of the β -thalassemia alleles in each population. The primary purpose of the study was to identify the prevalence of different types of beta-thalassemia mutations in the study group, and to establish a genotype-phenotype correlation. Complete blood count, peripheral blood smear, capillary electrophoresis and multiples arms refractory mutation system based polymerase chain reaction (MARMS) were performed on the peripheral blood samples to detect beta mutations at Decode Genomics and Research Centre, Sinamangal Kathmandu. The MARMS-PCR technique was applied for the detection of nine common mutations IVS1-5(G>C), 619 bp del, FS8/9 (+G), IVS1-1 (G>T), FS 41/42 (-CTTT), C-15 (G>A), FS 16 (-C), C-30 (G>C) and C-5 (-CTCT). In the study group, three mutations, C-15(G>A), C-26 (Glu-Lys) and IVS1-5 (G>C) were detected. The study revealed a characteristic mutational profile in the five ethnic groups of eastern Nepal. It is the first report of HbE in the Koch Rajbanshi of eastern Nepal. The mutation C-15 reported as rare by earlier workers was the most common mutation identified in this research work.

Keywords: MARMS, PCR-polymerase chain reaction, Beta-thalassemia, Haemoglobinopathies, Mutations

1. INTRODUCTION

Thalassemia and some structural variants like HbS and HbE are the most common autosomal recessive disorders of haemoglobin. These blood disorders are mostly prevalent in malaria prone parts of the world, including Africa, all Mediterranean countries, the Middle East and South-East

Asia (Modell & Darlinson 2008; Weather all & Clegg 2010). Annually over 50,000 new patients are born world-wide with a severe form of thalassemia (HbE- β -thalassemia). The co-inheritance of beta-thalassemia and HbE is the most common form of thalassemia in the Asian countries. Whereas HbE is the most prevalent haemoglobin variant in the eastern part of the Indian sub-continent, Bangladesh and South Asia (Olivieri *et al.* 2011). Most of the information on thalassemia in South Asia comes from research works conducted in India. The overall prevalence of beta-thalassemia carriers has been estimated to be between 2.78 and 4% in India (Mohanty *et al.* 2013).

The haemoglobin is composed of two α - and two β - chains ($\alpha_2\beta_2$). The thalassemias are a heterogenous group of disorders that result from lowered or absent synthesis of either α or β chain. Defects in the β -globin gene lead to a lowered rate (β^+) or absence (β^0) of β -chain synthesis and subsequent decreased production of haemoglobin (Weatherall 2001). The lowered production or absence of synthesis of two β -chains (β^0/β^0) is known as β -thalassemia major/Cooley's anemia. This condition leads to severe microcytic hypochromic anemia. The person requires a lifelong blood transfusion. β -thalassemia minor is absence or lowered synthesis of one β -chain (β^0/β , β^+/β). This condition is asymptomatic and results in microcytosis with mild anemia and increased HbA2 level. Thalassemia intermedia is a condition between major and minor forms. Although, in the Human Gene Mutation Database, more than 200 β -thalassemia mutations have been reported in each population, few ethnic group-specific alleles accounts for 90-93% of the β -thalassemia alleles (Weatherall 2010).

It was a population based cross-sectional study among the five ethnic groups of eastern Nepal, at which 1500 individuals have been screened for thalassemia and to establish a genotype phenotype correlation. Complete

blood count, peripheral blood smear, capillary electrophoresis and multiplex amplification refractory mutation system- based polymerase chain reaction were performed on peripheral blood samples to detect beta mutations.

2. MATERIALS AND METHODS

The ethical approval to conduct this study was granted by the Ethical Review Board of Nepal Health and Research Council (NHRC), Ramshah Path, Kathmandu (Registration No: 07/2017). Blood samples (N=1500) were collected in ethylenediaminetetraacetic acid (EDTA) vials. Initial screening tests of haematological parameters by an automated electronic cell counter was done, peripheral blood morphology was examined microscopically. The haemoglobin electrophoresis of samples with microcytic target cells was conducted for quantification of HbA2. Samples with increased HbA2 (>3.2) were selected for genetic analysis by simple PCR involving a multiplex amplification mutation system (MARMS). ARMS is one of the one of the most commonly used techniques for the diagnosis of this disease. The MARMS-PCR test was conducted at the Decode Genomics and Research Center, Sinamangal, Kathmandu. The DNA (n=187) was extracted using phenol chloroform method from the buffy coat of anticoagulated blood (Old J 1982) dissolved in distilled water and stored at 200 C till use. The method applied for genotyping the mutations for beta thalassemia was derived from the original method of Old, used by many centres in India. The method was developed for the detection of nine core mutations, i.e. IVS1, 5(G>C), 619 bp del, FS 8/9(+G), IVS1,1 (G>T), FS 41/42(-CTTT), C15 (G>A), FS16 (-C), C30 (G>C) and C5(CTCT), which were prevalent in the Indian population. To get more efficiency of the MARMS-PCR method, different modes of primer pairing was adopted so that the standard primers participated equally in amplification of the internal control and mutant target DNA. The groups, mutations, primers and amplicon sizes are shown in Table 1.

Table 1: Groups, amplicons, pairing and sequences of primers for amplification of the 9 mutations by MARMS

| Mutation | Amplicon Bp | Primer Pairs Mutants + common | Primer Pairs Internal control | Primer sequence 5'>3' (Primer number) |
|------------------|-------------|-------------------------------|-------------------------------|---|
| (Group A) | | | | |
| Internal control | 676 | | 11&12 | CTCCTTAAACCTGTCTTGTA-ACCTTGTTAG(1) |
| IVS1,5(G>C) | 285 | 1&11 | | TGAGGAGAAGTCTGCCGTTACTG-CCCAGTA(2) |
| C15(G>A) | 500 | 2&12 | | TAACTTGATACCAACCTGC-CCAGGGCGTT |
| C26(G>A) | 301 | | | |
| (Group B) | | | | |
| Internal control | 861 | 3&11 | 4&5 | |
| FS 8/9 (+G) | 225 | 6&11 | | CCTTGCCCCACAGGGCAGTAAC-GGCACACC(3) |
| 129del | 242(del) | | | CAATGTATACATGCCTCTTTG-CACC(4) |
| Forward | 281 | | 4&5 | GAGTCAAGGCTGAGAGATGCAG-GA(5) |
| 619 del | 439 | 7&11 | | TTAAACCTGTCTTGTAACCTTGA-TACGAAA(6) |
| (Reverse) | | | | GAGTGGACAGATCCCCAAAG-GACTCAACCT(7) |
| IVS1,1(G>T) | | | | |
| FS 41/42 (-CTTT) | | | | |
| Group C | | | | |
| Internal control | 676 | 8&11 | 11&12 | ACAGGGCAGTAACGG-CAGACTTCTCCGCGA (8) |
| C5 (CT) | 206 | 9&11 | | TCACCACCAACTTCATCCAC-GTTCAGTTC (9) |
| FS 16 (-C) | 239 | 10&11 | | TAAACCTGTCTTGTAACCTTGA-TACCTACG (10) |
| C30 (G>C) | 279 | | | |
| common forward | 676 | | 11&12) | ACCTCACCTGTGGAGCCAC (11) CCCCTTCTATGACATGAACT (12) |

Group A: This group was screened for IVS1, 5(G>C), C15 (G>A) and cC26 (G>A) mutations by duplex PCR using 4 primers. The primers 11 and 12 (→ & ←) were used to get an internal control of 676 bp. The primers 11 + 1 and 12 + 2 were used to amplify mutant target DNA fragment due to {IVS1,5(G>C)-285 bp} and {C15 (G>A)-500 bp} mutations.

The group A* had primers 11 and 6 (←) amplifying {IVS1,1(G>T)-281bp} instead of IVS1,5(G>C) Fig 1.

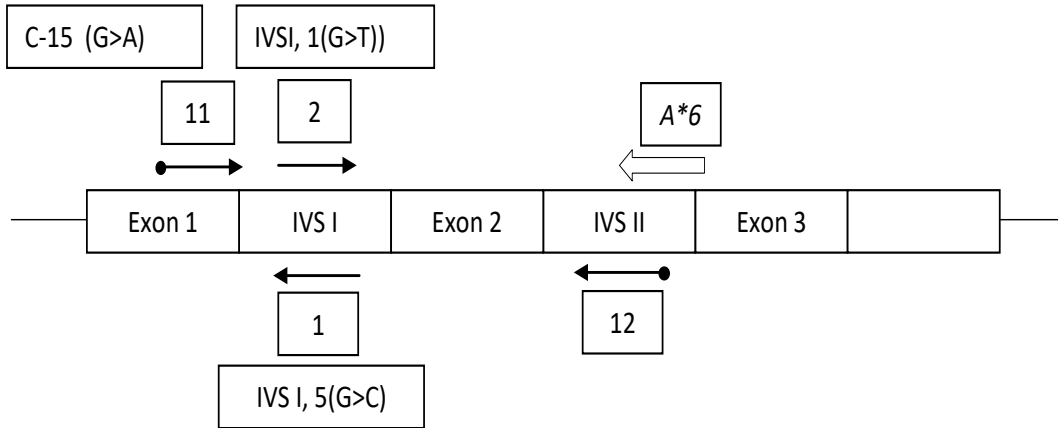


Fig. 1: Schematic diagram of group A/A* showing primers and mutations in the Beta globin gene

Group B: This group has screened 619 bp del, FS 8/9(+G), IVS1,1(G>T) and FS 41/42(-CTTT) mutations by quadruplex PCR using 6 primers. The primer 4 and 5 (←&→) were used for getting an internal control of 861 bp and the fragment due to 619 del (242 bp). The primers 11 + 3, 6 & 7 were used to amplify target DNA fragment due to {FS 8/9(+G)-225bp, IVS1,1(G>T)-281bp and FS 41/42(-CTTT)-439bp} mutations, respectively as shown in Fig2.

The GroupB* had primers 11 and 1 amplifying { IVS1,5(G>C) -285bp} instead of IVS1,1(G>T) Fig(3).

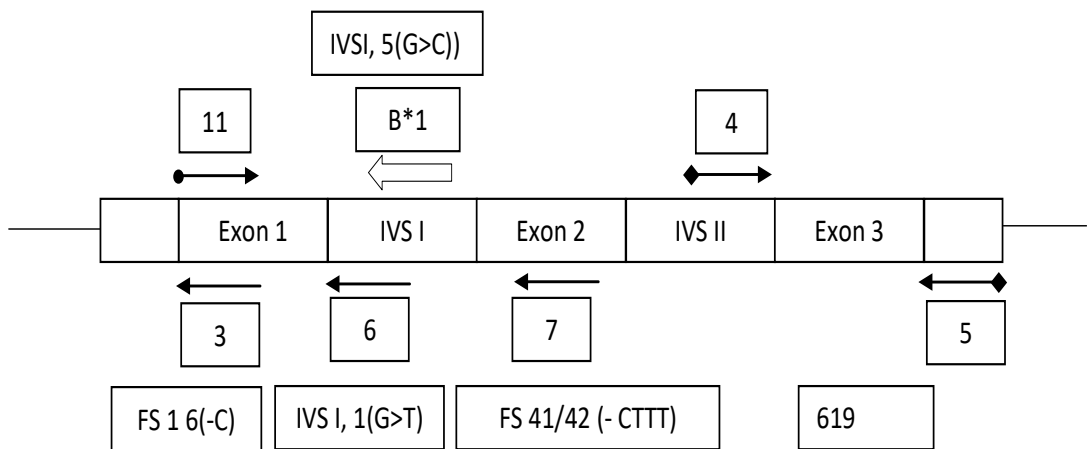


Fig. 2: Schematic diagram of group B/B* showing primer and mutations in the Beta globin gene

Group C: This group was screened for C5 (-CTTT), FS 16 (-C) and C30 (G>C) mutations by triplex PCR using 5 primers. The primers 11 and 12 were used for getting an internal control of 676 bp. The primers 11+8, 9 and 10 were used to amplify mutant target DNA fragment due to mutations {C-4(-CTCT)-206 bp, FS 16(-C)-239bp and C30 (G>C)-279 bp} respectively as shown in Fig 3.

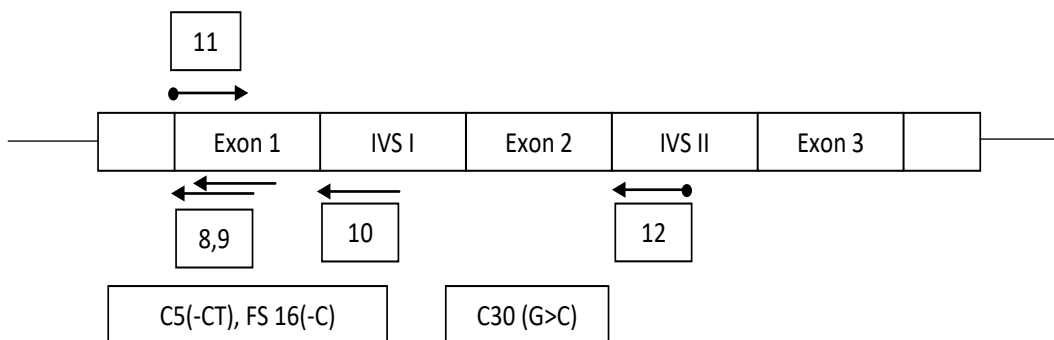


Fig. 3: Schematic diagram of group C showing primer and mutations in the Beta globin gene

The ARMS-PCR was done using the protocol from Old et al, 1990 and Varawalla *et al.* 1991. The PCR was optimized to obtain all possible amplicons. PCR amplification was carried out in 25 μ L reaction mixture containing 10X PCR assay buffer containing 10mM Tris HCl (pH 8.3), 1.5mM MgCl₂, 50mM K₂Cl, 250 mM of each dNTP, 1 unit of Taq DNA polymerase and 0.1 nmole of each primer, 500ng purified DNA template was added to the reaction mixture. The PCR was done in

locally available thin-walled PCR tubes in a thermal cycler, using initial denaturation at 94°C for 1 min, annealing a 65°C for 1min, extension at 72°C 1.5 min, final extension at 72°C for 3 mins. 20 μ L of PCR product was laid on 3.0% Agarose gel for electrophoresis in 1XTAE buffer at 150 volts for 1 hour. The gel was stained with Syber Safe, visualized under UV illuminator and photographed in a gel documentation system (Fig 4).

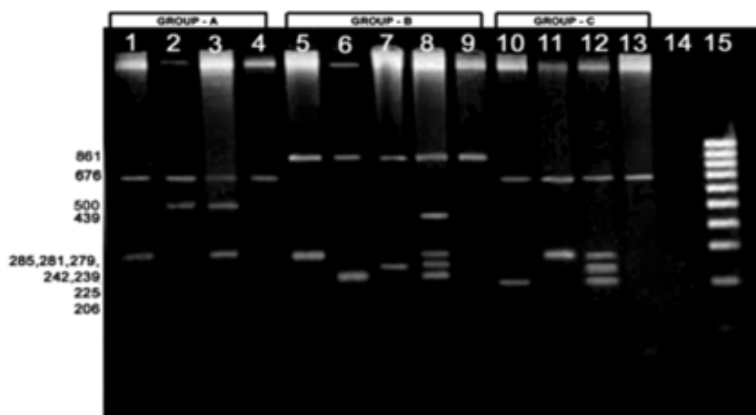


Fig. 4: Agarose gel electrophoresis pattern to detect 9 common mutations. Lanes 1-4-Group A (duplex) - Lane 1: IVS1,5(G>C)-285 bp, Lane 2: C15(G>A)-500 bp, Lane 3: +ve control(2 mutations of group A), Lane 4: -ve control. Lanes 5-9-Group B (quadruplex) - Lane 5: IVS1,1(G>T)-281 bp, Lane 6: FS 8/9 (+G)-208 bp, Lane 7: 619 bp del-242 bp, Lane 8: +ve control(3 + FS 41/42(-CTTT)-439 bp of group B), Lane 9: -ve control. Lanes 10-13- Group C (triplex) - Lane 10: C5(-CT)-208 bp, Lane 11: C30(G>C)-239 bp, Lane 12: +vecontrol(2 + FS16(-C)-225 bp mutation of group C), Lane 13: -ve control. Lane 14: reagent blank, Lane 15: 100 bp DNA marker.

3. RESULTS

The analysis was carried out on 184 alleles by multiplex simplification refractory system for the detection of nine common mutations IVS1.5 (G>C), 610bp del, FS8/9 (+G), IVS1,1 (G>T), FS 41/42(-CTTT), C-15(G>A), FS 16(-C), C30(G>C) AND C-5(-CT). The mutations detected by MARMS-PCR were C15, IVS1,5 and C26glu-lys.

The molecular study revealed that C-15(G>A) as the most common gene present in Nepal's ethnic groups. This mutation comprised around 50% of the total population with a frequency of 7.06% in the Kochila Tharu, 19.56% in the Santhals, 6.52% in the Musahars and 16.85% in the Muslims.

C-26 (Glu-Lys) was the second most common thalassemia gene in this study. It comprised 46.73% of the total study population with 30.97% Koch Rajbhanshis, 15.21% Muslims and 0.54 % Kochila Tharus.

The third common thalassemia gene observed during the study was IVS1,5 (G>C), it comprised 14.67% with a frequency of 4.89% in Santhals and 9.78% Musahars.

The co-inheritance of two mutations, C-15 and IVS1,5, was observed in two groups, Santhals and Musahars only with a total frequency of 9.78% (4.89% in Santhals and Musahars respectively).

The mutation FS8/9 was encountered in only one case.

4. DISCUSSION

This study has discovered the prevalence of beta-thalassemia trait and HbE gene especially in the Koch Rajbhanshi ethnic groups of Eastern Nepal. The mutations identified in five ethnic groups were C-15 (G>A), IVS1,5 (G>C), FS 8/9 and C-26 glu-ly. The presence two mutations {C-15 (G>A) and IVS1,5 (G>C)} of beta-thalassemia trait was detected in eighteen cases.

Nepal bordered in the east, west and south by India and north by China shares several similarities in the two neighboring countries mutational profile. In India beta-thalassemia overall prevalence was 2.78% and varied from 1.48%- 3.64% (Mohanty *et al.* 2013). Although the nationwide prevalence

of beta-thalassemia was 1.65 in China, 2.5-20% incidence of globin disorders has been reported from some parts of South China (Xiaoxiao 2020).

The estimated population of Nepal is 26,494,504 (CBC 2011). According to a WHO report on global distribution of haemoglobin disorders, there are 0.2-0.99 births per 1000 infants with haemoglobinopathy in Nepal.

Earlier research on thalassemia are hospital based on confirmed thalassemia patients. Population based data of thalassemia in Nepal is lacking. The cross-sectional study among the five ethnic groups of eastern Nepal provides interesting comparisons with earlier reports and the two neighboring countries. In the hospital based study on 22 beta-thalassemia patients, five mutations FS 41/42 (-CTTT), IVS1-5 (G>C), IVS1,1 (G>T), 619 bp del, FS 8/9 (G>T) were found to constitute 87.82% and remaining mutations, C88(C>T), C-16 (-C) and C-15 (G>A) had a combined frequency of 12.18% (Mishra *et al.* 2012).

In molecular studies on thalassemia in the Indian population also the same five mutations IVS1-5 (G>C), FS 8/9 (G>T), FS 41/42 (-CTTT), IVS1,1 (G>T), 619 bp del accounted for 93.5% (Varawalla 1991). However among the tribal populations in India, two mutations IVS1,5(G>A) and CD15 (G>A) accounted for over 90% of the mutant alleles presented a marked regional diversity (Colah *et al.* 2009). The same five mutations accounted 90% of beta thalassemia mutations in the Pakistani populations (Usman 2009).

The most common mutations in the Chinese populations include IVS-11-654 (47.89%), FS 41/42 (24.91%), FS-17 (13.81%), FS 28/29(2.89%) and FA 71/72 (2.46%) accounting for more than 90.0% of all beta thalassemia mutations (Xiaoxiao 2020). However, the distribution of mutations types was found to significantly different in different provinces of china. For example, C17 (40,22%) was the most common beta-thalassemia mutation in Baise, CD41/42 was most common in Guangdong, two mutations CD41/42 and IVS 11-654 were frequent in Hunan province, and CD 26, CD 17 and CD 41/42 were the top three mutations in Yunan province (Weatherall 2001).

In the light of the present study, C-15 (G>C) was the mutation with a frequency of 50%, whereas Mishra et al (2012) had reported this as rare mutation with frequency of 2.43%. The second common mutation was C26 (G>C) with a frequency of 46.73%. This mutation has not been reported earlier in Nepal.

The third common mutation in this study was IVS1,5 (G>C) with a frequency of 14.67% was the second common mutation reported by Mishra with a frequency of 17%. This mutation had a varying frequency of 27.85% in the Indian population.

Interestingly the most common mutation reported by Mishra was FS 41/42 (-CTTT) with a frequency of 31.71% was absent in the present study. In India FS 41/42 varies between 2 -15%, whereas in China, this is the most common mutation accounting for 24.91% (Zhu *et al.* 2020).

The detection of two mutations in eighteen cases of thalassemia trait in this study and more than one mutation in three cases in the previous study raise the likelihood of multiple mutations in Nepalese thalassaemic carriers. The most difference in the earlier and present study is in the mutation types. In molecular study by MARMS-PCR of 184 cases among five ethnic groups from east Nepal revealed three mutations. The earlier molecular analysis of 22 subjects belonging to ten different ethnicities showed five common mutations and three rare mutations. The mutation, C-15 reported very rare earlier was the most common mutation in the present study., Therefore, this researcha showed that beta-thalassemia mutations had strong regional and racial specificity with different distribution characteristics in different regions and ethnicities.

5. CONCLUSION

The study revealed that the mutational profile in five ethnic groups of eastern Nepal is very characteristic. It is the first report of the detection of HbE in eastern Nepal, especially in the Rajbhanshi ethnic group. Mutation C-15 reported as rare in earlier studies, was the most common mutation in this study. it can be concluded that haemoglobinopathy is emerging

as a health burden. The prevalence of Beta-thalassemia trait and HbE in the population demands prompt management to prevent birth of HbE/ β _thalassemia heterozygous. It is clinically a condition requiring regular blood transfusion and iron chelation. Therefore this research is instrumental in planning future large-scale prevention programmes based on prenatal diagnosis by MARMS-PCR technology.

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REFERENCES

1. Colah, R., A. Gorakshakar, A.H. Nadkarni and A.H. Phanasgaonkar. 2009. Regional heterogeneity of beta thalassemia mutations in the multi-ethnic Indian population. *Blood Cells Molecules and Diseases* 42(3): 241-246.
2. Galanello, R. and R. Origa. 2010. Beta thalassemia. *Orphanet Journal of Rare Diseases* 5:11. PMC2893117.
3. Hassan, T., M. Badr, U. El Safy, M. Hesham, L. Shefeif and M. Zakaria. 2016. B-thalassemia: Genotype and Phenotypes. *Epidemiology of Communicable and Non-communicable diseases. Attributes of Lifestyle: Nature and Mankind* Doi: 10.5772/6464.
4. Mahadik, C.T. 2012. Experience with Multiplex ARMS (MARMS)-PCR for detection of common Beta-thalassemia mutations in India. *Cardiovascular & hematological Agents in Medicinal Chemistry* 12: 14-24.
5. Mishra, A., A. Mukherjee, A. Roy, G. Singh, P. Shrestha, R. Singh. *et al.* 2012. Distribution of ethnic variation of beta thalassemia in Nepal. *Nepal Medical Journal* 4 (1): 49-52.
6. Modell, B. and M. Darlinton. 2008. Global epidemiology of haemoglobin disorders and derived service indicators. *Bull World Health Organ* 86: 480-7.

7. Mohanty, D., R.B. Colah, A.C. Gorakshakar, R.Z. Patel, J. Mahanta. *et al.* 2013. Prevalence of beta-thalassemia and haemoglobinopathies in six cities in India: a multicenter study. *J community Genet* 4(1): 33-42.
8. Old, J.V. 1990. Rapid detection of prenatal diagnosis of B-thalassemia: studies in the Indian & Cypriot population. *Lancet* 336 (8719): 834-37.
9. Olivieri, N.F., Z. Pakbaj, E. Vinchinsky. *et al.* 2011. HbE/beta thalassemia: a common & clinically diverse disorder. *Indian J Medical Research* 134(4): 522.
10. Usman, Md., M. Moiuddin, R. Ghani and S. Uman. 2009. The screening for five common beta thalassemia mutations in the Pakistani populations: a basis for prenatal diagnosis. *Sultan Qaboos University Med J* 3: 305-310.
11. Weatherall, D.J. & J.B. Clegg. 2001. Inherited haemoglobin disorders: an increasing global health problem. *Bull Health Organ* 79(8): 704-712.
12. Weatherall, D.J. 2010. The inherited disorders of haemoglobin are an emerging global health burden. *Blood* 155(22): 4331-4336.
13. Xiaoxiao Xu and Wu. Xiaoxiao. 2020. Epidemiology and treatment of beta thalassemia major in China. *Pediatric investigation* 4(1): 43-74.
14. Zhu, Y., N. Shen, X. Wang, J. Xiao and Y. Lu. 2020. Alpha and beta thalassemia mutations in Hubei area of China. *BMC Medical Genetics*. 21(6): 5p. <https://doi.org/10.1186/s12881-091-0925-5>.