BIO-MEDICAL AND GENETIC ANALYSIS OF BETA THALASSEMIA IN THE ETHNIC GROUPS OF EASTERN NEPAL

A THESIS SUBMITTED TO THE

CENTRAL DEPARTMENT OF ZOOLOGY

INSTITUTE OF SCIENCE AND TECHNOLOGY

TRIBHUVAN UNIVERSITY

NEPAL

FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

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BY

GITA SHRESTHA

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DEAN'S OFFICE

Kirtipur, Kathmandu, Nepal

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Kirtipur, 2345

EXTERNAL EXAMINERS

The Title of Ph.D. Thesis: "Bio-Medical and Genetic Analysis of Beta Thalassemia in the Ethnic Groups of Eastern Nepal "

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May 12, 2023

Dr. Surendra Kumar Gautam
Asst. Dean

Dedicated to

My Beloved Parents

Late Smt. Bhawani Laxmi Pradhan

&

Late Shri Krishna Hari Pradhan

DECLARATION

Thesis entitled "**Bio-medical and Genetic Analysis of Beta-thalassemia in the Ethnic Groups of Eastern Nepal"** which is being submitted to the Central Department of Zoology, Institute of Science and Technology (IOST), Tribhuvan University, Nepal for the award of the degree of Doctor of Philosophy (Ph.D), is a research work carried out by me under the supervision of Prof. Dr. Nanda Bahadur Singh of Central Department of Zoology, Tribhuvan University.

This research is original and has not been submitted earlier in part or full in this or any other form to any university or institute, here or elsewhere, for the award of any degree.

……

Gita Shrestha

RECOMMENDATION

This is to recommend that **Gita shrestha**has carried out research entitled "**Bio-medical and Genetic analysis of Beta-thalassemia in the Ethnic Groups of Eastern Nepal"**for the award of Doctor of Philosophy (Ph.D.) in **Zoology** under my supervision. To my knowledge, this work has not been submitted for any other degree.

She has fulfilled all the requirements laid down by the Institute of Science and Technology (IOST), Tribhuvan University, Kirtipur, for the submission of the thesis for the award of Ph.D. degree.

……

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Supervisor

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LETTER OF APPROVAL

Date:……..

On the recommendationof **Prof. Dr. Nanda Bahadur Singh,** this Ph.D. thesis submitted by **Gita Shrestha**, entitled "**Bio-medical and Genetic analysis of Beta-thalassemia in the Ethnic Groups of Eastern Nepal**" is forwarded by Central Department Research Committee (CDRC) to the Dean (IOST),T.U.

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……

Gita Shrestha

January 2022

ABSTRACT

Hemoglobinopathies and thalassemias are the most common monogenetic disorders in Southeast Asia. These are the only genetic disease where carriers can be detected using hematological findings rather than genetic analysis. The detection of Beta-thalassemia relies on estimation of red blood cell parameters that reveal microcytic hypochromic anemia. Carriers of this disorder are usually asymptomatic and non-transfusion dependent. However, marriage between two carriers produces severe transfusion dependent Thalassemia major off-springs who seldom survive beyond two years of age. The affected families suffer psychological, emotional, social and economical stress. In Nepal, this disorder is common in the malaria endemic regions of the Terai. Therefore, this study was aimed to assess the status of hemoglobinopathies and thalassemias in some ethnic groups of the Terai region in Eastern Nepal. This descriptive cross-sectional study was done from 06/01/74 to 29/12/77. Total 1500 individuals were enrolled for screening. The blood samples and demography were collected randomly from asymptomatic individuals belonging to the Koch Rajbanshi, KochilaTharu, Musahar, Musalman and Santhal ethnic groups of Jhapa, Morang and Sunsari districts. Haematological parameters were derived from automated coulter counter, peripheral blood smear was examined microscopically, hemoglobin electrophoresis was done by Capillary electrophoresis (Sebiaminicap flex piercing) and genotyped with multiplex amplification refractory mutation system-polymerase chain reaction for nine mutations.

Out of the total 1500, 285 (19%) cases suffered from microcytic hypochromia anemia. Abnormal haemoglobinopathies was detected in 184 (12%) cases. In which β-thalassemia trait was (44.44%), followed by HbE trait (34.56%) and HbE Homozygous (20.98%) in overall population. In the Koch Rajbanshi ethnic group Hemoglobin E variant was the only abnormal hemoglobinopathy found. Whereas inthe Santhals only Beta-thalassemia heterozygous was detected. The mutationsidentified were C-15 (G→A), IVSI-5 (G→C), FS 8/9(-TCCT) and C-26 $^{glu-ly}$.

The results of this study revealed that the ethnic groups of eastern Nepal are a rich reservoir for thalassemia and hemoglobinopathies. A regional and ethnic specificity in the mutational profile among the studied population was evident.

LIST OF ACRONYMS AND ABBREVIATIONS

AA : Normal haemoglobin

AFSC control : control for normal hemoglobin A and F and abnormal hemoglobin S and C.

ARMS : Amplification refractory mutation system

AS : Heterozygous

AS-PCR : Allelic specific Polymerase Chain Reaction

BTT : Beta thalassemia heterozygous/ trait/ carrier

CAE : Cellulose acetate electrophoresis

CBS : Central Bureau of Statistics

CDZ : Central Department of Zoology

CZE : Capillary zone electrophoresis

DF : Discriminant Functions

DF1 : Mentzer's index (MI=MCB ÷ RBC)

DF2 : Shine & Lal Index (S&LI= MCV2 × MCH ÷ 100)

DF3 : Srivastava Index (SI= MCH÷ RBC)

DF4 : RDW (Red cell distribution width) Index (MCV× RDW÷RBC)

DF5 : Green 7 King Index (G&KI= MCV2× RDW÷100×Hb)

DNA : De-oxy ribonucleic acid

FN : False Negative

FP : False Positive

Hb : Hemoglobin

HbA : Adult hemoglobin: α2β2

HbA2 : Adult hemoglobin: α2δ2

HbF : Fetal hemoglobin

HbE : Hemoglobinopathy E

HEE : Hemoglobinopathy E homozygous

HEA : Hemoglobinopathy E heterozygous

HbS : Sickle cell Hemoglobin

HPLC : High performance liquid chromatography

IDA : Iron deficiency anemia

IFN : Interferon

LCR : Locus Control Region

MARMS-PCR: Multiplex amplification refractory mutation system-polymerase chain reaction

MCH : Mean corpuscular hemoglobin

MCHC : Mean corpuscular hemoglobin concentration

MCV : Mean corpuscular volume

Non-BTT : Normal / Absence of beta-thalassemia

PBS : Peripheral blood smear

PCR : Polymerase chain reaction

RBC : Red blood cell

RDW : Red cell distribution width

SE : Sensitivity

SP : Specificity

TN : True Negative

TP : True Positive

WHO : World health organization

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CHAPTER 1

1. INTRODUCTION

1.1Background

The thalassaemia syndromes comprise a heterogeneous family of blood disorders that arise from defective synthesis of alpha or beta chains which form the globin component of hemoglobin (Galanello and Origa 2010). Beta-thalassemia, the most commonly inherited monogenetic disorder is an emerging global health problem. An estimated 300,000 – 400,000 children are born every year with severe hemoglobin disorders worldwide, 80% of these births are in developing countries (WHO 2007). Mutation leads to diminished or absence of globin chain synthesis and subsequent reduction in production of hemoglobin. The trait confers a degree of protection against *Plasmodium falciparum* malaria and selective survival advantage over carriers, thus the mutation has been perpetuated over generations. Traditionally high frequencies of thalassemias were prevalent in malaria prone parts of the world: Africa, all Mediterranean countries, Middle East, Indian sub-continent, South-east Asia. However, due to global population migration, it has spread all over the world. The Maldives has the highest incidence of Thalassemia in the world with a carrier rate of 18% of the population (Waheed et al. 2016). The estimated prevalence is 16% in people from Cyprus, 1% in Thailand, and 3 - 8% in Bangladesh, China, India, Malaysia and Pakistan (Colah et al. 2010; Modell and Darlinson 2008).

It became apparent only in the 1930's -1940's, that the disease described as severe anemia with splenomegaly and bone changes resulted from defective synthesis of one of the two globin chains (α & β), that constitute adult hemoglobin. Deficiency of hemoglobin leads to severe anemia requiring lifelong blood transfusions to asymptomatic cases (Origa 2018). Mutation in the genes encoding α-globin chain causes α-thalassemia and mutation in genes encoding β-globin chain causes β-thalassemia.

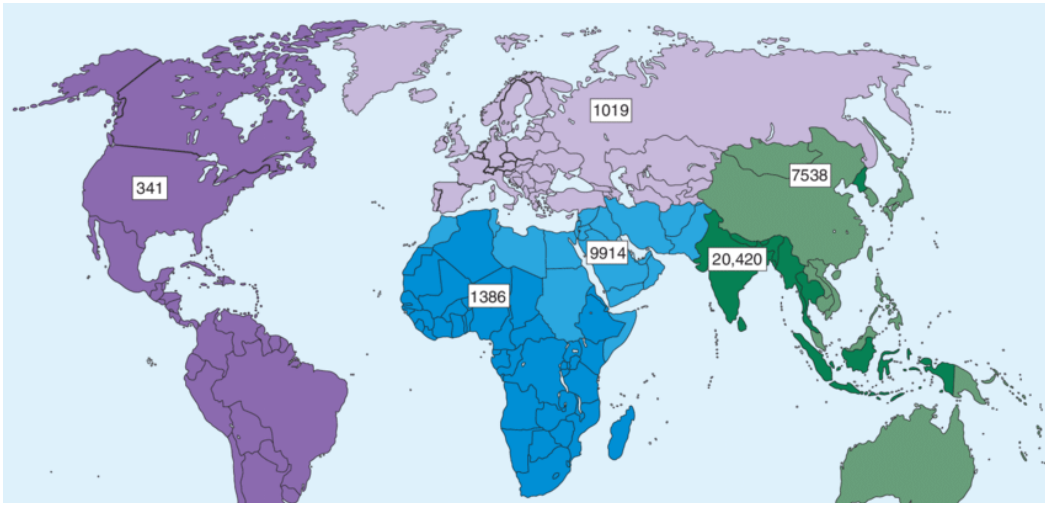


Figure 1: Estimated annual affected births of children with Beta-thalassemia world wide.

(source: Colah et al. 2010)

1.1.1Biology of Hemoglobin

Hemoglobin is the oxygen transport metallo-protein or the red pigment present in the red blood cells of all vertebrates. It is made up of two entities, heme and globin. Heme is a tetrapyrole ring composed of porphyrin with a ferrous iron in the centre of the ring as shown in Figure 2. Oxygen binds to the iron atom to form an unstable compound oxyhemoglobin travelling between the lungs and tissues. Each molecule of hemoglobin has four iron atoms that bind four oxygen molecules. The globin comprises of two linked polypeptide chains, alpha and beta globin chains. The normal human adult hemoglobin (HbA) consists of two parts of globin chains, $\alpha^2\beta^2$; their synthesis is normally tightly co-ordinated to ensure equal production. Thalassemia occurs when this balance between the globin chains is disturbed (Cao and Galanello 2010).

There are different types of hemoglobin depending on the type of globin chain. The functional and physical properties of hemoglobin depend on the composition of these globin chains. In normal adults, four types of globin chains are produced: α, β, δ and γ. A pair of α-chains combines with a pair of β, δ and γ to form three types of hemoglobin HbA ($\alpha^2\beta^2$), HbA2 ($\alpha^2\delta^2$), HbF ($\alpha^2\gamma^2$). HbA is the adult hemoglobin comprising 97% of total hemoglobin. HbA2 and HbF are minor hemoglobins. Production of HbA starts as early as nine weeks of pregnancy, increases after birthand the adult level achieved by the end of first year of life. HbA2 appears late in

fetal life <1% at birth of the total hemoglobin and reaches normal value (1.3-3.5%) at the end of first year (Cao and Galanello 2010).

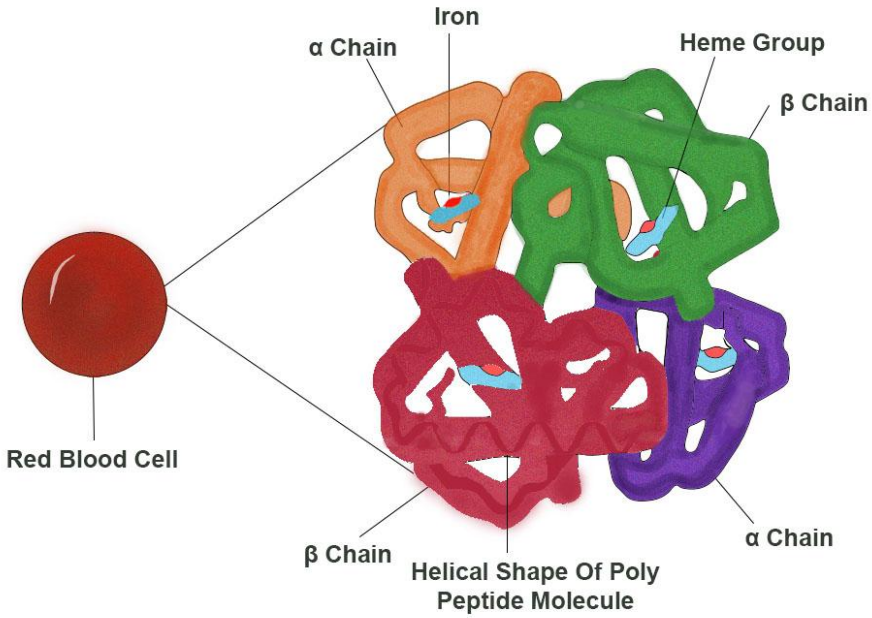
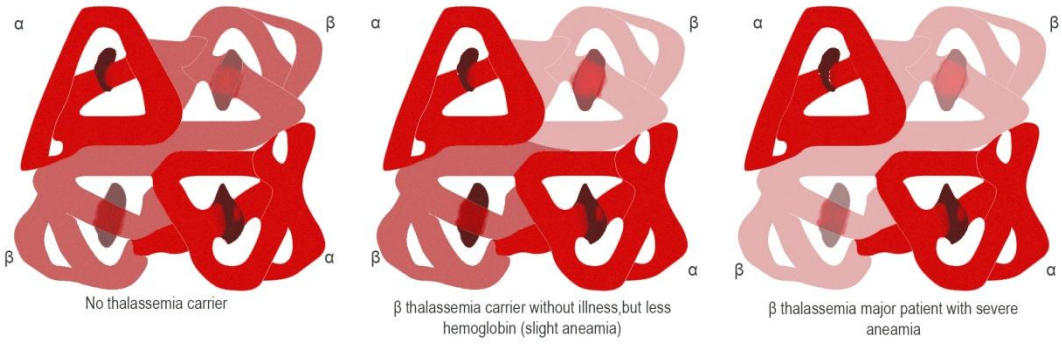


Figure 2: Structure of Haemoglobin Molecule

Source:Zeb A. Structure and function of hemoglobin. 2015 June 19 [cited 2017 Apr 6]. In Slideshare [Internet]. Lahore: Slideshare.net; c2017.Available from: https://www.slideshare.net/asifzeb2/structure-and-function-of-hemoglobin.

The selective deficiency of one or more globin chains has two consequences; Decreased hemoglobin synthesis due absence of one globin chain leads to hypochromasia with slight anemia as shown in Figure 3b. Mutations that result in absence of bothglobin chain synthesis cause severe anemia as shown in Figure 3c. The absence of complementary globin chains to bind (Figure 3c) results in precipitation of normally formed globin chain aggregates that cause damage of red cell membranes andpremature destruction (Weatherall 2013).



3a: No mutation normal Hemoglobin

3b: Single mutation less Hemoglobin

3c: Double mutation severe anemia

Figure 3: Haemoglobin molecules in different types of Thalassemia

Source: (Weatherall 2013).

1.1.2Pathophysiology

Beta-thalassemia occurs due to reduction or absence of β-globin chain synthesis. This results in excessive α-globin chains, which cannot form tetramers and therefore precipitate in the developing erythroid cell as large intracellular inclusion bodies. Some of these α-chains are removed by pairing with γ- globin chains as HbF (foetal haemoglobin). The remaining α-chains precipitate quickly within the red cells as Heinz bodies. The precipitated α-chains damage the red cell membrane. Ultimately, this leads to destruction of developing erythroid precursors in the intramedullary space by a number ofmechasnism including oxidative damage to cell erythropoiesis. These irreparably damaged red cells are phogocytosed by the reticulo-endothelial cells of spleen and liver causing anemia, hepato-splenomegaly and excess of tissue iron stores. Hence, the anemia in thalassemia syndromes is due ineffective erythropoiesis, shortened life span of red cells and haemodilution due to increased plasma volume (Mohan 2015).

1.1.2.1 Classification

Thalasemia syndromes can be subdivided according to their phenotypic presentation or genetic causation. Normal hemoglobin production results from a balanced synthesis of α-globin chains from duplicated genes on chromosome 16 and single β genes on chromosome 11. The following description will be focused mainly on β-thalassemia.

1.1.2.2 Classification according to phenotype

The simplest and commonly used classification is according to the clinical severity of the anemia. Thalassemia Minor/trait/heterozygotes are carriers of the defect with asymptomatic anemia. Thalassemia Major/ usually homozygotes or compound heterozygotes suffer from severe blood transfusion dependent anemia. Thalassemia Intermediate/ genetically heterogeneous in which the severity of anemia may or may not require blood transfusion support (Mohan 2015).

1.1.2.3 Classification of beta-thalassemia according to genotype

Beta-thalassemia mutations can be classified according to the amount of globin chain production. In some mutations globin chain synthesis is absent and is termed as β^0thalasasemia. The mutations with reduced globin chain synthesis are known as β^+thalassemia. Most of the β-thalassemia mutations are due to point mutations rather than deletions. However, because the δ and the duplicated γ genes lie upstream of the β gene, large deletional mutation of the β gene may also involve deletion of the δ and γ genes. The δβ-thalassemia, €γβ-thalassemia, γ-thalassemia and Hereditary Persistance of Foetal Hemoglobin (HPFH) will not be discussed individually but will be discussed so far as their role as modifiers of thalassemia phenotype. B-thalassemia like syndrome may also result from a heterozygous state of a β globin mutation with a beta hemoglobin variant present in reduced amounts such as Eβ-thalassemia syndromes (Mohan, 2015).

1.1.2.4 Genetic basis of β-thalassemia

More than 1000 mutations affecting the globin genes have been identified to date (Thein 2004) with more than 200 of these leading to β-thalassemia syndromes of varying severity (Flint et al 1998). A variety of mutations can occur in the globin genes ranging from simple point mutations to large deletions, alterations and insertions resulting from more than one mutation. The mutational site also affects the abnormal globin chain, reduced amounts of normal globin chain or absence of globin chain synthesis. Mutations occurring at initiation site will result in no globin chain synthesis, whereas mutation at one of the promoter sites will result in reduced production of globin chains. Due to abolition of normal splicing, the splice junction mutation (donor or acceptor sites) results in absent globin chain production. The reduced normal splicing due to mutations at the consensus splice sites results in some

globin chain synthesis. Mutations at the PolyA site also leads to decreased globin chain production. Sometimes mutations can occur outside the globin genes leaving the genes intact but result in reduced globin production. Deletions at the LCR (Locus control Region) of the globin genes lead to $\varepsilon\gamma\delta\beta^{0}$-thalassemia (Panigrahi and Agarwal 2013).

1.1.2.5 Molecular structure of Beta-thalassemia

β globin is encoded by a structural gene found in a cluster with the other β-like genes on chromosome 11 (11p 15.15) (Stamatoyannopoulos 2005). As shown in Figure 4, the cluster contains five functional genes, ε (*HBE*), Gγ (*HBG2*), Aγ (*HBG1*), δ (*HBD*), and β (*HBB*), which are arranged along the chromosome in the order of their developmental expression to produce different Hb tetramers: embryonic (Hb Gower-1 ($\zeta_2\varepsilon_2$), Hb Gower-2 ($\alpha_2\varepsilon_2$), and Hb Portland ($\zeta_2\beta_2$)), fetal ($\alpha_2\gamma_2$), and adult (HbA, $\alpha_2\beta_2$ and HbA_2, $\alpha_2\delta_2$). Expression of the globin genes is dependent on local promoter sequences as well as the upstream β globin locus control region (β-LCR) which consists of five Dnase 1, hypersensitive (HS) sites (designated HS1 to HS5) distributed between 6 and 20 kb 5' of *HBE* gene. There is one HS site at approximately 20 kb downstream of *HBB* gene (Panigrahi and Agarwal 2013).

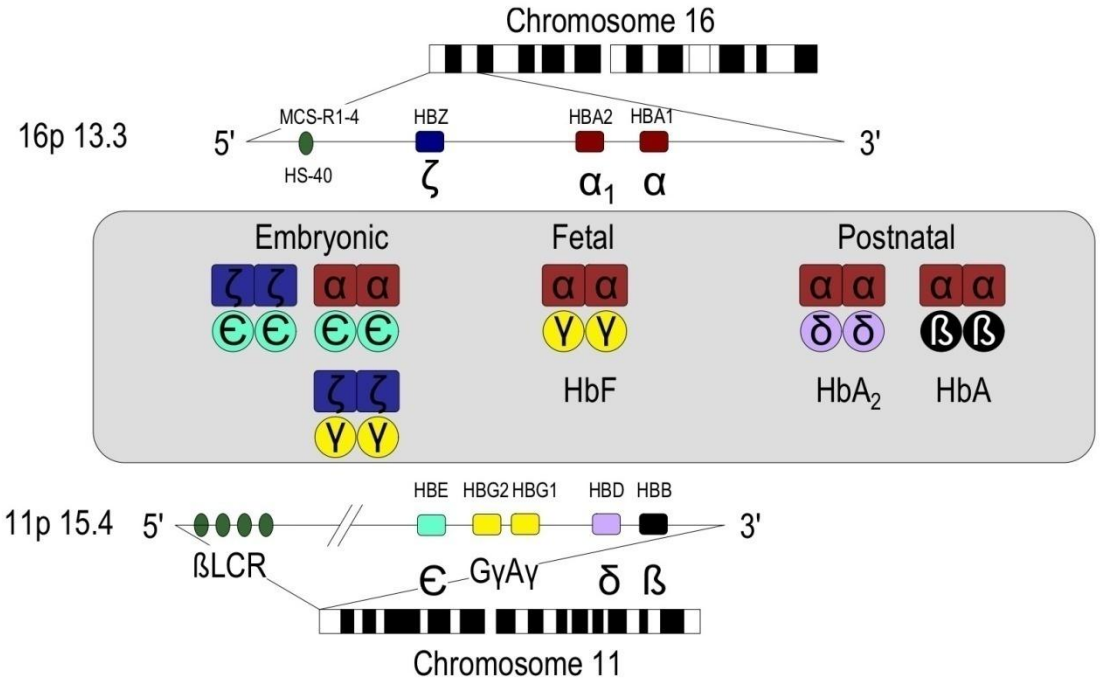


Figure 4: Schematic representation of the Beta-globin gene loci

(Source: Farashi & Harteveld 2018)

In Figure 4, the schematic representation of globin gene loci is depicted. The panel shows the beta locus that resides on chromosome11. The two gamma globin genes are active during fetal growth and produce hemoglobin F. The "adult" gene, beta, takes over after birth

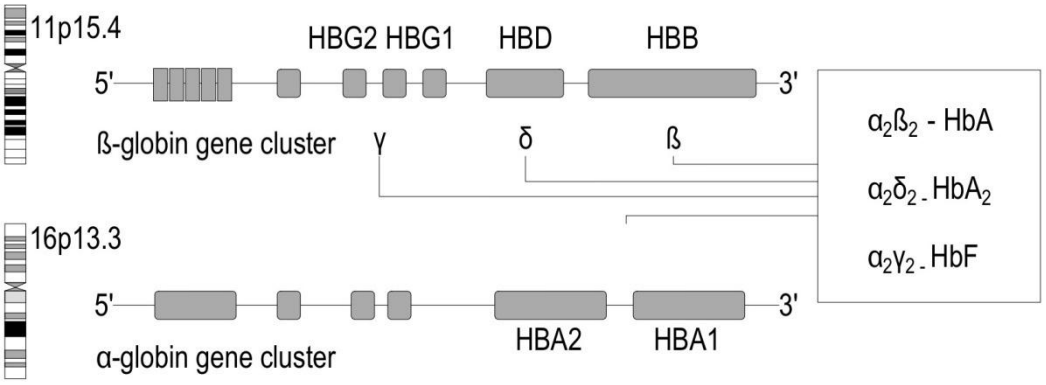


Figure 5: Organization of the human globin gene clusters on chromosome 11(β-globin gene cluster) and 16 (α-globin gene cluster)

Source: Farashi & Harteveld 2018)

1.1.3 Inheritance of Beta Thalassemia

Thalassemia heterozygous and homozygous is inherited in the Mendelian autosomal recessive pattern. Both copies of HBB genes in a cell have mutations. Thalassemia parents of an individual carry one copy of the mutated gene, but they donot show any signs and symptoms of the condition. Most of the β-thalassemia alleles are inherited in the Mendelian recessive manner (Figure. 6). But, there is a sub group of β-thalassemia alleles that behave as dominant negatives. These individuals with an autosomal recessive condition carry one copy of the mutated gene in an autosomal dominant manner. In these cases, one copy of the altered gene in each cell is sufficient to cause signsand symptoms of beta Thalassemia (Ghada and Khalda 2015).

Marriage between two carriers produces one normal child, two β-thalassemia carriers and one β-thalassemia major child (severe).

1.1.4 Signs and symptoms of beta-thalassemia

β-thalassemia major or Cooley's anemia appears before a child's second birthday. The severe anemia related to this condition maybe life threatening, other symptoms

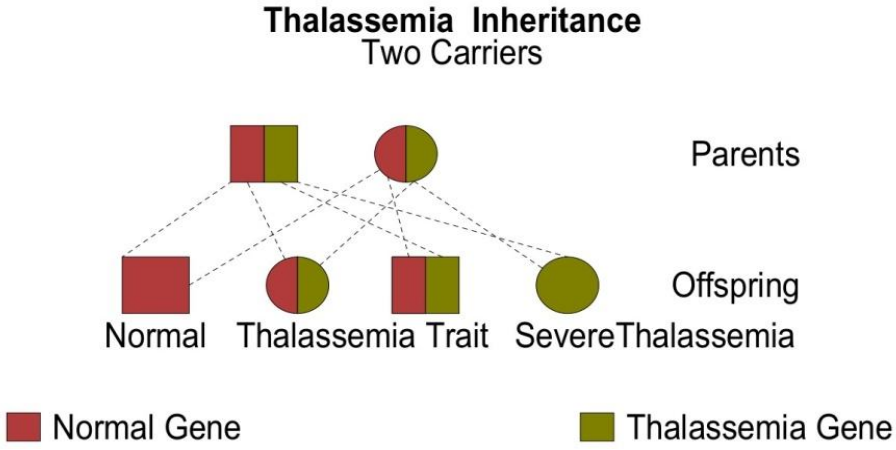


Figure 6: Inheritance of Beta Thalassemia (Carrier Parents)

include fussiness, paleness, frequent infections, poor appetite, failure to thrive, jaundice and enlarged organs (Mohan 2015).

1.1.4.1 Clinical manifestations appear insidiously and are as follows

Anemia: Anemia starts appearing within the first 4-6 months of life when the switch over from γ-chain to β-chain occurs, but is asymptomatic. Sometimes people with only one HBB gene mutation in each cell develop mild anemia (Mohan 2015).

Splenomegaly: β-thalassemia is often accompanied by destruction of large number of red blood cells and the task of removal of cells causes the spleen to enlarge. Splenomegaly can make the anemia worse and reduce life of the transfused red cells. Severe spleen enlargement may necessitate the removal of the spleen (Mohan 2015).

Iron overload: People with β-thalassemia major get an overload of iron in their bodies, either from the disease itself or from frequent blood transfusions. Excess iron damagesthe heart, liver, endocrine systems which include glands that regulate the processes throughout the body. The damage is characterized by excessive deposits of iron without adequate iron chelation therapy; almost all patients with β-thalassemia accumulate potentially fatal iron levels (Mohan 2015).

1.1.5 Diagnosis of beta thalassemia

The general diagnosis of beta thalassemia is as follows:

1.1.5.1 Complete blood count with red cell indices

The diagnosis of β-thalassemia relies on measuring red blood indices that reveal microcytic hypochromic anemia, haemoglobin concentrations below 11dL/gm, Mean cell volume (MCV) < 75fL, Mean cell haemoglobin (MCH) < 27pg (Mohan 2015).

1.1.5.2 Peripheral blood smear

Affected individuals manifest morphologic changes of erythrocytes displaying microcytosis, hypochromia, anisocytosis, and poikilocytosis, speculated tear-drop, elongated cells and erythroblast. The presence of erythroblastsis the diagnostic feature of thalassemia. The number of erythroblasts is related to the degree of anemia and is markedly increased following splenectomy (Mohan 2015).

1.1.5.3 Qualitative and quantitative hemoglobin analysis

Thehemoglobin analysis by cellulose acetate electrophoresis and DE-52 microchromatography or high-performance liquid chromatography (HPLC) identifies the amount and type of hemoglobin present. The following hemoglobin types are most relevant to β-thalassemia.

1.1.5.4 Osmotic fragility characteristically reveals the resistance to saline haemolysis

1.1.5.5 Iso-electric focusing

Abnormal and normal HbA, HbS and other proteins can be separated by iso-electric focusing which is a highly sensitive method. It is a technique which is based upon iso-electric points for separating proteins. In this HbA, HbA2, HbF and HbS are electrophoresized at different pH gradients. It is used as screening or confirmatory test.

High Performance Liquid Chromatography (HPLC): This is a highly developed technique used both for screening and confirmatory test. In this, haemoglobin samples are loaded, which are pumped with high pressure into HPLC column where different Hbs are seperated into bands which are detected and displayed by computerized system. It is useful in detection of Hb variants (Jha 2015).

1.1.5.6 Genetic Analysis

Genetic Analysis is applicable to investigate alterations and mutations in the genes that produce haemoglobin. DNA samples are obtained from red cells, bone marrow cells and amniotic fluid. The Polymerase chain reaction (PCR) method is a high means of detecting beta globin alleles in routine genetic engineering (Skogerboe 1991, Atkin 1998). Antenatal Screening: The diagnosis of beta thalassemia before birth is called ante-natal which is to find out the risk of passing of the abnormal gene or disease to the off-spring. Ante-natal testing is of the following two types:

1.1.5.7 Chorionic villus sampling

This is a prenatal test usually done around the 1st week of pregnancy. A sample of the chorionic villi is removed for testing.

1.1.5.8 Aminocentesis

This test is usually done around 16th week and involves taking a sample of the fluid that surrounds the fetus (Quinlan 2008).

1.1.5.9 New-born screening

This is the diagnosis of beta thalassemia after birth. It is carried out to avoid risk of complications in the future. Early detection of beta thalassemia by new-born screening programs enables early provision of comprehensive care, which in itself improve the quality of life and survival of the patient population (Srivastava and Shaji 2017).

1.1.5.10 Assisted reproductive technology

This is a form of technology that combines pre-implantation, gene diagnosis with in-vitro fertilization that may help parents who have thalassemia or are carriers of a defective haemoglobin gene give birth to healthy babies. The procedure involves retrieving mature eggs from a woman and fertilizing them with a man's sperm in a dish in the laboratory. The embryos are tested for defective genes, only those without genetic defects are implanted in the woman (Kamel 2013).

1.1.6 Treatment and management of beta-thalassemia

The therapeutic approach to thalassemia differs between thalassemia heterozygous and thalassemia homozygous.

1.1.6.1 β-thalassemia heterozygous

This condition usually does not require specific treatment. Pre-information about their condition must be given to patients. Sometimes this disorder is mistaken for iron-deficiency. Some pregnant patients with β-thalassemia heterozygous may develop concurrent iron deficiencies and severe anemia. Blood transfusion might be required if there is no response to iron medicines (Ali et al. 2021).

1.1.6.2 β-thalassemia major

Treatment for patients with β-thalassemia major includes chronic transfusion therapy, iron chelation therapy, splenectomy, allogenic haemopoietic stem cell transplantation and supportive measures. Emerging therapies include pharmacological agents to induce fetal haemoglobin. Gene therapy uses a viral vector to deliver beta-globin genes into the cells (Ali et al. 2021).

1.1.6.3 Long-term transfusion therapy

Blood transfusion maintains haemoglobin level at 9-10 g/dL, which improves the well-being of the patient while enhanced erythropoiesis is suppressed simultaneously. The anemia gets corrected and inhibits endogamous erythropoiesis so that extra medullary hematopoiesis and skeletal changes are prevented. Transfusion of washed red cells with reduced leukocyte at approximately 8-15 ml RBCs per body weight over 1-2 hours is recommended (Ali et al. 2021)..

1.1.6.4 Haemopoietic stem cell transplantation

HSCT involves intravenous infusion of hematopoietic stem cells to restore normal blood cell production in affected individuals (Ali et al. 2021).

Sharma et al. (2020) and Jha (2015) reported that Beta –thalassemia trait, sickle cell anemia along with some other hemoglobinopathies are common in Nepal. Jha observed a very high peak of beta-thalassemia among the Tharu community. The abnormal hemoglobins and Thalassemia were mainly seen in the ethnic groups of the Terai. Sickle-cell anemia was found to be prevalent in Western Nepal mainly among the Tharu population. Although eastern Nepal is home to many ethnic groups, recorded data on the prevalence of Thalassemia and hemoglobinopathies is lacking.

Thalassemias, sickle cell anemia along with some other hemoglobinopathies are most common in the Terai regions of Nepal and India. (Nigam et al. 2020). Numerous hospital-based studies have reported the prevalence of hemoglobinopathies in the Nepalese population especially the Tharu ethnic group residing in the western and far western Nepal (Sharma et al. 2020. Jha 2015). The incidence of malaria among the Tharusof Southern Nepal was seven-fold lower than that on sympatric non-Tharupeople (Modiano et al.1991). The mutation causing Thalassemia is associated with decrease malaria morbidity. Some these mutations are ethnic group specific and highly prevalent among certain ethnic groups of the Terai. These innocent people are unaware that this genetic disease can be prevented through proper management. Accurate epidemiological data on the frequencies of β-thalassemia in Eastern Nepal are not available. Beta-thalassemia is a congenital blood disorder caused by mutation in the β-globin gene. It is inherited in an autosomal recessive manner which can be prevented.Therefore I undertook to do this descriptive community-basedresearch work to find out the status of Beta Thalassemia among some ethnic groups of Eastern Nepal. The study population comprised of five ethnic groups, Koch Rajbanshi, KocilaTharu, Musahar,Musalman and Santhal ethnic groups of Eastern Nepal.

1.1.7 Ethnic groups

1.1.7.1 Koch Rajbanshi ethnic group

Koch Rajbanshi ethnic group are indigenous people settled in Jhapa, Morang and in some number in Sunsari districts of Nepal. Those residing in Jhapa and Morang are traditionally and culturally the oldest indigenous ethnic people. It was assumed that they are a mixed race of Austroasian or Dravidian and Mongolian. Their height is above average, eyes and forehead a mix ofAryan-Mongloid, Austric, Negrito and Dravidian.Theirroots can be traced back to Assam, Bengal and Bihar of India. Even today, most of the Rajbanshis are found in Assam, Meghalaya, Tripura, Nagaland, Manipur in India (Shrestha 2064). These peoples entered Morang as Rajbanshis, the name given after AD1515. Their original tribal name was Koch or Koche which was changed after coming into contact with Hindus (Sharma 2045 B.S.; Upadhyaya 2051-2052 B.S.). The largest settlement of Kochesas Rajbanshi is in Nepal only. There are different kinds of Rajbanshis such as Koch Rajbanshi, Poundra Rajbanshi,

Meche Rajbanshi, Newar Rajbanshi, and Khataha Rajbanshi. Thesub-castes of the Koch Rajbanshis in North Bengal, India are Paliya, Sadhupaliya, Babupaliya, Deshi, Domasir, Modasi, Jaluwa, Tongriya, Khopriya, Gobriya, Kantai, Dhalai and Koch are not found in Nepal.

The word Koach (Koch or Kooch) is associated with the formerly "Princely State of Cooch Behar" (1586-1949) under the British Raj. Some sources state that Kooch and Rajbhanshi had entirely different origin. The Kooch were Mongoloid whereas the Rajbhanshi were Dravidians. However, today it is impossible to separate these two communities of separate historical origin.

According to a study the blood samples from Koch people of Golpara (Borigaon area) district Assam (1978- 1979), disagree with the tribal population (like Gabo, Kacheri and Rabho who form the parental stock) in most characters. It has also been observed that the Rajbhanshis living in the area of Hindu Castes showed 'dilution' of HbE gene frequency and thereby showed similarity with the Assamese caste in this regard. The HbE gene frequency is very high in the Rajbhanshis. It is believed to be maintained through an adaptive advantage in the malarious environment. According to the latest census the population of Rajbhanshis is very small, in Nepal it is 115,252 (CBS 2012). The literacy rate is 67.19%.

The language of Koch Rajbanshisknown as Rajbanshi Bhasais a mix of Bengali and Maithiliand shares commonalities with the language of Garo (Bista 1996)

The Rajbanshis were nature worshippers.In Nepal, a mix of nature worship and Hindu culture seems to have developed. They are the most Hinduized aboriginal people of eastern Terai who also celebrate Dasain and Tihar. Their traditional festival is Pawani.

1.1.7.2 KochilaTharu ethnic group

The Tharu people are an ethnic group that spans the entire Terai in southern Nepal. The word Tharu was derived from 'sthavir' meaning follower of Theravade Buddhism (Skar 1995). The Tharus was recognized as an official nationality by the Government of Nepal and India (Lewis 2014). In actuality there are several endogamous sub-groups under the umbrella term Tharu scattered over most of Terai. Among the most recognized groups are the Rana, Dangaura, Kochila/Morangia,

Chitwania and Kathariya (Boehm 1997). The origin of the Tharus is not clear. The Rana Tharus claims to be of Rajput origin that have migrated from the Thar Desert, Rajasthan, India to Nepal's Far Western Terai region, hence the name 'Tharu.' The Tharu of Far East claim to be descendants of Shakya and Koliya people of Kapilvastu (Skar 1995) and a literacy rate of 64.41%.

There are several endogamous sub-groups of Tharus culturally and linguistically. Culturally, the Tharus of Jhapa, Morang and Sunsari district are known as Morang Kochila, regarding the ethnonym of their language name, Krauskopff says, "The ethnonym Koshila or Kuchila could be related to the name of the river Koshi on the bank of which they used to live" (1995: 190). The Tharus living in Udaypur, Saptari and area west to it are called western Kochila, those living in central and mid-western Terai are called Kotharia, Dangaha (Dangoura, Dagaura) and those living in the far western region are known Rana Tharu of Kailali and Kanchenpur (Eichentopf 2013; Khania 2010).

Tharus are rich in cultural heritage and have their own special costumes, unique ways of living, language, religious belief which make them different from other indigenous communities in Nepal.

Regarding religious practices, Tharu people are mostly animistic, however many claim to be Hindu). "Most Tharu practice an indigenous form of animism, in which shamanism, ancestor worship and tattooing plays pivotal roles. This older religionhas been overlaid with a veneer of Buddhism and has been influenced by Hinduism" Their verbally communicated literature is rich. They are animists and have belief in forest goddess Bandevi- and deity. They celebrate a number of festivals, of them, Maghi; a festival of New Year in mid-January is celebrated for three days with special dance performances, songs in mother tongue and animal sacrifice. The main festival is the new year of Baisakh called Siruwa Pavan or JurSittal (Krauskopff 1995). Tharus are the second largest ethnic group in Nepal after Magar (CBS 2012), but still very backward in the society.

Contempary research of different ethnic groups living nearby found an incidence of malaria nearly seven times lower among Tharus. Such a large difference pointed to genetic factors rather than dietary differences or behavior. This observation was

confirmed by finding gene for thalassemia in nearly all Tharu studied (Modiano 1991).

1.1.7.3 Musahar Dalit/Ethnic group

Out of the one hundred and twenty-six ethnic castes/groups found in Nepal, Musahar is one of the small ethnic groups of Terai and inner Terai. The Musahars are the secluded and isolated ethnic group. They have been given the name "Musahar" because of their dietary inclination to rats (local name 'musa'). They are highly marginalized, economically exploited and backward, politically violated, socially humiliated and treated as untouchables. Their educational status is very low:21.9% with males: 27.5, Females: 40.8%. compared to the National Literacy rate. (CBS 2011). There is uncertainty regarding the origin of the Musahars. Modern genetic studies have found Musahar clusters very closely related with the Munda tribes like Santhals and Hos with similar haplogroup like 02 and M40. Therefore, one theory is that the Musahar were originally a hunter-gatherer Munda speaking tribal group whoworked in the fields of Bhojpuri landlords. Hence, they learnt Bhojpuri to communicate better with their masters and slowly underwent a language shift away from their Munda language towards Bhojpuri and the social stigma of their tribal roots resulted in becoming a Dalit caste. The Musahars are divided into two groups according to the genealogy, Maghaiya and Tirutiha. One major difference between them is that the Maghaiya's bury their dead but the Tirutiha cremate the dead.

Etymological meaning of the word 'Musahar' was derived from their dietary inclination to mouse. Literally, 'Musahar' means one who eats rats (Jha 1998; Kumar 2006). Musahars are Dalit, fully backward, untouchable, highly marginalized, excluded group, very poor and landless (Chaudhry 2008). They were included in the untouchable's category of caste system without specific ritual-based caste occupation. Being socio-politically weakest and economically poor resource group of the Terai, Musahars practiced various strategies of livelihood (Giri 2012). In the whole country Musahars are in the lowest priority from local to state level.By some estimates in about 85% of some villages of Musahars health centers are scant and mal-nutrition, malaria, kala-azar, cholera, encephalitis in summer and cold wave in winter are prevalent.

The Sanskritzed Musahars claim a superior position in relation to the rest of the community. Musahars, one of the largest Mahadalit castes, is regarded as the lowest in the caste hierarchy (Kunnath 2013). But the Musahar themselves do not agree with that, because their oral traditions and stories speak that they were the descendants of great sage Balmiki, who is believed to be the author of the Hindu religious epic Ramayana.

The total population of Musahar in Nepal is 234,490 (0.76%) (CBS 2011) and is the second largest group among the Terai Dalits. The Literacy rate is 27.0% (males- 40.8%, females- 21.9%).

1.1.7.4 Musalman

Nepalese Musalmans are the people residing in Nepal following the religion of Islam and constitute most distinct and well-defined minority group in Nepal. Besides their adherence to Islam, their ethno-cultural affiliation too gives Muslims a distinct identity in a pre-dominantly Hindu Muslim set up. Their ancestors entered Nepal from different parts of South Asia, Central Asia and Tibet during different epochs.

About 97% of the Muslim community lives in the Terai region, the rest 3% are found mainly in Kathmandu and the western hills. According to Nepali historians, Kashmiri traders were the first Muslims to settle in Kathmandu. It was during King Ratna Malla's reign in the late 15th century. These were later followed by Afghans, Persians and even Arabian. The Bajar in Indra Chokwas named after the Iraqi merchants. A Kashmiri saint to build the first mosque, Kashmiri Taquia, in 1524, came with the first batch of Muslims.(Writes Shamima Siddika in her book Muslims of Nepal).

The Chaubise rajas of west Nepal employed Afghan and Indian Muslims to train Nepali soldiers to use firearm, ammunitions, to work as courtiers and counselors, musicians and specialists on perfumes and ornaments. During the economic blockade imposed by Prithivi Narayan Shah, many Kashmiri traders fled the country from fear of prosecution. The remaining handful was helpful as spies and informants.

However, following Nepal's unification, Muslim traders were encouraged to settle down with their families.The construction of the Nepali Jama Masjid, Ghantaghar, near Tri-Chandra campus was permitted by Prithivi Narayan Shah. Muslims from

Afghanistan and India were experts in manufacturing guns, cartridges, cannons. Their knowledge of Persian and Arabic aided in international diplomacy

During Jang Bahadur Rana's regime, mass migration of Muslims from India occurred. This was to avoid persecution by the British army during the Sepoy Mutiny in 1857. A senior courtier to Delhi, Emperor Bahadur Shah Zafar renovated the Jama Masjid and was buried there. Begum Hazrat Mahal, wife of Nawab Wajid Ali Shah of Lucknow, entered Nepal through Nepalgunj. She had settled in Thapathali Darbar and was later buried in the Nepali mosque.

Dresses: Religiously and culturally, dresses or outfits of Muslim women are loose in nature that covers their head, arms and leg. In someparticular areas women wear "Borkha" and "Hejab". Men wear Panjabi, Pajama, Lunge and "Topi" at the time of Namaz.

Culture: Aadab or etiquette is characteristic of Muslim culture. The greeting AssalamWaleykum ("peace be with you"), with the required response, WaleykumAssalam ("and with you") initiates all interactions. Respect is expressed after a handshake by. Muslims in Nepal do not shake hands with each other.Theyplace the right hand over the heart to express respect and take leave with the phrase "Khoda Hafez". Before meals "Bismillah" (in the name of God) and "Alhamdulillah" (all praise to Allah) is chanted after meals. Circumcision of male offspring is practiced by the Musalmans of Nepal also (Dahlen 2004).

Population comprises of 1,164,255, about 4.4% of the total population of Nepal

1.1.7.5 Santhal ethnic group

Satar/Santhals are the oldest inhabitants of the eastern Terai region of Nepal. They are one of the first people to settle in Jhapa and Morang district by clearing the charkose Jhadi.

The term Santhal is a derivation of 'Saontar,' a place in Midnapur, India, where they are known as Santhal (Gautam 2011). Dark complexion, black curly hair and stout, muscular body, they are called Satar or Santhal in Nepal. They are categorized as a highly marginalized indigenous people of Nepal.

The ancestral stronghold of Santhals also called themselves 'Hor'. They belong to the atic group of human families and are also known as a subgroup of the Munda

family (Dahal BS 2050/52). Their language called Santhali belongs to the Austro-Asiatic language. They Santhal script called "Olichiki" was developed by Dr. Raghu Murmu in 1925. The Santhals prefer to live near forests. Bow and arrow are their favorite weapons. Pork is their favorite meat.

Figure 7: A Santhali group dancing in a festival during Dashain

Dress: the females wear green or blue check jhelah or saree. Males wear hand loom loin cloth/ kacha, banion, shirts and gamchha (napkin).

Population: Satars are the most backward ethnic groups of Nepal. Their population is 51,735 people or 0.19% of the total population of Nepal (CBS 2012).

Education: The Literacy rate of Santhal is 48.30% which is lower by 17.6% compared to the national average (NPCS, 2014).

1.2 Rationale

Beta-thalassemia is a congenital blood disorder caused by mutations in the globin gene. It is not an infectious disease but a heritable disease. It is an autosomal recessive disorder which can be prevented by avoiding marriage between two carriers. Genetic counselling before marriage and before planning a family plays a very important rolein preventing the disease. There is no provision for anti-natal screening in Nepal. Pathological Tests are expensive; therefore the patients are facing the problems of wrong diagnosis and wrong treatment.The prevalence of anemia is high among the ethnic groups of the Terai where it has decreased malaria associated morbidity. These ethnic groups used traditional medicines to cure certain common diseases cough, cold and fever, diarrhea, dysentery, jaundice, bath, allergies, toothache, diabetes, headache, indigestion and gastric. The ethnic groups

are unaware of genetic diseases. The traditional medicines for the treatment of Beta – thalassemia and hemoglobinopathies were unknown. Therefore a relation between ethno-biology and Beta-thalassemia was not observed.

Beta-thalassemia homozygote generates a considerable financial, psychological and social burden on the affected individuals, family, society and the country at large.

1.3 Objectives

1.3.1 General Objective

To assess the prevalence of Beta-thalassemia in some ethnic groups of Eastern Nepal.

1.3.2 Specific Objective

i. To examine the red blood cell abnormalities in Beta-Thalassemia

ii. To study the prevalence of Beta-thalassemia and haemogobinopathies among the ethnic groups.

iii. To document the status of mutational profile causing Beta-thalassemia among the selected ethnic groups**.**

1.4 Limitations

Only five ethnic groups were considered for study purpose. The study area included three districts of eastern terai Nepal. Sample size was limited to 300 samples per ethnic group, altogether 1500 samples including three districts. Due to financial constraints only nine core mutations were selected for Multiplex Amplification Refractory System-Polymerase Chain Reaction.

CHAPTER 2

2. LITERATURE REVIEW

Beta-thalassemia once restricted to the Mediterranean region is now the commonest monogenetic disorder globally. Population migration and intermarriage between different ethnic groups has introduced Thalassemia to almost every country of the world, including North Europe where the disease was previously absent. An approximate 1.5% of the global population (80 to 90 million people) ascarriers of beta-thalassemia with an annual birth of 60,000 symptomatic individuals has been estimated. Most of the births occurr in developing countries. The annual incidence of 1 in 100,000 throughout the world and 1 in 10,000 people was reported in the European Union (Vinchinsky, 2005). Worldwide only 200,000 homozygotes for thalassemia/Cooley's anemia/thalassemia major has been registered alive and under regular treatment (Thalassemia Federation, 2008). The prevalence of beta thalassaemia ranges from <1% to 16% in different regions (Colah, 2010).

The study on Thalassemia, a genetic blood disorder causing defective production of hemoglobin and anemia started in 1925. However, a peculiar type of anemia accompanied by splenomegaly andleucocytosis was reported by Von Jakschin (1889). It was named as anemiaInfentum subsequently known as JakschHayemLuzet'sanemia. By the beginning of the twentieth century, Europeans had become aware of an anemia syndrome in infancy associated with enlargement of the spleen (Marengo-Rowe, 2007).

In 1925, Thomas, a Detroit paediatrician was the first to describe thalassemia. Although this condition had been referred by some ancient Greek and early Italian writers, the disease had been overlooked as a separate clinical entity by Mediterranean physicians who must have frequently encountered it among their patients. Thomas (1925), presented his findings on five children with severe anemia, splenomegaly, peculiar bone deformities and abundant red cells in the peripheral blood smear at the annual meeting of the American Paediatric Society. He named the disease erythroblastic anemia and thought that it was similar to the entity described by Von Jaksch in 1889. Later it was termed as Cooley's anemia or Mediterranean anemia. Cooley and Lee (1927) continued their investigations and

refined their concept of erythroblastic anemia. Their research work was considered one of the most significant contributions to hematology that laid the foundation for thalassemia research and treatment options in the following decades. Although Cooley was aware of the genetic disorder, he did not investigate the apparently healthy parents of the affected children.Thomas's anemia was recognized as the homozygous form of mild hypochromic and microcytic anemia described by Riette and Wintrobe (Yaish, 2010).

The disorder described by Cooley was later named Thalassemia by Whipple.The term thalassemia was derived from two Greek words “Thalassa" meaning sea and "emia" meaning blood due to the fact that the early cases were reported in children from the area of the Mediterranean Sea endemic to malaria.The clinical and autopsy findings of several cases including identical twins concordant for the syndrome were reviewed by George Hoyt Whipple and W.L. Bradford, professor of paediatrics at the University of Rochester. They were the first to describe the pathology of this condition in their classical paper in 1932 and concluded that it was a hereditary disease in people of Mediterranean descent; hence they chose the name thalassemia (Whipple & Bradford, 1932).The trait conferred a degree of protection against malaria and selective survival advantage over carriers, thus perpetuating the mutation. The reason for protection of heterozygotes from malaria is still not clearly understood. It has been proposed that the red cell membrane in thalassemia heterozygotes is particularly susceptible to damage by oxidation and the infection with the malarial parasite provides sufficient oxidative stress to perturb intracellular metabolism in a manner that leads to premature death of the parasite (Bunn, 1986). This caused the disease to proloiferate massively throughout the Old World, where it is presently a huge health problem, straining blood supplies and causing enormous problems for medical and public health facilities in that part of the world (Nathan, 1998).

The presence of a genetic determinant underlining the disease was suggested by Caminopetros but it was only after 1940, that the genetic nature of Thalassemia was truly appreciated. It became clear that the disease described by Cooley and Lee is the homozygous state for a recessive autosomal gene (Cooley and Lee 1927).

The pioneer work to decipher the pattern of genetic transmission of beta Thalassemia was done by Dr. Neel who observed that the parents of thalassemia major children had thalassemia minor with one beta thalassemia gene. Neel and Valentine introduced the term Thalassemia minor for the milder carrier heterozygous state and major for the severe homozygous state (Valentine & Neel 1944). In 1952, detailed research by Sturgeon and co-workers revealed that chronic haemolytic anemia was associated with thalassemia and sickling trait.Thalassemia major patients had severe anemia with splenomegaly whereas thalassemia minor patients had mild symptoms. But there were a group of cases that were too mild to be termed major and too severe to be termed minor. Sturgeon, *et al.* (1955a), studied the genetic aspects of thalassemia and for the first time suggested the term thalassemia intermedia (TI) to describe patients with symptoms too mild to be termed thalassemia major and too severe to termed thalassemia minor. This improved the understanding of the genetic mutation that leads to thalassemia intermedia.

The analysis of hemoglobin pattern of patients with different types of Thalassemia by Vernon Ingram and Anthony Strettonled to the suggestion that there might be two types, α & β Thalassemia (Weatherall, 2005).

That the patients with Cooley's anemia were more alkali resistant than normal was noticed by Vecchio in 1946 and suggested that the amount fetal hemoglobin was more in Cooley's anemia than normally present after the first year of life. This was supported by Rich whoproposed that defect in HbA synthesis with persistent synthesis of HbF resulted in thalassemia.

By the end of the 1940s, it was apparent that thalassemia was not a single disorder but a complex syndrome resulting from the interaction of many genetic factors.

The developedtechnologyintroduced electrophoresis that revealed the detailed composition of different types of hemoglobin.The common of hemoglobinswere HbA, HbA2, HbE, HbF, HbC, HbH, HbM&HbS. In healthy adults, a significant level of only HbA and HbA2 occurred. In an unborn baby's bodyfoetal hemoglobin / HbF was the main type of hemoglobin. After birthwithin a year, HbA replaced HbF. In some cases, significant amount of HbF persisted in adult life. Significant changes in the quantity of different types of hemoglobin lead to pathological conditions. of) An increased HbF in beta thalassemia major was reported Huisman et al. (1956. In

the following year Kulkel et al. (1957)disclosed twice the normal quantity of HbA2 in beta-thalassemia patients.Paul (1954) and Itano (1957) proposed the idea that the primary structure of Hb determined its rate of synthesis. With the help of these findings Ingram and Stretton (1959) defined two major classes of thalassemia, namely α-and β- thalassemia. They suggested that an undetected amino-acid substitution caused the inherited defect in α-and β- globin chains. This amino acid substitution was the basis for thalassemia. The further advancement in biosynthesis provided experimental evidence of the imbalanced synthesis ofhemoglobin in these two disorders. This imbalance produced heterogeneity among the thalassemic cases. Individuals with decreased or absent α- and β-globin chain synthesis were observed.

A few years later the obserevation of ragged inclusion bodies in the red cell precursors of thalassemic cases by Fesas (1963) created great confusion regarding the nature of globin gene synthesis. Therefore, a method to measure the in vitro synthesis of globin chains in a quantitative fashion was developed (Clegg, 1965. Weatherall et al.1965). This revealed that both α- and β-thalassemias had imbalanced globin chain synthesis. It was eitherabsent or at reduced rates. The excess α chain consequent to defective β-chain synthesis were highly unstable and precipitated rapidly on the red cell membrane (Bank & Marks, 1966). Clegg and Weatherall (1966- 1968) continued their investigations and analysis on the mechanisms and rates of globin chain synthesis. They concluded that the rate of initiation and elongation of the globin chains were normal in patients with α- and β-thalassemia. The likely cause of defective α- and β-chain production appeared to be the reduced amount of messenger RNA for the affected chains.

In the 1970s advanced DNA technology revealed decreased production in globin mRNA and identified deletions of individual globin genes in certain thalassemic patients. With the use of cDNA/DNA hybridization Old et al. (1978), showed the production of full-length β-globin mRNA. Chang and Kan (1979) applied similarapproach and revealed the first example of human non-sense mutation

In the second half of 1980s with further development of molecular biology techniques, especially with the introduction of PCR technique, almost complete characterization of the molecular defects underlying the thalassemias was possible.

Extensive studies in molecular pathology of thalassemia by the application of southern blotting, rapid DNA sequencing methods and restriction fragment-length polymorphisms of the α- and β-gene clusters was conducted by different researchers. A strong association between the different mutations the α- and β-genes was discovered (Orkin et al. 1982. Higgs et al.1986).Weatherall and Clegg (2001) identified more than 200 different β-globin gene mutations. With these advancements, successful screening and pre-natal diagnostic programs were developed.This formed the basis for future therapeutic modalities such as gene therapy and reactivation of γ-globin genes.

2.1 Prevalenceof Beta-thalassemia world wide

Worldwide studies in various populations to estimate the prevalence of thalassemia started in the mid-1940s. In the Mediterranean countries Beta-thalassemia was the most common genetic disorder (Weatherall 1981). The frequencies ranged from 5 to 20% with the highest carrier frequency in Cypriots (14.7%), Sardinians (12.6%), Greeks (8%), Albanians (7.1), Sicilians (5.9), Lybians (4.6%), Tunisians (4.4%) and Italians (3.7%) were reported. Lesser frequencies ranging from 2 to 3 % were reported from the populations of Turkey, Lebanon, Israel, Malta, Algeria, Morocco and Corsica. Lower frequencies in countries of the former Yugoalavia (1.4%) and Spain (0.5%) with the lowest frequency in Francewere observed.

In North African countries, the prevalence of Beta-thalassemia ranged from 2 to 9%. But, in the United Kingdom and America, Beta-thalassemia was mainly seen in the immigrant populations.

High prevalence was reported from South-East Asia and South Asia, Indonesia, Thailand, China, India, Maldives and Pakistan. In most of these countries Haemoglobin variant HbE was also seen. High frequencies of HbE reported. Indonesia, Myamar, Thailand and northeastern India reported high prevalence of HbE variant. In some ethnic groups' very high frequencies of 25 to 50% or more were seen (Modell & Darlinson, 2008).

Accurate epidemiological data on the frequencies of Beta-thalassemia was not found in many developing countries. Micro mapping showed regional variations in frequencies. The incidence of Beta-thalassemia and HbEvaried considerably in small geographic areas of Indonesia, Sri Lanka and Thailand. In different regions of

Indonesia frequencies of Beta-thalassemiaranged from 0 to 9% and HbE from 0 to 11% (Weatherall, 2001; Cao, 2008). In Portugal, distribution of Beta-thalassemia geographically varied from <0.5% in the north to 1.6% in the south (Martins et al. 1993).

In India Beta-thalassemia is the commonest single-gene disorder with ten percent of the total world thalassemics born annually (Bashyam 2004). It was estimated that the prevalence of pathological haemoglobinopathies in India was 1.2/1000 live births, which suggested the birth of 32,400 babies with serious haemoglobin disorder every year (Christianson 2006; PRB, 2010). A 1989 WHO Working Group on the guidelines for the control of hemoglobin disorders estimated a 3.9% carrier frequency for B-thalassemia in India (WHO 1989). An indication of similar overall frequency of 3 to 4% by a WHO update translated 35.6 to 47.5 million nationwide carriers of B-thalassemia in India (WHO 2008). In the most commonly affected communities like Gujaratis, Punjabis, Sindhis and Bengalis, the incidence of B-thalassemia varied from 1 to17% (Gupta et al. 2003).

In Pakistan,the findings of Black (2010), revealed that worldwide, Pakistan is amongst the countries with a high prevalence of B-thalassemia. The prevalence of heterozygous B-thalassemia minor/carrier/trait was 5-7%. An estimated 100,000 patients suffered from the severe form, homozygous B-thalassemia/major/ Cooley's anemia. An approximate increase of 5000 cases every year that depended on regular blood transfusions accompanied by iron chelation and other medical managements for survival was reported by Ansari (2011). The cost for thalassemia management of a single child amounted to US$4500, much higher than the per capita income in Pakistan. As a result, thalassaemia is a major healthcare challenge and places great psychological and financial stress on the affected families and is a huge burden on the national healthcare delivery system

In Bangladesh the prevalence of both beta thalassemia and HbE was found to be significantly high according to studies done by various researchers. In 2005, Khan had reported the overall prevalence of Beta thalassemia heterozygousas 4.1% and Haemoglobin E heterozygous as 6.1% among Bengali and 41.7% HbE in tribal school children.The findings of Uddin's (2012) investigation showed prevalence of 21.3% Beta-thalassemia minor, 13.5% E-Beta-thalassemia and 12.1% HbE trait,

9.2% HbE disease, 0.7% HbD/S, 0.5% Beta-thalassemia major and 0.5% Delta-Beta thalassemia. Waqar (2016) reported the carrier status of thalassemia trait as 10% with more than 7,000 thalassemic births every year.Whereas in the same year Sultana (2016) reported the carrier rate of 3.0% B-thalassemia, 4.0% HbE/b-thalassemia and the 0.01%birth of affected B-thalassemia and 3.0%HbE/B-thalassemia per thousand respectively.

Another report presented by Waqar, Banu, Sadiya and Sarwadi (2017) presented 17.94% B-thalassemia carrier, 4.02% B-thalassemia major, 12.50% HbE carrier and 2.50% HbE disease. Noor et al. (2020) conducted a nationwide thalassemia screening study by molecular approach in supplementation to hematological and electrophoretic indices. This gave a more precise result that differed from earlier studies. They reported carrier frequency of 10.92%HbE andB-thalassemia trait, with 9.68% HbE trait and 2.24% Beta-thalassemia trait.

In developing countries, the gradual control of malnutrition and communicable diseases has contributed to an epidemiological transition.Survival of children with severe thalassemias has increased. More patients are presenting for treatment, growing older and requiring multidisciplinary care. However, data on the large number of undiagnosed homozygote deaths is lacking. Worldwide, patients withcompound E-beta-thalassaemiaheterozygous (Hb E/β-thalassaemia) represent approximately 50 per cent of severe β-thalassaemia cases. The highest prevalence occurs in India, Bangladesh and Southeast Asia, particularlyThailand, Laos and Cambodia where inheritanceofhaemoglobin E (Hb E) and β-thalassaemiaalleles is common. Thailand has an annual birth of about 3,000 affected children and an estimated 100,000 living patient. (Nancy et al. 2011). The true prevalence has estimated 5-7% of the population worldwide carries clinically significant hemoglobin mutation. β-thalassemia is most commonly found in the population of southern Europe, southeast Asia, Africa and India, α-thalassemia is widespread in Africa and Mediterranean population, the Middle East and Asia (Navaneet et al. 2013).

In Nepal, few researchers in this field have reported prevalence of erythrocytic disorders. Sakai et al. (2000) had observed heterozygous HbE hemoglobin variant in the Danuwars and Tamangs.

Mishra analyzed the mutations causing beta-thalassemia in 22 patients belonging to different ethnic groups. They concluded that mutational profile observed in their study was similar to that of two neighbouring countries, China and India. More than one mutation was detectedin some cases. Therefore, prenatal screening program for Nepalese population must be developedcarefully.

Sherchand et al. (2013) studied 78 cases with consistent symptoms of Beta-thalassemia minor at the BPKIHS, Dharan, Nepal. Decreased hemoglobin, abnormal peripheral blood smear and abnormal hemoglobin pattern on electrogram confirmed Beta-thalassemia. They concluded that various laboratory parameters and hemoglobin electrophoresis had a decisive role in the detection of Beta-thalassemia. Amongst the different ethnic groups, Beta- thalassemia was predominant in the Tharus.

In her review article (Jha, 2014) has concluded that this genetic disease cannot be cured but it can be prevented.Beta-thalasemia minor is not a serious problem.But Thalassemia major patients did not live beyond five years without treatment. With regular blood transfusion accompanied by iron chelation life expectancy could be increased upto the second decade. In developed countries regular blood transfusions accompanied by iron chelation therapy has achieved decreased incidence of Thalassemia. Nevertheless, in less developed countries like Nepal safe blood transfusion and iron chelation are not available universally.Therefore, preventive protocols that provide acceptable safe blood transfusion accompanied by supportive iron chelation therapy have to be established. Jha also reported Beta-thalassemia traits in 26.8% and sickle cell disease in 21.6% of the 97 patients tested by Cellulose acetate electrophoresis at alkaline pH and High-Performance Liquid Chromatography (HPLC). The erythrocytic disorders occurred mainly in cases from the Terai region with a significant prevalence in the Tharu ethnic group.Consistent to this study, very high hemoglobin disorders of 71.13% was reported from the Terai region. Similarly, 75% sickle cell anemia and 33.93% Thalassemia was seen in the Tharu ethnic group (Sharma et al. 2020). Das et al. (2020) reported a much lower prevalence of 4.11% Beta Thalassemia in East Nepal. On the other hand, Bastola et al. (2017) revealed prevalence of thalassemias and hemoglobindisorders in non-Terai regions of Nepal from their research studies at Manipal Teaching Hospital, Pokhara

University. Beta-thalassemia heterozygote / trait in an asymptomatic male of Newar community was detected by Bhatt et al. (2017). This incidence manifested the presence of genetic disorders inasymptomatic people. Theseasymptomatic carriers produce severe thalassemic offsprings.Therefore, the general people must also develop an alertness and awareness regarding genetic disorders.

Shrestha et al. (2020) reported the prevalence of hemoglobinopathy and thalassemia prevalent in seven provinces. Data collected from five different hospitals of Nepal Government that used Capillary electrophoresis for screening of hemoglobin disorders revealed a drastic difference between province 5 and other provinces A highly significant prevalence of 64% was found in Province 5. The second highest with 10% was from Province 7 of which most belonged to the Tharu community. Provine 1, 2 and 3 had 7% each. The least prevalence of 3% was found in both Provinces 6 and 4. Sickle cell disease was most common among the Tharus of West Nepal and Beta-thalassemia among different communitiesin East Nepal. This drastic difference between in different regions is remarkable and calls for further investigation**.**

The genetic aspect of Thalassemia was analysed Lama *et al* (2021) by multiplex amplification refractory mutation system-polymerase chain reaction (MARMS-PCR). Nine mutations identified were IVA1-5 (G→C), C-26(G→A) (HbE), 619 deletion, Cd 8/9 (+G), Cd 16 (-C), Cd 41/42 (-tcct), IVS1-1 (G→T), Cd 19 (A→G0 and Cd 17 (A→T) were detected in 17 ethnic groups of Nepal, especially those whose ancestors originated from India and Central Asia. The mutational profile of Nepal was comparable with that of India.

There was variations in the prevalence of Thalassemia and hemoglobinopathy in neighbouring countries India, Pakistan and Banglsdesh.

In India, the prevalence of beta thalassaemia variedin different regions and communities. It was higher amongst Punjabis, Sindhis, Gujaratis, Bengalis, central, eastern and western parts of the country, but lower in the south (Kumar 2015). Mutations, IVS-I-5, 619 bp deletion, IVS-I-1 (G>T), codons 41/42 and codons 8/9 comprised 82.5% of all beta thalassaemia alleles. IVS-I-5 was the most common allele, it varied from 44.8% in the north to 67.0% in the south and 71.4% in the east.

In Pakistan, Sindhi, Urdu speaking, Punjabi, Baluchi and Pathanwere the five major ethnic groups with erythrocytic disorders. The five common mutations were IVS-I-5 (44.4%), codons 41/42(17.5%), codons 8/9(14.6%), IVS-I-1 (G>T) (7.5%) and the 619 bp (0.6%) deletion. These mutations comprised 90% of the total beta thalassemia genes in the Pakistani population. However, frequency of the mutations varied from region to region (Usman 2009).

In Bangladesh variability in the genotypic profile of β–Thalassemia was seen. The most common were IVSI -5: G > C (81.4%), CD 26: G>A (72.85%), CD 2: T>C (57.1%) and IVSII-16: G>C (57.1%). The less common were -90 C>T (1.43%), CD1 T>A (2.86%), CD2 C>A (5.71%), CD30 G>C (1.43%), and IVS-II-81 C>T (1.43%). The most common mutation for β- thalassemia was IVS1-5: G>C and a combination of IVS1-5: G>C and CD26/E: was the commonest mutationfor E β-thalassemia disease.In the coming years hemoglobin disorders will be a major genetic problem in Bangladesh.

India and Pakistan shared very similar mutational profile with each other which was different from Bangladesh.

CHAPTER 3

3. MATERIALS AND METHODS

3.1 Study area

The study area included three districts of eastern Nepal: Jhapa, Morang and Sunsari.

Figure 8: Map of Nepal showing Study Sites

3.1.1 Jhapa

JhapaDistrictis located in Province 1 ofthe easternmost district in the Mechi Zone, Eastern Development Region of Nepal. Jhapa stands at latitude 26^0 38'25.80" North, longitude $87^0$59'.80" East and covers an area of 1,606 km^2 (north south). Altitude: 58 to 500 m. Nepal's lowest land Kechana Kawal (58 m from sea level) and longest concrete bridge Kankai Bridge (702 m) is located inJhapa.It lies in the fertile Terai plains and borders with Ilam in the north, Morang in the west, the Indian states of Bihar and West Bengal to the south, southeast and east respectively.

Jhapacomprises of 8 municipalities and 7 rural municipalities. Jhapa is home to many indigenous ethnic groups such as the Limbu, Tajpuriya, Gangai, Dhimal, Dhangad, Santhal, Tamang, Uraon, Magar, Gurung, Sunuwar, Brahmins, Chettri and Newar. Many Nepali language speaking Indian immigrants from Assam have also settled in Jhapa. Indian Madhesis (majority) from Indian states of U.P. and Bihar come for trade and work. As per the Census 2011, total population of Jhapa is

812,650 comprising of 427,554 females and 385,096 males residing in 184,552 households. The average literacy rate is 75% (68% female and 82% males) which is higher than the National average.

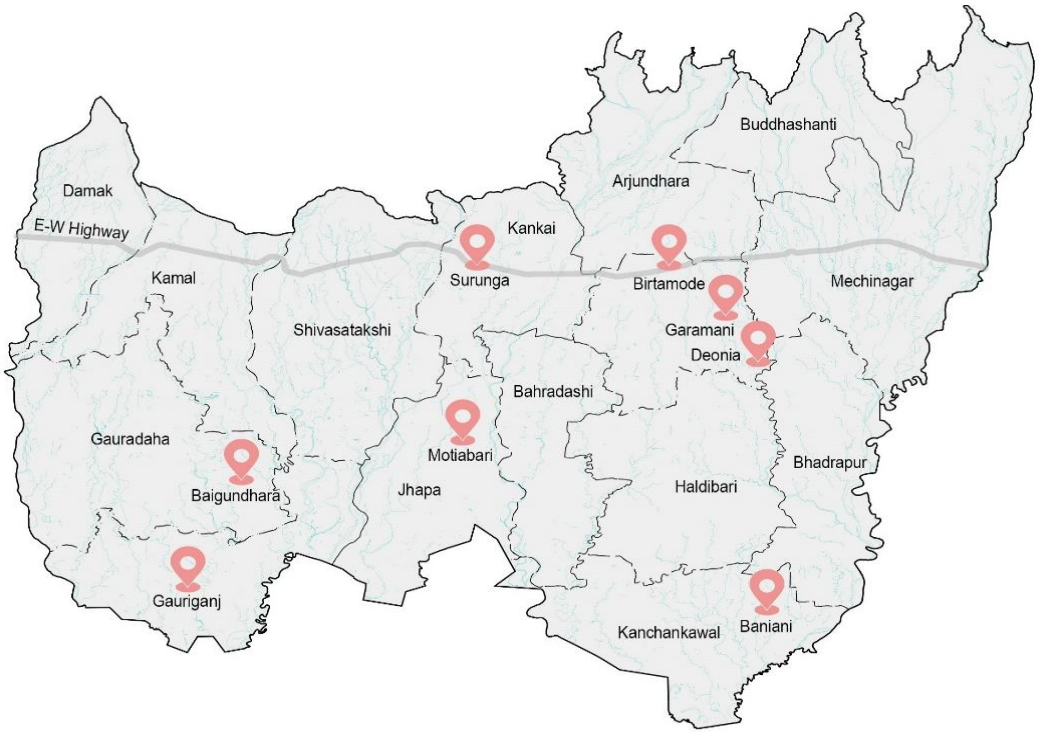


Figure 9: Map of Jhapa district showing study sites

3.1.2 Morang

Morang a district in the Koshi Zone, Eastern Development Region of Nepallies in the southern Terai plains of eastern Nepal. The name Morang was derived from the name of the Kirat King Mawrong Mung hang, who established the Morang Kingdom in the beginning of seventh century. It has been renamed Morang District Co-ordination Committee (DCC), located in the Province 1 of eastern region Nepal. It borders with Jhapa to the east, Dhankuta and Panchthar to the north and Sunsari to the west and the Indian state of Bihar to the south. The total area of Morang is 1,855 km^2 (9716 sq miles). The lowest elevation point is 60 meters and the highest is 2410 literacy rate of Morang is 69%.Ttal population of Morang is 1,147,186 comprising of 589,669 females and 557,627 males) residing in 193,728 households (Census 2011). Average literacy rate is 66.33% (57.12% females and 75.54% males).

Biratnagar, the headquarters of Morang is the second largest city of the country. The Morang plains are one of most culturally diverse regions in Nepal. The languages

spoken are Nepali (36.9%), Maithali (22.12%), Tharu (7.27%), Rajbhanshi (5.12%), Limbu (4.22%). Bantawa (4%), Urdu (2.22%) and Magar (2.15%). The ethnic groups are: Brahmin Hill (13.04%), Chhetri (11.22%), Tharu (7.55%), Rai (5.25%), Muslim (4.39%), Rajbhanshi (4.11%), Newar (4.03%), Magar (2.04%) and Tamang (2.25%).

Figure 10: Map of Morang district showing study sites

3.1.3 Sunsari

Sunsari district is located inKoshi Zone of Eastern Development Region of Nepal. The total area of the district is 1,257 km2 (485sq miles) lies mostly in the Terai and partly in the mid-hills at 260 38’ 29” N and longitude 870 07’ 44.76” E. As per the 2021 Nepal Census the population was 994,090 (596,594 females and 477,496 males)It borders with Morang district to the East, Udaypur, Sagarmatha zone to the West, Dhankuta to the North and the Indian state of Bihar to the south. The lowest elevation is 610 meters and highest level 11430 meters from mean sea level. The district headquarters is located in Inaruwa.

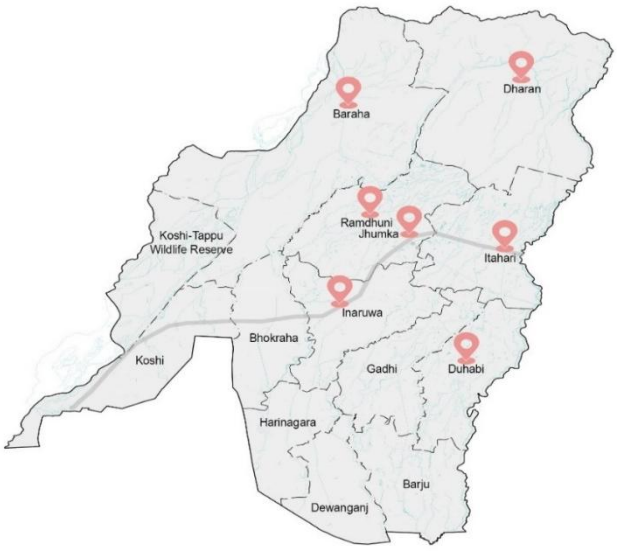


Figure 11: Map of Sunsari district showing study sites

According to the 2011 Nepal census, the population was 763,487 (1). The languages spoken are Nepali (29%), Maithali (28%), Tharu (12%), Urdu (10%), Urao (3%), Limbu (2%), Rai (2%), Newar (2%), Tamang (2%) and others 10%. the ethnic groups are Tharu (12%), Musalman (12%), Chettree (9%), Brahmin Hill (8%), Rai (7%), Yadav (4%), Newar (4%), Musahar (3%) and others 37%.

3.2 Materials

3.2.1 The materials required:

i. For collection and storage of blood samples: disposable syringes, refrigerator, EDTA vials.

ii. For preparation peripheral blood smears: Wright's Geimsa stain, slides, coverslip and microscope.

iii. For Haemoglobin electrophoresis:

3.2.2 Reagents required

i. Capillary's hemoglobin kit

ii. Two haemoglobin buffers (250 ml per vial); long stored at 2-8^{0}C for use. One month accumulated at room temperature.

iii. Hemolysing solution (250 ml) stored at 2-8^{0}C.

iv. Concentrated wash solution (25ml): Prepared at 1 in 10 dilution (25ml concentrated solution and +225 ml distilled water). Stable 3 monnths after reconstitution. Clean Protect added 35µl/dl of distilled water.

v. Normal A2 control: stored at 2-8^{0}C in lyophilized form.

vi. Pathological A2 control

vii. AFSC control (Analytical control systems for normal Hemoglobin A and F and abnormal hemoglobin S and C)

3.2.3 Equipments and Acessories

i. Capillarys Flex Piercing instrument Sebia

ii. Samples racks (supplied with instrument)

iii. Container kit (supplied with instrument)

iv. Collection tube with 75 nm length and 13

Materials for Amplification Refractory Mutation System-Polymerase Chain Reaction (ARMS-PCR)

DNA isolation kit (Quiagen): -

i. Ethanol

ii. 1.5 ml microcentrifuge tube

iii. Pipette tips

iv. Microcentrifuge tube with rotar for 1.5 tubes

v. vortexer

vi. Waterbath

vii. Phosphate buffer

viii. Saline

Polymerase Chain Reaction (PCR) reagents:

i. Taq DNA polymerase
ii. Mg free Taq buffer
iii. $MgCl_2$
iv. dNTPs
v. primers
vi. double ionized water
vii. TBE buffers
viii. Thermo- cycler

3.3 Methods

Ethical consent: 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: 17/2017). A community-based cross-sectional descriptive studywas conducted in different areas of the study sites. The study population comprised of persons aged 5-80 years.

Study Sites : Jhapa, Morang and Sunsari Districts

Sampling Technique: Random Sampling

3.3.1 Preliminary meetings

On 06/1/2074 a meeting was organized with the local population and well-known persons of a small village Motiabari, Jhapa district. Similar meetings were organized prior to sample collection with the help of influential personalities of the respective sites, Paglibari, Garamani, Sainik Mode, Tengra, Baigundhura, Baniani, Gauriganj, Itahari, Ramdhuni, Jhumka, Inaruwa, Duhabi, Baraha, Dharan, Rangeli, Kerabari, Pathari, Belbari, Kathahara, Biratnagar, Jahdaha. The purpose of the meeting was to share knowledge about anemia and related health issues along with an introduction about genetic disorders particularly beta-thalassemia. It was a very important and sensitive topic since the purpose for blood sample collection had to be convinced. Oral consent was obtained from all participants ranging from age 5years to 80 years of both sexes.

Inclusion criteria: Healthy inividuals of the study ethnic groups between age 5-80 years.

Exclusion criteria: Individuals with a history of blood transfusion within the last 1 month and suffering from any type of chronic diseases were excluded. Since parents were reluctant very young children below the age of five years was excluded. Blood samples were collected over a period two years. The dates, sites and number of samples collected are depicted in Table 1.

Blood samples were collected with the expert help of a nurse and two laboratory assistants. Approximately 3-5 ml of peripheral blood were collected and equally distributed in two ethylene diamine tetrachloride acetate (EDTA) vials and analysed. The EDTA vials were labeled as A & B and. Vial A was kept aside for identification of β-thalassemia mutations.Vial B was used for estimation of red cell indices and Hemoglobin fractions. Initially a CBC was performed and within 2 days the remaining blood volume of Vial A and Vial B were transported to the National Public Health Laboratory, Teku, Kathmandu for further analysis by maintaining the cold chain. Vial A was stored at a temperature of -4^0C for DNA analysis. Vial B was used for hemoglobin electrophoresis.

Table 1: Field work schedule

Date	Jhapa District	No of samples	Date	Sunsari District	No of samples	Date	Morang District	No of samples
6/1/74	Motiabari	63	9/8/74	Itahari	70	9/3/75	Rangeli	74
7/2/74	Paglibari	62	10/9/74	Ram dhuni	72	10/4/75	Kerabari	74
6/3/74	Garamani	61	9/10/74	Jhumka	69	9/5/75	Pathari	70
7/4/74	Sainik Mode	64	9/11/74	Inaruwa	75	9/6/75	Belbari	70
10/5/74	Tengra	62	12/12/74	Duhabi	68	8/7/75	Katahara	72
11/6/74	Baigundhura	62	9/1/75	Baraha	72	9/8/75	Biratnagar	70
8/7/74	Baniani	57	8/2/75	Dharan	74	10/9/75	Jahdaha	70
9/8/74	Gauriganj	69						

For the systematic analysis of RBC abnormalities a CBC (Complete blood Count) was performed.An approximate 0.5ml of blood from each of the 1500 samples collectedwas analyzed with Sysmex, United States of America (USA) automated cell counter for RBC parameters. The parameters considered for the screening of β-thalassemia were Hb concentrations, Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC), Red cell distribution Width (RDW), Red blood cells (RBC). The cases with Hb level < 13dl in males and <12dL in females along with low MCV <80 fL, low MCH <27 pg were considered positive for Beta-thalassemia.

On spot peripheral blood smear (PBS) of each sample was prepared and stained with Leishman's stain. The slides of CBC positive cases were observed microscopically for red cells morphology for the supporting diagnosis of beta-thalassemia and hemoglobinopathies. Those slides presenting target cells, anisocytosis and poikilocytosis indicated β-thalassemia. These positive cases were confirmed by hemoglobin electrophoresis test.

To find the prevalence of hemoglobinopathies and to compare the different types among the five ethnic groups, Capillary electrophoresistest was performed using a SebiaMinicap Flex Piercing system according to the manufacturer's guidelines.Thisautomated analyser performed a complete hemoglobin profile for quantitative analysis of normal hemoglobin fractions A, A2 and F and for the detection of major hemoglobin variants S, C, E and D. The instrument was equipped to re-suspend, lyse, separate, and analyse EDTA whole blood for hemoglobin variants. It was a multiparameter instrument for hemoglobin analysis on parallel capillaries that used 8 capillaries. The entire procedure was divided into two steps, manual and automated. The manual steps were as follows:

- Capped sample tubes were placed in racks positions 1 to 8 with the bar code of each tube visible at the openings of the sample racks;
- New dilution segments were placed in sample racks
- The racks were slided into the Capillarys 2 Flex-Piercing instrument
- Sample racks were removed after analysis
- The dilution segments were removed and discarded

The sequence of automated steps included

- A built-in bar codereader tracked the samples and produced electrograms automatically.
- The blood samples were mixed before analysis.
- The samples were hemolysed and diluted from primary tubes into dilution segments.
- The hemolysed blood was injected by aspiration at the anodic end of the capillary.
- The lysed red cells were electrophoresed in an alkaline buffer (pH 9.4). Separation of hemoglobin variants was directed by pH and endosmosis. The separation of different hemoglobin fractions took place in eight silica capillary tubes of 25 µm internal diameter.
- The migration of hemoglobin fractions was conducted at a high voltage of 9,800 volts and a Peltier device maintained a tight temperature control of 34^0C. The Hb migration was on the X-axis from 0-300.The migration of each Hb was normalized relative to the standardized position of HbA (position 150) and HbA2 (position 243). In the presence of HbA and /or HbA2, the electrophoretic profile was divided into 15 migration zones. The division into 15 zones enabled discrimination between the most common Hb variants: HbS, HbE, HbC and HbD-Punjab.
- The Hb fractions were detected directly at a specific absorption wavelength of 415nm (specific to hemoglobin) at the cathodic end of the capillary. The presumptive identification of any abnormal fraction was obtained according to the migration zone in which the abnormal fraction migrated. This method allowed the measurement of HbA2 in the presence of HbE. The position of the Hb variant in each sample was recorded.
- The optical detector consisted of a deuterium lamp, optical grating, CMOS (complementary metal-oxide semi-conductor), optical fibres and diode array detectors.
- The electrogram of each sample wasgenerated automatically.

To find the spectrum of mutation causing β-thalassemia among the five ethnic groups Molecular analysis was conducted. This comprised of two steps: A. DNA extraction

and B. Multiplication Refractory Mutation System-Polymerase Chain Reaction (MARMS). DNA extraction: DNA was extracted from the buffy coat by Ethanol method which involved the following steps.

A. Preperation of buffy coat

The whole blood was centrifuged at 2500 x g for 10 min at room temperature and incubated at room temperature for 1 imn. DNA eluted at room temperature. After centrifugation 3 different fractions were distinguishable: upper clear layer of plasma; intermediate layer of buffy coat containing concentrated leukocytes; and bottom layer of concentrated erythrocytes. The upper layer of plasma discarded. The buffy coat was pippetted out carefully into a fresh microcentrifuge tube.

Preliminary preperations

1. Proteinase K was dissolved in 1.3 ml of nuclease free water and stored at -20^{o}C.
2. 10 ml of Ethanol 100% was added to the Desinhibition buffer and tightly closed to avoid evaporation of ethanol.
3. 40ml of ethanol 100% was added to the wash buffer and tightly closed to avoid evaporation.
4. Elution buffer was preheated at 70^{o}C.

Protocol for Genomic DNA extraction from buffy coat

1. 25 µl of proteinase K was pipeted into the bottom of a 1.5 ml microcentrifuge tube.300 µl of sample was added to the microcentrifuge tube.
2. 300 µl of Lysis/binding buffer to the sample material. This was vortexed 1-2 seconds and incubated at room temperature for 10 minutes.
3. The mixture was centrifuged at maximum speed for 1 minute. Supernatent was aspirated by micropipette to eliminate small non-visible cell pellet. 10-20 µl of residual liquid was left in the microtube. This was vortexed to resuspend the pellet.
4. This was incubated at 56^{o}C for 15 minutes.
5. 200 µl of Lysis / Binding buffer was added. Vortexed and incubated at 72^{o}C for 10 minutes.
6. 200µl of Ethanol (96 – 100 5) was added and vortexed.
7. The sample was transferred to a Microspin column with its collection tube.
8. Centrifuged at 8.000 rpm for 60 seconds. Collection tube was removed.

9. Spin column was placed in a new collection tube and 500 µl of Desinhibition buffer was added. Centrifuged at 8.000 rpm for 60 seconds. Liquid was removed.700 µl of Wash buffer was added to the reservoir of the Spin colomn. Centrifuged at 12.000 -14.000 rpm for 60 seconds. Liquid was removed.
10. Centrifuged at maximum speed for 2 minutes to remove residual ethanol.
11. Collection tube was removed and Spin column was and placed on 1.5 ml microtube.50 – 200 µl of Elution Buffer (preheated at 72^{o}C) was added to the reservoir of spin column. The buffer was directly dispensed onto the silica membrane. Incubated at room temperature for 1 min.
12. Centrifuged at maximum speed for I min. The microtube now contained the genomic DNA ready for PCR test.

B. Multiplication Refractory Mutation System-Polymerase Chain Reaction (MARMS).

The confirmed cases were subjected to molecular analysis of mutations IVSI, 5(G>C), 619 bp del., FS8/9(+G), IVSI,1(G>T), FS 41/42(-CTTT), C15(G>A, FS16(-C), C30(C>C), C5(-CT), and C26 $^{glu\text{-}lys}$ at Decode Genetics and Research Centre, Cinamangal, Kathmandu.The multiplex was optimized with minor modifications and allele specific mutation was performed individually with internal control primer according to the protocol of Old et al. and Varawalla et al. The PCR master mix was homemade, the recipe was as follows: 62.5 mmol/ l KCl, 12.5 m mol/ l Tris (pH 8.3), 1088 mml/ l $MgCl_2$, 0.265mmol/ l of four dNTPs each, 0.01% gelatin and 0.001% Spermidine. To 20µl of the prepared PCR master mix, 5 pm/ µl of primers, 1 unit of Taq DNA polymerase and I µg of DNA were added only at the time of PCR test. The PCR Buffers and molecular biology reagents were stored at -20^{0}C. The PCR was conducted in thin-walled PCR tubes in a thermal cycler. The steps included were: denaturation at 94^{0}C for 1 min, Annealing at 65^{0}C for 1 min, extension at 72^{0}C for 1.5 min. and the final extension at 72^{0}C for 3minutes. For mutation detection 20µl of PCR product was laid on 3.0% Agarose gel and electrophoresis at 150 V was run for 1 hour. The gel was stained with Ethidium Bromide and results were observed on the monitor and printed on black and White Polaroid film.

Table 2: Group, amplicons, pairing & sequence of primers for amplification of 9 core mutations by MARMS-PCR

Mutation	Amplicon base pair	Primer pairs Mutant Control	Primer pairs Internal Control	Primer sequence 5'>3' (Primer number)
Group A Internal control IVSI,5(G>C) C15 (G>A)	676 285 500	 1 &11 2&12	11&12	CTCCTTAAACCTGTCTTGTAACCTTGTTAG (1) TGAGGAGAAGTCTGCCGTTACTGCCCAGTA(2
Group B Internal Control FS8/9(+G) 619bp del Forward 619bp del reverse IVSI,1(G>T) FS41/42-(CTTT)	861 225 242 (del) 861-normal 281 439	 3&11 4&5 6&11 7&11		CCTTGCCCCAGGGCAGTAACGGCACAAA(3) CAATGTATCATGCCTCTTTGCACC (4) GAGTCAAGGCTGAGAGATGCAGGA (5) TTAAACCTGTCTTGTAACCTTGATACGAAA(6) GAGTGGACAGATCCCCAAAGGACTCAACCT(7
Group C InternalControl C-5(-CT) FS16 (-C) C-30(G>C)	676 206 239	8&11 9&11 10&11		ACAGGGCAGTAAACGGAGACTTCTCCGCGA8 TCACCACCAACTTCATCCACGTTCACGTTC-9 TAAACCTGTCTTGTAACCTTGATACCTACG 10

	279			
Common forward Common reverse	676		11&12	ACCTCACCTGTGGAGCCAC 11 CCCCTTCCTATGACATGAACT 12

[Group A: IVSI,5(G>C) and C15 (G>A) mutations by duplex PCR using 4 primers was screened. Primers 11 and 12 (→&←) were used in getting an internal control of 676 bp. Primers 11+1 and 12+2 were used to amplify mutant target DNA fragment due to {IVS1, 5(G>C)-285 bp} and {C 15 (G>A)-500bp} mutations. The Group A* had primers 11 &6 (→) amplifying {IVSI,1 (G>T)-281 bp}

GroupB: 619 bp ing 6 primers was screened. The group B* had primers 11 and 1 amplifying {IVSI,5 (G>C bp} instead of IVSI,1(G>T).

Group C: C5 (-CT), FS16 (-C) and C30 (G>C) mutations by triplex PCR using 5 primers were screened. 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-5(-CT)-206bp, FS16 (-C)-239 bp and C30(G>C)- 279 bp} respectively].

Data Collection Tool: A self constructed tool was used to collect socio-demographic data and blood examination findings of the study participants.

Statistical Analysis: The raw data were managed on Microsoft Excel sheet then exported into 3.6.1 (R Core Team 2019) for further analysis. The red cell indices below the normal range indicated the positive betathalassemia. The positive sign represented by "1" and the negative sign by "0". The chi-squared test was applied to individual ethnic group and their district of residence. The statistical significance ($p \leq 0.05$) indicated the anemia but not the type. Descriptive statistics such as means, frequencies and proportions were reported. Tables and graphs were used to summarize and present the data. Point prevalence of microcytic hypochromic anemia and haemoglobinoathies and mutations were reported. Mean Hb level with standard deviation was reported.

The prevalence analysis was performed on the basis of total number of positive samples per indices per total population studied by the application of prevalence package (Devleesschauwer et al. 2014) under R (R Core Team, 2019).

3.3.1.1 Estimation of the Prevalence

To assess the prevalence of thalassemia and haemoglobinopathies in East Nepal,a cross-sectional study was conducted in three districts (Jhapa, Morang and Sunsari) of East Nepal. Blood samples were collected in EDTA vials and the Red Cell Indices was estimated in a fully automated electronic cell counter. Out of the 1500 blood samples tested for Microcytic Hypochromia, 285 tested positive. Because no confirmation was available to assess the true status of the disease, an estimate of the true prevalence was obtained by combining the apparent prevalence with prior information on test characteristics in a Bayesian model. Two cases were considered for the test. In the first test sensitivity was modelled as a uniform (0.60, 1.00) distribution, and the specificity as a uniform (0.90, 1.00) distribution. In the second case, the specificity was modelled as uniform distribution ranging from 75% to 100%, reflecting the potentially high proportion of positive test results.

CHAPTER 4

4. RESULTS AND DISCUSSION

4.1.Districtwise gender distribution

A total of 1500 samples were taken with equal number of cases (33%) from the three districts of Jhapa, Morang and Sunsari. Females comprised 58% of the study population and the rest were males. The districtwise distribution also revealed that the majority were female subjects in the study population in all three districts comprising of 61% from Sunsari, 58% in Morang and 54% from Jhapa (Figure 12).

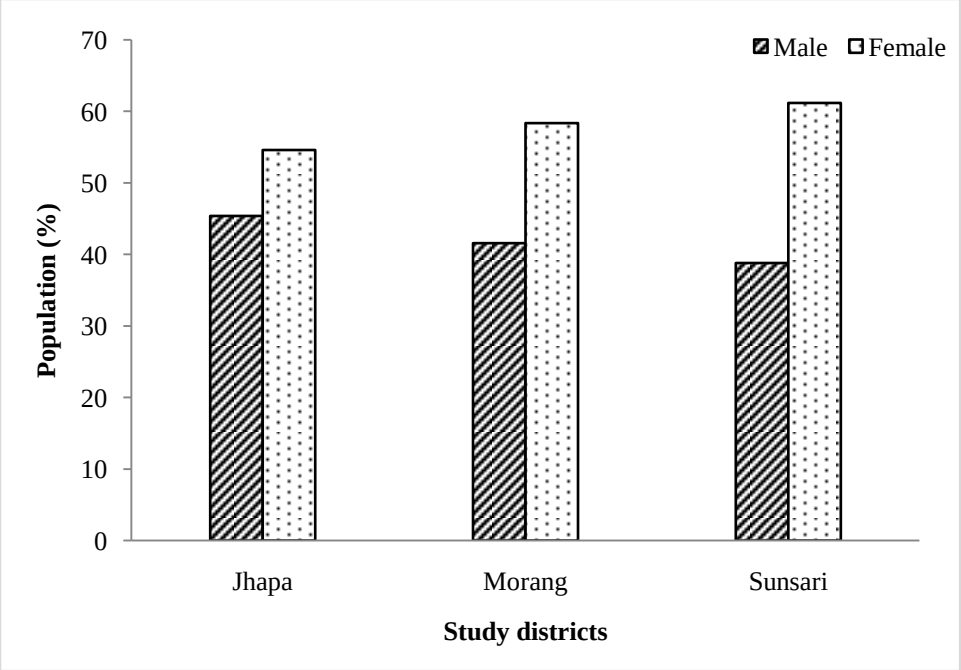


Figure 12: Districtwise sex distribution among studied samples

4.1.1. Distribution of gender in the ethnic groups

Random sampling of the five ethnic groups was done. In the different ethnic groups females comprised of 59%in Koch Rajbanshi, 55% in Kochila Tharu, 51% Musahar, 53% Musalman and 81% Santhal ethnic group respectively (Figure 13).

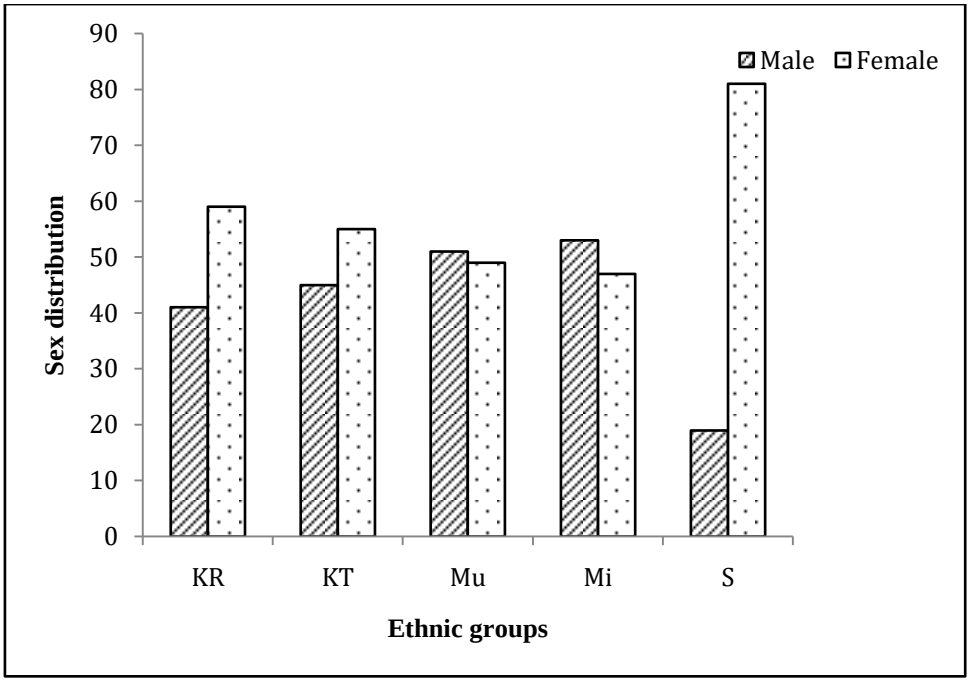


Figure 13: Sex distribution among ethnic groups

KR Koch Rajbanshi, KT KochilaTharu, MU Musahar, MI Musalman, S Santhal

4.1.2 Distribution of population according to age

Maximum samples were from the age group 11 to 20 years (34 %). Age10 years and below comprised of 19% of the total samples. Age group between 50 to 60 years represented the least (4%) of the total samples (Figure 14).

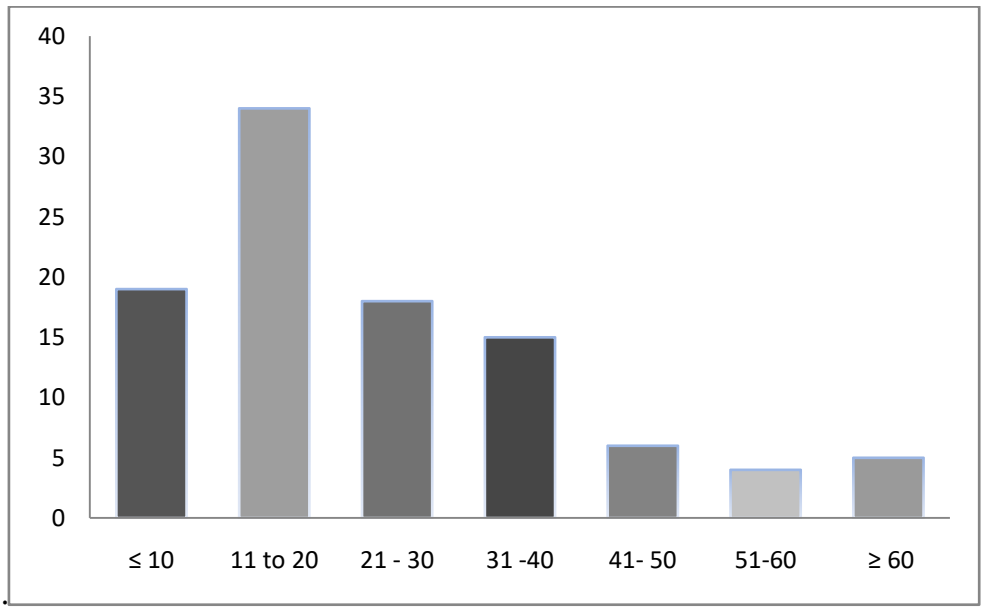


Figure 14: Distribution of population according to age groups

4.2 Systematic analysis of Red Cell abnormalities in Beta-thalassemia

4.2.1.Red blood cell parameters of the studied population

Out of the total 1500 samples, in 285 samples the level of mean corpuscular volume(MCV), mean corpuscular hemoglobin(MCH), mean corpuscular hemoglobin concentration(MCHC), red cell distribution width- standard deviation (RDW-SD) and mean red blood cell(RBC) counts was below the cut–off level for Beta-thalassemia diagnosis.These 285 samples were diagnosed as microcytic hypochromic anemia. The cut-off values applied for each parameter wasHb:12g/dl,MCV:75fl, MCH:27pg, MCHC:35g/dl, RDW-SD:35 and RBC: 5.0% respectively. The mean values in these 285 microcytic hypochromic cases were: Hb 10.48 ± 1.77, mean corpuscular volume(MCV) was 68.36±7.87 fl, mean corpuscular hemoglobin(MCH) was 22.03 ±4.76 pg,mean cell hemoglobin concentration(MCHC)was 31.47± 3.21 g/dl, red cell distribution width- standard deviation (RDW-SD) was 32.84± 9.88 and mean red blood cell RBC 5.5 ± 0.87 % (Table 3).

The prevalence of microcytic hypochromic anemia was found to be 19% in the total study population (285/1500).

Table 3: RBC parameters among persons with microcytic hypochromic anemia (n=285)

Variable	Mean± SD	Minimum	Maximum	Median	IQR
Age in years	25.46 ± 17.13	2	90	20	23
Hb	10.48 ± 1.77	6.3	35	10.5	1.2
MCV	68.36 ± 7.87	9.1	79.9	69.1	7.75
MCH	22.03 ± 4.76	15.1	74.8	31.3	3.1
MCHC	31.47 ± 3.21	19.6	45.5	32.3	5.35
RDW-SD	32.84± 9.88	11.3	62.10	35.4	7.8
RBC	5.55 ± 0.87	3.4	11.8	5.4	1

(Hb hemoglobin, MCV mean corpuscular volume, MCH mean corpuscular hemoglobin, MCHC mean cell hemoglobin concentration, RDW-SD red cell distribution width- standard deviation, RBC red blood cell).

4.2.1.1. Analysis of Hemoglobin

Anemia by definition is low hemoglobin concentration. All the cases with hemoglobin level below 12g/dL wereconsidered for screening of Beta-thalassemia.In this study 285 cases suffered from anemia.

4.2.1.2 Relationship between gender and hemoglobin concentrationin the studied samples

The mean ±standard deviation of Hb in 104 males with microcytic hypochromic anemia was 11.0± 3.0, in 181 females it was 10.0 ±1.0, median 10.5 with of p = 0.5 (Table 4).

Table 4: Sexwisehemoglobin estimation

Variable	Category	Number	Mean±sd	Median	IQR	Z/Chi-square	p-value
Sex	Male	104	11.0 ± 3.0	11.0	1.0	0.6	0.5
	Female	181	10.0 ±1.0	11.0	1.0		

4.2.1.3 Hemoglobin found among anemic samples by their districts

Out of 500 studied in each district anemia was detected in 158 samples of Jhapa and 74 samples of Sunsari with Hb 11g/dL± sd. In 53 samples of Morang Hb was 10g/dL± sd (Table 5).

Table 5: Districtwisehemoglobin estimation

Variable	Category	Number	Mean± SD	Median	IQR	Z/Chi-square	p-value
District	Jhapa	158	11.0 ±2.19	10.0	1.0	17.4	0.0
	Morang	53	10.0 ±1.03	10.0	1.0		
	Sunsari	74	11.0 ± 0.99	11.0	1.3		

4.2.1.4 Ethnicity wise hemoglobin analysis

Out of total 300 samples from each ethnic group, Hb was 11.0g/dL ± sd in 57 samples from Koch Rajbanshi. In the three ethnic groups Kochila Tharu, Musahar and Santhal the hemoglobin level was 10.0g/dl ± sd. It was found to be at border line

level of 12g/dl ± sd in the Musalman ethnic group.The p-value 0.02 was found (Table 6).

Table 6: Ethnicity-wise hemoglobin estimation

Variable	Category	Number	Mean ± sd	Median	IQR	Z/Chi-square	p-value
Ethnic groups	KR	57	11.0 ± 3.3	10.0	10.0	11.1	0.02**
	KT	48	10.0 ± 2.0	11.0	11.0		
	Mu	28	10.0 ± 1.0	10.0	10.0		
	Mi	116	12.0 ± 2.0	11.0	11.0		
	S	36	10.0 ± 1.0	11.0	11.0		

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4.2.1.5 Age-wise hemoglobin analysis

An average hemoglobin level of 10.0g/dl ± sdwas recorded in samples from following five age groups ≤10, 11-20, 21-30, 41-50 and 51-60 years. In the 31-40 and ≥ 60 years group it was 11.0g/dl ± sd. The overallp-value 0.9was obtained (Table 7).

Table 7: Hemoglobin estimation among categorized age groups

Variable	Category	Number	Mean±SD	Median	IQR	Z/Chi-square	p-value
Age in years	≤ 10	53	10.00 ± 0.79	10.5	0.8	1.6	0.9
	11-20	96	10.00 ±1.09	10.5	1.2		
	21-30	50	10.00 ± 1.08	10.45	1.6		
	31-40	43	11.00 ± 3.85	10.4	1.4		
	41-50	16	10.00 ± 1.29	10.9	0.97		
	51-60	14	10.00 ± 1.41	10.5	1.53		
	>60	13	11.00 ± 0.44	10.5	0.4		

4.2.2 Analysis of mean corpuscular volume (MCV)

The MCV test measures the average size of the red blood cells. Low MCV means the red blood cells are small in size. This indicated microcytic anemia. In 285 samples the red blood cells were small in size with low MCV (<80fL).

4.2.2.1 Sex-wise analysis of mean corpuscular volume (MCV)

Out of the 285 microcytic anemic samples, 104 were males with MCV 69fL± sd and 181 females with 68.0 ± sd. The p-value 0.00 was recorded.

Table 8: Sex-wise comparison of mean corpuscular volume

Variable	Category	Number	Mean ± sd	Median	IQR	Z/Chi-square	p-value
Sex	Male	104	69.0 ± 10.0	70.0	10.0	-2.9	0.00**
	Female	181	68.0 ± 6.0	69.0	10.0		

4.2.2.2 District-wise estimation of mean corpuscular volume

Out of the 500 samples from each district, 158 individuals (MCV = 66.0 ± sd) in Jhapa, 53 individuals (MCV = 69.0 ± sd) in Morang and 74 individuals (MCV = 72.0 ±sd) in Sunsari suffered from microcytic anemia (Figure 9).

Table 9: District-wiseestimation of mean corpuscular volume

Variable	Category	Number	Mean± SD	Median	IQR	Z/Chi-square	p-value
District	Jhapa	158	66.0 ± 8.0	68.0	8.0	50	0.00**
	Morang	53	69.0± 6.0	71.0	8.2		
	Sunsari	74	72.0 ± 6.4	74.0	8.37		

4.2.2.3 Ethnicity wise analysisofmean corpuscular volume

Among the five ethnic groups the mean corpuscular volume ranged from 69.0fl to 72fl (Table10).

Table 10: Ethnicity-wise estimation of mean corpuscular volume

Variable	Category	Number	Mean±SD	Median	IQR	Z/Chi-square	p-value
Ethnic groups	KR	57	69.0 ±6.05	70.0	9.7	47.2	0.00**
	KT	48	72.0 ±4.37	69.0	1.5		
	Mu	28	70.0 ±11.74	61.0	11.9		
	Mi	116	70.0 ±4.19	69.0	7.2		
	S	36	69.0 ±5.23	72.0	7.3		

KR Koch Rajbanshi, KT KochilaTharu, MU Musahar, MI Musalman, S Santhal

4.2.2.4 Age-wise analysis of mean corpuscular volume

The size of the red blood cells was found to be small in all the 285 samples of different aged groups which revealed low MCV. The mean corpuscular volume was 65.0fl ±sd in the 41-50 years category,67.0 ±sd in 31–40 years, 68.0fl ±sd in the ≤ 10and>60 respectively,70.0± sd in the 11-20 years and 71.0fl ±sd in the 51 – 60 years age categories (Table11).

Table 11:Age-wise estimation of mean corpuscular volume

Variable	Category	Number	Mean±SD	Median	IQR	Z/Chi-square	p-value
Age in years	≤ 10	53	68.0 ±9.0	70.0	8.0	8.4	0.2**
	11-20	96	70.0 ±5.0	70.0	5.0		
	21-30	50	69.0 ± 6.0	68.0	11.0		
	31-40	43	67.0 ±11.0	68.0	14.0		
	41-50	16	65.0 ± 8.0	68.0	15.0		
	51-60	14	71.0 ±8.0	73.0	14.0		
	>60	13	68.0 ±7.97	68.0	17.0		

4.2.3Analysis of mean corpuscular hemoglobin (MCH)

The mean corpuscular hemoglobin (MCH) test indicated the amount of iron in the blood. All of the 285 microcytic anemic cases had low MCH (≤ 27pg).

Sex-wise analysis of mean corpuscular hemoglobin concentration (MCH)

The MCHin the male was 23.0pg±sd and in the female 22.0pg± sd (Table 12).

Table 12: Sex-wise estimation of mean corpuscular hemoglobin

Variable	Category	Number	Mean±SD	Median	IQR	Z/Chi-square	p-value
Sex	Male	104	23.0 ±7.0	22.0	4.0	-2.5	0.011*
	Female	181	22.0 ± 3.0	21.0	3.0		

4.2.3.1 Districtwise analysis of mean corpuscular volume (MCH)

Low MCV (≤27pg) was observed in all the 285 microcytic anemic cases. It was found to be 22.0 ± sd in Jhapa and Morang. In Sunsari reports disclosed 23.0 ±sd (Table 13).

Table 13: Districtwisecomparision of mean corpuscular hemoglobin estimation

Variable	Category	Number	Mean± SD	Median	IQR	Z/Chi-square	p-value
District	Jhapa	158	22.0 ± 6.0	21.0	3.0	21.14	0**
	Morang	53	22.0 ± 3.0	22.0	3.0		
	Sunsari	74	23.0 ± 3.0	23.0	4.0		

4.2.3.2 Ethnicity wise estimation of mean corpuscular hemoglobin (MCH)

Among the five ethnic groups the value of MCH was 21.0 ±sd in the Musalman and KochilaTharu, 22.0± sd Koch Rajbanshi and Santhal and 23.0± sdMusahar ethnic groups with p-value 0.70.

Table 14: Ethnicity-wise estimation of mean corpuscular hemoglobin

Variable	Category	Number	Mean ± sd	Median	IQR	Z/Chi-square	p-value
Ethnic groups	KR	57	22.0 ± 3.0	21.0	4.0	2.0	0.7
	KT	48	21.0 ± 3.0	21.0	3.0		
	Mu	28	23.0 ± 9.0	21.0	2.0		
	Mi	116	21.0 ± 2.0	21.0	3.0		
	S	36	22.0 ± 3.0	22.0	3.0		

(KR Koch Rajbanshi, KT KochilaTharu, MU Musahar, MI Musalman, S Santhal)

4.2.3.4 Age-wise estimationof mean corpuscular hemoglobin (MCH)

In different age groups MCH was below the cut-off (27pg) level for Beta-thalassemia. Between≤ 10 -30 years and 41- 50 years it was 21.0± sd and 22.0 ± sd between 31-40,51-60 and ≥60 years age category.

Table 15: Age-wise estimation of mean corpuscular hemoglobin

Variable	Category	Number	MCV± sd	Median	IQR	Z/Chi-square	p-value
Age in years	≤ 10	53	22.0 ± 6.0	21.0	3.0	5.0	0.54
	11-20	96	22.0 ± 3.0	21.0	4.0		
	21-30	50	22.0 ± 3.0	21.0	3.0		
	31-40	43	23.0 ± 8.0	22.0	4.0		
	41-50	16	21.0 ± 2.0	21.0	3.0		
	51-60	14	22.0 ± 3.0	22.0	3.0		
	>60	13	23.0 ± 3.0	22.0	2.0		

4.2.4Association of abnormal red cell parameters between BTT cases

The comparison of red cell parameters between BTT and Non-BTT revealed that the the RBC count was > 5.0 million/cumin in 38.04% of BTT cases while only 7.92% non-BTT cases showed increased RBC count, which was statistically significant with p= 1.026e-07. However, the Hb was below 11gms in 84.78% of BTT cases compared to 23.76% non-BTT cases. MCV was lower <80fL in 82.60% cases of BTT with a significant p-value of 6.735e-05. In 81.52% BTT cases MCH was low (<27pg). RDW was significantly lower in 61.95% i.e., 114 out of 184 BTT cases. Hence high RBC (p =1.026e-07), low Hb (p =<2.2e-16), low MCV (p =6.725e-05) and low MCH (p =9.041-05) were significantly associated with BTT.

Table 16: Association of Red Cell parameters with BTT

Lab parameters	BTT		NON-BTT		Chi-square	P = value
	N=184	%	N=285	%		
RBC (>5.0)	70	38.04	78	27	28.2	1.026e-07
Hb (<11g/dl)	156	84.78	56	20	101.7	<2.2e-16
MCV (<80fl)	152	82.60	213	75	15.8	6.725e-05
MCH (<27pg)	150	81.52	210	74	15.3	9.041e-05
MCHC (<31g/dl)	26	14.13	38	13	0.12	0.7247
RDW-sd<15	114	61.95	164	58	3.6	0.056

4.2.4.1Coparision of mean values of red cell parameters between BTT and Non-BTT cases

The mean values of different laboratory parameters between BTT and Non-BTT cases were analysed. The mean RBC count and MCHC was significantly higher in BTT, where as mean hemoglobin concentrations and RDW were slightly lower in BTT and MCV and MCH were significantly reduced in BTT when compared to non-BTT as depicted in Table 17.

Table 17: Comparision of Red Cell Parameters of BTT and NON-BTT

RBC INDICES	BTT(n=184)	NON-BTT(n=101)	P =value
RBC $\times 10^{12}$ cells/l	5.64 ± .747	5.09 ± 1.579	0.0001
Hb(g/dl/dl)	10.20 ± 1.076	10.85 ± .879	0.0002
MCV(fl)	65.88 ± 4.925	74.84 ± 2.892	0.0001
MCH(pg)	21.01 ± 2.576	23.15 ± 2.361	0.0001
MCHC(dl)	31.49 ± 3.070	31.11 ± 3.269	0.2221
RDW(%)	14.70 ± 2.013	14.89 ± 2.210	0.1981

4.2.5 Analysis of the co-relation co-efficient of the red cell parameters

Most of the red cell parameters of the 285 microcytic hypochromic cases showed positiveco-relation with each other.

The correlation co-efficient values between Hb and MCV was significantly positive (Figure 15 r = 0.49). Likewise the correlation co-efficient values between Hb and MCH was 0.26 which was positive and significant. The correlation co-efficientbetween Hb and MCHC was 0.51 and with RBC was 0.14, both of which were significant.

There was a significant positive co-relation between MCV and MCH (r=0.36), between MCV and MCHC (r=0.32), between MCV and RDW-SD (0.32) andbetween MCV and RBC (0.43).

Similarly, the correlation co-efficient values between MCH and MCHC was significantly positive (r = 0.25) and between MCH and RBC was also significant positive (r=0.14, p < 0.05, Figure 15).

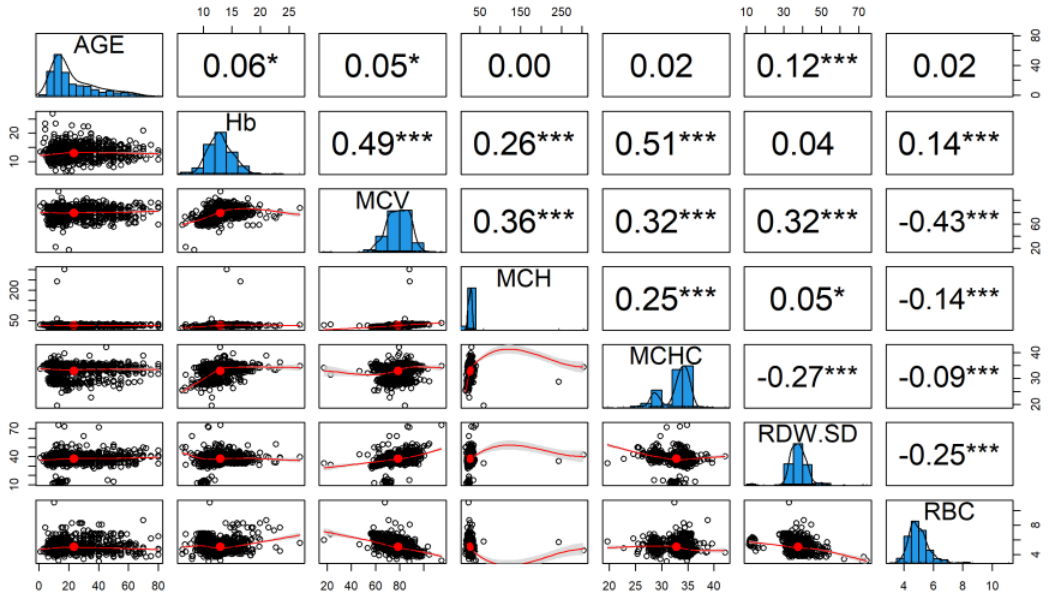

Figure 15: Co-relation between different Red cell parameters in BTT cases of the study area.

4.2.5.1 Co-relation co-efficient of Red cell parameters amongst ethnic group

4.2.5.1.1 Koch Rajbanshi

In the Koch Rajbanshiethnic group: Age did not have a positive co-relation co-efficient with any of the red cell parameters, however there was a strong negative co-

efficient between age and MCV(r= -0.15), MCH (r=0.16) and RBC (r=0.18).The hemoglobin level was seen to be highly significantly co-related to MCV (r =0.52), MCH (r=0.57), MCHC (r=0.30) and RBC (-0.21, Figure16)..

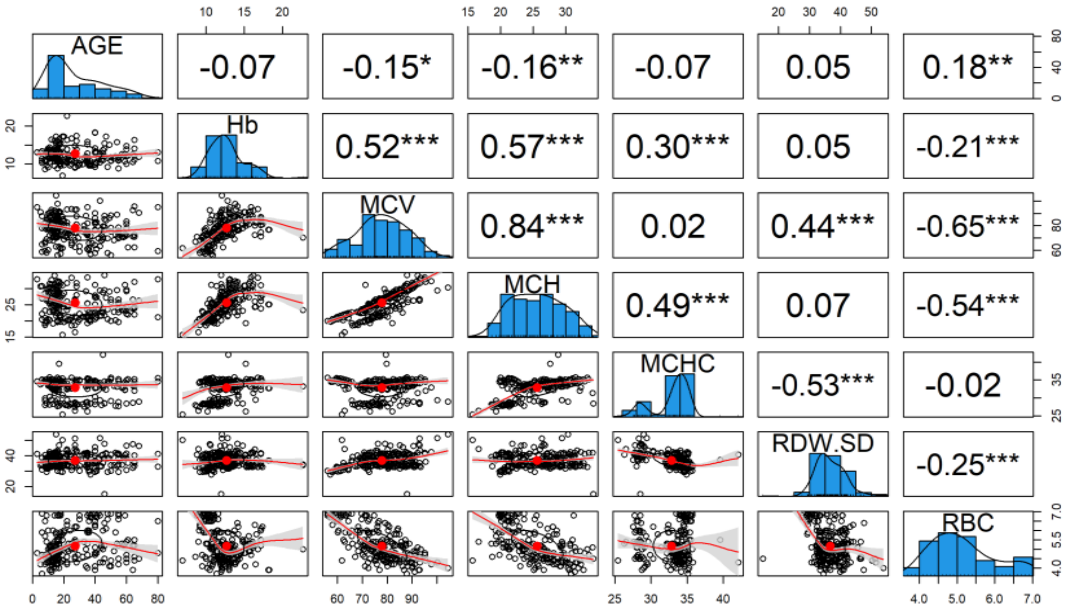

Figure 16: Rajbanshi ethnic group red cell parameters

4.2.5.1.2 KochilaTharu ethnic group

In the Kochila Tharu a significant positive correlation co-efficient between Hemoglobin and MCV (r= 0.50), MCH (0.62), MCHC (0.60) and negative correlation with RBC (r= -0.21) was observed. Likewise MCV was positively correlated significantly with MCH (r= 0.79), MCHC (0.35) and negatively co-related with RBC (r= -0.28). MCH had a positive correlation with MCHC (r= 0.74) and negative with RBC (r= -0.26). MCHC showed negative correlation with RDW (r= 0.62) and RBC (r= -0.17). RDW also had a negative correlation with RBC (r= -0.06) which was not very significant (Figure17).

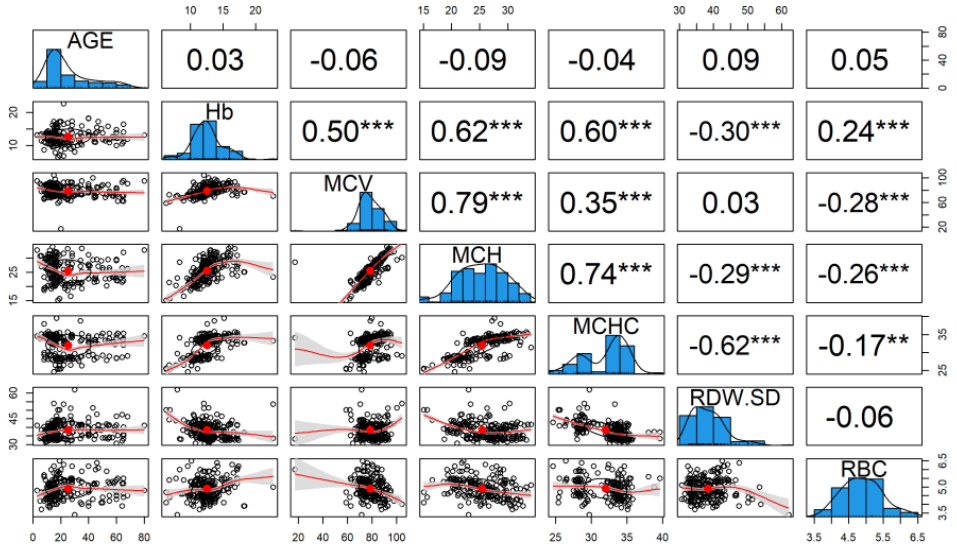

Figure 17: Co-relation between the Red cell parameters in KochilaTharu

4.2.5.1.3 Musahar ethnic group

Among the ethnic groups, the Musahars had a significant positive correlation co-efficient between age and Hb (r= 0.14), MCV (r= 0.13), MCH (r= -0.19), MCHC (r=0.18) and RDW (r= 0.23). Hemoglobin had a strong positive co-relation with MCV (r= 0.25), MCH (r = 0.23), MCHC (r= 0.32), RBC (0.34), and RDW (0.12, Figure 18).

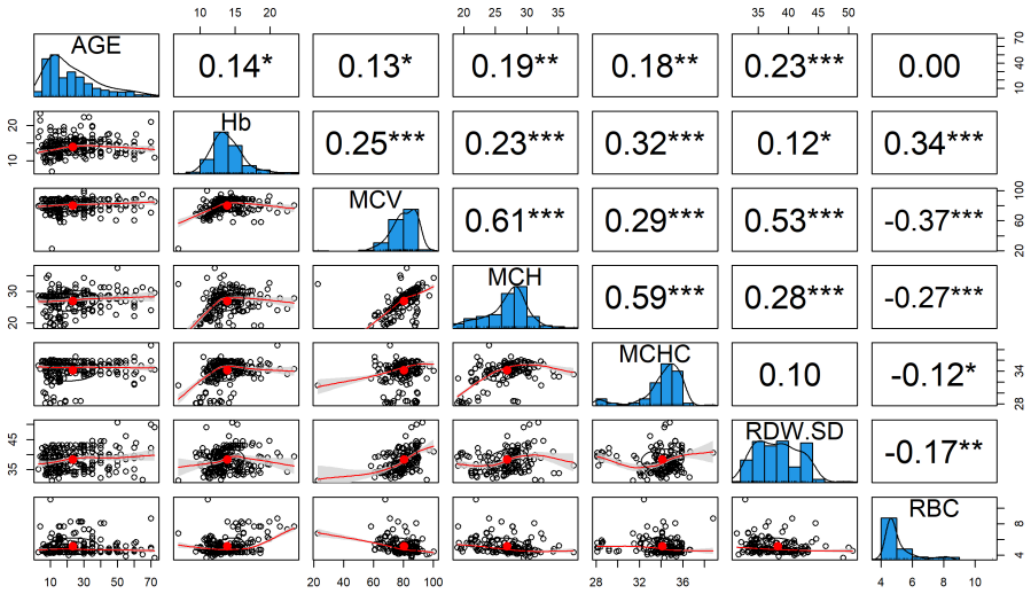

Figure 18: Co-relatiion between different Red cell parameters in Musahars

4.2.5.1.4 Musalman ethnic Group

In the Musalmans correlation co-efficient between age and Hb, MCV,MCH, MCHC and RDW-SD was significantly positive (r=0.14, r=0.13, r=0.19, r=0.18 & r=0.23 respectively). The correlation co-efficient between Hb and MCV (r=0.25), MCH (0.23), MCHC (0.32), RDW-SD (0.12) 7 RBC (0.34) was significantly positive. Likewise between MCV and MCH (r=0.61), MCHC (r=0.29), RDW-SD (r=0.53) & RBC (r=0.37) a significantl positive correlation co-efficient was seen. Similarly between MCH and MCHC (r=0.59), RDW-SD (r=0.258) a strong positive and a strong negative correlation with RBC (r=-0.27) was found. There wasa very strong negative correlation between MCHC and RBC (r= -0.12) and between RDW-SD and RBC (r= -0.17, Figure 19).

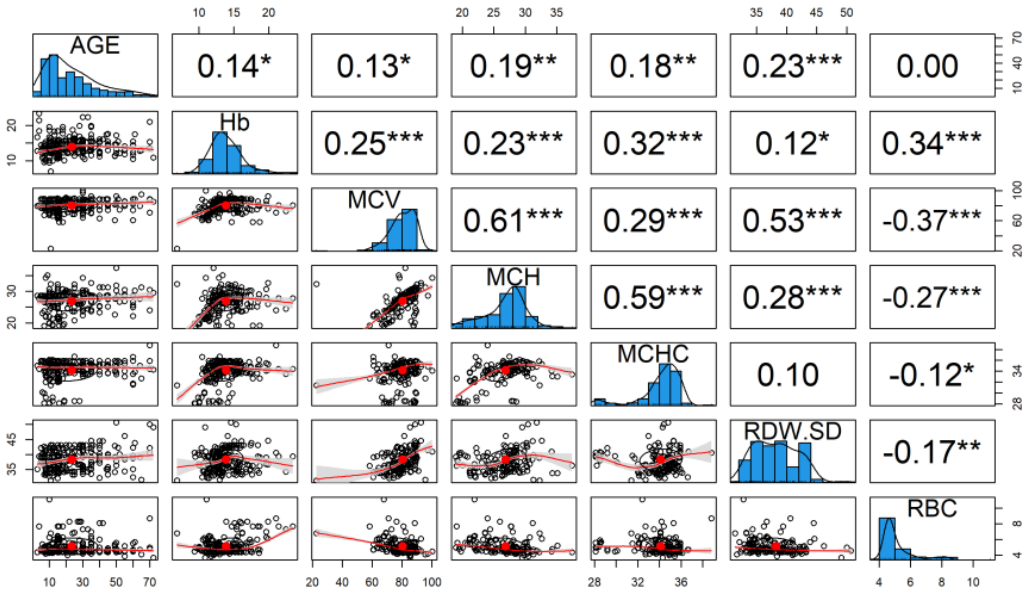

Figure 19: Co-relation between different Red cell parameters in Muslims.

4.2.6 Discriminant functions for identifying BTT

Five Discriminant Functions were applied for the screening of 285 cases with microcytic hypochromic anemia for discrimination of BTT were: DF1-Mentzer's Index <13, DF2-Shine & Lal Index value of <1530, DF3-Srivastava Index or DF3 positive value < 3.8, DF4-RDW Index indicative value ≤220 & DF5-Green & King Index positive value <65. The association of Discriminant Functions with BTT and Non-BTT cases was analysed. The cases that suggested BTT were further subjected

to hemoglobin electrophoresis. The number of cases suggested by DFs and those confirmed by hemoglobin electrophoresis is displayed in Figure 20 and described as follows

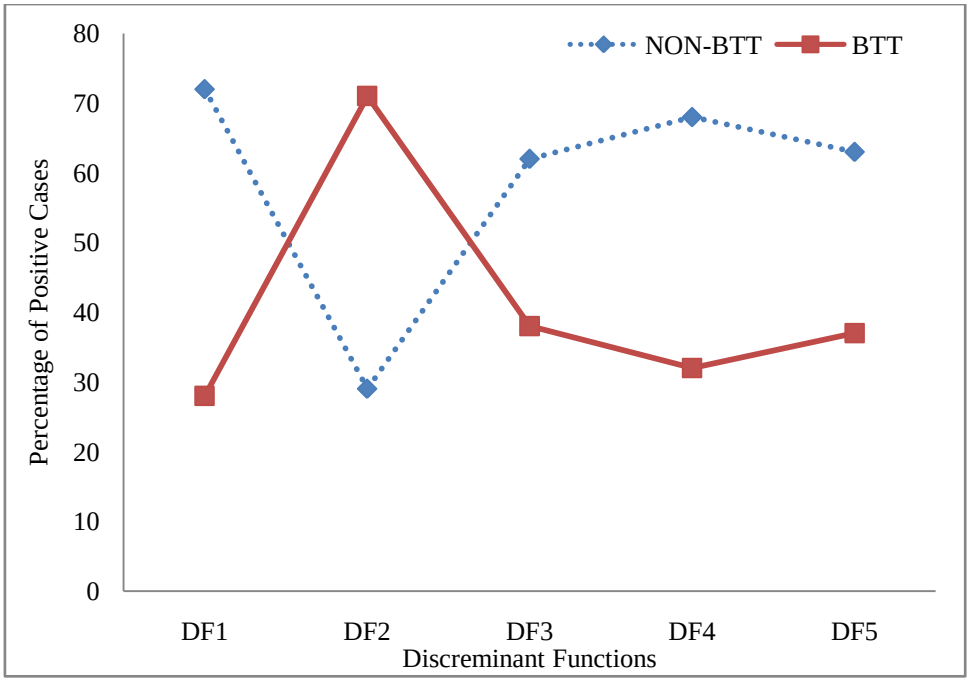

Figure 20: Association of Discriminant Functions with BTT & Non-BTT

(DF1=Mentzer's index, DF2= Shine & Lal Index, DF3= Srivastava index, DF4= RDW index), DF5= Green & King index)

DF1(Mentzer's Index) <13 was considered positive for BTT. 168 cases showed values <13. About 121 out of these168 cases turned out to be BTT.

DF2 (Shine & Lal Index) value of <1530 indicated BTT. In 264 cases DF2 value was <1530 of which 180 cases were confirmed as BTT by hemoglobin electrophoresis.

DF3 Srivastava Index or DF3 positive value < 3.8 was seen in 137 cases, of which 84 cases were confirmed as BTT.

DF4 (RDW Index) indicative value≤220 for BTT was seen in 205 cases. Out of these 137 turned out to be BTT

DF5 (Green & King Index) positive value <65 was seen in 173 cases, of which 109 turned out to be BTT.

All the five Discriminant Functions were positively associated with BTT significant p-values

The mean scores of discriminant functions exhibited a significant difference between BTT and Non-BTT cases, DF1 (p=0.0001), DF2 (p=0.0062), DF3 (p=0.0001), DF4 (p=0.0016) and DF5 (p=0.0001).

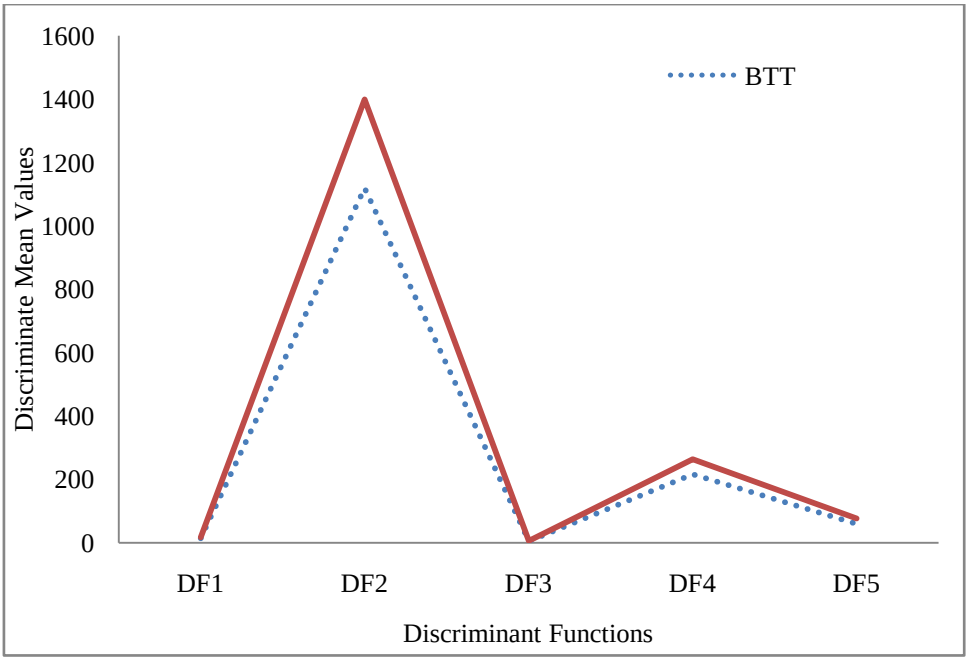

Figure 21: Mean Pattern of Discriminant Functions between BTT & Non-BTT

(DF1=Mentzer's index, DF2= Shine & Lal Index, DF3= Srivastava index, DF4= RDW index, DF5= Green & King index)

4.3 Peripheral Blood Smear

The positive findings on peripheral blood smear such as numerous target cells, anisocytosis, poikilocytosis and a few cells with basophilic stippling were seen among 184 of the blood samples examined out of the 285 Microcytic hypochromic positive cases as shown in in Fig11. These cases were considered as indicative of Thalassemia and hemoglbinopathy.

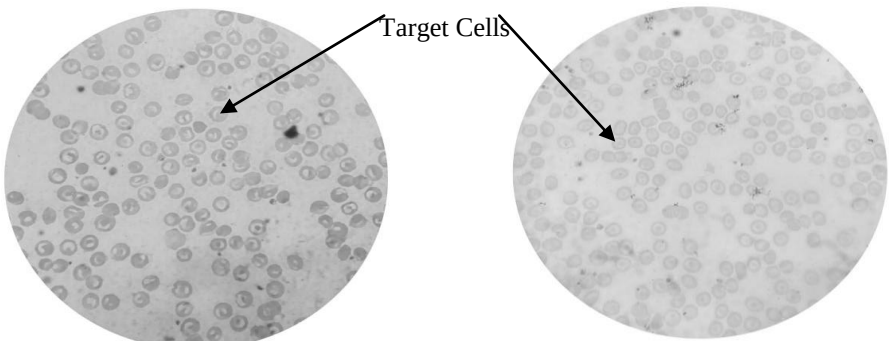

Figure 22: Microcytic Hypochromic Red Blood Cells in Beta-Thalassemia Heterozygous

The comparison of the peripheral blood smear findings with the hematological parameters is depicted in Table 18.The hematological parameters such as hemoglobin (p= 0.004), mean corpuscular volume (p=0.000), mean corpuscular hemoglobin concentration (p= 0.000), red cell distribution width-standard deviation (p = 0.000) and red blood cell (p = 0.000) was statistically significantly different. This suggested a positive association of the red cell parameters and PBS with Beta-Thalassemia Hemoglobin variants.

Table 18: Association of peripheral blood smear findings with red cell parameters

Red cell parameters	PBS	Number	Mean ± sd	z-value	p-value
Hb	Positive	184	10.4 ± 2.0	-3.5	0.00
	Negative	101	10.6 ± 1.1		
MCV	Positive	184	66.6 ± 8.5	0.5	0.00
	Negative	101	71.6 ± 5.3		
MCH	Positive	184	22.0 ± 5.5	0.8	0.41
	Negative	101	22.0 ± 3.1		
MCHC	Positive	184	32.0 ± 3.1	-4.7	0.00
	Negative	101	30.5 ± 3.1		
RDW	Positive	184	31.3 ± 9.8	-4.7	0.00
	Negative	101	35.6 ± 9.5		
RBC	Positive	184	5.7 ± 0.8	-5.1	0.00
	Negative	101	5.3 ± 0.9		

PBS (peripheral blood smear), Hb (hemoglobin), MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration, RDW-SD (red cell distribution width-standard deviation) RBC (red blood cell).

4.4 Prevalence of Beta-Thalassemia heterozygous/trait and Haemoglobinopathies

The diagnosis of beta-thalassemia and hemoglobinopathies was based on the percentage of different types of hemoglobin revealed by the electrophoretic profileproduced by capillary electrophoresis. A normal electrogram (no

hemoglobinopathy) revealed a high peak at the Zone A (>97%), prominent peak at Zone A2 (2.4%), absence of a peak at zone F and Zone E (Figure 23).

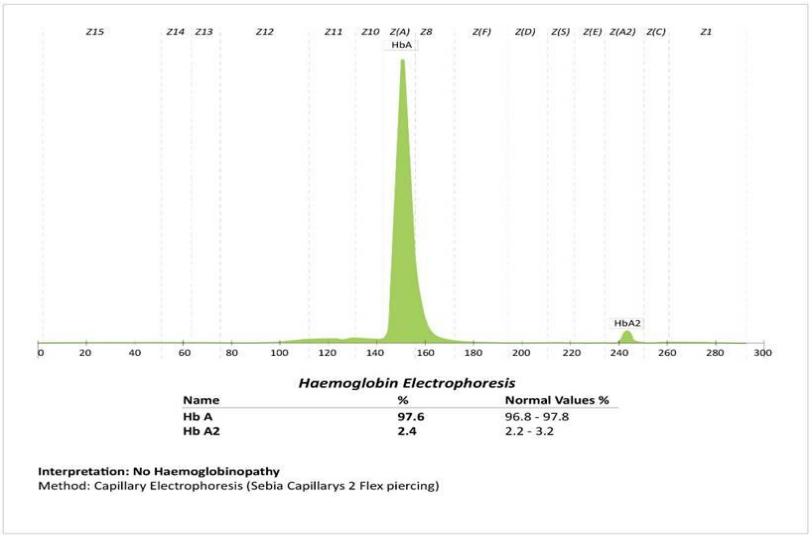

Figure 23: Normal Electogram

Beta-thalassemia heterozygous was diagnosed by a high peak at zone A (>90%), Zone F visible (1.0%) and Zone A2 prominent (6.5%) absence of any peak at Zone E.The prominent peak at Zone A2 was the gold standard for the identification of Beta-thalassemia heterozygous (Figure 24).

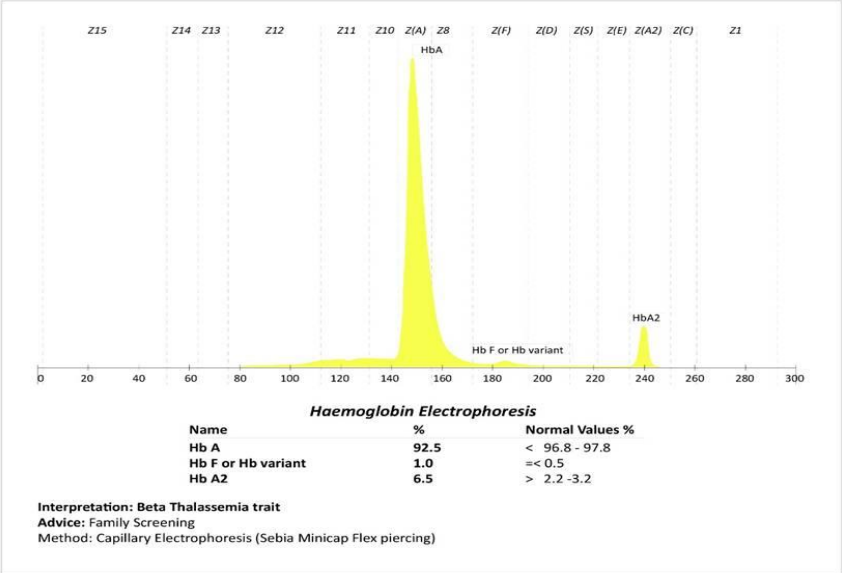

Figure 24: Beta Thalassemia Heterozygous/trait

The high peak at Zone E (94%), absence of peak at Zone A, HbA2 (5.2%) and slightly visible peak at Zone F (0.7%) confirmed HBE homozygous (Figure 25).

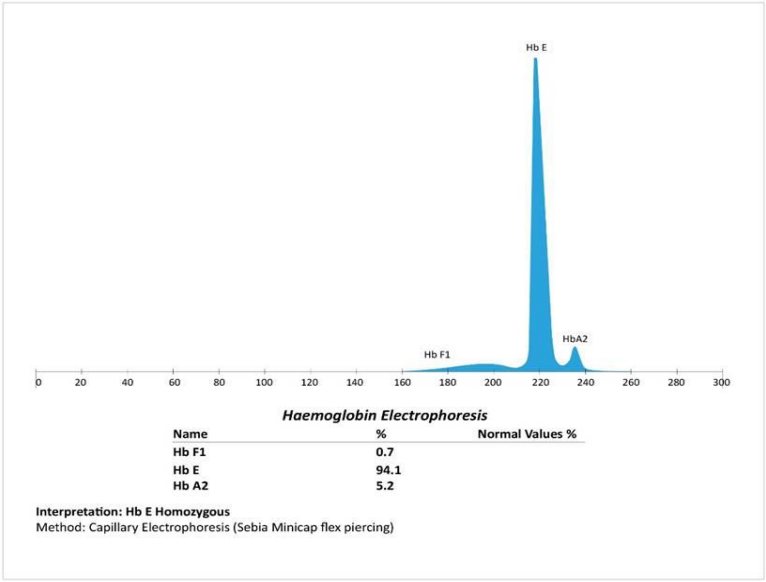

Figure 25: HbE Homozygous

A relatively high (>70%) at Zone A, (> 25%) at Zone E, increase peak (3.7%) at HbA2 confirmed Hb/e heterozygous (Figure 26).

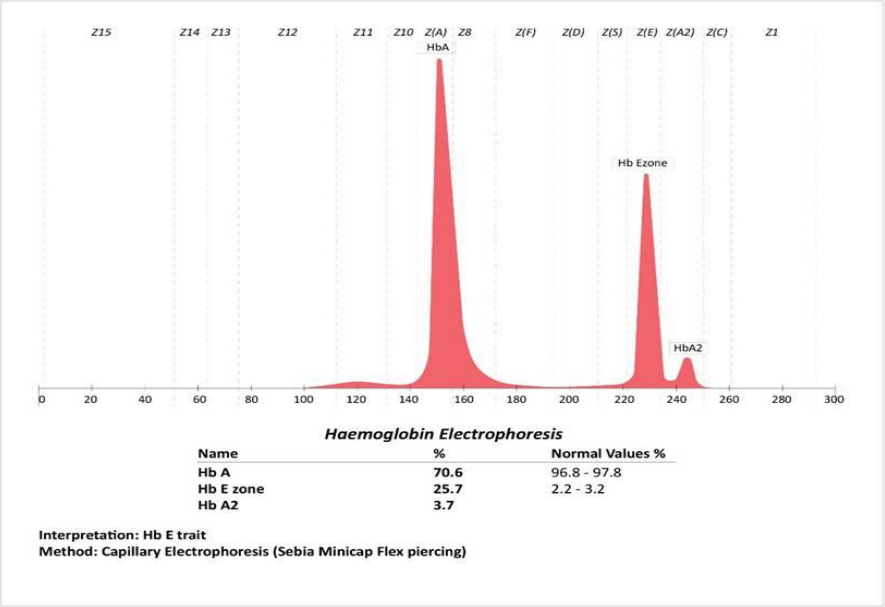

Figure 26: HbE Heterozygous/trait

Out of 285 microcytic hypochromic cases 184 samples presented abnormal (HbA2 >3.2) electrograms in the hemoglobin electrophoresis test and the remaining 101 samples showed normal (HbA2 2.0 -3.2) electrogram. Out of the 184 abnormal hemoglobin samples 98 showed Beta-thalassemia trait, 36 HbE homoygote/disease and 50 HbE trait/heterozygous (Table 19).

Table 19: Findings of Electrophoresis among studied population

EG	Type	HbA	HbA2	HbF	HbE%
KR	HbEE	Absent	>3.3	0.7- 6	90.5
	HbEA	68- 70	3.0- 5	0.3-0.8	22- 25
KT	BTT	94.4	5.0- 7.6	Absent	Absent
	HbEA	70- 72	3.0- 4.0	0.3	20- 25
Mu	BTT	94.4	6.0	Absent	Absent
	HbEE	3.0	5.5	2.3	89.9
Mi	BTT	96.0	4.3	Absent	Absent
	HbEE	Absent	>3.3	0.7- 6	90.8
	HbEA	70-72	3.0- 4.4	0.3- 0.8	24
S	BTT	95	5.0	0.7	Absent

(E.G. Ethnic Group, KR Koch Rajbanshi, KT KochilaTharu, Mu Musahar, Mi Muslim, S:Santhal N number, HbEE Hemoglobin homozygous, HbEA Hemoglobin E heterozygous, BTT Beta-thalassemia heterozygous)

According to the quantification of hemoglobin by electrophoresis the level of HbA2 differed in Beta-thalassemia heterozygous and Non-Beta Thalassemia cases. In Beta-thalassemia heterozygous the range was 3.52% – 7.59 % with mean 4.91% +/- 0.93, whereas in Non-Beta Thalassemia cases0.3%- 3.5% with mean 2.62 ± 0.69 was revealed (Figure 20).

Table 20: Mean HbA2 levels in BTT and Non-BTT

	HbA2	
	BTT	**Non-BTT (%)**
Range	3.52 – 7.39	0.3- 3.5
Mean±sd	4.91 ± 0.93	2.62 ± 0.69
CI 95%		

HbE variant was detected by a prominent peak at the E zone of the electrogram graph. The proportion of HbE was significantly different in HbE homozygous (HbEE 94.0% - 97.0%) and HbE heterozygous (HbEA20.0% - 25.0%)

Table 21: HbE level in HbE homozygous (HbEE) andHbEheterozygous(HbEA)

	HbEE	HbEA
Range	94.0% - 97.0%	20.0% - 25.0%
Mean±sd	3.45 ± 0.94	4.5 ± 1.5
CI 95%		

4.4.1 Prevalence of Beta-thalassemia heterozygous and HbE variant among ethnic groups

An overall prevalence of 13% Beta-thalassemia heterozygous and HbE variant hemoglobin was found in this study. Out of the total abnormal cases 53% comprised of Beta-thalassemia heterozygous and 47% HbE variant. The HbE variant consisted of 20% HbEE (Hemoglobin E homozygous/disease) and 27% HbEA (Hemoglobin E heterozygous/trait). Among the studied population (n=184) a varied frequency of abnormal cases was observed. In Koch RajbanshisHbEE (Hemoglobin E homozygous/disease) 17% and HbEA (Hemoglobin E heterozygous/trait) 14% was prevalent. Beta-thalassemia heterozygous/trait 8% and HbEA 0.5% was identified the Kochila Tharu. The Musahars had 9% Beta-thalassemia heterozygous and 0.5%HbEE.Likewise Beta-thalassemia 17%, HbEE 2.0% and HbEA 13% was confirmed in theMusalman and in Santhals only Beta-Thalassemia heterozygous/trait 20% was detected (Table 20).

300 samples per ethnic group (five ethnic groups) was studied. In Koch Rajbanshis HbEE (Hemoglobin E homozygous/disease) 10% and HbEA (Hemoglobin E heterozygous/trait) 9% was identified. Beta-thalassemia trait/ carrier/ heterozygous was not detected. Beta-thalassemia heterozygous/trait 5% and HbEA 0.3% was identified the KochilaTharu. The Musahars had 6% Beta-thalassemia heterozygous and 0.3%HbEE. Likewise Beta-thalassemia10%, HbEE 1.0% and HbEA 8% was confirmed in the Musalman and in Santhals only Beta-Thalassemia heterozygous/trait 12% was detected (Table22).

Table 22: Prevalence of Beta-thalassemia heterozygous, HbE homozygous, HbE heterozygous in the studied population

E.G.	Number	N=184 (%)	N=300 (%)	N=1500 (%)	Type
K.R.	31	17	10.33	2.06	HbEE
	26	14	9.3	1.87	HbEA
KT	14	8	4.67	0.93	BTT
	1	0.5	0.33	0.06	HbEA
MU	17	9	5.67	1.13	BTT
	1	0.5	0.33	0.06	HbEE
MI	31	17	10.33	2.06	BTT
	4	2	1.33	0.26	HbEE
	24	13	8.00	1.6	HbEA
S	36	20	12.0%	2.4%	BTT

(EG. Ethnic Group, K R Koch Rajbanshi, K T KochilaTharu. Mu Musahar, Mi Muslim, S Santhal. N: number, Type: Beta Thalassemia or Hemoglobin variant, HbEE Hemoglobin homozygous, HbEAHemoglobin E heterozygous, BTT Beta-thalassemia heterozygous)

4.4.2 District-wise prevalence ofBeta-thalassemia heterozygous, HbE variant

Among the studied districts, the highest prevalence of hemoglobinopathy occurred in Jhapa (58%) followed by Morang (22%) and Sunsari (20%) (Figure 27).

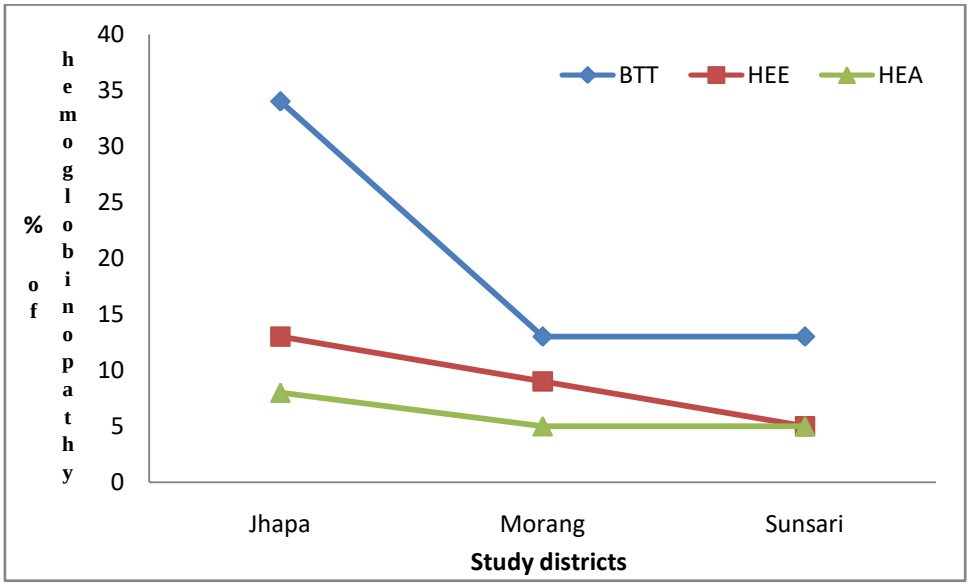

Figure 27: Haemoglobinopathies in the Study Districts¬

Table 23: Haemoglobinopathies in the Study Districts

District/Type	BTT	HbEE	HbEA	Total
Jhapa	34	13	11	58
Morang	10	9	5	24
Sunsari	8	5	5	18
Total	52	27	19	100

4.4.3Association of Discriminant Functions with Electrophoresis

Five Discriminant functions (DF) were applied for the screening of Beta-thalassemia and hemoglobinopathies.DF1 showed highest PPV (71.46%)

DF2lowest PPV (30.38%).whereas DF2 showed highest false positivity (69.88%). DF2 showed lowest False negativity (13.68%)DF2 (Shine & Lal) with sensitivity of 88.32%,

DF3(Srivastava Index) showed highest Specificity (95.43%)but lowest sensitivity (15.8%). and DF3 showed lowest NPV (76.94%) (Table 22).Lowest False positivity (3.46%) was seen in DF3whereas DF3 showed highest False negativity (82.12%).

The most sensitive DF was found to be DF2 (Shine & Lal) with sensitivity of 88.32%, however the specificity was the lowest (32.03%). DF3(Srivastava Index) showed highest Specificity (95.43%) but lowest sensitivity (15.8%). Lowest False positivity (3.46%) was seen in DF3, whereas DF2 showed highest false positivity (69.88%). DF2 showed lowest False negativity (13.68%) whereas DF3 showed highest False negativity (82.12%). DF1 showed highest PPV (71.46%) and DF2lowest PPV (30.38%). DF4 showed highest NPV (95.15%) and DF3 showed lowest NPV (76.94%) (Table 24).

DF2 can be a good screening test due to its high sensitivity. DF1 and DF3 are good diagnostic tests due to their high specificity. DF4 and DF5 with high sensitivity and high specificity can be used for both screening and diagnostic tests.

Table 24: Diagnostic values of discriminant functions with respect to electrophoresis

Discriminant functions	**Diagnostic values in relation to electrophoresis**					
	SE	**SP**	**FP**	**FN**	**PPV**	**NPV**
DF1 (<13)MCV/RBC	34.21	94.6	5.4	64.56	71.46	80.78
DF2 (<1530) **$(MCV)^2$× MCH× 0.01**	88.32	32.02	69.88	13.68	30.88	80.16
DF3 (< 3.8)MCH/RBC	15.8	95.43	3.46	82.12	60.45	76.95
DF4 (≤220) **$(MCV)^2$×RDW/RBC**	83.63	87.43	15.58	14.48	68.58	95.15
DF5 (<65) **$(MCV)^2$×RDW/100×Hb**	81.28	85.62	16.38	20.62	64.33	93.78

(SE Sensitivity, SP Specificity, FP False positive, FN False negative, PPV Positive predictive value, NPN Negative predictive value)

4.4.4 Association between peripheral blood smear and thalassemia and hemoglobinopathies

Beta-thalassemia Major (BTM) and Beta-thalassemia Intermedia (BTI) were not detected out of 184 PBS positive Beta-thalassemia and Hemoglobin E variant. There was a statistical significance difference between the microscopical examination of the peripheral blood smear with Beta-Thalassemia heterozygous and hemoglobin variants ($p<0.005$, Table 24).

Table 25: Association between peripheral blood smear and thalassemia and hemoglobinopathies (n=285)

Variable	Category	PBS		Total	p-value
		Positive	**negative**		
BTM	Positive	ND	ND	……..	--------
BTI	Negative	ND	ND	…….	--------
BTT	Positive	91 (97.85%)	2 (2.15%)	93	0.000
	Negative	93 (48.44%)	99 (51.56%)	192	
HBEE	Positive	37 (94.87%)	2 (5.13%)	39	0.000
	Negative	147 (59.76%)	99 (40.24%)	246	
HBEA	Positive	45 (100%)	0 (0%)	45	0.000
	Negative	139 (57.92%)	101 (42.08%)	240	
C-26	Positive	82 (98.8%)	1 (1.2%)	83	0.000
	Negative	102 (50.5%)	100 (49.5%)	202	
C-15	Positive	87 (97.75%)	2 (2.25%)	89	0.000
	Negative	97 (49.49%)	99 (50.51%)	196	
IVSI-5	Positive	22 (100%)	0 (0%)	22	0.001
	Negative	162 (61.60%)	101 (38.40%)	1478	
FS 8/9	Positive	1 (100%)	0 (0%)	1	
	Negative	183 (64.44%)	101 (35.56%)	284	

4.5Analysis of the spectrum of mutation causing Beta-Thalassemia among the ethnic groups

A total of four mutations identified were C15(G>A),IVS1.5IVS1.5 and , C-26 $^{glu\text{-}lys}$5. The mutations were identified with the help of the DNA ladder. For the positive mutations bands appeared at specific horizontal base pair (bp) lines that were matched against the DNA marker ladder. The bands at 500bp identified mutation C-

15, bands at 285 identified mutation IVS1-5, band at 225bp identified mutation FS 8/9 responsible for Beta-thalassemia heterozygous and bands at 310 bp identified muatation C-26 $^{gly\text{-}lys}$for HbE gene. As presented by the agarose gel images in Figure 28 the presence of band 285in lane 2,3,4,5,6,7 and 8 confirmed mutation IVS-.5.

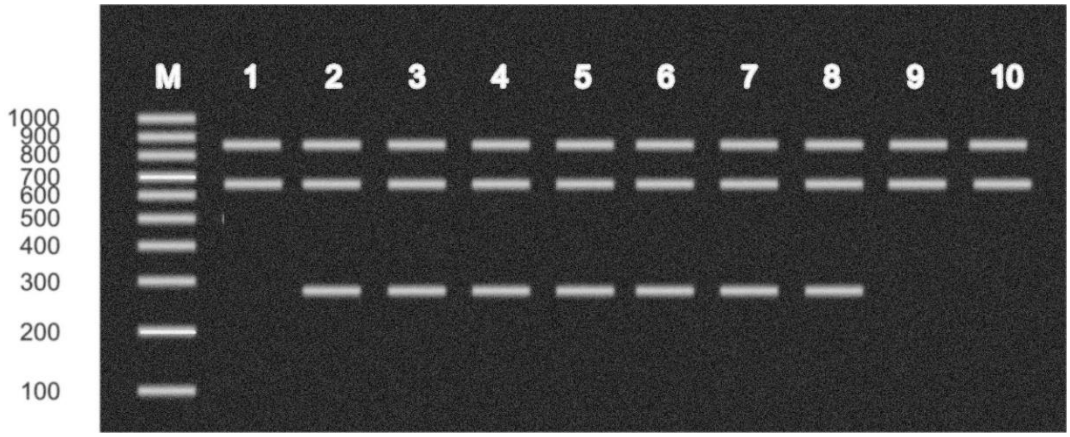

Figure 28: Agarose gel image of Thalassemia Mutation IVS1-5 (band- 285). Internal control IC-861, IC- 676

M= 100 bp DNA ladder. Lane 1 Normal control

Lane 2, 3, 4, 5, 6 & 7 DNA sample carrier for IVS1-5 mutation

in Figure 29, presence of band 500 in lane 2, 7 an10 identified mutation C-15. The presence of band 285 in lanes 4 and 5 identified Mutation IVS1-5. The presence of both band 500 and band 285 in lanes 2,3,6,7,8,9 and 10 identified double heterozygous mutations C-15 and IVS1-5 in the same individual,

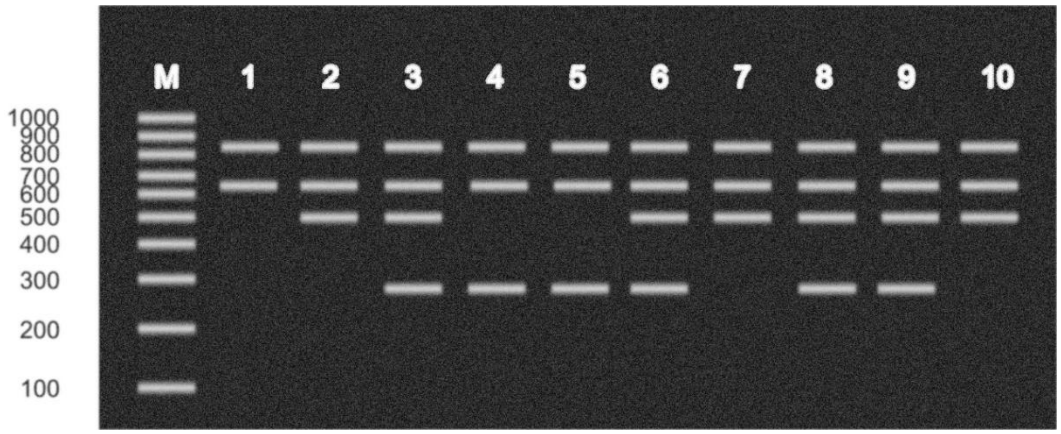

Figure 29: Agarose gel image of Heterozygous Mutation C15 and IVS 1-5 (band 500& band-285).

Internal control IC 861, IC 676 (M= 100 bp DNA Ladder.

Lane 1 normal control

Lane 2,7 & 10 DNA sample carrier for C-15 mutation

Lane 3,6,8 7 9 DNA sample carrier for double mutation IVS1-5 and C-15 nutations

Lane 4 &5 DNA sample carrier for IVS1-5 Mutation)

As depicted in Figure 30, a single band 225 in lane 8 identified mutation FS 8/9. Band 500 in lanes 9and 10 identified Mutation C-15. Band 285 in lane 2,3,4,5,6 and 7 identified mutation IVS 1-5.

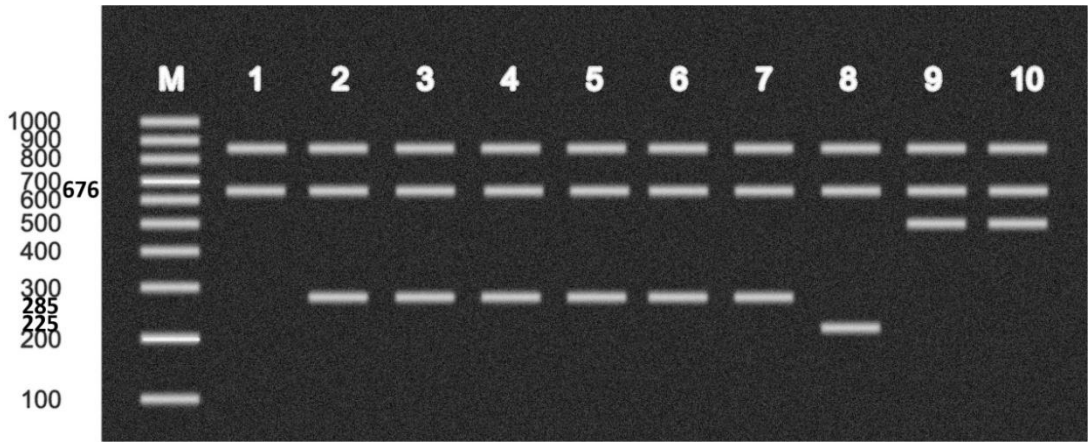

Figure 30: Agarose gel image of Mutation C15 (band-500) IVS 1-5 (band- 285)and FS 8-9 (band- 225).

Internal control: IC 861, IC 676.

Lane M: 100 bp DNA ladder. Lane 1 normal control

Lane 9&10 DNA sample carrier for C-15 mutation,

Lane 2, 3 , 4, 5, 6&7 DNA sample carrier for IIVS1-5 mutation.

Lane 8 DNA sample carrier for FS8/9 mutation)

The presence of band 310 at lanes 2,3,4,5,6,7,8,9 and 10 confirmed mutation C-26 responsible for HbE variant (Figure 31).

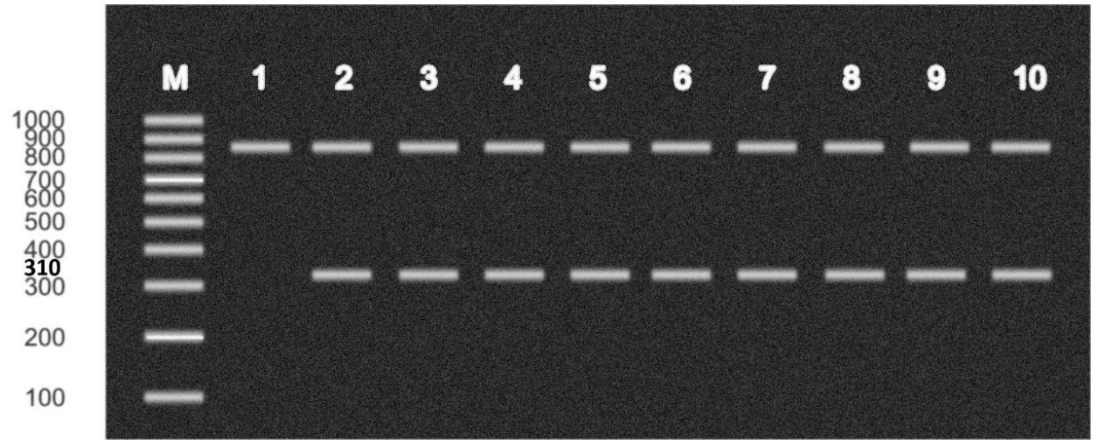

Figure 31: Agarose gel image of Mutation-C26(band-310)

M: 100 bp DNA ladder. Internal control IC 861

Lane 1 Normal control

Lane 2, 3, 4, 5, 6, 7, 8, 9 &10 DNA sample carrier for C-26 HbE variant)

4.5.1 The distribution of the mutations district wise

The different types of mutation found in the studied area are depicted district wise in Table 26.

Jhapa: This was the most affected area with both BTT and HbE gene. Prevalence of mutation C-15 (34.0 %), IVSI-5 (7.0 %) and C-26 (24.0 %) was observed.

Morang: Prevalence of C-26 (9.0%), C-15 (2.0 %), IVSI-5 (12.0%) & co-inheritance of C-15 + IVSI-5 (5.0 %) was detected.

Sunsari: C-26 (12.0%), C-15: (8.0%), IVSI-5 (2.0%) & co-inheritance of C-15 + IVSI-5 (5.0%) was observed. Single individual with mutation FS8/9 was identified from this district.

The mutation C26-$^{glu–lys}$ was highly significant in all three districts; Jhapa (p-2.20e-16), Sunsari (p-2.90e-07) and Morang (p-1.19e-06).

The mutation IVSI-5 had significance only in Morang (p-1.00e-02) district.

C-26 $^{Glu-Lys}$ (41%) was the second most common mutation in this study.

C-15 (G>A) was the most common mutation with a frequency 38%

The third common thalassemia mutation was IVS1-5 (G>C), which comprised 21%.

Table 26: Prevalence of BTT mutations in the studied area

District	Mutation	No	%	Chi-squared	Df	p-value
Jhapa	C-26	43	23.91	97.29	4	2.20E-16
	C-15	54	33.69	39.88	4	4.581e-08
	IVSI-5	14	8.69	25.44	4	4.105E-05
Morang	C-26	18	8.69	21.82	4	2.00E-04
	C-15	06	1.63	12.04	4	1.00E-02
	IVSI-5	14	11.41	33	4	1.19E-06
Sunsari	C-26	15	11.41	35.9	4	2.91E-07
	C-15	10	8.15	6.9	4	0.14
	IVSI-5	10	1.63	12.07	4	0.02
	FS 8/9	01		0	4	1

4.5.2Mutational profile of Beta-thalassemia in the study population

Amongst the five ethnic groups studied, the mutations prevalent in each ethnic group were as follows: - Koch Rajbanshi: Only one mutation detected was C-26: 30.97%.

KochilaTharu: Two mutations detected were C-26 (0.54%) and C-15 (7.06%).

Musahar: In the Musahars 10.32% BTT mutations present were C-15 (6.52%) & IVSI-5 (9.23%) & C-26 (0.54%). A co-inheritance of the mutations (C-15 + IVSI-5) was present in 4.86%.

Musalman: A prevalence of 32.06% was found in the Musalmans which comprised of 16.85% BTT cases and 15.2E gene (HbE homozygous + HbE heterozygous).

Santhal: Of the 19.56% mutations, the BTT mutations identified were C-15 (19.02%) and IVSI-5 (4.89%). A co-inhritance of these two mutations in was found in 4.89%.

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

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

4.2 Discussion

Beta-thalassemia the most frequently transmitted erythroytic disorder globally is widespread in eastern Nepal. The classic carrier of Beta-thalassemia known as Beta Thalassemia trait/heterozygous is asymptomatic. The detection is made through evaluation of positive family history and during population screening. Although importance has to be attributed to family history, a significant number of patients do not have previously affected family members. Considering the seriousness of Beta-thalassemia homozygous/ major, correct detection of Beta-thalassemia trait is of utmost importance for correct screening and genetic counseling.

The aim of this study was to analyse the RBC abnormalities in Beta-thalassemia and to find the prevalence of the spectrum of mutation causing Beta-thalassemia among the five ethnic groups.

To achieve the first objective an algorithm based on screening for microcytic hypochromic anemia was established. Microcytic hypochromic anemia is one of the commonest types of anemia in children and adolescents. World Health Organization (WHO 2007) estimated that 66-80% of the global population suffered from microcytic hypochromic anemia. It is a very diverse group of diseases that may be either genetic or acquired usually due to iron deficiency. The common causes of microcytic hypochromic anemia are Beta-thalassemia heterozygous (BTT) and Iron Deficiency Anemia (IDA). However, BTT must be excluded in all microcytic cases to prevent birth of Beta thalassemia homozygous/major (Lafferty 1996). In the present study an algorithm was used to segregate cases of possible BTT from cases of non-BTT by cost effective discriminant testsDF1(Mentzer' Index <13), DF2 (Shine & Lal Index <1530, DF3 (Srivastava Index<3.8), DF4 (RDW Index ±220) and DF5 (Green & King Index<65) as screening

In the present study comprising of 1500 cases, abnormal red cell parameters were observed in 285 cases.

In these 285 cases mean Hb level was 10.48±1.77 which was comparable to the findings of 10g/dl by Jha (2015),whereas a lower level of 8.57 g/dl by Sharma (2020) and 8.1 g/dl by Sherchand *et al* (2013) was reported respectively in studies conducted at tertiary care hospitals in Kathmandu and Dharan. On the other hand, much higher levels of Hb was presented by Bhatt et al. (2017) Hb: 12.6g/dl, Das et al (2020) Hb:11.56±1.24 and Bastola et al. (2017) Hb: 11.4±1.8,in the studies conducted in Kathmandu and Dharan.

In the present study the Mean Cell Volume was significantly different ($p< 0.005$). It was lower than 80fL with a mean of 63.36±7.87 fL. In various studies conducted in Nepal the MCV value was seen to be in the lower side.In consistence to my findings, Bhatt et al (2020) reported 62 fL, Jha (2015) 64fL and Sharma (2020) reported 64.71 fL respectively. A much higher level of MCV: 74.67 fl by Bastola et al (2017) and 74.8 fl by Das et al. (2020) have been reported. A variation in the results produced by different authors was noticed, however an overall decreasd MCV (<80fL) suggested hemoglobinopathies.

A mean value of MCH 22.03 ±4.76 was found in my study which was in concordance with result of MCH 22fL by Bhatt et al. (2017). Whereas a lower level of MCH 20.3fL was seen by Jha (2015). Higher levels were observed by following authors 23.5fl by Bastola et al. (2017) and 24.83fL by Das et al (2020).

The mean RBC value of 5.55±0.87 was found in this study. This was similar to 5.8 found by Bhatt et al. (2020) and 5.2 reported by Jha (2015). The slightly increased RBC was suggestive of Beta-thalassemia heterozygous.Usually, low RBC <5 is indicative of Beta-thalassemia homozygous. However lower than normal values of 3.9±1.7 by Sherchand et al. (2013) and 4.4 by Jha has been reported.

The values of mean MCHC were near to normal with 31.47 fL comparable to the findings of 32.82 by Das et al. (2020).

The RDW-SD value of 32.84±9.88 was comparable to 32.82 ±1.80 reported by Das et al. (2020).

The BTT cases was segregated from non-BTT cases by the application of Discriminant functions. In this study only five discriminant functions were implemented.DF1 (Mentzer's Index.<13), DF2 (Shine & Lal Index<1530), DF3

(Srivastava Index<3.28), DF4 (RDW Index ±220) & DF5 (Green & King Index<64). All the five Discriminant Functions were in concordance with the Hb electrophoresis which confirmed BTT. In 168 cases the DF1 or Mentzer Index was <13, out of which 121 cases were confirmed as hemoglobinopathies by hemoglobin electrophoresis. Out of 260 cases with DF2 or Shine & Lal Index <1530, hemoglobin electrophoresis confirmed Thalassemia in 180 cases.The 256 cases with DF3 or Srivastava Index < 3.8, 178 cases were identified as hemoglobin disorders. Similarly 260cases showed DF4 or RDW Index ≤ 220, which was confirmed by electrophoresis in 181 cases. Of the 258 cases confirmed byDF5 or Green & King Index <65, 180 were identified as Thalassemia by electrophoresis. The maximum number of cases correctly diagnosed was shown by Mentzer's Index with 121 cases (72%, n=168) followed by Srivastava Index 69.23% (n=260). The association of discriminant functions between BTT and Non-BTT that indicated Beta Thalassemia were significantly statistically different.

In this study DF2 was found to be a potentially good screening test due to its high sensitivity (88.32%). DF1 (94.60%) and DF3 (95.43%) are good diagnostic tests due to their high specificity. However, DF4 and DF5 with high sensitivity (83.63%& 81.28% respectively) and high specificity (87.43%& 85.62% respectively) can be used for both screening and diagnostic tests.

Peripheral blood smear morphology provided key information to create the differential diagnosis for patients with anemia. The microscopic examination of 285 peripheral smearswith microcytic hypochromic anemia cases revealed target cells, anisocytosis, poikilocytosis, ovalocytes, elliptocytes and a few basophilic stipplings in184 cases. The presence of target cells, anisocytosis and poikilocytosis supported the abnormal readings of the red cell parameters suggestive of beta thalasemia and hemoglobin variants. In concordance with the present study, Sherchand and Tiwari both had revealed microcytosis, hypochromia, anisocytosis, poikilocytosis, and target cells in the peripheral blood smear examination. Bhat et al. (2020) revealed predominance of normocytic normochromic cells with mild anisocytosis, few microcytes and hypochromic cells. K.C. (2017) had found 42.3% (n=65) normocytic normochromic cells, 39.9% (n=65) microcytic hypochromic cells and 17.8% (n=29) on the peripheral smears. Kafle et al. (2021), revealed that poikilocyosis was in the form of tear drop cells, target cells and elliptocytes.

There was a female preponderance showing 66.85% females and 60.58% males.The association between peripheral blood smears with Hb, MCV, MCHC, RDW and RBC was significantly statistically different (P<0.005).

Given the same level of anemia (Hb), microcytosis with mild anisocytosis, target cells, fine basophilic stippling was observed in BTT cases when compared to IDA. Morphological abnormalities of erythrocytes with target cells and fine basophilic stippling were encountered more frequently in peripheral blood (PB) smears of patients with BTT (Walters & Abelson 1996). England & Fraser opined that the presence of significant target cells and basophilic stippling in RBCs in microcytic cases strongly suggested BTT (Mentzer 1973).

The final confirmation of Beta-thalassemia was done by performing Capillary electrophoresis in an alkaline medium. This technique was able to differentiate the Hb variants such as HbA1, HbA2, HbF, HbE and HbS. Based on this differentiation in this study, the hemoglobinopathies identified were Beta Thalassemia heterozygous, HbE homozygous and HbE heterozygous.The gold standard for the diagnosis of Beta thalassemia heterozygous is increased HbA2. The electrophoretic findings of normal HbA1(mean 94%), increased HbA2 (>3.2%) in this sample confirmed. the Beta Thalassemia heterozygous. However HbF level was found to be either nil or low percentage (0.7 – 1.0%).However in the study conducted by Das et al. (2020) the confirmed Beta-thalassemia had high HbF (3.61%) whereas the levels of HbA1 & HbA2 were similar to that of the present study. Likewise KC et al (2017) also noted mean HbF levels of 2.31% in the confirmed Beta-thalassemia heterozygous population of his study. A highly increased HbF is characteristic of Beta Thalassemia homozygous which was not found in this study conducted in three districts (Jhapa, Morang and Sunsari) of eastern Nepal. But in another study conducted in Kathmandu by KC et al. (2017) showed increased level of HbF (93.60%) with low HbA (9.65%) and HbA2(2.0%) confirming the presence of few cases of Beta-thalassemia homozygous.

HbE homozygous was confirmed by absent HbA1, HbE>90%. increased HbA2 (5.0%), HbF (0.5%) and HbE heterozygous was confirmed by decreased HbA1 (71%), increased HbA2 (4%), HbF (0.5%) and HbE (25%).

In this study the prevalence of hemoglobinopathies was 64.56% (n=184/285). The prevalence Beta Thalassemia Homozygous, Beta Thalassemia intermedia and compound E-beta Thalassemia heterozygous was not detected in this study. Bet-thalassemia heterozygous was the most common constituting 44.49% of the total cases followed by HbE homozygous 20% and HbE heterozygous 24.46% In the other studies conducted in Nepal a varied picture of the prevalence of hemoglobinopathies was observed. A very low incidence of 4.11% (19/462) beta-thalassemia was encountered by Das et al. (2020). In the same year findings of Nisha et revealed 27.71% hemoglobinopathies with 57.71% thalassemias as the most common hemoglobinopathies.KC et al. (2017) reported 47.2% hemoglobinopathies, whereas Jha (2015) Sherchand et al (2013) reported 26.8%, 10.25% cases of Thalassemiarespectively. Hemoglobin E (HbE) is estimated to affect at least one million people worldwide. The carrier frequency of hemoglobin E/β-thalassemia (HbE/β-thalassemia) is the highest in Southeast Asia, reaching a high as 60% in Laos, Thailand and Cambodia. In the Indian subcontinent, highest frequency is observed in the Northeast regions, but relatively rare in rest of the country. In the present study prevalence of HbE homozygous was 20% and HbE heterozygous was 24.46%.

Amongs the ethnic groups of this study were, Musalman and Koch Rajbanshi had the highest hemoglobinopathy of 19.66% &19.0% respectively followed by Santhal (12%), Musahar (6%) and KochilaTharu (5%). In our neighbouring country India, Goswami et al, had found the highest prevalence of hemoglobinopathies among Rajbanshis (72.1%) and Muslims (54.9%) an equal prevalence in tribes like Santal and Oraowo, Bengali, Marwari, Behari (34% each) and the least in Mongoloid like Nepalis and other Hill-man population (17.5%) in northeast India.

In Nepal Nisha, Tiwari, Shrestha et al and Jha had drawn the conclusions that the Terai janajati (Tharu/Chaudhary and Dhimal communities) had the highest and a substantial risk of hemoglobionopathies with high prevalence of both thalassemia and sickle cell disorders.In BPKIHS, Dharan Sherchand et al. observed that the Tharu population had significant Beta-thalassemia ($p<0.05$) as compared to other ethnic groups like Musalman, Newars and Brahmins

Mohanty et al (2013) reported a prevalence of 23.9% in Dibrugarh and 3.92% in Kolkata. In six ethnic groups in Assam prevalence of HbE ranged from 41.1% to 66.7%, but was relatively rare in rest of the India. In the present study prevalence of HbE homozygous was 20% and HbE heterozygous was 24.46%.

The highest prevalence of β-thalassemia was found in the Santhal (12%) followed by Musalman (10.33), KochilaTharu (6%) and Musahar (5%) respectively. Koch Rajbhanshi had the HbE homozygous (10.33%), Musalman (4.0%) and Musahar (0.33%). Prevalence of HbE heterozygous was Koch Rajbanshi (9.33%), Musalman (24%) and KochilaTharu (0.33%).

HbE variant (19.0%) was the only hemoglobinopathy identified in the Koch Rajbanshi ethnic group. Likewise in the Santhals Beta-thalassemiaheterozygous (12%) wasidentified. Whereas, in the Musalmans,Musahars and KochilaTharus Beta-thalassemia heterozygous and HbE variant were both detected.

This is the first study on the ethnic groups of Eastern Nepal that used the MARMS-PCR genotyping method. This method enabled the simultaneous detection of 3 β-thalassemia variants and Cd-26(G→A), a hemoglobin E (HbE) variant. The genotyping results of the present study revealed mutations C-15 (G→A), IVSI-5 (G→C), FS 8/9 (+G) and C-26 (G→C) as prevalent among Koch Rajbanshi, KochilaTharu, Musahar, Musalman and Santhal ethnic groups of Eastern Nepal. Single mutation per individual was commonly encountered. But, the co-inheritance of two mutations C-15 (G→A) and IVSI-5 (G→C) of beta- thalassemia trait was detected in eighteen cases. The findings of this research study differed from those of previous studies on the Nepalese population conducted by Lama et al, Mishra et al and Thapa. This might be attributable to the ethnicity and sample size, used in all four studies and the difference in the variants genotyped. As depicted in Table 16 four β-thalassemia mutations were detected in the present study, whereas Lama et al had detected the following nine β-thalassemia mutations in 57 Nepalese thalassemic patients, IVSI-5 (G→C), C-26 (G→A), 619 del, Cd 8/9 (+G), Cd 16 (-C), Cd 41/42 (-TCCT), IVSI-1 (G→T), Cd 19 (A→G) and Cd 17 (A→T).

The two mutations common in both studies were IVSI-5(G→C) and C-26 (G→A). Mutation C-26 (G→A) at 18(23%) was the commonestin Lama's study, the second most common mutation at 77(42%) of this study. Prevalence of IVSI-5(G→C) was

37 (20%) in the present, 18 (23%)in Lama's findings. Although C-15 was the commonest mutation 69(37.49%) in the present study, it was undetected in Lama's study.The five mutations 619 del 07 (11.8%), C-16 –C 6 (9.8%), FS 41/42(-TCCT) 2 (3.3%), FS-19(A→G) 02 (3.3%) and IVSI-I(G→T) 01 (1.6%) detected by Lama were not detected in this study. These undetected five mutations had been genotyped in this research work as well.

In the findings of Mishra et. al., mutation C-26 (G→A) was not detected, mutation FS8/9 (+G) at 4(9.8%) found common was encountered in only one individual of the present study. Mutation C-15 (G→A) at 69 (37.5%) a common mutation of the present study was detected in 1(3.7%) in Mishra's study. Similarly,IVSI-5(G→C) at 37 (20%) in the present was detected in 07 (17.1) cases by Mishra et al.

In concordance to the present study, Thapa et al also reported only 1(3.84%) case of FS8/9(+G) and IVSI-5(G→C)13 (50%) as the most common Beta-thalassemia heterozygous mutation. Similar to the studies of Lama et al and Mishra et al (2012) C-15 (G→A) was reported in only single individuals.

Compound heterozygous mutations were detected in both researches. As depicted in Table 16 one double compound heterozygous mutation detected in this study was C-15(G→A) & IVSI-5(G→C) at 7.78%. It is an interesting point to be noted that although Lama et al's had also found nine compound heterozygous mutations but the above mentioned in particular was not found in his study.In his study some of the mutations were double compound heterozygous such as 619 del & FS 8/9(+G) 3 (4.9%), C-17(A→T) & FS 8/9(+G) 5(8.3%), IVSI-5(G→C) & FS 41/42 (-TTCT) 1 (1.6%) whereas some had multiple compound heterozygous conditions such as IVSI-5(G→C)+ 619 del +FS 8/9(+G)1(1.6%), IVSI-5(G→C)+ 619 del + IVSI-I(G→T)1(1.6%), C-26 (G→A)+ FS 8/9(+G) + IVSI-5(G→C)1(1.6%)

The above difference between the two researches can be attributed to the fact that while the present study was community based on apparently asymptomatic population, the other study was hospital based on diagnosed thalassemic patients. The occurrence of more variants is attributable to a larger number of ethnic groups genotyped by Lama et al. These patients belonged to 17 ethnic groups, with 38 of them carrying single mutations and 19 carrying various combinations of compound heterozygous mutations.

The findings of Mishra et al. and Thapa were comparable to the present study. Three mutations, C-15 (G→A), IVSI-5 (G→C) and FS 8/9 (-+G) was common in all threeresearch work. The mutation C-26 (G→A) hemoglobin E variant was not detected by 74 both Mishra et al. and Thapa. Mutation IVSI-I (G→T) and FS 41/42(-TCCT) found by Mishra et al. was not detected by Thapa and also in this study.Whereas, 619 del was found common in both studies except this current study. Although, the three studies shared three common mutations, there was great difference in their prevalence. The β-thalassemia mutation, C-15 (G→A) was at 77(42%) in this study, both Mishra et al. and Thapa identified only one patient each with this mutation The most common mutation in the finding of Mishra et al., FS 41/42(-TCCT) at 13 (31.7%) was not detected by Thapa and also in this study. IVSI-5(G→C) at 13 (50%) was reported as the most common by Thapa, with a good prevalence in the present and Mishra's study at 37(20%) 37 and 07(17.1%) respectively.

The prevalence of β-thalassemia and HbE variants in this study was categorized based on five ethnic groups (Koch Rajbanshi, KochilaTharu, Musahar, Musalman and Santhal). The highest prevalence of β-thalassemia was found in the Santhal (12%) followed by Musalman (10.33), KochilaTharu (6%) and Musahar (5%) respectively. Koch Rajbhanshi had the HbE homozygous (10.33%) followed by Musalman (1.3%) and Musahar (0.33%). HbE heterozygous was also highest among the Koch Rajbanshi (9.33%) followed by Musalman (8%) and KochilaTharu (0.33%).

In the research carried by Mishra et al. a high frequency of β-thalassemia mutations was found in groups of patients whose ancestors originated from India (e.g., such as Brahmin, Chhetri and Yadav), and (e.g., Rai/Limbu, Tamang and Sherpa) those from Central Asia and Tibet.

Comparatively, Lama at al. found the highest prevalence of β-thalassemia and HbE/ βthalassemia variants in Brahmin (19.3%), Chettri (17.5%) and Chaudhary (12.3%) ethnic groups that originated from India and Rai/Limbu (12.3%) and Tamang (8.8%), which originated from Central Asia. The aboriginals from Nepal such as Newars, had a prevalence of 8.8%.

However, this study focused more on core variants that are more prevalent in the Southeast Asia regions. For a more thorough analysis of β-thalassemia variants prevalent in Nepalese ethnic groups, further studies should be conducted by including variants found in neighbouring countries as intermarriages and migration across the border produce a more diverse combination of the most common mutation identified in the present study was C-15 (G→C) with a frequency of 50%. The second common mutation was C26 (G→C) with a frequency of 46.73% followed by IVS1- 5 (G→C) with a frequency of 14.67%, Earlier in Nepal, the following 8 beta-thalassemia mutations FS 41/42 (-CTTT), IVS1-5 (G→C), IVS1,1 (G→T), 619 bp del, FS 8/9 (G→T), C88(C→T), C-16 (--C) and C-15 (G→A) had been identified (Mishra, 2012). Amongst these, 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 three mutations, C88(C→T), C-16 (--C) and C-15 (G→A) had a combined frequency of 12.18% C-15 (G.A), the commonest mutation of this study was regarded as rare by Mishra et al (2012). The second common mutation was C-26 (G→C). IVSI-5 (G→C) was the least common mutation in Mishra's study.

Interestingly the most common mutation reported by Mishra was FS 41/42 (-CTTT) with a frequency of 31.71% was not detected in the present study. In India prevalence of FS 41/42 varies between 2 -15%, whereas in China, this is the most common mutation accounting for 24.91% (Zhu et al. 2020).

In the present study, β-thalassemia mutations identified among the KochilaTharu was a single mutation C-15, whereas, mutations C15 & IVAI-5 (G→C) were Cd 41/42 (- TCCT), Cd 8/9 (+G), IVAI-5 (G→C), 619 del, Cd-26 (G→A) were found among the Chaudhary / Tharu in the study conducted by Lama et al. Similary difference was observed among the Musalmans also. In the present study of Eastern Nepal, a single mutation C-15 (G→A) occurred; in the study lama also a single mutation IVSI-5 (G→C) was genotyped. A difference in the prevalence was also noticed, Kochila Tharu 14 (8%) and Musalman 31(10%) in the present study and Chaudhary/ Tharu 7 (12.3%) and Musalman 2 (3.5%) were observed in the findings of Lama et al. Furthermore, a as shown in Table, there was only one compound heterozygous mutation (C15 & IVSI-5) in the current study, whereas multiple compound heterozygous mutations were present in Lama's study.

Comparatively, Lama at al. (2021) found the highest prevalence of β-thalassemia and HbE/ β-thalassemia variants in Brahmin (19.3%), Chettri (17.5%) and Chaudhary (12.3%) ethnic groups that originated from India and Rai/Limbu (12.3%) and Tamang (8.8%), which originated from Central Asia. The aboriginals from Nepal such as Newars, had a prevalence of 8.8%. However, this study focused more on core variants that are more prevalent in the Southeast Asian regions. For a more thorough analysis of β-thalassemia variants prevalent in Nepalese ethnic groups, further studies should be conducted by including variants found in neighbouring countries as intermarriages and migration across the border produce a more diverse combination of mutations. In the research carried by Mishra et al. a high frequency of β-thalassemia mutations was found in groups of patients whose ancestors originated from India (e.g., such as Brahmin, Chhetri and Yadav), and (e.g., Rai/Limbu, Tamang and Sherpa) those from Central Asia and Tibet.

CHAPTER 5

5. CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

Thalassemia and HbE variant are widespread in Eastern Nepal. In this study the maximum number of cases was found in the age group 6 to 29. The RBC indices were valuable in the provisional diagnosis of Thalassemia and hemoglobinopathies. Automated hematology analyzer-based red cell indices and certain formulae derived from these indices had a high predictive value in screening BTT. According to the results, the percentage of correctly diagnosed cases was highest with the RDW index (98.3%) followed by Shine and Lal index and Green & King index both at (97.8%), Srivastawa index at 96.73%. In this study Mentzer index had the least percentage of correctly diagnosed cases. It can be concluded that cell-count based parameters and formulae, particularly the MCV, Hb concentration and RBC counts and their related discriminating indexes (RDW index, Shine and Lal index and Green & King index), have a good discrimination ability in diagnosing Beta-thalassemia heterozygous.

On peripheral blood smear examination, the presence of target cells was diagnostic feature of Beta-thalassemia.

Quantification of HbA2 level by capillary zone electrophoresis was simple, rapid and cost- effective for the fast determination of human globin chains. This was an important diagnostic tool for confirming Thalassemia and hemoglobinopathies. Beta-thalassemia was most prevalent followed by HbEheterozugous and HbE homozygous. Jhapa, the easternmost district had the highest prevalence of Thalassemia and hemoglobinopathy. This could be related to the fact that Jhapa is bordered to the North-eastern states of India with high incidence of Thalassemia and hemoglobinopathies. Among the Koch Rajbanshi ethnic group, only HbE variant was found. From this study it can be concluded that Thalassemia and hemoglobinopathies were more common in non-Tharu ethnic groups.

A significant variation of Beta-mutations was observed within the different the ethnic groups. Among Koch Rajbanshi ethnic group exclusively mutation C-26 (G→A) and in the Santhal mutation C-15 (G→C) was found. The distinctive genetic profile could be due to their isolated existence, an intricate caste system and practice

of endogamy over centuries. The results of this study revealed a regional and ethnic specificity in the mutational profile among the ethnic groups of eastern Nepal. Beta-Thalassemia homozygous generates considerable financial, psychological and social burden on the affected individual, family, society and the country at large.

5.2 Recommendations

A nationwide concentrated effort towards education and awareness generation at the level of the government is urgently needed in this complex multiethnical Nepalese population.

Education and awareness about genetic diseases at schools, colleges, universities and municipal ward offices.

Mass population screening and simple inexpensive tests like Complete Blood Count. Analyses of Red Cell Parameters, microscopical examination of Peripheral blood smear. Mandatory confirmatory tests of positive samples by hemoglobin electrophoresis.

PCR Test (Polymerase Chain reaction) in all Government Hospitals must be provided at minimal costs.

Genetic counselling, pre-marital screening and carrier screening of at risk couples.

New-born screening:The diagnosis of Beta Thalassemia after birth helps avoid risk of complications in the future and enables early provision of comprehensive care, which in itself improves the quality of life and survival of the patient population

CHAPTER 6

6. SUMMARY

Beta thalassemias and hemoglobinopathies are heterogenous groups of inherited hemoglobin disordes. They are prevalent in the Mediterranean countries, Gulf countries, South-east Asia, Indian sub-continent, Pakistan, Bsngladesh, Nepal. Beta-thalassemia homozygous/major causes severe health problems like heart failures, multi organ failures, hepatomegaly, splenomegaly, enlarged kidneys. Affected child seldom lives beyond two years of age. Beta thalassemia heterozygous and HbE hemoglobinopathy are mild and not life threatening. But a combination of Beta-thalassemia and HbE results in compound E-β-thalassemia heterozygous. This condition is more serious than Beta thalassemia homozygous. The thalassemiasarehereditary diseases inherited in an autosomal recessive manner. Therefore this genetic disease can be prevented genetic counseling and proper management.

A descriptive cross-sectional study of Koch Rajbanshi, KochilaTharu, Musahar, Musalman and Santhal ethnic groups of Jhapa, Morang and Sunsari districts of Eastern Nepal was performed. A total of 1500 samples consisting of 100 samples per ethnic group per district were collected in 3ml EDTA vials for investigations. Peripheral blood smears were stained with wright, sgeimsa stain on spot. Initially Red Cell parameters were estimated by an electronic cell counter. Out of thetotal cases, 285 samples with MCV (<80fL), MCH (27pg), Hemoglobin (10g/dL) below standard cut-off level were considered positive. Microscopical examination of Peripheral Blood Smear presented 184 morphological abnormalities. Microcytic hypochromic cells, target cells and anisocytosis indicated Beta thalassemia &hemoglobionoapthies. Estimation of HbA2 by capillary electrophoresis of the positive cases was conducted. Analysis of the electrograms confirmed Beta-thalasemia and hemoglobinopathies in all the 184 cases. District wise Jhapa district had the highest prevalence with 57.60% (n=184) followed by Morang 22.28% (n=184) and the lowest was found in Sunsari 21.10% (n=184). Among the five ethnic groups, high prevalence was seen Musalman (32.06%) and Koch Rajbhanshi (31%) followed by Santhal (19.56%), Musahar (9.78%) and the lowest in KochilaTharu (8.15%). This study has identified high occurrence of HbE variant among Koch

Rajbhanshi (31%) and Musalman (15.22%). Mutation analysis was performed by multiplex amplification refractory mutation system polymerase chain reaction for genotyping nine core mutations. Among the study groups the following mutations were identified: C-15 (G>A), IVSI-5 (G>C), FS 8/9(+CCCT) and C-26 $^{glu\text{-}ly}$ the mutation responsible for HbE variant. The study group presented a characteristic mutational profile. The only mutation identified in the Rajbanshi ethnic group was C-26 $^{glu\text{-}ly}$. Double mutations C-15 (G>A), IVSI-5 (G>C) was found in the Musahars and Santhal Ethnic groups. Mutation C-15(G>A) was prevalent in 50%, C-26 $^{glu\text{-}ly}$in 43.73%, IVSI-5 (G>C) in 14.67% and single case FS8/9 was identified.

It can be concluded that Beta-thalassemia and HbE is highly prevalent among Koch Rajbanshi, Kochilatharu, Musahar, Musalman and Santhal ethnic groups of Jhapa, Morang and Sunsari districts of Eastern Nepal. The birth of life-threatening transfusion-dependent Beta-thalassemia homozygote and compound E-beta thalassemia heterozygote is preventable by population screening and genetic counseling.

Capillary electrophoresis system is a simple and automated procedure for accurate prenatal diagnosis of severe thalassemia diseases which could readily be performed in routine setting.HbE disease and E trait are homozygotic and heterozygotic conditions of a β-chain variant, where a lysine residue is substituted by glutamic acid. This monogenic disease also has a broad distribution throughout the Mediterranean, the Middle East, and the Indian subcontinent [12, 13]. This study found 9.2% Hb E disease and 12.1% Hb E trait among the overall study subjects.

An effective strategy of preventing the progression of the disease in Nepal might be a nation-wide screening program employing more sophisticated techniques like polymerase chain reaction (PCR) followed by direct sequencing, genetic counseling, and creating public awareness.

REFERENCES

Aggrawal, S., Salaya, S., Bhesan, S., Sharma, M., Gupta, D.K. andGupta, B. (2011). HbE variants: Retrospective analysis in a tertiary care unit. *Journal Indian Academy of Clinical Medicine*,**12**(4): 263-5.

Agarwal, M.B. (2009). Advances in management of thalassemia. *Indian Pediatr*ics, **76**(2):177-84. DOI: 10.1007/s12098-009-0048-7. Epub 2009 Mar 28.

Allen, S.J., O'Donnell, A., Alexander, N.D.E., Alpers, M.D., Peto, T.E.A.,Clegg,J.B. et al.(1997). α+ thalassemia protects children against disease caused by other infections as well as malaria. *Proceedings of National Academy of Sciences of United States of America*,94: 14736 – 14741.

Ali, S., Mumtaz, S., Shakir, H.A., Khan, M., Tahir, H. M., Mughal, T. A. et al. (2021). Current status of beta Thalassemia and its treatment strategies. *Molecular Genetics and Genomic Medicine*, **9**(12): e1788. DOI: 10.1002/mgg3.1788.

Ali, N., Moiz, B., Azhar, W.B., Zaidi, N. and Memon, R. (2012). Carrier detection for beta-thalassemia trait in general Pakistani population: a way forward.*Hematology*,**17**(4):237-240. DOI:10.1179/1607845412Y.0000000002.

Al-Ali, AK., Al-Ateeq, S., Imamwerdi, B.W., Al-Sowayan, S., Al-Madan, M. Al-Muhanna, F. et al. (2005). Molecular bases of beta-thalassemia in the eastern province of Saudi Arabia. *Journal of Biomedicine and Biotechnology*,**2005***(*4):322–325. DOI: 10.1155/JBB.2005.322

Al-Suliman, A. (2006). Prevalence of Beta thalassemia trait in pre-marital screening in Al-Hassan, Saudi Arabia. *Annals of Saudia Medicine*,**26** (1): 14-16.https://doi.org/10.5144/0256-4947.2006.14

Ambedkar, S.S., Aphodka, M.A., Makashi, G.D., Bankar, M.P., Akhsdkar, L.A., Venkat, V. etal. (2001). Pattern of hemoglobinopathies in Western Maharastra. *Indian Pedeatric*,**38**:530-534.

Ansari, S. H., Shamsi, T. S., Ashraf, M.,Bohray, M., Farzana, T., Khan, M. T. R.et al. (2011).MolecularepidemiologyofBeta-thalassemia in Pakistan: far

reaching implications. *International Journal of Molecular Epidemiology and Genetics*, **2**(4):403–408.PMID: 22200002.

Ahmed, S., Saleem, M., Sultana, N., Raashid, Y., Waqar, A., Anwar, M., et al.(2000). Prenatal diagnosis of beta-thalassaemia in Pakistan: experience in a Muslim country. *Prenatal Diagnosis*, **20**(5):378–83. PMID: 10820404

Ahmed, S., Saleem, M., Modell, B. & Petrou, M. (2002). Screening Extended Families for Genetic Hemoglobin Disorders in Pakistan.*The New England Journal of Medicine*, **347**(15):1162-1168. DOI: 10.1056/NEJMsa013234.

Angastinotis, M. (2005). Epidemiology. In;Galanello, R, Eleftheriou, A. Traeger-Synodinos J. et al. eds. Prevention of thalassemia and other hemoglobin disorders. Vol 1. Nicosia, Cyprus. *Thalassemia International Publications* 10-13.

Angastinotis, M., and Eleftheriou, A. (2016). National prevention strategies for the control of haemoglobin disorders. A special reference to the countries of the Middle East, in Jan Cools (Eds.), Proceedings and Abstract Bookof 2nd Megma Conference on Thalassaemia& other Haemoglobinopathies, Amman, Jordan, Nov. 11-12, 2016, pp. 1-5.

Arthur, W. N. and David, G. N. (2012).Pathophysiology and Clinical Manifestations of the B-thalassemias. *Cold Spring Harbperspect Med*,**2**(12). 2012; 2:a0117262012;2:a011726.

Asif, N. and Hassan, K.(2016). Management of Thalassemia in Pakistan. *Journal of Islamabad Medical & dental College*, **5**(4): 152-153.

Bain, B.J. (2006). The alpha, delta and gamma thalassemias and related conditions. Hemoglobinopathy diagnosis. 2nd EDT. Blackwell Publishing. London. U.K. 63-138.

Balgir, R.S. (2008). Haematological profile of twenty nine compound cases of haemoglobinopathies and G6-PD deficiency in rural Orissa. *Indian Journal of Medical Sciences*, **62**(9): 364-372.DOI: 10.4103/0019-5359.43423.

Balgir, R.S. (2005). Spectrum of hemoglobinopathies in the state of Orissa, India: a ten years cohort study.*Journal Associaton Physicians India*,**53**: 1021-26. PMID: 16572956.

Balgir, R.S. (2000). The burden of hemoglobinopathies in India and the challenge ahead. *Current Science*,**79**(11): 1536-1547.

Barua, T., Das, A.K., Sultana, R., Das. D. Mahmood, A. and Chowdhury, A. (2020). Socio-demographic Profile of patients admitted in thalassemia Care Centre. *Chattagram Maa Shishu-O-General Hospital Medical College Journal*,**19(**1): 33-37. www.banglajol.info/index.php CMOSHMCJ.

Baruah, M.K., Saiki, M. and Baruah, A. (2014). Pattern of hemoglobinopathies and thalassemias in upper Assam region of North eastern India: high performance liquid chromatography studies in 9000 patients.*Indian journal of Microbiology*,**57**(2): 236-243. PMID: 24943756 DOI: 10:4103/0377-4929.134680

Bastola, G., Acharya, R., Dhakal, N. & Gupta, U.P.(2017). Study on thalassemia and hemoglobinopathies in Pokhara, Nepal. *Journal of Clinical and Diagnostic Research*,**11**(1): 15-18. DOI: 10.7860/JCDR/2017/29845.10916

Bashyam, M.D., Bashyam, L.&Savithri, G.R. et al.(2004). Molecular genetic analyses of beta thalassemia in South Indiareveals rare mutations in the beta globin gene. *Journal of Human Genetics*,**49**(8):408-413. Doi:10.1007/s10038-004-0169. Epub 2004 Jul 24

Bhatt, R.D., Koju, S. and Risal, P. (2017). Report of Bet Thalassemia in Newar ethnicity. *Annals of Cinical Chemistry and Laboratory Medicine*,**3**(1): 30-34. DOI: 10.3126/acclm.v3i1.17298.

Bhattacharya, D.M., Mukhopadhya, A. &Basek, J. (2014). Descriptive profile of beta thalassemia mutations in West Bengal population: a hospital based study. *International Journal of Hematology*,**99**(3): 345-353. DOI: 10.1007/s12185-014-1511-4.

Bista, D.B. (2034 B.S.). Sabai Jatko Fulbari. Chapter 1: Pg 2. Sajha Prakashan, Kathmandu.

Bista, D.B. (2000). Rajbhanshi. People of Nepal: Ratna Pustak Bhandaar. 4th edition. Part11: 134- 137.

Bista, D.B. (2000). Musalman. People of Nepal: Ratna Pustak Bhandaar: 4th edition. Part11: 150-155.

Bista, D.B. (2000).Satar. People of Nepal: Ratna Pustak Bhandaar: 4th edition. Part11: 138-141.

Bista, D.B. (2000). The Tharu. People of Nepal: Ratna Pustak Bhandaar. 4th Edition. Part 11: 118-127.

Cao, A. &Kan, Y.W. (2013). The prevention of thalassemia. *Cold Spring Harbor Perspectives in Medicine*,**3**(2): a011775.DOI: 10.1101/cshperspect. a011775

Cao, A. &Galanello, R.(2010). Beta thalassemia, *Genetics in Medicine*,**12**(2): 61-76 doi.org/10.1097/GIM.0b013e3181cd68ed

Caminopetros, J. 1938Recherches sur Tanemiaerythroblastique infantile des peuples de la Méditerranéeorientale, eduteanthropologique, etiologiqueetpathogenique; la transmission héréditaire de la maladie. Ann. Med., 43:104.

Central Bureau of Statistics. (2012). A Household of population by districts. Kathmandu: Central Bureau of Statistics Government of Nepal.

Central Intelligence Agency. The World Factbook Washington, DC: Central Intelligence Agency; (2016). Available from: https://www.cia.gov/library/publicatios/resources/theworldfactbook/geos/gt.ml.

Chakrabarti, S., Mandal,K., Pathak,S., Patra, A. and Pal, S. (2016). Hemoglobinopathies among the tribal and non-tribal mothers in a tertiary care hospital of rural West Bengal, India. *Bangladesh Journal of Medical Sciences*,**5** (1): 90-94. DOI: 10.3329/bjms.v15i1.18647

Chandrasekhar, V. and Soni, M. (2011). Hemoglobin disorders in South India. ISRN *Hematology*,6 pages. Epub. Article ID 748939. DOI: 10.5402/2011/948939.

Chang, J. C. and Kan, Y.W. (1979). Beta 0 thalassemia, a non-sense mutation in man. *Proceedings of National Academy of Sciences*, 76(6): 2886-2889. DOI:10.1073/pnas.76.6.2886

Chaudhary, J. K. (2008). Landless and its Impact in the life of Musahar: A case of Madhesi Dalit people at Pothiyahi VDC in Rautahat district Nepal. Research Report

Chopra, G.S., Nair, V., Gupta, P.K., Mishra, D.K., Sharma, A., Mathew, O.P. et al.(2008). Spectrum of Hemoglobinopathies in a Tertiary Care Hospital of Armed Forces. *Medical Journal Armed Forces India*, 64(4); 311-314.DOI: 10.5402/2011/748939 Corpus ID: 17415905

Chen, W., Zhang, X., Shang, X., Cai, R., Li, l. and Zhou, T. *et al*. (2010). The molecular basis of beta-thalassemia intermedia in southern China: genotypic heterogeneity and phenotypic diversity. *BioMed Central Medical Genetics*,11(31):10 pageshttps://doimedcentral.com/1471-2350/11/31

Clark, B. E. and Thein, S. L. (2004). Molecular diagnosis of hemoglobin disorders. Clinical and Laboratory Hematology, 26(3): 159-176. DOI: 10.1111/j.1365-2257.2004.00607.x

Colah,R., Italia, K. and Gorakshakar, A. (2017). Burden of thalassemia in India: the road map for control. *PediatricHematology Oncology Journal*, **2**(*4*): 79-84. DOI: 10.1016/j.phoj.2017.10.002

Colah, R., Gorakshakar, A. &Nadkarni, A. (2010). Global burden, distribution and prevention of β-thalassemias and haemoglobin E disorders. *Expert Review of Hematology*,**3**(1): 103-107. DOI: 10.1586/ehm.09.74

Colah, R., Gorakshakar, A., Nadkarni,A.&Phanasgaonkar, S. (2009). Regional heterogeneity of beta-thalassemia mutations in the multi ethnic Indian population. *Blood Cells Molecules Diseases*,**42**(3): 241-6. DOI: 10.1016/j.bcmd.2008.12.006

Colah, R.B., Surve, R.,Sawant, P., et al. (2007). HPLC studies in hemoglobinopathies. *Indian Journal Pediatrics*,**74**(**7**): 657-62. DOI: 10.1007/s12098-007-0117-8 source PubMed.

Colah, R.B., Gorakshakar, R.C. &Nadkarni, A.H. (2011). Invasive & non-invasive approaches for prenatal diagnosis of haemoglobinopathies: Experiences from India. *Indian Journal Medical Research*, **134**(4): 552-560. PMID: 22089620 PMICD: PM3237256

Cooley, M. D. and Lee, P. (1927). A series of cases of splenomegaly in children and peculiar changes in bones. *American Journal of Diseases of Children*, 34(3): 347-364. DOI: 10.1001/archpedi.1927.04 13021002202

Das, M.K., Nepal, N. and Chaudhary, P. (2020). Screening for Thalassemia in healthy population at a tertiary care hospital in eastern Nepal. *Journal of Nobel Medical College*,**9**(16): 41-45. DOI: 10.3126/jonmc. v9i1.29529.

Demir, A., Yarali, N., Fisgin, T., Duru, F. and Kara, A. (2002). Most reliable indices in differentiation between thalassemia trait and iron deficiency anemia. *Pediatric International*,44(6): 612- 616. DOI: 10.1046/j.1442-200x2002.01636.x.

Eichentopf, S.R. and Mitchell, J. R. (2013). Linguistic Survey of Nepal (LinSuN). Central Department of Linguistics. Tribhuvan University, Nepal and SIL International. Chapter 1 Pg 2.

Farashi, S. and Harteveld, C. L. (2018). Molecular basis of α-thalassemia. *Blood Cells, Molecules and Diseases*, 70: 43-53. DOI: 10.1016/j.bcmd.2017.09.004

Fesas, P. (1963). Inclusions of hemoglobin in erythroblasts and erythrocytes of thalassemia. *Blood*,21(1): 21-32. PMID: 13944958

Fuchareon, S. &Weatherall, D.J. (2012). The haemoglobin E thalassemia. *Cold Spring Harbor Perspectives in Medicine*,**2**(8): 1-14. www.perspectivesinmedicine.org.

Fuchareon, G., Sanchaisurya, K., Sae-ung, N., Dangibul, S.&Fuchareon, S. (2004). A simplified screening strategy for thalassemia and Hemoglobin E in rural communities in south-east Asia. *Bulletin of World Health Organisation*,**82**(5): 364-72. Ref No:02.000521

Flynn, M., Thomas, S.R. &Bhagavan, N.V. (1986). Limitations of red blood cell distribution width (RDW) in evaluation of microcytosis. *American Journal*

ofClinical Pathology,**85** (4): 445-449. PMID:3953499 DOI: 10.1093/ajcp/85.4.445

Galanello, R. &Origa, R. (2010). Beta thalassemia. *Orphanet Journal of Rare Diseases*,**5** (11): 1-5. https://www.ojrd.com/content/5/1/11

Galanello, R. &Campus, S. (2009). Deferiprone chelation therapy for thalassemia major. *Acta Haematologica*,**122**(2-3):155-164. PMID: 19907153 DOI: 10.1159/000243800.

Ghada, Y. E. and Khalda S. A. (2015), Thalassemia- From Genotype to Phenotype. Inherited Disorders of Hemoglobin. Chapter 2. DOI: 10.5772/61433

Ghosh, K., Colah, R.B. and Mukherjee, M.B. (2015).Haemoglobinopathies in tribal populations of India. *Indian Journal Medical Research*,**141**(5): 505-508.DOI: 10.4103/0971-5916.159488.

Ghosh, N., Chakrabarti, I. &Goswami, B.K. (2018). Study to assess prevalence of hemoglobinopathies by HPLC among Class 1X and X students of a rural block, Darjeeling district, West Bengal. *International Journal of Medicine and Public Health*,8(2): 71-76. DOI: 10.5530/ijmedph.2018.2.6.

Giambona, A., Passarello, C., Renda, D. &Maggio, A. (2009). The significance of hemoglobin A2 value in screening for hemoglobionpathies. *Clinical Biochemistry*,**42**(18): 1786-96. DOI: 10.1016/j.clinbiochem.2009.06.026. PubMed PMID:19591816.

Giordano, P.C. (2013). Strategies for basic laboratory diagnostics of the hemoglobinopathies in multi-ethnic societies: interpretation of results and pitfalls. *International Journal of Laboratory Hematology*,**35**(5): 465-479. PubMed PMID: 23217050. DOI: 10.111/ijlh.12037.

Giri, M. (2012). Politico Economic History of Marginalization and Change among the Musahars of East-Central Tarai. *Contributions to Nepalese Studies*, **39**: 69 – 94.

Green, R. and King, R. (1989). A new red blood cell discriminant incorporating volume dispersion for differentiating iron deficiency anemis from Thalassemia minor. *Blood Cells*, **15**(3): 481-495. PMID: 2620095

Goswami, B.K., Pramanik, R., Chakrabarty, S., Pal, P.P. &Bandyopadhyay, A. (2014). A spectrum of hemoglobin variants in the population of northern region of West Bengal: an ethnogenetic proposition. *Journal Family Mecicine Primary Care*,**3**(3): 219-213. DOI: 10.4103/2249-4863.141614

Grow, K., Vashist, M., Abrol, P., Sharma, S. and Yadav, R. (2014). Beta Thalassemia in India: Current status and the challenges ahead: *International Journal of Pharmacy and Pharmaceutical Sciences*; **6**(4): 28-33. 11 ISSN-0975-1491https://www.researchgate.net/publication/271642486

Garewal, G. and Das, R. (2003). Spectrum of β-Thalassemia Mutations in Punjabis *Int J Human Genetics*,**3**(4): 217-219 DOI: 10.1080/09723757.2003.11885855.

Gupta, A., Hattori, Y. & Gupta, U, R.*et al*.(2003). Molecular genetic testing of beta-thalassemia patients of Indian origin and a novel 8bp deletion mutation at codons 36/37/38/39. *GenetTest,* **7**(2):163-8. DOI: 0.1089/109065703322146894

Harrington, A.M., Ward, P.C. &Kroft, S.H. (2008). Iron deficiency anemia, β-thalassemia minor, and anemia of chronic disease. A morphological reappraisal. *AmericanJournal of Clinical Pathology*,**129**(3): 466-471. DOI: 10.1309/LY7YLUPE7551JYBG

Hashmi, M.R., Nasir, A., Rashid, A., Moatter, T. and Moiz, B. (2012). Hemoglobin E syndromes in Pakistani population. *Blood disorder*,**12** (3): 6 pages.

DOI: 10.1186/1471-2326-12-3.

Hoppe, C. C. (2009). Newborn screening for non-sickling hemoglobinopathies. *Hematology*, 2009(1): 19-25. DOI: 10.1182/asheducation-2009.1.19.

Hossain, M.S., Raheem, E., Sultana, T.A., *et al*. (2017). Thalassemia in South Asia: clinical lesson learnt from Bangladesh. *Orphanet Journal of Rare Disease*,**12**(93): 9pgsdoi: 10.1186/s13023-017-0643

Higgs, R. (2013). The molecular basis of α-thalassemia. *Cold Spring Harbor Perspectives in Medicine*,**3**(1): a011718. DOI: 10.1101/cshperspect.a011718.

Huisman, T.H.J., Carver, M.F.H. and Baysal, E. (1997). A syllabus of thalassemia mutations. The Sickle Cell Anemia Foundation in Augusta, GA, USA, Available from: http://globin.cse.psu.edu [accessed on 6 February 2008].

Ingram, V.M. &Stretton, A.O.W.(1959). Genetic basis of the thalassaemia disease. *Nature*,**184**:1903-9 DOI: 10.1038/1841903a0

Islam, M.M., Hossain, F., Sakib, N., Zeba, A., Bhuiyan, A.K.M.I. and Mamun, M.A. (2021). Distribution of β-thalassemia and other hemoglobinopathies in Bangladeshi University students and Ready Made Garment workers. *Risk Management and Healthcare Policy*,**14**: 2707-2714. DOI http://doi,org/10.2147/RMHP.S317852.

Jahangiri, M., Rahim, F. and Malehi, A.M. (2019). Dagnostic performance of haematological discriminating indices to discriminate between B-thalassemia trait and iron deficiency anemia and using cluster analysis: Introducing two new indices tested in Iranian Population. *Scientific Reports*,**9**: 18610. DOI: 10.1038/541598-0154575.

Jameel, T., Baig, M., Ahmed, I., Hussain, M.B. &Alkhamaly,M. D. (2017). Detection of beta thalassemia trait from iron deficiency anemiaby hematological indices. *Pakistan Journal of Medical Science*, **33**(3): 665-669. PMID: 28811791 DOI: 10.12669/pjms.333.12098

Jha, R. and Jha, S. (2014). Beta-thalassemiaa review. *Journal of Pathology of Nepal*,**4**(8):663-71. www.acpnepal.com

Jha, R. (2015). Distribution of hemoglobinopathies in patients presenting for electrophoresis and comparision of result with high performance liquid chromatography. *Journalof Pathology of Nepal*,**5**(10): 850-58.

John O. (2013). *Emery and Rimon's Principles and Practices of Medical Genetics. Hemoglobinopathies and thalassemias.* Chap. 71:1-44. U.K. Academia Press.DOI:10.1016/B978-0-12-383834-6-00075-6.

Joshaghani, H.R., Parvizi, S., Kalavi, K.,Behnampour, N., Joshaghani, H. &Ayatollahi, A.A. et al. (2015). Hemoglobin D is the most common hemoglobinopathy in south of Iran. *Medical Laboratory Journal*,**9**(5):28-32. DOI:10.18869/acadpub.mij.9.5.28.

Kafley, R., Bastola, A., Samdurkar, A. P., Gautam, N., Poudel, A. and Shahi, A. (2021). Hematologica profile in microcytic hypochromic anaemia in children. *Journal of University College of Medical Sciences*, **9**(2): 3-7. DOI: 10.3126/jucms.v9i02.41941.

Kamel, R. M. (2013). Reproductive technology after the birth of Louise Brown. *Journal of Reproduction and Infertility*, **14**(3): 96-109.PMC 3799275.

Kan, Y.W. & Nathan, D.G. (1970). Mild thalassemia: the result of interactions of alpha and beta thalassemia genes. *Journal of Clinical Investigations*,**49**(4): 635-42. DOI: 10.1172/JCI106274.

KC, S. R., Basnet, S. and Gyawali, P. (2017). *Nepal Medical College Journal*, 19(3): 121-126.

Khan, W.A.,Banu, B., Amin, S.K., Selimuzzaman, M., Rahman, M., Hoaasin, B. *etal.*(2005). Prevalenceof beta thalassemia trait and HbE trait in Bangladesh school children and health burden of thalassemia in our population. *Dhaka Shishu (Child) Hospital Journal*,**21**(1):1-7. https://www.researchgate.net/publication/200176529

Khan, W.A., Banu, B., Sadiya, S. and Sarwadi, G. (2017). Spectrum of types of thalassemias and haemoglobinopathies: study in tertiary level children hospital in Bangladesh. *Thalassemia Reports*,**7** (1): 18-20. DOI: 10.4081/thal.2017.6354

Khania, P R. (2010). Socio-Economic condition of the Tharuu community in Pathani VDC, Chitwan. *Tribhuvan University Journal*, XXVII (1-2): 97-102. https://www.nep.info

Kim, J.E., Kim, B.R., Woo, K.S., Kim,J.M., Park, J.I. and Han, J.Y. (2011). Comparision of capillary electrophoresis with cellulose acetate

electrophoresis for the screening of hemoglobinopathies. *Korean Journal of Laboratory Medicne*,**31**(4):238-243 DOI: 10.3343/kjlm.2011.31.4.238

Kiran, S.S., Saraswathy, A. &Charalingappa, S.B. (2016). HbE hemoglobinopathy in an adult from Assam with unusual presentation: A diagnostic trauma. *Journal of Laboratory Physicians*,**8**(2): 116-9 DOI: 10.4103/0974-2727.180793

Korber,C., Wolfer, A., Neubauer, M. &Robier, C. (2017). Red blood cell morphology in patients with β-thalassemia minor. *Journal LaboratoriumsMedicine*, **41**(1): 49-52. https://doi.org/10.1515/labmed-2016-005.

Kumar, R., Arya, V.&Agrawal, S.(2015). Profiling beta thalassemia in consanguinity and non- consanguinity for prenatal screening and awareness program. *Advances in Hematol*, 1-5pgs Article ID 625721 DOI: 10.1155/2015/625721.

Kumar, A. (2006). Culture, Development and the Cultural Capital of Farce: The Musahar community in Bihar. *Economic and Political Weekly*, 41 (401): 4281-4285.

Kunkel, G.H.,Ceppellini, R., Muller-Eberhard, U. &Wolf, J. (1957). Observations on the minor basic hemoglobin component in the blood of normal individuals and patients with thalassemia. *Journal of Clinical Investigations*,**36**(11):1615-25 DOI: 10.1172/JCI103561

Kunnath, G. (2013). Compliance or defiance. The case of Dalit and Mahadalit. 36-59. http://tiny.cc/dhzfozCorpus ID: 35045790.

Lama, R., Yusof, W., Shrestha, T.R. and Hanafi, S. (2021). Prevalence and Distribution of Major β-thalassemia Variant in Nepalese Ethnic Groups. *Hematology/Oncology and Stem cell Therapy;* 15(1): 7 pgs. DOI: 10. 1016/j.hemonc.2021.02.004.

Langlois, S., Ford, J.C., Chitayat, D., Desilets, V.A., Farrell, S.A., Ganguly, M. *et al*. (2008). Carrier screening of thalassemia and hemoglobinopathies in Canada. *Journal of Obstetrics and Gynaecology Canada*, **30**(10):950-71.DOI: 10.1016/S1701-2163(16)32975-9.

Lafferty, J.D., Crowther, M.A., Ali, M. A. and Levine, M. (1996). The evaluation of various mathematical indices and their efficacy in discriminating between thalassemic and non-thalassemic microcytosis. *American Journal of Clinical Pathology*, **106**(2):201-205.DOI: 10.1093/ajcp/106.2.201

Lewis, M.P., Simons, G.F. and Flennig, C.D. 2013. Ethnologue: Languages of the world (17^{th} ed.). Dallas, TX: SIL International.

Local Governance and Community Development Programme (LGCDP) – II*Ministry of Federal Affairs and General Administration (MoFAGA)* GIS District Map.

Lund, S.K., Rajput, B.R.H.M., Kumar, D., Rajput, M.S., Memon, A. &Qaiser, F. (2014). Frequency ofVarious Hemoglobinopathies. *IOSR Journal of Dental and Medical Sciences*, **13(**2): 68-70 e-ISSN: 2279-0853, p-ISSN:2279-0861

Madan N., Sharma S., Sood S.K., Colah R.,& Bhatia H.M. (2010). Frequency of beta thalassemia trait and other hemoglobinopathies in northern and western India. *Indian Journal of Human Genetics*,**16**(1): 16-25. DOI: 10.4103/0971-6866.64941

Mandal, P.K. &Majhi, S.K. &Dolai, T.K. (2014). Present scenario of hemoglobinopathies in West Bengal, India: Analysis of a large population. *International Journal of Medicine and Public Health*,**4**(4): 496-499. DOI: 10.4103/2230-8598.144127

Mannan, A., Kawser, J., Ahmed, A.M.A., Sikder M.O.F., Islam, M.J. &Choudhury, M.A. (2013). A Demographic approach for understanding the Prevalence of β-thalassemia pattern and other Hemoglobinopathies: Selective study in Chittagong city Perspective. *Asian Journal Biological Science*, **6**(2): 124-130. DOI: 10.3923/ajbs.2013.124.130.

Majhi, S., Mishra, A., Mehta, K.D., Baral, N.& Shrestha, P. (2007). Detection of beta thalassemia (homozygous) by haemoglobin electrophoresis on agar gell and citrate agar medium: a case report. *Nepal Medical College Journal*, **9**(1):70-71

Marengo-Rowe, A.J. (2007). The thalassemias and related disorders. *Proceedings* (*Baylor University Medical Center*,**20**(1): 27-31. DOI:10.1080/08998280.2007.11928230.

Marengo-Rowe, A. J. (2006). Structure-function relations of human hemoglobins. *Proceedings (Baylor University Medical center Proceedings),* 19(3): 239-245. DOI: 10.1080/08998280.2006.11928171.

Martins, M.C., Olim, G., Melo, J., Magalhaes, H.A. &Rodriques, M.O. (1993). Hereditary anemias in Portugal: epidemiology, public health significance and control. *Journal of Medical Genetics*,**30**(3), 235–239 DOI: 10.1136/jmg.30.3.235

Mehdi, S.R. &Dahmash, B.A. (2013). Analysis of haemoglobin electrophoresis results and physicians investigative practices in Saudi Arabia. *Indian Journalof Human Genetics*,**19**(3):337-41 DOI: 10.4103/0971-6866-120829

Mentzer Jr.W.C. (1973). Difference of iron deficiency anemia from thalassemia. *Lancet*,**21**(1)7: 808- 882. Doi: 10.1016/s0140-6736(73)91446-3.

Mishra, A., Mukherjee, A., Roy, A., Singh, G., Shrestha, P. & Singh, R.R. *et al*. (2012*).* Distribution and ethnic variation of beta thalassemia mutations in Nepal.*Nepal Medical College Journal*, **14**(1): 49-52.PMID: 23441495

Modell, B., Darlison, M., Birgens, H., Cario, H., Faustino, P., Giordano, P.C. *et al*.(2007). Epidemiology of Haemoglobin Disorders in Europe: an overview. *Scandinavian Journal of Clinical Laboratory Investigations*, **67**(1): 39-69.doi: 10.1080/00365510601046557.

Modell, B. &Darlinson, M. (2008). Global epidemiology of haemoglobin disorders and derived service indicators. *Bull world Health Organ*,**86** (6): 480-487.Doi: 10.2471/blt.06.036673.

Moiz, B., Hashmi, M.R., Nasir, A., Rashid, A. &Moatter, T. (2012). Hemoglobin E syndromes in Pakistani population. *Hematol*,**12**(3): 1-6. DOI:10.1186/1471-2326.

Mondiano, G., Morpurgo, G., Terrenato, L., Novelletto, A., Di Rienzo, A., Colombo, B. et al. (1991). Protection against Malaria morbidity: near fixation of Alpha

thalassemia gene in a Nepalese population. *American Journal of Homan Genetics*, **48** (2): 390-397. PMID: 1990845.

Mohan, H. (2010). Textbook of Pathology 7th ed.Thalassemia. Chap. 10:298-301. Jaypee Brothers Medical Publishers (P) Ltd., Chandigarh, India..

Mohanty, D., Colah, R.B.,Gorakshakar, A.C., Patel, R.Z., Mahanta, J. et al. (2013). Prevalence of beta-thalassemia and haemoglobinopathies in six cities in India: a multicentre study. *Journal of Community Genetics*,**4**(1): 33-42

Moiz, B., Hashmi, M.R., Nasir, A. et al. (2012). Hemoglobin E syndromes in Pakistani population. *Hematol*, **12**(3): 6pages.https://doi.org/10.1186/1471-2326-12-3.

Mondal, S.K. & Mandal, S. (2016). Prevalence of thalassemia and hemoglobinopathy in eastern India: A 10 year high-performance liquid chromatography study of 119,336 cases. *Asian Journal of Transfusion Science*,**10**(1): 105-110. Doi: 10.4103/0973-6247.175424.

Mondal, S.K., Dasgupta, S., Mondal, S., Das, N. (2014). Spectrum of thalassemias and hemoglobinopathies in West Bengal: A study of 90,210 cases by cation exchange high-performance liquid chromatography method over a period of 8 years. *Journal of Applied Hematology*.**5**(3): 91-95. DOI: 10.4103/1658-5127.141993

Mondal, P.K., Majhi, S.K. and Dolai, T.K. (2014). Present scenario of haemoglobinopathies in West Bengal, India: Analysis of a large population. *International Journalnd of Medicine and Public Health*, **4**(4): 496-499. DOI: 10.4103/2230-8598.144127.

Mukhopahyay,D., Saha, K., Sengupta, M., Mitra, S., Datta, C. and Mitra, P.K. (2015). Spectrum of hemoglobinopathies in West Bengal, India. A CE-HPLC study on 1047 subjects.*Indian Journal ofHematology and Blood Transfusion*,**31**(1): 98-103. DOI: 10.1007/s12288-014-0373-5.

Mulchandani, D.V., Fularr, M.B., Zodpet. S.P. &Vasudes, N.D. (2008). Prevalence of some epidemiological factors of beta-thalassemia trait in Sindhi

Community of Nagour city, India. *Indian Journal of Public Health*,**52**(1): 11-15. PMID: 18700715

Herbert, L., Muncie, Jr. and Campbell, J. (2009). Alpha and beta thalassemia. *American Family Physician*, **80**(4): 339-344.PMID:19678601

Naseeb. U., Shafi, M.K., Syed, M.I., Khan, M.A., Husain, A., Majis, A. *et al.*(2019). Quantitative determination of HbA2 level in β-thalassemia trait by using Capillary Zone Electrophoresis in Balochistan. *Pak-Euro Journal of Medical and Life Sciences;* **2**(3): 56-61. ISSN: 2707-4471 (print), 2707-448X (online). www.readersinsight.net/pjml.

Neel, J.V. & Valentine, W.N. (1947). Further studies on the genetics of thalassemia. *Genetics*,**32**(1): 38-63. DOI: 10.1093/genetics/32.1.38

Nepal human rights Year book 2018. Musahar of Mahotari: measured in the perspective of socio-economic and cultural rights. First edition. Informal Sector Service Center. Chapter 3: 99- 108. ISBN: 978-9937-8964-9-8.

Nigam,N., Kushwaha, R., Yadav,G., Singh, P.K., Gupta, N., Singh, B. *et al*. (2020). A demographic prevalence of β-thalassemia carrier and other hemoglobinopathies in adolescent of Tharu population. *Journal of Family Medicine and Primary Care*, **9**(8): 4305-10.DOI: 10.4102/jfmpc.jfmpc-879-20.

Noor, F.A., Sultana, N. &Bhuyan, G.S. *et al.* (2020). Nationwide carrier detection and molecular characterization of β-thalassemia and hemoglobin E variants in Bangladeshi population. *Orphanet Journal of Rare Diseases*, **15**(1):15.https://doi.org/10.1186/s13023-020-1294

Old, J.M., Varawalla, N.Y. & Weatherall DJ. (1990) Rapid detection and prenatal diagnosis of beta-thalassemia: studies in Indian and Cypriot populations in UK. *Lancet*, **336**(8719):834–7. DOI: 10.1016/0140-6736(90)92338-i

Olivieri, N.F., Pakbaj, Z. and Vinchinsky, E. (2011). HbE/ beta thalassemia: a common and clinically diverse disorder. *Indian Journal of Medical Research*,**134** (4): 522-531.PMID: 22089616

Orkin, S., Kazazian, H. &Antonarakis, S. *et al.* (1982). Linkage of *β*-thalassaemia mutations and *β*-globin gene polymorphisms with DNA polymorphisms in human *β*-globin gene cluster. *Nature,* **296**(5858): 627–631. doi.org/10.1038/296627a0.

Panja, A., Ghosh, T.K. &Basu, A. (2012). Genetics of thalassemia in Indian population. *Journal of Community nutrition and health,*1(1)*:*39-46.

Panigrahi, I. and Agarwal, S. (2013). Genetic determinants of Phenotype in betathalassemia. *Hematology*, 13(4):247-252. DOI: 10.1179/102453308X316031.

Panyasai, S., Sakkhachornphop, S. &Pornprasert, S. (2018). Diagnosis of compound heterozygous Hb Tak/β-thalassemia and HbD-Punjab/ β-thalassemia by HbA2 levels on capillary electrophoresis.*Indian Journal of Hematology and Blood Tranfusions,* **34**(10): 110-114. PMID: 29398808.

Patel, J., Patel, A., Kaur, A. & Patel, V. (2009). Prevalence of hemoglobinopathies in Gujarat, India. A Cross-sectional Study. *The Internet Journal of Hematology*, **5**(1) 6pgs. ISSN: 1540-2649.

Piga, A., Gaglioti, C., Fogliacco, E., and Tricta, F. (2003). Comparative effects of deferiprone and deferoxamine on survival and cardiac disease in patients with thalassemia major: a retrospective analysis. *Haematologica,* **88**(95):489-496. PMID: 12745268

Pranpanus, S., Sirichotiyakul, S., Srisupundit, K. &Tongsong, T. (2009). Sensitivity and specificity of mean corpuscular haemoglobin (MCH): for screening alpha-thalassemia-1 trait and beta-thlassemia trait. *Journal of Medical Associations Thai*,**92**(6):793-43. https://doi.org/10.1111/j. 1447-0756.2005.00280.x

Population Reference Bureau. World population data sheet. 2010. Population /reference Bureau, Washington D.C

Poudel, B. and Kattel, S. (2019). Social Changes in Musahar community: A Case Study of Dhanusa District of Nepal. *Nepal Journal of Multidisciplinary Research*, 2(4): 9 – 16. ISSN: 2705-4691.

Quinlan, M. P. (2008). Aminocentosis: Indications and risks. *Virtual Mentor*, **10**(5): 304-306. DOI: 10.1001/virtualmentor.2008.10.5.cprl 1-0805.

Rahman, S., Ahmed, T., Rahma, A.S., Alam, N., Ahmed, A,M.S.,Ireen, S. *et al*. (2016). Determinants of iron status and Hb in the Bangladesh population: the role of groundwater iron. *Public Health Nutrition*,**19**(10): 1862-74. PMID: 26818180 DOI: 10.1017/S1368980015003651

Rahman, M.J. and Rahman, M.H.(2003). Prevention & control strategy of thalassemia in Bangladesh. *The Orion Medical Journal*,**16**: 121-122.

Rajpoot, A., Kumar, V.P. & Sharma, J.(2016) Current health status of Uttarakhand, Tharutribe on the basis of blood clinical parameters: a bio-cultural perspective. *International Clinical Pathology Journal*,**3**(3):219–223. DOI: 10.15406/icpjl.2016.03.00077

Rathod, D.A., Kaur, A., Patel, V., Patel, K., Kabrawalla, R., Patel, V. *et al*. (2007). Usefulness of cell counter based parameters and formulas in detection of beta thalassemia trait. In areas of high prevalence. *American Journal of Clinical Pathology*,**128**(4): 285-289. DOI: 10.1309/R1YL4B4BT2WCQDGV

Rees, D.C. and C.J. 1998. Is haemoglobin instability important in the interaction between HbE and beta thalassemia. *Blood*, **92**(6): 2141-6. PMID: 9731073

Rahman, M.J. and Rahman, M.H. Prevention & control strategy of thalassemia in Bangladesh., Pakistan. KJMS,**13**(1): 39-42.

Rich, A. 1952. Studies on the hemoglobin of Cooley's anemia and Cooley's trait. *Proceedings of National Academy of Sciences USA*; **38**(3): 187-9. DOI: 10.1073/pnas.38.3.187

Rigas, D.S., Koler, R.D., and Osgood, E.E. (1955). New hemoglobin possess- ing a higher electrophoretic mobility than normal adult hemoglobin. *Science*, **121**(3141):372. DOI: 10.1126/science.121.3141.372

Roberts, G.T. and Badawi, S.B.El. (1985). Red blood cell distribution width index in some hematologic diseases. *American Journal of Clinical Pathology*,83(2): 222-226. DOI: 10.1093/ajcp/83.2.222

Roth, I.L., Lachover, B., Koren, G., Levin, C., Luci, Z. &Koren, A. (2018). Detection of B-thalassemia carriers by Red Cell Paramaters obtained from Automatic counters using mathematical formulas. *Mediterranean Journal of Hematology and Infectious Diseases*,**10**(1): e:2018008. Doi: 10.4084/MJHID.2018.008

Ryan, K., Bain, B.J., Worthington, D., Plews, D., Mason, A. et al. (2010). Significant hemoglobinopathies: guidelines for screening and diagnosis. *British Journal of Haematology*, **149**(1): 35-49. Doi: 10.1111/j.1365-2141.2009.08054.x.

Sadiya, S., Khan, W.A., Banu, B., Akhter, N., Sarwadi, G. &Babu, M. (2015). Role of RBC Indices in screening of thalassemia and other haemoglobinopathies in children. *Dhaka Shishu (Child) Hospital Journal*,**31** (2): 101-108

Sakai, Y., Kobayashi, S., Shibata, H., Furuumi, H., Endo, T.,Fuchareon, S. *et al*. (2000). Molecular Analysis of Alpha thalassemia in Nepal: correlation with malaria endemicity. *Journal of Human Genetics*, **45**(3):127-132.

Saliba, A.N., Atouni, A., Labban M., Hamade, H., Bou-Fakhredin, R., Mufarrij, A. &Taher, A.T. (2020). Thalassemia in the emergency department: special considerations for a rare disease. *Annals of Hematology*, **99**: 1967-77. Doi: 10.1007/s00277-020-04164-6.

Sandhya, P., Vijayakumar, S., Vijaykumar, G. and Chandana, N. (2013). Beta thalassemia mini review. *International Journal of Pharmocology Research*; **3**(2): 71-79

Samira, Z.S., Basma, A.A.&Amr, S.A. (2013). The early cardiac involvement in patients with β-thalassemia major. *The Egyptian Heart Journal*, **65**(3): 243-51. doi: 10.1016/j.ehj.2013.01.0002

Shabbir, S., Nadeem, M., Sattar, A., Ara, I., Ansari, S.,Farzana, T. et al. (2016). Type and frequency of hemoglobinopathies, diagnosed in the area of Karachi, Pakistan. *Cogent medicine*, 3: 6 pgs.http://dx.doi.org/10.1080/2331205X. 2016.1188875 ISSN: (Print) 2331-205X (Online) Journal homepage:https://www.tandfonline.com/loi/oamd20

Shamshad. G.U., Mustaq, F., Khan, S.A. & Ali, N. (2017). Diagnosing Beta Thalassemia trait in a developing country. *Acta Hematologica Polonica*,**48**: 18-22. https://dx.doi.org./10.1016/j.achaem.2017.01.001.

Sharma, N., Pant, A.D. &Manadhar, U. (2020). Spectrum of hemoglobinopathy in a tertiary care centre.*Journal of Pathology of Nepal*,**10**: 1645-1649. DOI: 10.3126/jpn.v10i1.27369:1645-1740. www.acnepal.com.

Sherchand, O., KC, R., Majhi, S., Lamsal, M. and Baral, N. (2013). Laboratory parameters of Beta-thalassemia patients visiting BPKIHS as diagnosed by Agoarose gel electrophoresis, *Journal of Institute of Medicine*, **35**(1): 28-31. DOI: 10.3126/jiom.v35i1.8894

Shine, I. and Lall, S. (1977). A Strategy to detect β-thalassemia Minor.*The Lancet*,**39**(8013): 692-694. DOI: 10. 1016/S0140-6736(77)92128-6.

Shokrani, M., Terrell, F. & Turner, E.A. et al. (2000). Chromatographic measurements of hemoglobin A2 in blood samples that contain sickle hemoglobin. *Annals of Clinical Laboratory Sciences*, **30**(2): 191-194. PMID:10807164.

Shrestha, R.M., Pandit, R., Yadav, U. K. and Das, R. (2020). Distribution of Hemoglobinopathy in Nepalese Population, *Journal of Nepal Health and Research Council*.**18**(46): 52-58. DOI: 10.33314/jnhrc.v18i1.2303.

Shrivastava, A., Umang. P., Joshi, J.R. & Kaur, A. (2013). Study of hemoglbinopathies and Hb variants in population of Western India using HPLC: A report of 7,000 cases. *Journal of Applied Hematology*, **4**(3): 104-109. DOI:10.4103/1658-5127.123308. http://www.jhjournal.org. IP: 348.211.86.12.

Sickle cell anemia. Agenda item 11.4. In: *59th World Health Assembly, (27 May2006)*.WHA59.20.http://www.who.int/gb/ebwha/pdf_files/WHA59-REC1/e/WHA59_2006_REC1-en.pdf [p. 26; accessed on 6 February 2008].

Srivastava, A. and Shaji, R.V. (2017). Cure for Thalassemia major-from allogenic hematopietic stem cell transplantation to gene therapy. *Hematologica.* 102(2): 214- 223. DOI: 10.3324/haematol.2015.141200.

Stamatoyannopoulos, G. (2005). Control of globin gene expression during development and erythroid differentiation. *Experimental Hematology*, 33(3): 259-271. DOI: 10.1016/j.exphem.2004. 11.007

Stephton-Smith, R. (1962). Iron excretion in thalassemia major after administration of chelating agents. *British Medical Journal,* **2**(5319): 1577- 1580. DOI: 10.1136/bmj.2.5319.1577

Singh, J., Saxena, M., Ahmad, F., Kumar,A., Awasthi, S. &Dutta, S. (2016). Spectrum of hemoglobinopathies and thalassemias diagnosed on HPLC in a tertiary teaching hospital of North India. *Natural Journal of Laboratory Medicine*,**5**(3): 70 -75. DOI: 10.7860/NJLM/2016/19972.2149.

Skar, H. O. 1995. Contributions to Nepalese Studies. *CNAS Journal*, **22** (1): 31-42.

Srivastava, P. C. and Bevington, J. M. (1973). Iron deficiency and-or Thalassemia trait. *Lancet*, 1(7807):832. DOI: 10.1016/ S0140-6763(73)90673-5.

Stepton-Smith, R. 1964. Chelating agents in the diagnosis and treatment of iron overload. *Annals of New York .Academy of Sciences*, **119**: 776.

Sturgeon, P. & Finch, C.A. 1957. Erythrokinetics in Cooley's anemia. *Blood*,**12**(1): 64- 73. PMID: 13382990

Sultana, G.N.N., Begum, R., Akhter, H., Shanmim, Z., Rahim, M.A. &Chubey, G. (2016). The spectrum of beta (β) thalassemia mutations in Bangladeshi population. *Austin BiomarkDiagnosis*, **3**(1): 1024.

Tahura, S. (2017). Thalassemia and other Hemoglobinopathies in Bangladesh Children. *Imperial Journal of Interdisciplinary Research*,**3**(4): 180-184. ISSN: 2454 1362. http://www. online journal.in.

Terrenato, L. (1998). Decreased Malarial Morbidity in Tharu people compared to Sympatric population in Nepal. In Terrenato, L. *Annals of Tropical Medicine and Parasitology*,**62**(1): 1-11.DOI: 10.1080/00034983.11812202.

Thapa, S. (2016). Spectrum of mutations in Hbb gene among Thalassemia major patients in a cohort of Nepalese population. *Journal Proteomics Bioinform*, 9(10): 1pg. DOI: 10.4172/0974-276X.C1.089.

Thalassaemia and other haemoglobinopathies. World Health Organisation. Agenda item 5.2. In: *59th World Health Assembly, 27 May 2006*. EB118/5.http://www.who.int/gb/ebwha/pdf_files/EBSS-EB118-2006-REC1/english/Res/res-eb118_2006_rec1-en.pdf

Thein, S. (2004). Genetic insights into the clinical diversity of β-thalassemia. *British Journal of Hematology*, 124: 264- 274. Doi: 10.1046j 1365-2141.2003.04769x

Thein, S. (2013). The molecular basis of beta thalassemia. *Cold Spring Harbor Perspectives Medicine*,**3**(5): e 011700. DOI:10.1101/cshperspect.

Uddin, M.M., Sharif, T., Rahman, T. &Hossain, U.S. (2012). Pattern of beta thalassemia and other hemoglobinopathies: a cross-sectional study in Bangladesh. *ISRN Hematology*; 2012: 6 pages. Article ID 659191. Https://doi.org/10.5402/2012/659191.

Ukyab, T. &Adhikari, S. (2000). The Nationalities of Nepal. Library of Congress, New Delhi.

Ullah, A., Lal, A., Ahmed, S., Nawab, A., Khan, S.(2020). Frequencies of Abnormal haemoglobin Electrophoretic Patterns in hemolytic Anemia cases in District Swat.*Medical Laboratory Journal*,**10**(2): 160-163

U .S. National Library of Medicine. (2015). Genetic Home. Reference. http:// ghr.nlm, n.h.nih-gov. condition/beta thalassemia, accessed on 10 Dec2015

Varawalla, N.Y., Old, J.M., Sarkar, R., Venkatesan, R. &Weatherall, D.J. (1991). The spectrum of beta-thalassaemia mutations on the Indian subcontinent: the basis for pre-natal diagnosis. *British Journal of Hematology*, **78**(2): 242-7. DOI: 10.1111/j.1365-2141.1991.tb04423.x.

Verma S.C. (2010). The eco-friendly Tharu tribe: a study in socio-cultural dynamics. *Journal of Asia Pacific Studies*, **1**: 177-187.

Vehapoglu, A., Ozgurhan, G., Demir, A.D., Uzuner, S., Nursoy, M.A. Turkmen, S. et al. (2014). Hematological indices for differential diagnosis of beta thalassemia trait and iron defficiency anemia. *Anemia*, **2014**: 7 pages. Article ID 576738 | https://doi.org/10.1155/2014/576738

Vincenzo, D. S., Christos, K., Duran, C., Ashraf, T.S., Heba, E,Menran, K. et al. (2017). Betathalassemia distribution in the world: an ancient disease seen from a historical standpoint. *Mediterranean Journal of Hematology and Infectious Diseases*, **9**(1) 0:e2710708.

Vinchinsky, E.F. (2005). Changing patterns of thalassemia worldwide. *Annals of the New York Academy Sciences,***1054**: 18-24.doi: 10.1196/annals.1345.003

Vinchinsky E. (2007). Hemoglobin E syndromes. *Hematology*. 79-83. DOI: 10.1182/asheducatio-2007.1.9.

Waheed, U., Satti, H.S., Farooq, N.& Zaheer, H.A. (2012). Frequencies of hemoglobinopathies: a single-centre, cross-sectional study from Islamabad, Pakistan. *Eastern Mediterranean Health Journal*,**18**(12): 1257-1259. DOI: 10.26719/2012.18.12.1257.

Walters, M.C.&Abelson, H.T. (1996). Interpretation of the complete blood count. *Pediatric Clinics of North America*,**43**(3): 599-622.DOI: 10.1016/soo31(05)70424-7.

Wajcman, H. and Moradkhani, K. (2011). Abnormal haemoglobins: detection & characterization. *Indian Journal Medical Research*. **134**(4): 538-46. PMID: 22089618

Weatherall, D.J. (2013). The role of inherited disorders of haemoglobin, the first "Molecular Diseases" in the future of human genetics. *Annual Review of Genomics of Human Genetics*,**14**:1-24.

Weatherall, D.J. (2010). The inherited diseases of hemoglobin are an emerging global health burden. *Blood*,**115**(22): 4331-6. Doi:10.1182/blood-2010-01-251348

Weatherall, D.J. and Clegg, J.B. (2001). The thalassemia syndromes. 4thedt. Oxford: Blackwell Science Ltd.

Weatherall, D.J. and Clegg, J.B.(2001). Inheritedhaemoglobin disorders: an increasing global health problem. *Bull Health organ*,**79** (8): 704-12. Epub 2001 Oct 24. PMID: 11545326

Whipple, J.H. and Bradford, W.L. (1932). Mediterranean disease-thalassemia (erythroblastic anemia of Cooley); associated pigment abnormalities simulating hemochromatosis. *TheJournl of Pediatrics*, 9:279, DOI: 10.1016/S0022-3476(36)80021-3

WHO.Guidelines for the control of hemoglobin disorders: report of the VIth Annual Meeting of the WHO Working Group on Hemoglobinopathies, Cagliari, Sardinia, 8-9 April. Geneva, World Health Organization 1989

Wintrobe, M.M., Mathews, E. &Pollack, R. *et al*. (2017). A Familial Hemopoietic Disorder. In Italian Adolescents And Adultsresembling Mediterranean Disease (Thalassemia)DOI:10.1001/jama.1940.02810160032008

World Health Organisation. 16-18 Nov. (2007). Management of Hemoglobin disorders: report of a joint WHO-TIF meeting Nicosia, Cyprus.

Williams, T.N. and Weatherall, D.J. (2012). World distribution, population genetics, health burden of the hemoglobinopathies. *Cold Spring HarborPerspectives in Medicine*,**2**(9): a011692. DOI: 10.1101/cshperspect.a011692.

Guidelines for investigation of the Alpha and Beta Thalassemia traits. The Thalassemia Working Party of the BCSH General Hematology Task Force. *Journal of Clinical Pathology*. 1994; 47: 289-295

Xiaoxiao, Xu and Xiaoxiao, Wu. (2020). Epidemiology and treatment of beta thalassemia major in China. *Pediatric Investigation*,**4**(1): 43-74.DOI: 10.1002/ped4.12154

Zaghloul A., Al-bukhari, T.A.MA.,Bajauifer, N., Shalaby, M., Al-Pakistani, H.A. and Halawani, S.H. (2016). Introduction of new Formulas and evaluation of the previous red blood cell indices and formulas in the differentiation be.tween beta thalassemia trait and iron deficiencyanemia in the Makkah region. *Hemoglobinopathy*, **21**(6): 351-358. doi.org/10.1080/10245332.2015.1133753

Zhu, Y., Shen,N., Wang, X., Xiao, J. &Lu, Y. (2020). Alpha and beta thalassemia mutations in Hubei area of China. *BMC Medical Genetics*; **21**(6):5pgs. https://doi.org/10.1186/s12881-091-0925-5.

APPENDIX

Publications

1. Gita Shrestha and Nanda Bahadur Singh.2021. Pattern of Beta thalassemia and Other Hemoglobinopathies: A Cross-sectional Study in the Ethnic Groups of Eastern Nepal. Integrating Bilogical Resource s for Prosperity. A proceeding of the National Conference, Biratnagar, 2021, 402-407. ISBN: 978-9937-0-9013-1

2. Gita Shrestha and Nanda Bahadur Singh. 2021. Role of Red Cell Indices in the screening of National Conference o n Zoology, Kathmandu. 78-84. Isbn: 978-9937-0-9119-0

3. Gita Shrestha and Nanda Bahadur Singh, 2020. Hypochromic microcytic anemia: differential diagnosis of Heterozygous beta-thalasseia. Adhyan Journal. 3(8):36-40. ISBN: 2091-2722

4. Gita Shrestha and Nanda Bahadur Singh. 2020. Distribution of Thalassemia and Hemoglobinopathy in the Koch Rajbanshi ethnic group of Jhapa, Nepal. International Research Journal of Biological Sciences. 9(2): 35-38. ISSN 2278-3202. Online at: www.isca.in, www,isca.me

5. Gita Shrestha, Nanda Bahadur Singh,Priti Shrestha and Chitra Baniya. Detection of common β-globin gene mutation in eastern Nepal by multiplex amplification refractory mutation system polymerase chain reaction. Nepal Journal of Science & technology; 20(1): ISSN-2282-5359. (Online) 1994-1412 (print) DOI:https://doi.org./10.3126/njstN21il.4358.

Participations and Presentations

1. 2019/02/5-6 International Conference on "Sustainable Materials for Advanced Research in Technology" (SMART-2019) 2018/12/8-9. Title: Distribution of Haemoglobinopathies in East Nepal BaburaojiGolap College Sangvi, Pune India

2. 8th International Science Congress Maharishi Markandeshwar (deemed to be University) Mullana, Ambala, Haryana, India. 2021/03/13 Title: Distribution of Thalassemia Haemoglobinopathies in the Koch Rajbanshi ethnic group of Eastern Nepal

3. Province 1 Zoological Seminar. Integrating Biological Resources for Prosperity. 2020/20/20. Title: Detection of Beta Thallasemia Mutations in the ethnic groups of Eastern Nepal by Multiplex Amplification Refractory Mutation System Polymerase Chain Reaction

4. First National conference on Zoology.Biodiversity in a Changing World. 2020/11/28-30. National Health and Research 4 th National Summit of Health and Population Scientists in Nepal Council 2018/04/12. Title: Distribution of Haemoglobinopathies in the ethnic groups of Eastern Nepal National Health and Research Council.

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Short Communication

Distribution of Thalassemia and Hemoglobinopathy in the koch Rajbanshi ethnic group of Jhapa, Nepal

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Abstract

Thalassemia and haemoglobinopathies are the most frequent monogenetic haemolytic disorders worldwide. Beta-thalassemia is an emerging global health burden. The study in the different ethnic groups of Nepal regarding incidence of various types of thalassemia is scarce, despite Nepal being included in the World Thalassemia Belt. The current study was done in the Koch Rajbanshi ethnic group of eastern Nepal to determine different varieties of thalassemia and haemoglobinopathies present in them. Three hundred (300) individuals were randomly selected for screening. The screening was done by complete blood count test and microscopical study of peripheral blood smear. The positive samples were further analysed by haemoglobin electrophoresis. Out of total 300 cases 57 cases (19%) showed abnormal red cell indices. Based on the level of haemoglobin A2 and haemoglobin E levels, 26 cases with HbE heterozygote/trait and 31 cases with HbE homozygote/ disease were diagnosed. Hence it was deciphered that haemoglobin variants are a common genetic problem in the Koch Rajbanshi ethnic group.

Keywords: Thalassemia, Haemoglobin E, haemoglobin disorders, electrophoresis, Koch Rajbanshi.

Introduction

Haemoglobinopathies, the common autosomal recessive single gene disorder worldwide, fall into two main categories, the thalassemia syndrome and structural variant haemoglobin[1]. As per an estimated data by the World Health Organisation (WHO), 7% of the people in the world were carriers of various haemoglobinopathies, and 300,000 to 500,000 of the paediatric population were born with significant disorders of haemoglobin on a yearly basis and majority of them belonged to the developing nations[2].

Among the hemoglobinopathies, beta thalassemia is an emerging global health problem. The clinical manifestations of beta thalassemia are highly variant. At one end of the spectrum there area symptomatic cases with mild (silent) mutations, while at the extreme end there are cases with severe lifelong transfusion-dependent anaemia and multi-organ involvement. The treatment of affected individuals presents substantial disease burden.

In North East India, Bangladesh and South-East Asia, HbE is the most prevalent haemoglobin variant[3]. This could be a logical explanation for its high prevalence in Nepal, as India and China border Nepal. According to a WHO report on Global distribution of haemoglobin disorders there are 0.2 to 0.99 births per 1000 infants with a major haemoglobinopathy[4]. This study is the first to report the prevalence of HbE in Rajbanshis of Nepal. Rajbhanshis are an indigenous group of people that migrated to Nepal from adjacent states of West Bengal and Assam in India about 250 years ago. Anthropologists suggest them to be kith and kin of the peripheral Koch people of North East India.

Materials and methods

Study sites: The study design is cross-sectional, observational study and was conducted at three districts of eastern Nepal: - Jhapa, Morang and Sunsari. Jhapa is the easternmost district of Nepal, it lies between $26^0$48' to $27^0$47'06'' latitude and $85^0$19'14'' to $87^0$85' longitude. Morang lies between $26^0$39'59'' latitude and $87^0$29'59' longitude. Sunsari lies between $26^0$38'29'' and $26^0$50' latitude and $87^0$07'44.76'' longitude. These districts lie in the Terai belt bordered by Assam and West Bengal of the northeastern parts of India.

Methods: Priorethical consent from Health and Research Council, Kathmandu and informed consent from the participants was taken. Samples were collected randomly from both males and females between 6 to 80 years with a median age of 23 years. Samples were not collected from individuals with a history of blood transfusion within the last 1 month.

Complete Blood Count: Three hundred (300) samples comprising of 192 females and 108 males were screened for beta thalassemia. About 3-5mL intravenous blood was collected

in EDTA (ethylene diamine tetra acetic acid) vials and analyzed with Sysmex, United States of America (USA) automated cell counter for complete blood counts. The blood samples were stored at 4^0C for further investigations. The presence of microcytic hypochromic anaemia, screened positive for Thalassemia and these were further subjected to Haemoglobin Electrophoresis for confirmation of the type of haemoglobinopathy. On the other hand those with normocytic normochromic anaemia ruled out the presence of Thalassemia.

Peripheral Blood Smear: Peripheral blood smear (PBS) of each patient was prepared on spot, stained with Leishman stain and the red cell morphology was microscopically observed for supportive diagnosis of hemoglobinopathies. Based on the presence of target cells Capillary electrophoresis was performed to confirm the type of haemoglobinopathy.

Haemoglobin Electrophores: Capillary electrophoresis (Sebia Minicap flex piercing) of all the cases with low MCV and target cells were performed to confirm the Hb variant. This is widely accepted as an important diagnostic tool for the quantification of HbA in the detection of thalassemia and haemoglobinopathies[4]**.**

Results and discussion

A total of 300 cases was studied, of these 243 cases showed normal red cell indices and 57 had lowered haemoglobin concentration and MCV with presence of target cells. The haemoglobin concentration was below cut-off level (<15 dL). On the basis of this 19 (33.3%) patients had mild severe anemia (Hb 6-10g/dL) and 38(66.7%) had mild anemia (10-16g/dL) with statistical significance (Table-1). The mean haemoglobin concentration 10.56 +/- 1.019 (mean +/- SD) was observed. In these 57 cases microcytic hypochromic anemia was also evident. The mean corpuscular volume (MCV) was 70.47 +/- 11.7 (mean +/- SD) (Table-2). Target cells were seen in peripheral blood smear.

Heamoglobin electrophoresis of all 57 cases performed, confirmed haemoglobinopathy. These 57 abnormal electrograms included 2 types of cases, HbE homozygotes and HbE heterozygotes (Table-3). On the basis of the level of HbA, HbA2, HbE and HbF obtained through capillary electrophoresis, 31 HbE homozygotes and 26 HbE heterozygotes were diagnosed (Figure-1, 2,3).

Table-1: Distribution of patients on severity of anemia.

Group, Hb g/dL	No of patients	Percentage (%)
Mild severe (6-10)	19	33.3%
Mild (10 – 15)	33	66.7%

Table-2: Haematological parameters of Koch Rajbanshi Ethnic Group.

Variables	Hb g/dL	MCV fL	MCH pg	MCHC g/dl	RDW-SD
Minimum	9.0	31.0	19.0	27.0	28.0
Maximum	14.0	91.0	72.0	36.0	48.0
Range	5.0	60.0	53.0	9.0	20.0
Mean	10.561	70.474	23.123	31.667	36.696
SD	1.0180	11.6988	6.8636	3.3452	5.8120

Table-3: Haemoglobin pattern and quantification in normal adult, HbE homozygote and HbE heterozygote case.

No of cases	HbE	HbA2	HbA	HbF	Haemoglobinopathy
243	……	2.2- 3.2	96.8 - 97.8	……..	Normal
31	>90 %	>3.2 %	……	O.7 – 5.9 %	HbE homozygote
26	24 25 %	3.0 - 4.0 %	< 71%	0.3 – 0.8%	HbE heterozygote

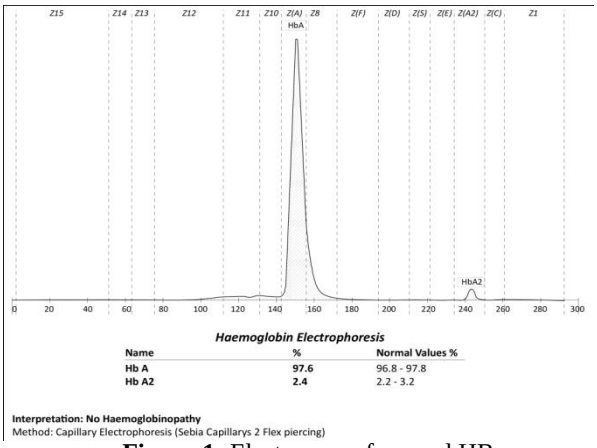

Figure-1: Electogram of normal HB.

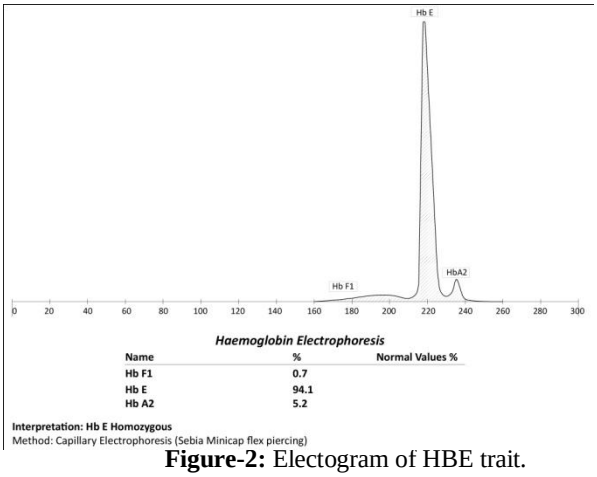

Figure-2: Electogram of HBE trait.

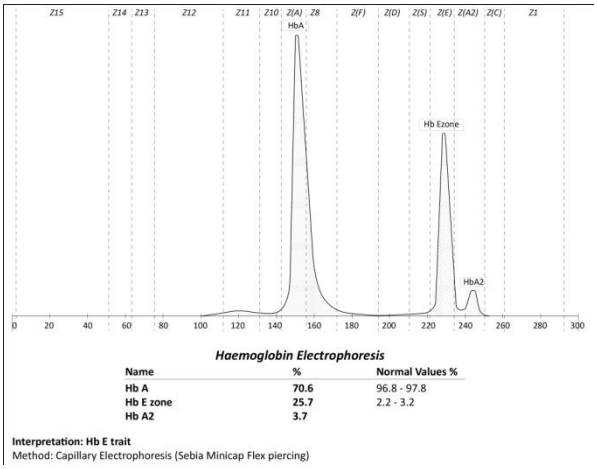

Figure-3: Electogram of HBE disease.

Discussion: The presence of thalassemia and HbE in Nepal has been reported earlier[7-9], but due to the lack of any screening programme, population based data regarding haemoglobin disorders in Nepal is scarce. In a study done by Sakai et al, thalassemia was found to be prevalent in the Danuwar and Tamang ethnic groups of Nepal. Jha et al further reported thalassemia in the Tharu community of western Nepal (prevalence of 37%). The current study done in the Koch Rajbanshi ethnic group of eastern Nepal is the first of its kind to be conducted in this geographical region. Studies done in India by Goswami et al[10] reveals that the presence of hemoglobinopathy was highest among Rajbanshis (72%) followed by Muslims (54%). In the Santhal and Oraowo, Bengali/Hindu and Marwari/Bihari approximately equal percentage (34%) of hemoglobinopathies was observed, whereas the least percentage was found in the Mongoloids like Nepali and other Hill-men populations (17.4%)[11]. Similarly in Nepal too, thalassemia is more prevalent in the Terai ethnic community as compared to the Mongoloid and Hill-men population. The similarity in the distribution amongst various communities is attributed to migration and constant mixing of people from different regions of India.

HbE the commonest structural variant in South East Asia is the second most prevalent worldwide. Das et al.[12] has reported a variable incidence of HbE in different states of north-eastern regions of India ranging from 16.2% to 47.3% reported.Others have reported an average HbE gene frequency of 10.9% in North Eastern India[13] and 25.48% in upper Assam[14]. However the frequency of HbE trait in Western India and North India was reported to be < 0.2% and 1.1% in Delhi, north India and 3.92% in Assam and 23.9% in West Bengal[15]. HbE thalassemia is a rare finding amongst the Nepalese, so far three cases have been reported in the Danuwars by Sakai et al, while two cases were reported in a district hospital in Pokhara by Bastola et al, however, in the current study 57 cases of HbE (HbE homozygote10.33% and HbE heterozygote 9%) was reported in the Koch Rajbanshis, which is a significant finding considering that the study was limited to East Nepal and Koch Rajbanshi of this area.

Conclusion

The findings of HbE gene in the Koch Rajbanshi of Nepal is being reported for the first time. This study suggests a high prevalence (19%) of Haemoglobin disorders in the study population. These findings can be applied as baseline data for future preventive measure programmes. A wider community based initiation for carrier detection and awareness generation must be seriously considered to prevent the transmission of haemoglobinopathies.

References

1. Weatherall, D.J. (2007). Haemoglobin and inherited disorders of globin synthesis. Post Graduate Haematology 5th edt. Oxforf: Butterworth Heinimann. Chapter 6. 85-103.
2. Kuhansu, W., Schimidt, K.J. (1999). Identification and clinical presentation of beta thalassemia mutations in theeastern regions of Saudia Arabia. *J Med Genet*., 36, 937-937.

3. Olivieri, N.F., Pakbaj, Z., Vinchinsky, H. et al. (2011). Hbe / beta thalassemia a common and clinically diverse disorder. *Indian J Med Res*., 134(4), 522-31.

4. World Health Organisation (2008). Management of haemoglobin disorders: report of a joint WHO-TIF meeting Nicosia. *Cyprus*, 16-18 Nov. 2007.

5. Kim, J.E. Kim, B.R. Woo, K.S. Kim, J.M. Park, J.L. Han, J.Y. (2011). Comparision of capillary electrophoresis and cellulose acetate electrophoresis for the screening of haemoglobinopathies. *Korean J. Lab Med.*, 31(4), 238-43.

6. Pranapus, S., Siricholiyakul, S., Srisopundit, K., Tongsong, T. (2009). Sensitivity and specificity of mean corpuscular volume (MCV) for screening alpha thalassemia-1-trait and beta thalassemia trait. *J Med Assoc India*., 6, 739-43.

7. Bastola, G., Acharya, R., Dhakal, N., Gupta, U.K., (2017). Study of thalassemia and haemoglobinopathies in Pokhara, Nepal. *Journal of Clinical and Diagnostic Research*., 11(11), 15-18.

8. Jha, R. (2015). Distribution of haemoglobinopathies in patients presenting for electrophoresis and comparison of result with high performance liquid chromatography. *Journal of Pathology Nepal*., 5(10), 850-858.

9. Sakai, Y. (2000). Molecular Analysis of Alpha thalassemia: co-relation with malaria endemicity. *Journal of Human Genetics.* 45(3), 127-132.

10. Goswami, B., Pramanik, R., Chakrabarty, S., Pal, P.P., Banerjee, S., Bandhopadhyay, A. (2014). Spectrum of haemoglobin variants in the population of northern region of West Bengal: An Ethnogenetic Proposition. *J Fam Med Prim Care*., 3(1), 219-233.

11. Mondal, S. K., Dasgupta, S., Mondal, S., & Das, N. (2014). Spectrum of thalassemias and hemoglobinopathies in West Bengal: A study of 90,210 cases by cation exchange high-performance liquid chromatography method over a period of 8 years. *Journal of applied Hematology*, 5(3), 91.

12. Das, B., Sengupts, S. (2008). HbE genotypes and fertility; a study on Tibeto-burmesepopulation in upper Assam, India. *Annals of human biology*., 35(4), 422-31.

13. Kalita, B. and Mehdi, S. (2016). A cross-sectional study of the Haemoglobin variants in north east India. *Indian Journal of Basic and Applied Medical Research*., 5(2), 72-79.

14. Baruah, M. K., Saikia, M., & Baruah, A. (2014). Pattern of hemoglobinopathies and thalassemias in upper Assam region of North Eastern India: high performance liquid chromatography studies in 9000 patients. *Indian Journal of Pathology and Microbiology*, 57(2), 236.

15. Mohanty, D., Colah, R., Gorakshakar, A., and Patel, R.Z (2013). Prevalence of Beta-thalassemia and other haemoglobinopathies in six cities of India. A multi-centre study. *J Community Genet*, 4, 33-42.

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

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CONFLICT OF INTEREST: None

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 smeacr, 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 mutationsIVS1-5(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. Weatherall & 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 (α2β2). 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 (βo) of β-chain synthesis and subsequent decreased production of haemoglobin (Weatherall 2001). The lowered production or absence of synthesis of two β-chains (β0/β0) 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 (β0/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 allaeles (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)
(GroupA)				
Internal control	676		11&12	CTCCTTAAACCTGTCTTGTA-ACCTTGTTAG(1)
IVS1,5(G>C)	285	1&11		TGAGGAGAAGTCTGCCGTTACTG-CCCAGTA(2)
C15(G>A)	500	2&12		TAACCTTGATACCAACCTGC-CCAGGGCGTT
C26(G>A)	301			
(Group B)		3&11		
Internal control	861	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)
				CCCCTTCCTATGACATGAACT (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.

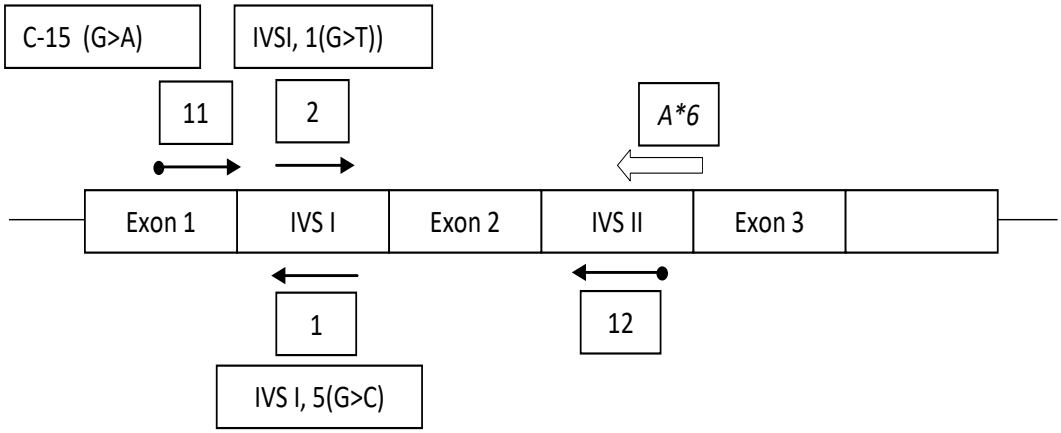

Fig. 1: Schematic diagram of group A/A* showing primers and mutations in the Beta globin gene

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).

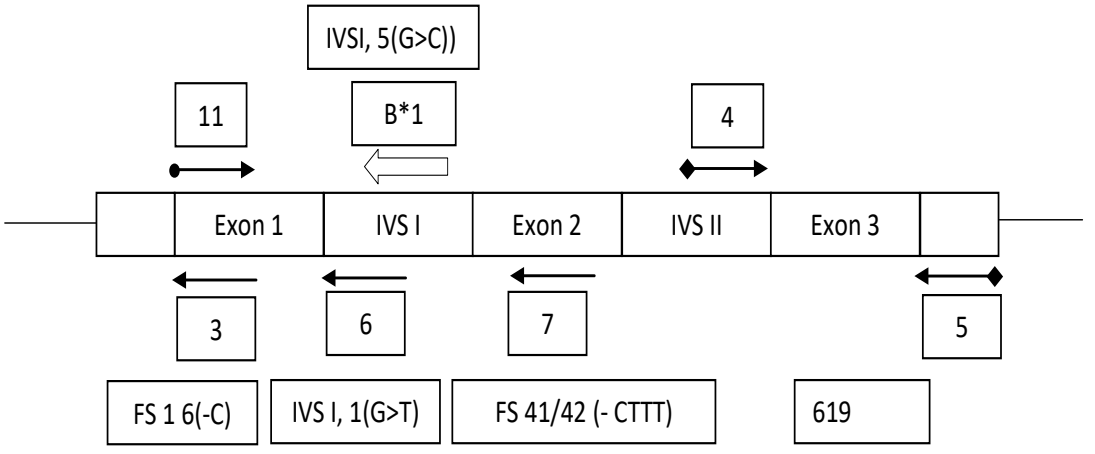

Fig. 2: Schematic diagram of group B/B* showing primer and mutations in the Beta globin gene

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.

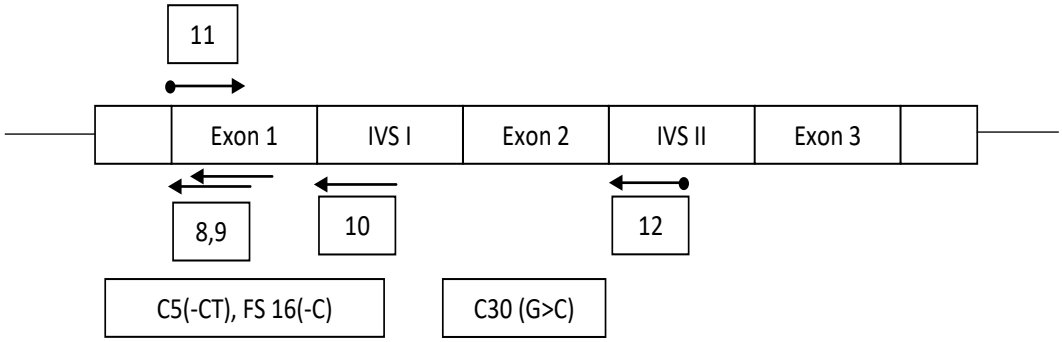

Fig. 3: Schematic diageam of group C showing primer and mutations in the Beta globin gene

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 25µL reaction mixture containing 10X PCR assay buffer containing 10mµTris HCl (ph 8.3), 1.5mµ MgCl2, 50mµ K2Cl,250 mµof each dNTP, 1 unit of Taq DNA polymerase and 0.1 nmole of each primer, 500mg 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 940C for 1 min, annealing a 650C for 1min, extension at 720C1.5 min, final extension at 720C for 3 mins. 20µL 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 geldocumentation system (Fig 4).

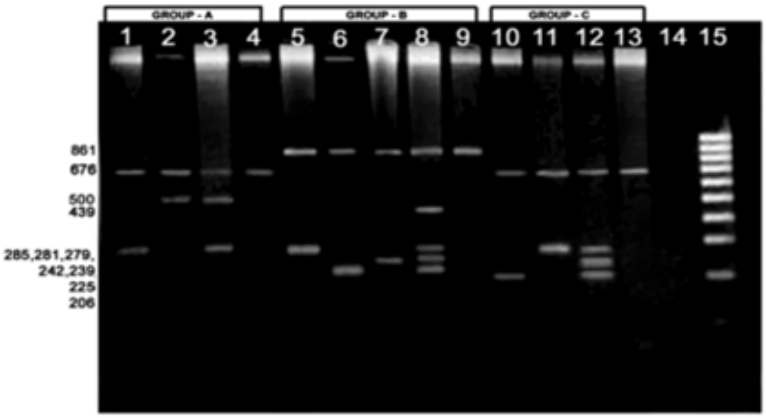

Fig. 4: Agarose gel electrophoresis pattern to detect 9 common mutations. Lanes1-4-Group A (duplex) - Lane 1: IVS1,5(G>C)-285 bp, Lane 2: C15(G>A)-500 bp, Lane 3: +ve control(2 mutations of group A), Lane 4: -ve control. Lanes 5-9-Group B
(quadruplex) - Lane 5: IVS1,1(G>T)-281 bp, Lane 6: FS 8/9 (+G)-208 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 pf 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), IVSI,5 (G>C), FS 8/9 and C-26 glu-ly . The presence two mutations {C-15 (G>A) and IVSI,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 I 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 46,735. 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 27-855 in the Indian population.

Interestingly the most common mutation reported by Mishra was absent in the present study 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 thalassemic 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. Darlinson. 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.