DETECTION OF THE PREVALENCE OF SICKLE CELL DISEASE IN THARU POPULATION OF FAR-WESTERN NEPAL BY RFLP METHOD

M. Sc. Thesis
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Submitted to
CENTRAL DEPARTMENT OF BIOTECHNOLOGY
Tribhuvan University
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Kapil Adhikari

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RECOMMENDATION

This is to certify that **Mr. Kapil Adhikari** has successfully completed his dissertation work entitled **"Detection of the Prevalence of Sickle Cell Disease in Tharu Population of Far-western Nepal by RFLP Method"** under my supervision**.**

This thesis work was performed for the partial fulfillment for award of Master of Science in Biotechnology under the course code BT 621. The result presented here is his original findings. I, hereby, recommend this thesis for final evaluation.

……

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ACRONYMS

%	Percentage
µl	Microliter
APS	Ammonium Persulphate
Bp	Base Pairs
β^S	Sickle Cell Allele
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetra Acetic Acid
EtBr	Ethidium Bromide
gDNA	Genomic DNA
Hb	Hemoglobin
HbF	Fetal Hemoglobin
HbS	Sickle hemoglobin
mL	Milliliter
mM	Millimolar
NFW	Nuclease Free Water
NHRC	Nepal Health Research Council
NO	Nitric Oxide
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
RBC	Red Blood cell
RFLP	Restriction Fragment Length Polymorphism
SCD	Sickle Cell Disease
TAE	Tris Acetate EDTA
TBE	Tris-Boric EDTA
TE	Tris- EDTA
TEMED	N, N, N' N' – Tetra Methyl Ethylene Diamine
UV	Ultraviolet
WHO	World Health Organization

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ABSTRACT

Sickle cell disease (SCD) is an autosomal recessive disorder of hemoglobin (Hb) affecting individuals of common malarial region in the world. The genetic cause of SCD is a transversion mutation at 6^{th} codon (GAG→GTG) of β-subunits of hemoglobin molecule known as sickle hemoglobin (HbS). The pathological complications in SCD arise due to HbS polymerization causing vaso-occlusion and hemolytic anemia which secondarily lead to other presentations. Because of poor health education and not well defined symptoms, the management procedure is costly, tedious and sensitive for developing country like Nepal. In this study, 116 samples were randomly collected from two Tharu sub-groups (Rana and Dangaura) of Kailali and Kanchanpur districts of Nepal. Initial screening of HbS was performed by hemoglobin solubility test, out of which only 26 showed positive results. To confirm the preliminary results genomic DNA was extracted and the targeted region of *β-globin* (539 bp) was PCR amplified. Validation of the point mutation that causes sickle cell disease was performed by restriction digestion of the amplified products. Twenty five samples were confirmed to be sickle cell carrier (heterozygous with only six exhibiting sickle cell trait) and none of the sample was found to be homozygous for the disease. The frequency of HbS carrier cases in male i.e. 13/60 (21.67%) was almost similar to that of female i.e.12/60 (21.43%). The highest frequency of HbS carrier cases were from >40 years groups that is 6/23 (26.08%) and least frequency from <20 years groups that is 2 (11.11%). Though the studied Tharu subgroups were originated from different geographical area and marriage practice, these groups were restricted and the distribution of HbS gene was found on both groups of people. The distribution of HbS was found to be higher in Rana compared to Dangaura. It demands a rapid, cheap and sensitive technique to perform the screening of the individuals living in the areas of high risks. RFLP technique has been widely used for detection of sickle cell disease. It can be performed in such disease risk population before the marriage and for the prenatal diagnosis of risks couple to predict, prevent and to reduce the frequency of diseased child born in near future.

Keywords: Sickle cell disease, Tharu population, *β-globin*, hemoglobin solubility test, PCR-RFLP, Prenatal diagnosis.

CHAPTER 1

INTRODUCTION

1.1 Background

Sickle cell anemia is a severe hereditary form of anemia in which a mutated form of hemoglobin changes red blood cells into a crescent shape at low oxygen level. Every year, around 300,000 children are born with sickle cell disease (SCD); >75% being from African countries (Yue *et al.,* 2014). This disease occurs due to the single amino acid substitution i.e glutamic acid to valine (GAG→GTG) at 6^{th} codon in first exon of the *β- globin* of hemoglobin molecule. This results in formation of sickle hemoglobin (HbS), which has the propensity to polymerize during the process of deoxygenation. Polymerization of HbS distorts the normal discoid erythrocyte into crescent shape thus the disease is termed as "sickle cell disease". Sickle cell erythrocyte blocks blood vessels in the microcirculation obstructing oxygen delivery to tissues. This tends to cause series of complication- severe pain episodes from bone marrow ischemia, central nervous system strokes, the acute chest syndrome, pulmonary hypertension, bacteremia, leg ulcers, growth failure, priapism and damage to the spleen, kidneys, liver and bones, heart failure etc. (Sankaran, and Orkin, 2013; Rees, *et al.,* 2010). Infection is major cause of death in children if left untreated (Abdelazim and Widaa, 2015). SCD is distributed in almost all malarial regions with greater frequency in Sub-African country, Mediterranean country, parts of India (Steinberg, 2008; Pagnier *et al.,* 1984) and in Nepalese Tharu community (Jha, 2015; Shrestha and Karki, 2013).

Though single point mutation is critical for precipitation and polymerization of sickle hemoglobin that induces sickling and injury to Red Blood Cells (RBCs), the phenotypic effect of disease severity is not limited to RBCs only. Multiple factors are reported to complex pathophysiology of SCD. Sickle Shaped Red Blood Cells (SSRBCs) are prone to adhere to endothelium resulting in the obstruction of blood flow called vaso-occlusion and causes pain and organ damage due to creation of hypoxy condition. While injury of sickle cell RBCs results hemolysis, which scavenge the nitric oxide (NO) biology in plasma reduces vassodilution, and set of abnormality such as assist of platelet activation and an endothelial dysfunction that enhance inflammation, hypercoagulability and increases the expression of adhesion molecule (Costa and Conran, 2016).

Mutation in only one β- globin chain of hemoglobin known as heterozygous (carrier) of sickle cell disease (HbAS), has protective mechanism against malaria (Taylor *et al.,* 2012). However, homozygous (HbSS) form tends to cause disease with complex phenotypical

clinical effects as described above. SCD is inherited in autosomal recessive pattern where synthesis of HbS occurs in place of normal adult major hemoglobin (HbA). The switching of HbS is initiated in birth after silencing of fetal hemoglobin (HbF). Thus children in earlier age are asymptomatic to SCD due to predominance of HbF, but are prone to infection. The adult hemoglobin is made of two alphas and two beta globin chains ($\alpha_2\beta_2$). Coinheritance of one (heterozygous) of sickle cell allele of gene with other *β-globin* mutation (beta thalassemia, HbC, HbD and HbE) called sickle cell disease define various degree of clinical severity and difficult to diagnosis (Frenette and Atweh, 2007). Thus accurate diagnosis of different forms hemoglobin is essential. Taking regular medicine, vaccination, blood transfusion and bone marrow transfusion therapy reduces the complication and increase the life span of the patients.

1.1.1 Tharu people of Nepal

Tharu peoples are one of the major indigenous ethnic groups of Nepal. They are distributed in the northern part of inner Terai with a dominated population in the middle and western regions of Nepal. According to the national population and housing census report 2011, the population proportion of Tharu is 6.56% and they were kept in top 4th position after Magar (National Population and Housing Census 2011, National Report). Majority of them live in rural area and are far from basic needs. They have their own language and culture with practice of endogamous marriage. Terai is malarial zone and settlement only has scattered by Tharu population before conducting malarial eradication program. Despite the Tharu people have long history being lived in malaria prevalent region, the incidence of malaria is very low as compared to other ethnic groups of people. It is believed that Tharu people have developed malaria protective gene as selective pressure from malarial infection (Ødegaard, 1997). The most common of such gene are sickle cell hemoglobin, β-thalassemia, glucose-6-phosphate dehydrogenase deficiency and alpha thalassemia gene with low frequency.

1.2 Epidemiology

A report of a systematic analysis for the Global Burden of Disease Study 2015 reported 4.4 million people have sickle-cell disease and 404 million have sickle-cell trait (Vos *et al.,* 2016). And majority of disease infants born in less developed country like Africa, India leads to significant mortality rates before five year (Aygun and Odame, 2012). Such disease associated with high morbidity and mortality leads to significant impact on social and

economic life so it has been recognized as global public health problem by the United Nations General Assembly (World Health Organization, 2006).

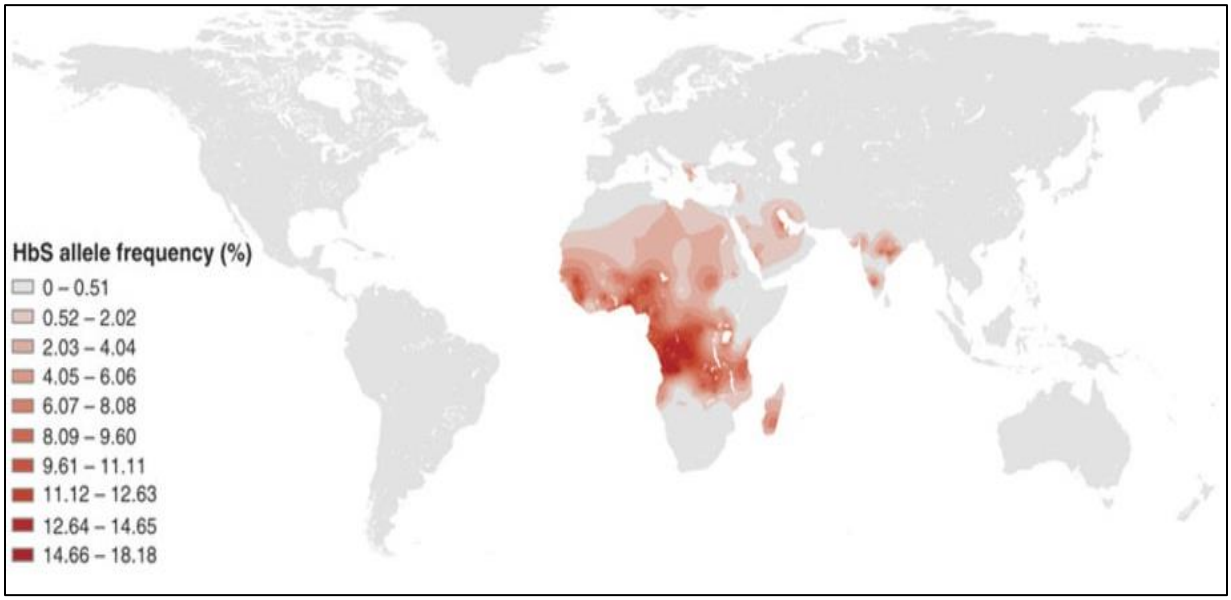

Figure 1.1: Global distribution of sickle hemoglobin allele frequency (adopted from Piel *et al.,* 2010).

Study shows that the epicenter of sickle cell disease is Africa. The incidence of the sickle cell trait in Cameroon, the Democratic Republic of Congo, Gabon, Ghana, and Nigeria ranges from 20% to 30%. Similarly in some parts of Uganda, the prevalence is up to 45% (Afolayan and Jolayemi, 2011). The frequencies of HbS are distributed up to 10 % both in the eastern and western coastal populations of Saudi Arabia (Lehmann *et al.,* 1963; El-hazmi and Warsy, 1993; El-hazmi and Warsy, 1987). In the United Arab Emirates and Oman the prevalence of SCD has reported to be 0.07% and 0.2% (Al Hosani, 2005; Al-Riyami and Ebrahim, 2003). The prevalence is also reported from the autochthonous populations of Iran and Pakistan (Farzana *et al.,* 1975; Rahgozar *et al.,* 2000).

The historical study suggests sickle cell carrier frequency ranges from 5 to 34 % among the isolated tribal populations in India (Shukla and Solanki, 1958; Balgir, 2006; Rao, 1988; Tewari and Rees, 2013; Colah *et al.,* 2014). It is thought that introduction of such mutation into Southern India may have been occurred through the migration of Dravidians from Nubia i.e. an ancient region of northeastern Africa (Winters, 2008).

1.2.1 Prevalence of sickle cell disease in Nepal

Although SCD is highly spread in Nepal, there is no solid data how many peoples are affected. Several hospital reports and other study state the disease is most prevalent among the Tharu people (Adhikari *et al.,* 2003). The SCD is found to be prevalent in (Chaudhary) sub-group and the Rana sub-group of Tharu frequent in Western Terai region (Shrestha and Karki, 2013).

1.3 Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR- RFLP)

Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (RFLP) based analysis (also known as Cleaved Amplified Polymorphic Sequence (CAPS), is a popular technique for genetic analysis that is useful for the detection of intraspecific as well as interspecific variation. The technique exploits Single Nucleotide polymorphisms (SNPs), Multi- Nucleotide polymorphisms (MNPs) and microindels that are often associated with the creation or abolishment of restriction enzyme recognition site (Narayanan, 1992). The first step in PCR-RFLP analysis is amplification of a fragment containing the variation followed by treatment of amplified fragment with an appropriate restriction enzyme. Since the presence or absence of the restriction enzyme recognition site results in the formation of restriction fragment of different sizes, allele identification can be done by electrophoretic separation of these fragments (Rasmussen, 2012).

Bsu36I is a type second restriction endonuclease and isoschizomer of restriction enzyme Mst II. Its restriction site is CC$^{\downarrow}$TNAGG. It has been produced in an *E. coli* strain that carries the cloned *Bsu36I* gene from *Bacillus subtilis* 36 (B. Zhou) (Husain *et al.,* 1995).

1.3 .1 Advantages of PCR-RFLP

1. Inexpensive
2. Easy to design
3. Applicable to analysis of SNPs as well as microindels
4. No requirement for expensive instruments
5. No requirement for extensive training of laboratory staff
6. Miniaturisable

1.3.2 Disadvantages of PCR-RFLP

1. Requires that a variation generates or abolish restriction enzyme recognition site
2. Some restriction enzymes are expensive
3. The exact genotyping can be achieved in the event that there is more than one nucleotide variation in the restriction enzyme recognition site
4. Requires relatively large amounts of hand on time.
5. Long time from start to completion of the analysis.

1.4 Rationale

Sickle cell disease is major public health problem of our country, Nepal. Several case studies and news reports state that SCD is common to Tharu community (Shrestha and Karki, 2013; Jha, 2015). Complex pathophysiology of SCD, phenotypically normal asymptomatic trait, common practice of endogamous marriage, lack of proper health education and lack of screening programs are the major cause for the high prevalence of SCD. There is limited data on the status of sickle cell disease in our country. Socio-economical, environmental factors, epistasis genes such as co- inheritance with alpha thalassemia, hereditary persistence of fetal hemoglobin gene also takes part in clinical phenotype. As this disease will inherit in autosomal recessive pattern, it is difficult to prevent easy manner. Taking regular medicine and regular blood transfusion therapy can increase the life expectancy of diseased patient but is costly process and may not be feasible for economically poor people. Molecular studies like PCR-RFLP is very useful in premarital screening program in disease population, prenatal screening of risk couple and neonatal screening of children which minimize the severe complications of the disease and also increasing the life expectancy. This study may be baseline for detecting SCD individuals, even carrier individuals in most frequent and rural area of Far-Western region that might help in future management of the disease and may also assist in predicting the frequency of HbS distribution.

1.5 Objectives

1.5.1 General objective

To detect the prevalence of sickle cell disease in Tharu population of Far-western Nepal.

1.5.2 Specific objectives

- To perform hemoglobin solubility test.
- To extract gDNA and perform PCR amplification of targeted region of *β-globin*.
- To perform Restriction Fragment Length Polymorphism of amplified product by Bsu 36I.
- To determine frequency of distribution of SCD based on socio demographic parameters.
- To determine the association between genotype and phenotype.
- To calculate gene frequency and allele frequency of sickle cell disease.

1.6 Research hypothesis

The distribution of HbS is common among the Tharu population of Far-western Nepal as they are living in malarial zone with the common practice of endogamous marriage.

CHAPTER 2

LITERATURE REVIEW

2.1 History of Sickle cell disease

There are no clear historical reports elucidating the discovery of sickle cell disease. At the middle of 18th century (1846), SCD was reported on adult African slaves struggling for life and death. While at the end of 19th century, it was revealed that individual with SCD acquires immunity against malaria (Ballas, 2015; Ballas *et al*., 2012). The first peculiar, elongated and sickle shaped red blood cells have been reported jointly by Ernest E. Irons and Professor James B. Herrick in 1910 during microscopic examination of human blood. The term "sickle cell anemia" was first used by Verne Mason (Mason, 1922; Savitt and Goldberg, 1989; Serjeant, 2010). In 1949, it was reported that an abnormal electrophoretic mobility of structural variant of hemoglobin molecules elucidate sickle hemoglobin (HbS) (Pauling *et al.,* 1949). The molecular cause for the SCD was first discovered by Ingram in 1956. He reported substitution of glutamic acid by valine at sixth codon of β-globin chain of hemoglobin (Ingram, 1956). During the period of 1960 to 1970 the mechanism of abnormal deoxy HbS polymerization and its various patho-physiological schemes were extensively elucidated (Bunn and Forget, 1986).

2.2 Structure and function of red blood cells

The major constituents of the human blood are Red blood cells (RBCs), white blood cells, platelets and plasma; each with specified function. Approximately RBC constitutes 42% of women's and 45% of men's total blood volume (red cell mass). A normal matured RBC is a biconcave-shaped flexible disc with a diameter of 7um. The biconcave shape is maintained by a network of proteins, one of which is spectrin, which attaches to the cytoplasm face of the plasma membrane. This spectrin net is deformable and thus allows the RBC to bend flexibly over itself without damage to its own structure or function. A significant property of RBC is its ability to distort its shape and can pass through small blood capillaries which are narrower than its own diameter. RBC is capable of regaining its original discoid shape once the narrow channels have been traversed. Thus under normal conditions, it is responsible for transporting of oxygen molecule to all parts of body efficiently. However, a genetic mutation of hemoglobin, such as sickle cell hemoglobin, alters the cell's ability to perform this vital function (Oni, 2007).

The depression on each flat surface of the RBC gives it a thinner central cortex with a thicker and denser outer ring. The central depression in RBCs results by losing its nucleus during its development in the bone marrow, from the immature nucleated cell to the mature enucleated cell, the cell mass caves in on itself to occupy the central space which was originally occupied by the nucleus. This central depression provides the RBCs to a wider surface area, promoting the cell's capacity to transport oxygen and maintain its flexibility. RBCs are also the main contributors to the blood viscosity. Where there is increase in viscosity, blood flows more slowly, conversely if there is decrease in viscosity blood flows more rapidly. This has implications in the biological and pathophysiological changes which occur in the red blood cells of those with SCD.

2.2.1 Hemoglobin structure and function

Hemoglobin constitutes one third of the cell mass and volume of RBC (Marieb, 2001). Hemoglobin contains heme-proteins. Its major function is to transport oxygen. As in other vertebrate organisms, human hemoglobin is found in high concentrations in erythrocytes (~640 million molecules per cells) (Shikama, 2006).

During the different course of human evolution, diversity of hemoglobin protein was evolved but it contains higher conserve region so molecular structure are very much similar with each other. The total hemoglobin protein is about 64,500 Daltons and is globular in shape formed by two pairs of polypeptide chains.

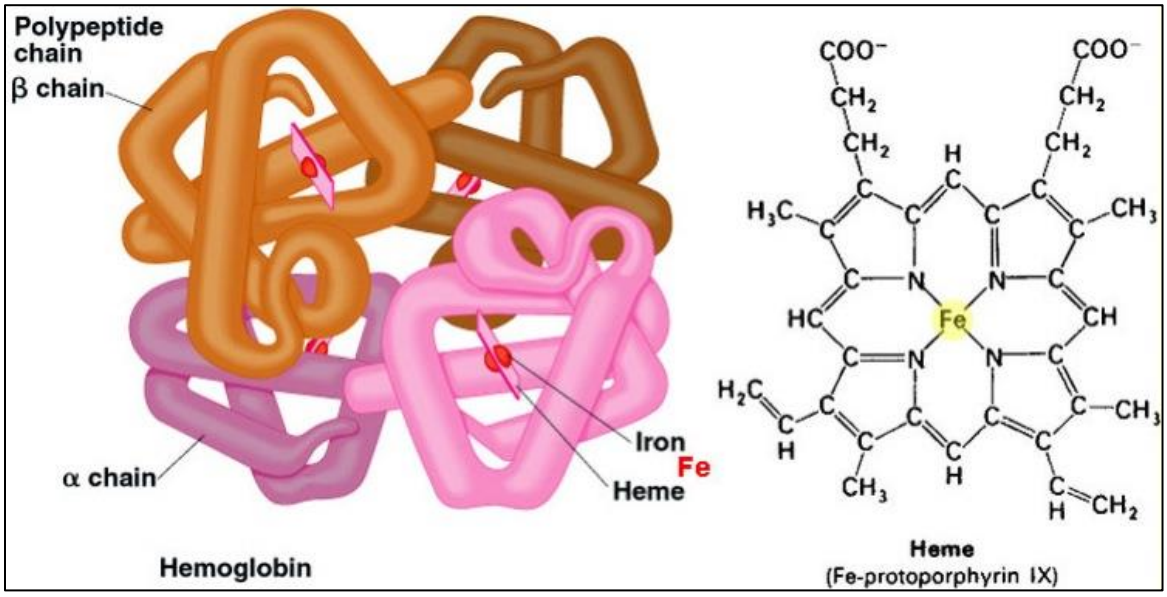

Figure 2.1: Structure of hemoglobin molecule

One pairs of alpha (α) like (α or ζ) chain located on chromosome 16 (Costa and Conran, 2016) and another pair of beta-like structure (ε, γ, δ or β) globin chain located on chromosome 11. All chains are linked with heme prosthetic group that has a tetrapyrrole ring (protoporphyrin IX) with a central ferrous atom ($Fe^{2+)}$ which can reversibly bind to one molecule of O_2 to transport it from lungs to tissues (Jorge *et al.,* 2016).

2.2.2 Structural organization of globin gene cluster

The formation of functional hemoglobin is by proper arrangement of two pairs of globin genes located on two different chromosomes as shown in figure 2. The beta globin gene cluster contains 5' Locus control region (LCR), similarly alpha globin gene cluster possess 5' HS region. The upstream elements, promoters, other regulatory elements and transcription factors determine the expression of these genes. In beta gene cluster, five genes (ε, G^{γ}, A^{γ}, δ and β) lies 5'→ 3' are positioned on short arm of chromosome 11. They are sequentially expressed during various developmental period of human, from embryonic hemoglobin to fetal and fetal to adult hemoglobin. The β–locus control region (β –LCR) is necessary for the high level of expression of those genes as shown in figure **2 A**. The genes of α -globin gene cluster has located on chromosome 16 (ζ, α_1, and α_2), also expressed in the same order as like β globin cluster genes, according various developmental period. HS-40 is a major regulatory element located far upstream of the genes of the α-cluster that is necessary for their high level of expression as shown in figure **2 B** (Bank, 2005).

The adult hemoglobin switching refers to the developmental process that leads to the silencing of γ globin gene expression HbF ($\alpha_2\gamma_2$) and the reciprocal activation of adult β-globin gene expression HbA and HbA_2 as shown in figure **2 C**.

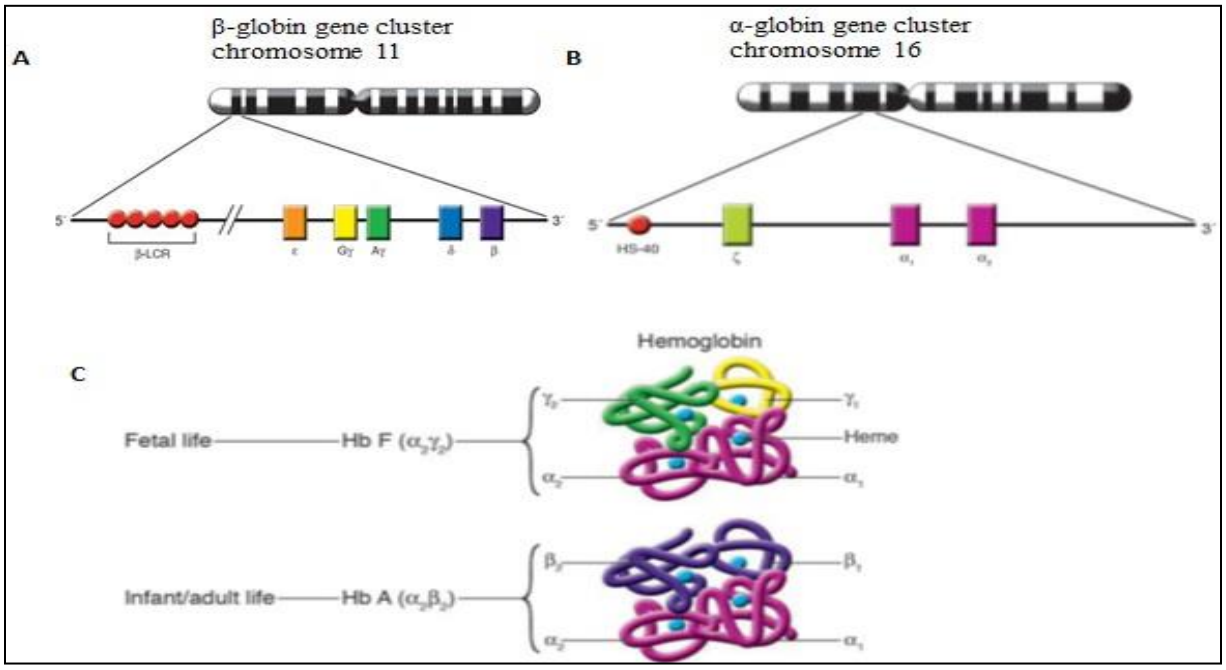

Figure 2.2: Chromosomal organization of the α- and β-globin gene clusters. **(A)** The genes of the β-globin gene cluster (ε, G^{γ}, A^{γ}, δ and β) and **(B)** The genes for α -globin gene cluster (ζ, α_1, and α_2) and **(C)** Fetal hemoglobin Hb F ($\alpha_2\gamma_2$) and adult hemoglobin HbA ($\alpha_2\beta_2$).

2.2.3 Hemoglobin synthesis

The α like globin chain contains 141 amino acids residues while β-like globin have 146 amino acids residues. The differential expression of α like and β-like chain and their arrangement define functional hemoglobin molecule.

Table2.1: Hemoglobin outline in healthy adult (Hoffbrand and Moss, 2011)

	HbA	HbF	HbA_2
Globin chains	$\alpha_2\beta_2$	$\alpha_2\gamma_2$	$\alpha_2\delta_2$
Normal range in (%)	95-98%	Up to 2.0%	2.0-3.0%

During the course of developmental period in human, hemoglobin undergoes various changes. The synthesis of Hb protein is initiated after third week of pregnancy within vitelline sac as primitive erythroblast that produce embryonic hemoglobins Grower1 ($\zeta_2\varepsilon_2$), Hb Gower 2 ($\alpha_2\varepsilon_2$), Hb Portland I ($\zeta_2\gamma_2$) and Portland II ($\zeta_2\beta_2$). These embryonic hemoglobins are replaced by fetal hemoglobin HbF ($\alpha_2\gamma_2$) after tenth week of pregnancy. At this stage of life, hemopoiesis occurs at the fetal liver and spleen. HbF becomes predominant during

entire fetal ages and its expression continues till 6th month after childbirth. While in adult human life, the major site of hemoglobin synthesis or erythopoiesis is bone marrow. These hemoglobins are of two types: adults major hemoglobin; HbA ($\alpha_2\beta_2$) and minor adult hemoglobin; HbA_2 ($\alpha_2\delta_2$). Even synthesis of HbA and HbA_2 initiates before birth, are efficiently expressed after 6th months of childhood. In healthy adult human, HbA make 95% as major hemoglobin and HbA_2 constitutes 2-3% as minor hemoglobin. While HbF <2% as residual Hemoglobin that is summarized in table 2.1(Makani *et al.,* 2013).

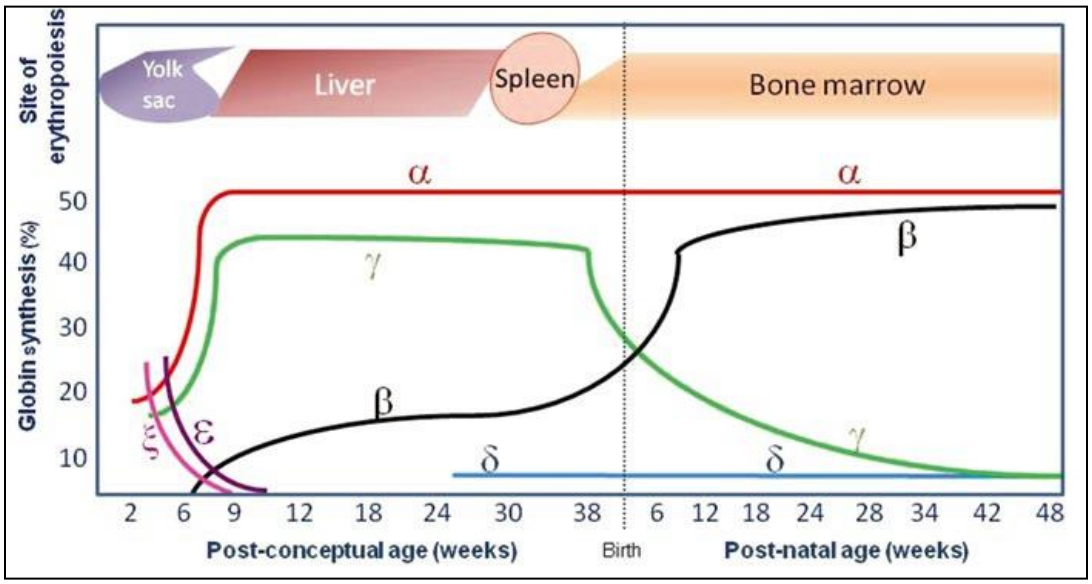

Figure 2.3: The timeline of human globin gene expression (Adopted from Hoffbrand and Moss, 2011).

2.2.3.1 Beta globin subunits

The β- globin subunits position to 3' site of β- globin gene cluster on short (P) arm of human chromosome 11 (11p 15.5). The total genome of *β globin* consists of 1606 base pairs (bp) nucleotides sequences whereas mRNAs transcripts consists of 626 bp nucleotides sequences including three exon two introns, 5'-UTR and 3'-UTR. The encoded protein is of 444 bp on β globin chain (Fagerberg *et al.,* 2014).

2.2.4 Common structural variants of hemoglobin gene

About 700 structural variants of Hb molecule have been identified but not all of them are clinically significant. The most common clinically significant structural variants are HbA, HbA_2, HbS, HbC, HbF, HbD, and HbO (Serjeant, 1992). Most of these variants arises single or

few amino acid substitutions as a result of point mutation, duplication and deletion of nucleotides.

Table2.2: Summary of single point mutation in β-globin gene responsible for clinical phenotype. β-thalassemia results from >300 mutations so is not included in this table.

Abnormal structural hemoglobin variants	**Amino acid residue change in β-globin subunits**
HbS	Glutamate to valine (GAG→GTG) 6th codon
HbC	Glutamate to lysine (GAG → AAG) at 6th codon
HbE	Glutamate to lysine (GAG → AAG) at 26th codon
HbD Punjab or Los Angeles	Glutamate to glutamine (GAG → CAG) at 121 codon
HbO Arab	Glutamate to lysine (GAG → AAG) at 121 codon

SCD is used to describe a wide range of hemoglobinopathies that are characterized by HbS in the presence of another variant beta globin chain. The majority of SCD cases are composed of four primary subtypes: HbSS, HbSC, HbSβ^0, and HbSβ$^+$ (Serjeant and Serjeant, 1992) as shown in Table 2.2.

2.3 Genetics of SCD

Sickle cell disease (SCD) is a hereditary hemoglobinopathy consequence from inheritance of a mutant form of the *β-globin* (HBB) on chromosome 11.5. This gene codes for assembly of the β- globin subunit of adult hemoglobin protein (HbAA). The mutant β-allele (β^S) codes for the production of the abnormal hemoglobin variant, known as sickle hemoglobin (HbS). The sickle cell disease is due to a transversion mutation in the 6th codon at the first exon in the β^A (HBB) gene that replaces adenine nucleotide with thymine (GAG→GTG) (rs334) (Marotta et *al.,* 977; Tamar *et al.,* 2014). Homozygous form of the sickle mutation (i.e., HbSS disease) is responsible for the most common and most severe variant of SCD, also known as sickle cell anemia. Individual carrying only a single copy of the HBB or *β-globin* (rs344) polymorphism (HbAS) generally asymptomatic often called sickle cell trait (Firth and Head, 2004). In carriers, each RBCs contains 30–40% HbS, polymer is not present under most conditions. Thus most of the cases carriers have normal life span and complication may

develop during vigorous exercise like military training, long time swimming and prolong spend on low oxygen environment like climbing mountain of high altitude, sickle cells can be found in the venous circulation (Martin *et al.,* 1989).Co-inheritance of one sickle cell gene and its interaction with abnormal hemoglobin gene that would results clinical complications is called sickle cell disease (SCD).

The common SCD are $S\beta^0$ thalassemia, $S\beta^+$ thalassemia, HbSC, HbSD, HbAD and HbSE. The disease is monogenic. By contrast, the phenotype of sickle cell anemia is multigenic (Chui and Dover, 2001) i.e. other genes or factor, unlinked to the β-globin locus contribute in relevant pathological events (e.g., rapid destruction of sickle cells, dense cell formation and adhesion to endothelium) that are controlled by many genes, known as pleiotropic or secondary effector genes (Stuart and Nagel,2004). Severity of sickle cell disease varies greatly among individuals, since not all disease individual have identical pleiotropic genes. Some carriers have mutated genes that can either ameliorate or exacerbate the phenotype.

2.4 Pathophysiology of SCD

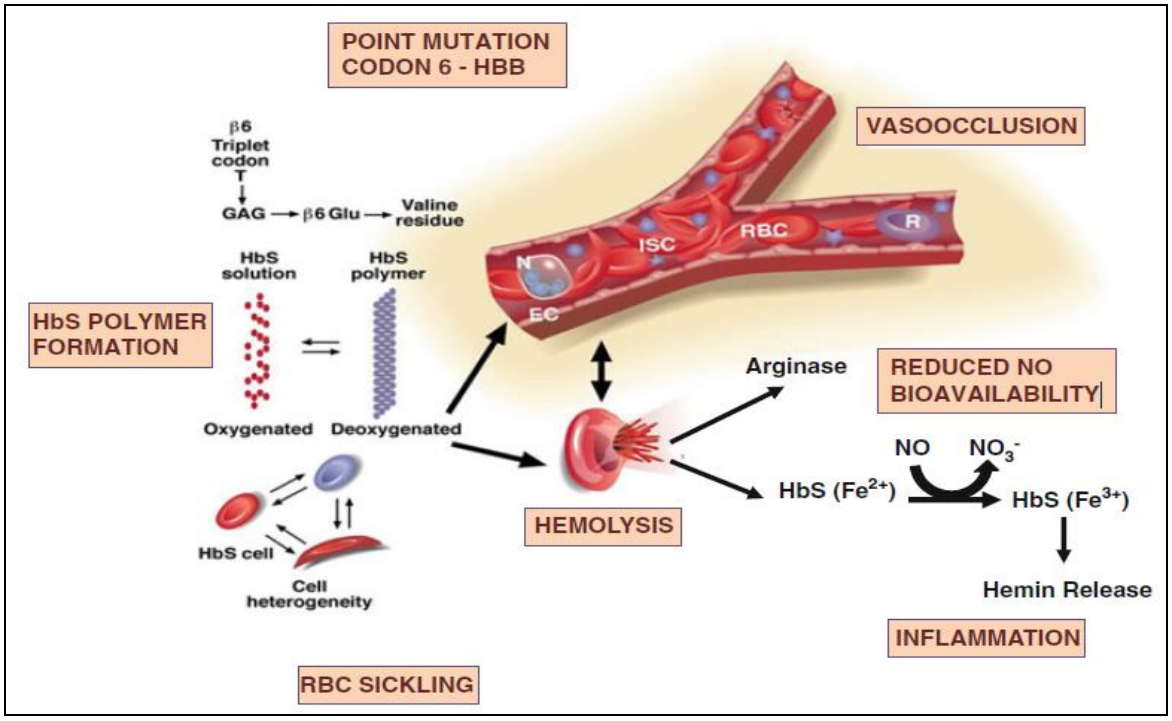

Figure 2.4: The pathophysiology of sickle cell disease (Adapted from Steinberg, 2006). EC = endothelial cell; N = neutrophil; R = reticulocyte; RBC = red blood cell, ISC = irreversible sickle cell).

The adenine (A) to thymidine (T) point mutation at codon 6 in the HBB (*β-globin*) substitutes a valine for the normal glutamic acid. This single and "simple" change leads to the synthesis of HbS that has the nearly unique property of polymerizing when it is deoxygenated. The deoxyHbS polymer is formed by homogeneous and heterogeneous processes of nucleation (Ferrone *et al.,* 1985; Eaton and Hofrichter, 1974). As sickle erythrocytes enter the microcirculation and an ambient oxygen tension falls, it creates molecules of deoxyHbS that have a quaternary structure which differs from oxygenated sickle hemoglobin.

The hemoglobin polymer formation is dependent on HbS concentration, pH, oxygen saturation and temperature (Eaton and Hofrichter, 1987). The hydrophobic site of the HbS mutation finds a properly registered hydrophobic receptor in another molecule forming a double strand. The lateral contacts of the fiber are the most crucial for polymerization where the β6 valine is within a hydrophobic pocket formed by β88 leucine, β85 phenylalanine and several heme atoms. As the densely packed deoxyHbS molecules collide, they interact and nucleate, rapidly growing into a structured polymer, with seven pairs of elementary fibers called homogenous polymerization. The exponential growth of the polymer formation needs certain times to be primed, called "delay time". DeoxyHbS polymer injures the erythrocyte and leads to a heterogeneous population of sickle cells with a damaged membrane. In the vasculature, sickle cells interact with endothelium and other blood cells leading to vaso-occlusion. If the microcirculation is successfully traversed, the return of the cell to the lungs and its exposure to high oxygen tensions allow the HbS polymer to melt. Cycles of polymerization and depolymerization of HbS cause irreversible damage to the sickle erythrocyte membrane cytoskeleton, accounting for the irreversibly sickled cells seen in the peripheral blood (lux *et al.,* 1976).

Damaged erythrocytes are short-lived and while most hemolysis is extravascular and 10–30% of hemolysis occurs intravascularly releasing hemoglobin into the plasma. Hemoglobin scavenges NO that acts as muscle vasodilation. Reduced endothelial NO bioavailability impairs the homeostatic vascular functions like inhibition of platelet activation and aggregation and transcriptional repression of genes transcribing cell adhesion molecules. Hemoglobin, hemin (or heme), and heme iron catalyze the production of oxygen radicals and protein nitration, which further limits NO bioavailability. Lysed erythrocytes also liberate arginase, which destroys L-arginine, the substrate for NO production, providing another mechanism for endothelial NO deficiency. Hemin is released from ferric hemoglobin (Fe^{3+}) and promotes inflammatory and oxidative effects (Steinberg, 2006).Multiple cellular molecules can account sickle erythrocyte prone to adhere

endothelium leading to vaso-occlusion in the post capillary venules.These adherence takes either sickle red blood cell directly interact to endothelium via P-selectin ($\alpha_4\beta_1$,$\alpha_v\beta_3$) (Hebbel 1984, 1987) or by the sickle erythrocytes and inflammatory mediators activate endothelium like integrin and their receptors; immunoglobulin family members (VCAM-1, ICAM-4) the endothelial soluble adhesion proteins such as thrombospondin, fibrinogen, fibronectin, von Willebrand factor and other exposed membrane components such as Band3 and sulfated glycolipids(Costa and Conran, 2016; Hebbel *et al.,* 2004).

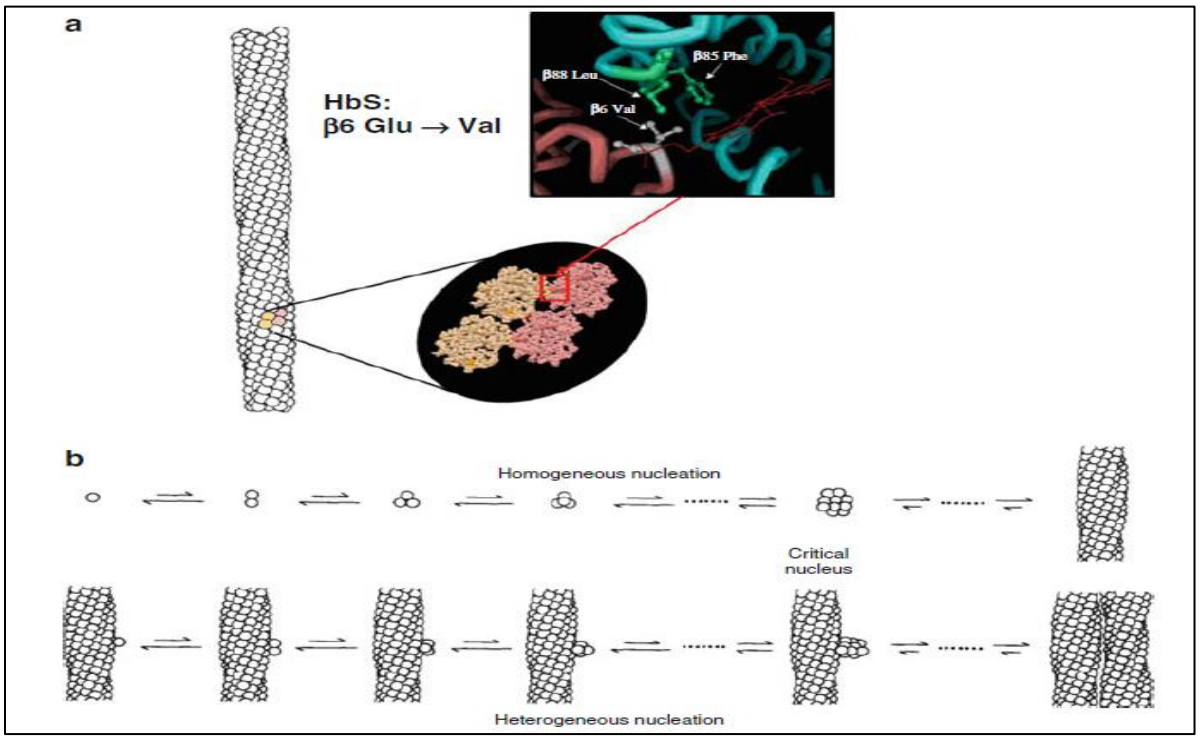

Figure 2.5: Process of HbS polymer formation (a) The 14-strand HbS fiber which is the basic unit of the HbS polymer. (b) The homogeneous and heterogeneous 2-phase model for sickle polymer growth (Costa and Conran, 2016).

Continuous cycle of polymerization and melting of HbS and also injury to sickle erythrocyte membrane results in earlier removal from circulation. The life span of sickle erythrocytes is reduces to 7-14 days as compare with 120 days for normal erythrocytes. To compensate this action, the hematopoietic bone marrow increases erythropoiesis and hypertrophies while spreading into long bones. But, this compensation is partial and less than expected. That causes the anemia. As compared with other types of anemia, erythropoietin levels are inappropriately low in sickle cell anemia and decrease further as renal function deteriorates (Sherwood *et al.,* 1986).

2.5 Modifier of sickle cell disease phenotype

Various genetic and non-genetic (like climate, infection and socioeconomic) factors are modulator of SCD (Piel *et al.,* 2017). First underlying mutation resulting in polymerization of HbS molecules causes structural change of red blood cells. Although the heterozygous sickle allele has strong protective effect for reduction malarial parasite, the homozygous mutation is more severe reducing the life expanses and also causing early death if untreated and unmanaged carefully. The co-inheritance of HbS with other hemoglobin variants such as thalassemia (α and β), HbF, HbC and HbE and HbD are of global importance (Kaur *et al.,* 2013).

2.5.1 Association of SCD and α- thalassemia

Alpha thalassemia is characterized by the absence or deficiency of α-globin chain synthesis and its combination with β-chain variants decreases the concentration of abnormal hemoglobin (Baysals *et al.,* 1994; Gonçalves *et al.,* 2003). The coexistence of α thalassemia and β-globin gene cluster haplotypes has shown changes in the severity of the clinical profile of SCD patients (Goncalves, 2014). The reduced synthesis of the a α- globin chain results in changes in hematological parameters, decreasing the degree of hemolysis and cellular dehydration, and increasing the ratio between the volume and the cell membrane area of red blood cells in sickle cell patients (Takekoshi *et al.,* 1995). Studies shown that α – thalassemia inhibit polymerization of HbSS and reduces hemolysis and cellular dehydration and decreasing in vital organ damage(Steinberg, 2005; Powars *et al.,* 1990). This also reduces mean corpuscular volume (MCV), percentage of dense cells and number of irreversibly sickle cells and increasing total hemoglobin level and hemoglobin A_2 levels (Nagel *et al.,* 1989; Higgs *et al.,* 1982). The mean corpuscular hemoglobin (MCH) alters the hematological profiles of HbSS patient as compared to individuals without α-thalassemia (Ballas, 2001). In certain population the increase in survival associated with α- thalassemia has been reported (Martinez *et al.,* 1996).

Although the co-inheritance of SCD and α-thalassemia known to decreases the hemolysis but may not decrease the chances of hematocrit and blood viscosity; an another risk factor of vaso-occlusion (Steinberg, 1996).

2.5.2 Association with β-globin gene haplotype

Study suggested that β-globin gene haplotype determines the production of HbF concentration. Excess HbF makes the sickle hemoglobin more soluble in deoxygenated state and reduces the clinical severity (Paunipagar *et al.,* 2010).

Five main haplotype has been characterized that is correlated with various level of HbS production (Bitoungui *et al.,* 2015). These are defined by restriction fragment length polymorphisms (RFLPs) in the β-globin locus and are associated with variation in HbF expression (Nagel *et al.,* 1985; Nagel, 1987). Four of them are Senegal, Benin, Cameroon and Bantu haplotypes distributed in African continents and the fifth one is Arab- Indian haplotype among the Indian continent and Arabian country. They are named according to the geographical region they were first identified, and have also been correlated to differential HbF levels (Chebloune *et al.,* 1988; Nagel and Ranney, 1990).

Among African haplotype, Bantu haplotype have the lowest HbF expression and Senegal haplotype has the highest. HbSS patients with the Senegal and Saudi-Indian haplotypes have a C–T polymorphism at 158 base pairs upstream of HBG_2. HBG_2 is one of the two γ-globin genes, that is associated with higher HbF levels (Miller *et al.,* 1987; Ballas *et al.,* 1991). However there is significant intra-haplotype variation. HbSS for the Saudi-Indian haplotypes have higher HbF levels than those HbSS for the Senegal haplotype (Akinsheye *et al.,* 2011, Pembrey *et al.,* 1978). This suggests that there might other heritable factors explaining the different HbF levels.

Similarly the co-inheritance of HbAS with β^+ thalassemia or β^0 thalassemia causes significant severity than β-thalassemia alone. Their double heterozygous inheritance sometime makes diagnostic problem in clinical field. For example $HbS\beta^0$ thalassemia produces visually indistinguishable electrophoretic pattern as sickle cell anemia, but a diagnosis can often be made by the presence of higher level of HbA_2 and a decreased mean corpuscular volume. However for clear distinction it requires detailed family history and DNA-based studies (Wang and Lukens, 2009).

Study showed co-inheritance of compound heterozygous sickle allele (sickle cell allele with hemoglobin D Punjab) in India reported variable clinical manifestations of HbSD disease (Tyagi *et al.,* 2000; Panigrahi *et al.,* 2000). While earlier studies done in Iran, Pakistan United Arab Emirates and Mexico have shown that the clinical presentation of HbSD disease cases is similar to that of patients with the severe form of sickle cell anemia (Perea et al., 1999; El-Kalla and Mathews, 1997). Similarly Sickle cell disease occasionally inherited with other

hemoglobin variants such as HbE results in clinically pathogenic variants (Bender and Seibel, 2014).

Table 2.3: Genotypes and phenotypes of different sickling disorders (Frenette and Atweh, 2007)

Genotype	**Interacting genes**	**Typical clinical severity**	**% of Hb type/total Hb in a typical patient [A,B]**				
			HbS	**HbA**	**HbF**	**HbC**	**HbA_2**
HbAA	β and β	None	-	96%	2%	-	2%
HbSS	βS and βS	Severe	95%	-	3%	-	2%
HbSC	βS and βC	Mild	48%	-	3%	47%	2%
$HbS\beta^0$	β^S and β^0 thalassemia	Severe	93%	-	2%	-	5%
$HbS\beta^+$	β^S and β^+ thalassemia (severe β thalassemia mutation)	Moderate	85%	6%	5%	-	4%
$HbS\beta^+$	β^S and β^+ thalassemia (mild β-thalassemia mutation)	Mild	70%	23%	3%	-	4%

[A]"Typical" refers to the most common presentation of a particular sickling genotype. It should be noted that in many patients, the genotype does not accurately predict the clinical phenotype. [B]Assessed by gel electrophoresis. Hb A_2, minor adult hemoglobin.

2.6 Inheritance of sickle hemoglobin

Sickle cell disease is inherited in autosomal recessive pattern that means at least one sickle hemoglobin gene (abnormal hemoglobin gene) should inherits from each parent. If an individual has one sickle gene and one normal gene then he/she is considered to have sickle cell trait or a carrier for SCD. If both parents are carriers for SCD then the inheritance chance will be 25% or (1/4) having homozygous sickle gene (Sickle cell anemia), 25% normal and 50% carrier trait in each pregnancy.

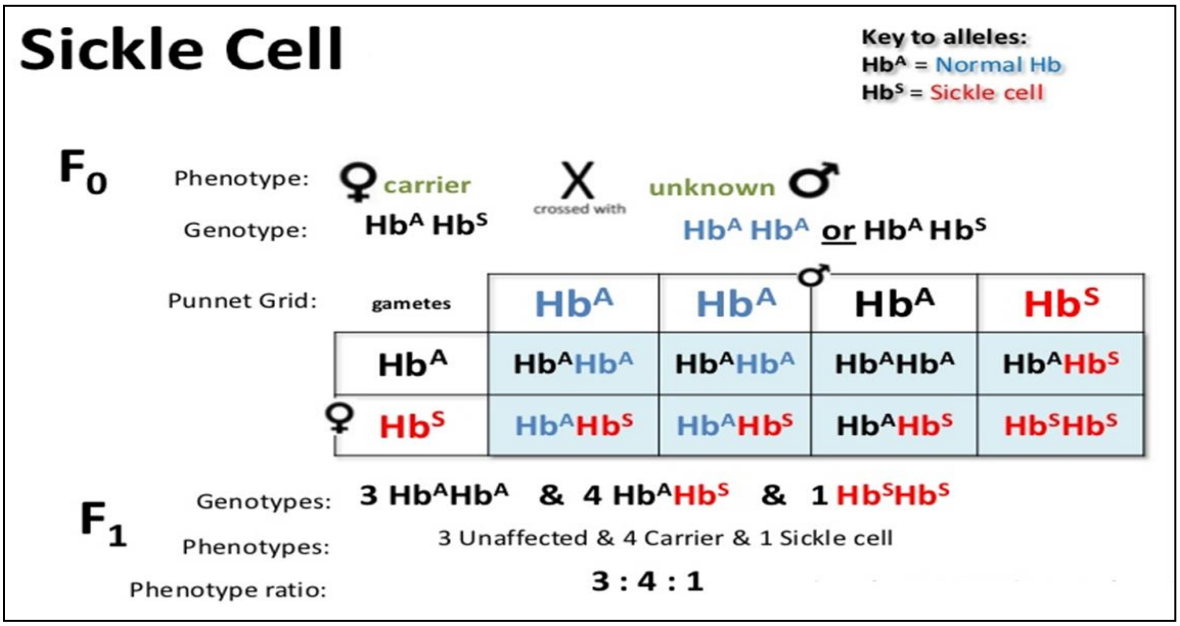

Figure 2.6: Schematic diagram of autosomal recessive inheritance pattern of SCD (https://www.slideshare.net/gurustip/theoretical-genetic).

2.7 Mechanism of malaria protection

Although it is known that the HbAS protects from malaria but the exact and full mechanism is still unclear. This may be due to a combination of mechanisms (López *et al.,* 2010; Gong e*t al.,* 2013; Bunn, 2013) as described below.

2.7.1 Enhanced Elimination of Parasitized Red Blood Cells

Parasitized heterozygous sickle RBCs has 2-8 times chances of sickling (Cholera *et al.,* 2008). This may enhance phagocytosis of infected RBCs and, consequently, decreases in parasitemia (Luzzatto *et al.,* 1970; Roth *et al.,* 1978; Ayi *et al.,* 2004). This process predominantly affects red blood cells having small parasite forms compared to larger schizon and trophozoite.

2.7.2 Decreased Rosette Formation

Non-infected RBCs can bind to infected RBCs with malarial parasites, a process called rosette formation, which adds to microcirculatory obstruction. Carlson *et al.,* 1994 has reported that the mechanical properties of modified RBCs containing HbS under deoxygenated state results in a declined ability to form rosettes. Because of this mechanism it could be mainly protective against malaria (Carlson *et al.,* 1994).

2.7.3 Reduced Cytoadherence

The parasite infected RBCs possess a specific membrane protein on their surface. For example P. falciparum erythrocyte membrane protein 1 (Pf EMP-1) enhance *P. falciparum* - infected red blood cells to adhere to the microvasculature endothelium, the process known as sequestration, and therefore it disturbs during clearance from the circulation by the spleen (Fairhurst *et al.,* 2012). Sequestration enhances endothelial activation and associated inflammation in the brain and other organs, and thus the progression of severe malaria. Reduced amount of Pf EMP-1 has been found in HbAS red blood cells in comparison to HbAA red blood cells, and to be associated with reduced binding properties (Cholera *et al.,* 2008; Opi *et al.,* 2014). It is possible that reduced cytoadherence of homozygous and heterozygous sickle hemoglobin of mature RBCs leads to increased splenic clearance, and may in part explain lower parasite densities and a lower manifestation of severe malaria in HbAS individuals.

2.8 Diagnosis of Sickle Cell Disease

The laboratory test of SCD includes the tests for the presence of sickle hemoglobin HbS and absence or considerable reductions of normal hemoglobin HbA along with the variation of other hemoglobin as - HbF, HbA_2 inside the RBCs.

2.8.1 Screening Tests

The generally used cost effective screening test is sodium meta-bisulphite sickling test. A thin film of blood under reduced oxygenated state is observed on light microscope. Another is Kleihauer Betke test used to characterize coexistent of hereditary persistence of fetal hemoglobin (HPFH) with sickle cell disease. Next, hemoglobin solubility test is used to check the presence or absence of HbS (Bender and Seibel, 2014).

2.8.1.1 The hemoglobin solubility test

The hemoglobin solubility test is a simple preliminary diagnostic test that can be performed in patients suspected of having a sickle cell syndrome. Hemoglobin solubility test is used to screen relative insolubility of deoxygenated HbS in solutions of high molarity phosphate buffer. It is based on the principle the HbS hemolysates precipitated in the test solution while other hemoglobins remain in solution. Sodium dithionite ($Na_2S_2O_4$) is used as reducing agent that rapidly reduces oxyhemoglobin to reduced hemoglobin, so it can be used to test erythrocytes for sickling. Dithionite ion tends to decompose to thiosulfate and

sulfite with formation of hydrogen ion, decreasing the pH. This favors the polymerization of RBCs.

The HbS in its homozygous and double heterozygous (with other hemoglobins, such as, HbC or HbD, or with thalassemia) form interferes the synthesis of normal hemoglobin. These different qualitative outcomes allow for the detection of sickle cell disease and its traits.

Different names of kits are available to test sickle hemoglobin such as Sickledex, Sickleprep, or Sicklequik.

The main advantage of the solubility tests is: low-cost and rapid screen for the presence of HbS prior to perform definitive testing; and emergent estimation of whether a clinically significant hemoglobinopathy exists or not (if combined with a complete blood count CBC, blood smear, and reticulocyte count).

And limitation of hemoglobin solubility test as: The false positives results may show in patient with erythrocytosis, hyperglobulinemia, extreme leukocytosis or hyperlipidemia. Coarse flocculation may occur in these samples due to elevated levels of total serum protein. False negatives or false positives may occur in patients with severe anemia (<15% hematocrit), in patients with a recent blood transfusion. The rare sickling hemoglobin subtypes such as HbC Harlem or HbC Georgetown also gives same results as sickle hemoglobin thus the chance of misdiagnosis exists (e.g., HbSC) (Fabry *et al.,* 2003).

Screening tests does not confirm homozygous sickle anemia from heterozygous carrier of Sickle cell phenotype (HbSS /HbAS/HbSC/HbSβ-thalassemia) therefore additional confirmatory tests are essential for the precise diagnosis of SCD from other hemoglobinopathies. The general routine confirmatory test to detect abnormal hemoglobin can be performed by hemoglobin electrophoresis test. More advance confirmatory tests are isoelectric focusing (IEF), capillary electrophoresis and HPLC. DNA based tests exactly define the genotype of disease frequently used in clinical diagnosis purpose.

2.8.2 Confirmatory Tests

These confirmatory tests have performed because different hemoglobin protein (isoforms) possesses different overall ionic charge according to which their migration pattern also varies with different velocities in an electric field. Hemoglobin electrophoresis can be performed on alkaline or acidic conditions. Various hemoglobin molecules such as- HbA, HA_2, HbF, and HbS migrate towards the anode when an electric field is applied. They possess different charge so their mobility is different so they can be confirmed. HbF and

HbS have poor resolution at alkaline electrophoresis while at acidic condition HbF relative migrate faster than HbA and HbS therefore it can be distinguish from them. The neonates possess predominant (i.e. >80%) of fetal hemoglobin and comparatively lesser amount of the HbA or HbS, because synthesis of HbA initiate only after birth and significant amount after 6^{th} month of birth so results of hemoglobin electrophoresis will difficult to interpret until few month after birth. Also conventional hemoglobin electrophoresis requires much time and is labor expensive, unable to identify and quantify abnormal hemoglobin.

The Isoelectric focusing technique can works under the same principal of hemoglobin electrophoresis. Though it is slightly more expensive than former, it is able to identify more Hb variants. The latest technology that can accurately quantify the variants of hemoglobin level is HPLC- the cation exchange chromatography. The additional advantage of HPLC is that it can quantify Hb fractions of the SCD patients undergoing blood transfusion and hydroxyurea therapy, which are the treatment of sickle cell anemia (Makani *et al.,* 2013).

2.8.2.1 Molecular Diagnosis of Sickle cell disease

Although the solubility test, sickling technique and peripheral blood film methods are available for screening of SCD and their reliability in the demonstration of patients with SS, however they showed variability in their ability to detect the carrier state of haemoglobin (AS) (Chasen *et al.,* 1999). Even though hemoglobin electrophoresis can't be used for prenatal screening and neonatal screening in which the major Hb is HbF (Grosse *et al.,* 2011). PCR technique has been used that increased sensitivity of reaction so it has potential uses for prenatal diagnosis and confirmation of genotype in neonates (Steinberg, 1993).The generally applied diagnosis methods is designation of primer sequence adjacent to 6^{th} codon of *β-globin* and its amplification by PCR. PCR amplification of the hotspot region of DNA followed by RFLP (using specific restriction enzymes DdeI and MS II) such as has become a widely used approach. Carriers can be distinguished from homozygous wild type or homozygous mutant by observing the banding pattern of the PCR products on a gel. This technique is simple, cost effective and superior to other screening methods (Makani *et al.,* 2013).

2.9 Complication of sickle cell disease

The high mortality rate of SCD patients in poor resource country are influenced by many factors including limited resources leading to poor access to care, and lack of comprehensive SCD management programs. While reducing rate in high resource countries

is associated with intervention of newborn screening, and prophylactic penicillin administration are not available in most low resource countries (Odame, 2010).

2.9.1 Morbidity

In SCD the complication may occur in multiple body organs. Chronic pain and intermittent painful episodes, musculoskeletal problems, stroke, pulmonary hypertension, and septicemia are the most common morbidity associated with SCD. These complications often co-exist, affecting the quality of life for patients, and if untreated, they may lead to death.

2.9.1.1 Pain and Stroke

Pain in SCD is associated with vasoocclusive crises; due to microvascular occlusions triggering the activation of nociceptive afferent nerve fibers (Stuart & Nagel, 2004). The most common pain are; hand-foot syndrome or dactylitis, intense abdominal pain Long bones and joints are often areas of necrosis leading to pain similarly - Micro vaso-occlusion in the mesenteric blood vessels(Stuart & Nagel, 2004). Vasoocclusive crises also leads to musculoskeletal complications due to avascular necrosis, osteomyelitis, and septic arthritis (Balogun *et al.,* 2010).

Another most serious complication of SCD is stroke which occurs due to occlusion in the cerebral microvasculation (Kolapo & Vento, 2011). Higher rate of stroke will develop after increasing age, other risks factors are low hemoglobin, leukocytosis, and the Bantu haplotype because of low level of HbF expression (Makani *et al.,* 2007).

2.9.1.2 Pulmonary hypertension

Pulmonary hypertension (PH) occurs with SCD as a result of chronic hemolysis. The risk factors for PH are HIV/AIDS, Hepatitis B and C, and malaria.

2.9.1.3 Septicemia/Infection

It is well reported that SCD are more prone to pneumococcal disease compared to those without SCD. *Streptococcus pneumoniae, Haemophilus influenza, Staphylococcus epidermis, S. pneumonia, Streptococcus viridans,* and *Escherichia coli* causes bacteremia. Bacterial infections leading to septicemia has been reported as a major cause of morbidity and mortality especially in the children below 2 years old (Battersby *et al.,* 2010; Mulumba and Wilson, 2015; Serjeant, 2005).

2.10 Management strategies of sickle cell disease

Effective management of SCD revolves around genetic counseling, neonatal screening and early diagnosis; prophylaxis with immunizations; antibiotics, and hydroxyurea; and prompt management of complications. Bone marrow transplantation in a selected segment of patients is the only potential cure for SCD to date (Walters *et al.,* 2001), but this is an expensive treatment procedure may not feasible as a public health approach in low resource countries (Serjeant and Ndugwa, 2003).

2.10.1 Treatment of complications

Treatment of SCD includes antibiotics, vitamins, blood transfusions, pain relieving medicines, and possibly surgery such as to correct vision problems or to remove a damaged spleen.

Vaccination and Antibiotics

The infection is pronounced more severely among SCD children. Immunizations in childhood could reduce the incidence of infections which is major cause of death (Obaro, 2009). Children with SCD need to get routine immunizations such as pneumococcal vaccinations and the annul flu shot to prevent disease and prophylaxis using penicillin. It has increased survival rates among children in the developed world (Makani *et al.,* 2007).

Sickle cell anemic children may always begin to take antibiotics like penicillin from two months old till five years. Infectious disease like pneumonia which can be life threating to SCD infants can be treated with continuous use of antibiotics. Similarly if sickle cell anemic adults has removed spleen or has pneumonia, they might need to take penicillin throughout their life.

Hydroxyurea (Droxia, Hydrea)

It is the potent drug that stimulates the production of fetal hemoglobin (HbF) which prevents the formation of sickle cells. Stimulated production of HbF reduces needs of blood transfusion and hospitalization. Besides, Hydroxyurea increases water content of red blood cells resulting in less cell deformity and adhesion to the endothelium. It is also believed to have antihemolytic properties (Aliyu *et al.,* 2008; Aneni *et al.,* 2013; Stuart and Nagel, 2004). Hydroxyurea also reduces hepatic sequestration and priapism reducing the need for blood transfusion; and lowers mortality from SCD related complications by 40% (Aliyu *et al.,* 2008).

Blood transfusion

In sickle cell anemia the red blood transfusion means the removal of RBCs from a supply of blood, then given intravenously to person with sickle cell anemia. Transfusion helps to relieve anemia that increases the number of healthy RBCs in circulation. Regular blood transfusion will generally reduce risk of stroke and prevent complications. However blood transfusions sometimes bring risks, like infection and excess iron buildup in SCD patients. The excess iron can damage heart, liver and other organs.

Bone marrow transplantation

The ultimate cure from sickle cell anemia is bone marrow transplant, a process of replacing affected (sickle cell) bone marrow cells with healthy bone marrow from a donor (*Walters et al.,* 1996).This procedure usually needs a specific HLA typing (matched donor) from their sibling, who doesn't have sickle cell anemia.

The application of radiation therapy or chemotherapy is used to destroy or reduce host's bone marrow cells and stem cells containing normal β-globin genotype are injected, where they migrate to the bone marrow and begin generating new blood cells. The early transplantation especially in children before (end organ damaged) occurs and HLA-matched sibling donor shows increases its successes up 85% (Talano and Cairo, 2015, Bernaudin *et al.,* 1993).

Future Perspectives for the Treatment of Sickle Cell Gene therapy

Successful gene transfer was demonstrated in sickle murine models with improvement in the SCD phenotype (Levasseur *et al.,* 2003; Pawliuk *et al.,* 2001). Research is being progressed regarding the possibility of turning off the defective gene while reactivating another gene responsible for the production of fetal hemoglobin HbF. Newborns possess significant HbF that prevents sickle cells from forming. Gene transfer therapy is being performed with several clinical trials to evaluate safety and expression of gene transfer using γ-globin and *β-globin* lentivirus vector (Cavazzana-Calvo *et al.,* 2010).

Drugs to boost fetal hemoglobin production

Nowadays research have been focused on various drugs such as histone deacetylase inhibitors, lysine-specific histone demethylase 1 (LSD1) inhibitors, and immunomodulatory drugs for the production of fetal hemoglobin. HbF halts sickle hemoglobin synthesis (Telen, 2016).

2. 10.2 Prevention and management

The management of SCD has remained a matter of concern in both developed and developing countries. A greater awareness and understanding of the communities and health care personnel about SCD and its detection has been found to be beneficial in the management of the disease (Armeli *et al.,* 2005; Treadwell *et al.,* 2006). Several studies have clearly shown that genetic counseling is considered as one of the best ways of controlling the genetic disease (Gustafson *et al.,* 2007).The preventive measures include:-

- Continued community education programs at high risk area by generating and strengthening the national sickle cell disease control programs.
- Setting up sickle cell screening and genetic counseling programs. The disease should be identified during the prenatal period or at birth as part of a routine screening program.
- Use of prophylactic drugs namely chloroquine and penicillin.
- Basic and clinical research.
- Provision of primary health care (access of sickle cell children to health centers).
- Improvement of living standard and better feeding for patients with SCD.

2.11 Present scenario of SCD in Nepal

Various types of malaria protective genes have been reported in Nepal. The prevalence of alpha thalassemia has reported at comparatively high frequency in central region than western region (Modiano *et al.,* 1991). Studies by Sakai *et al.,* reported the prevalence of alpha thalassemia among Danwar and Tamang ethnic groups while HbE has also been reported on Danwar inhabitants of malarial zone (Sakai *et al.,* 2000). Other malaria protective genes like HbE, alpha and beta thalassemia, sickle cell disease and their association with fetal hemoglobin percentage is also reported (Jha, 2015).

The first reports on sickle cell disease were published in 2003 on two Tharu patients of Nawalparasi district in which a five years child was diagnosed with compound heterozygous Sickle cell-beta thalassemia and other 17 year female was with homozygous sickle cell disease (Adhikari *et al.,* 2003).

Shrestha and Karki reported SCD is most prevalent in Tharu ethnic community inhabitant in malarial region, however sickle cell trait has also reported on few patients from non-malarial region (Shrestha and Karki, 2013). Jha detected greater number of hemoglobinopathies on Tharu ethnic group. Beta Thalassemia trait and sickle cell anemia both are common in Nepal. And most of SCD and hemoglobinopathies have been reported in people of Terai region. Double heterozygous condition of sickle cell beta thalassemia and Hb E –beta thalassemia are also prevalent in Nepalese cases. Now the government of Nepal has appreciated SCD as public health problem. And provides certain free treatment facilities if individuals were confirmed with the SCD. In most of the developed countries i.e. where hemoglobinopathies is high, has applied screening programme for the aim of identifying sickle cell disease and other hemoglobin disorders in order to measure the risk of a couple having a severely affected child and to provide information on the options available to avoid such an possibility. But in our country Nepal, neither population based study nor such screening programme has been held to find out the prevalence of sickle cell disease till (Jha, 2015). Thus, the aim of my study is to determine prevalence of SCD on detection of reasonable mutation at the beta globin gene resulting in diseased.

CHAPTER 3

MATERIALS AND METHODS

3.1 Selection of Study site

The study was designed as a descriptive cross-sectional study and conducted on Tharu population of Far-western Terai region of Nepal. It was carried out from April 2016 to October 2016. Malaria endemic regions of Kailali (Dhangadhi and Attariya) and Kanchanpur (Krishnapur) were selected as sample collection sites. Blood sample of Dangaura subgroups of Tharu were taken from Dhangadhi, Attariya and Krishnapur region and that from Rana subgroups were collected from Dhangadhi gaun (Dhangadhi) as shown in Figure 3.1. Positive samples included in this study were taken from sickle cell disease patients from Seti zonal hospital.

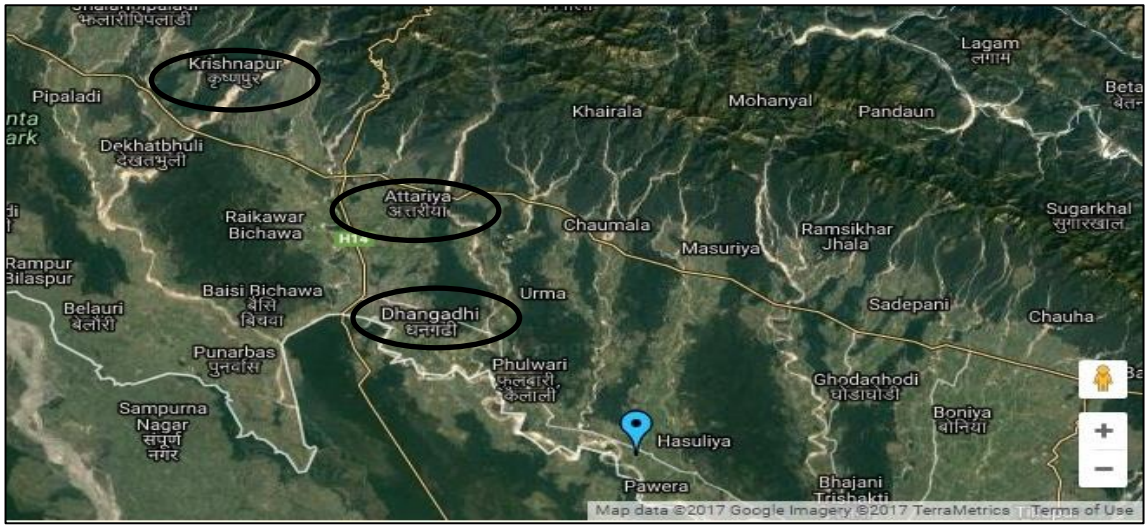

Figure 3.1: Google map showing sites of sample collection. Simple collection sites are Krishnapur (Kanchanpur), Attariya and Dhangadhi of Kailali (represented by black circle).

3.2 Collection and transport of samples

Prior to blood sample collection, the ethical approval was taken from Nepal Health Research Council (NHRC). Stratified random sampling technique was used to collect blood samples from two subgroups of Tharu. A total of 116 blood samples were collected from male and female individuals of age 18 years to 60 years by satisfying inclusion (healthy donors and

family members), and exclusion (individual having HIV, tuberculosis, other genetic or congenital disease) criteria.

Information regarding respective individual were filled up on consent form. Individual with clinical signs and symptoms of sickle cell disease were noted. Then 3ml of blood was withdrawn from inclusion criteria satisfied individuals by the help of registered health worker using sterile syringe and transferred into K_3EDTA Vacutainer followed by gentle shaking to prevent blood coagulation. Thus, collected blood samples were transported to Central Department of Biotechnology laboratory in ice box and stored at 4^{o}C.

3.3 Sickle Solubility test

3.3.1 Preparation of hemolysate

The blood sample was thawed to room temperature by gentle shaking on orbital shaker for homogenization. Four hundred microliters of blood sample was taken in 2ml of Eppendorf tube and 1500µl of normal saline was added. Then content was mixed by inverting tubes gently 10 times. Tube was then centrifuged at 1500 rpm for 4 minutes and supernatant was discarded. Red pellet was washed four times with normal saline by centrifuging at 1500 rpm for 4 minutes. The red layer of supernatant was discarded and pellet was vortexed for 3 minutes by adding 535µl of carbon tetrachloride. Then the content was centrifuged at 5100 rpm for 20 minutes to separate different constituents of blood based on their densities. Thus, obtained uppermost red layer of hemolysate was further used for solubility test.

3.3.2 Preparation of phosphate buffer

3.3.2.1 Stock (2.58M) phosphate buffer

Phosphate buffer was prepared by dissolving 239.66 gram of K_2HPO_4 and 164 gram of KH_2PO in 800ml of distilled water. The pH of solution was maintained 6.5 and distilled water was added to maintain the final volume of 1litre.

3.3.2.2 Preparation of high molarity phosphate buffer

High molarity phosphate buffer of concentration 2.24M was prepared by adding 66ml of distilled water into 434 ml of stock phosphate buffer (2.58M).

3.3.2.3 Preparation of low molarity phosphate buffer

Low molarity phosphate buffer (1.1M) was prepared by adding 213 ml of stock phosphate buffer (2.58M) into 287ml of distilled water.

3.3.3 Hemoglobin solubility test

Hemoglobin solubility test were performed according to the Old *et al.,* 2012 protocol for presence of sickle hemoglobin (Old *et al.,* 2012). At first clean dry glass test tube (12x75 mm) was taken. For each samples two test tubes were taken and marked as "H" and "L" for high molarity phosphate and low molarity phosphate buffer respectively.

One milliliter of high molarity phosphate buffer was transferred to "H" marked test tubes and 1ml of low molarity phosphate buffer was transferred into "L" marked test tubes. Forty microliter of carbon tetrachloride (CCl_4) hemolysates was added to each test tube and mixed well by pipetting. Thirty microgram of sodium dithionite powder was added to each test tube and the content was mixed by gentle shaking. It was then incubated at room temperature for 15 min and formation of precipitate was observed.

The hemolysate from SCD patient was taken as positive control while the hemolysate from healthy individual was taken as negative control.

3.4 Extraction of genomic DNA

Genomic DNA (gDNA) was extracted from white blood cells by osmolarity methods (Madhad and Senthei, 2014). First of all sample was thawed by incubating at room temperature for 15 minutes with gentle shaking on orbital shaker. Then 500µl of blood sample was taken in micro-centrifuge tube and 1ml RBC lysis buffer was added to it. It was then mixed gently by inversion. Content was centrifuged for 10 minutes at 2200 rpm. The supernatant containing lysed RBC was discarded and pellet was dissolved in 1 ml of RBC lysis solution. Tube was then centrifuged for 10 minutes at 2200 rpm in order to obtain residual hemoglobin free leucocyte. The supernatant was discarded and residual RBC lysis solution was completely removed by inverting tube over blotting paper. After that, RBC lysis free pellet was dissolved in 400µl of nuclei lysis buffer by gentle pipetting. Then 100µl of 5M NaCl, and 600 µl Chloroform were added and vortexed. It was then centrifuged for 3 minutes at 7000 rpm to separate aqueous DNA from other debris. The 400 µl of aqueous supernatant were transferred to a fresh microcentifuge tube containing 400µl of isopropanol. It was mixed by inversion and kept in -20^0C for half an hour to allow DNA precipitation. DNA was then pelleted by centrifuging the content at 12000rpm for 5 minutes. The supernatant was discarded and the pellet was washed with 70 % ethanol and allowed to air dry by incubating at room temperature for 15 minutes. The dried DNA sample was re-suspended in 50 µl TE buffer. Then quality of gDNA was checked in 0.8% agarose gel

(gel electrophoresis and photography on Gel documentation) and its purity was measured (absorbance 260/280nm) in spectrophotometer. Thus purified DNA samples were preserved at -20^{o}C until its use.

3.4.1 Gel electrophoresis of gDNA

Four hundred microgram (0.8%) of agarose was dissolved in 50 ml 1X Tris-acetate EDTA (TAE) buffer in 100 ml conical flask. The solution was boiled to make a clear solution. Thus melted agarose was allowed to cool to 50^{o}C. Then 2.5 µl Ethidium Bromide (EtBr) (10mg/ml) was added and it was casted on a gel casting tray then allowed to solidify. The gel was transferred to electrophoretic tank containing 1X TAE buffer and comb was removed. Then DNA samples were mixed with DNA loading (bromophenol) dye and loaded in a well of agarose gel. Fifty voltages electric supply was applied to separate the DNA in agarose gel for an one hour.

3.5 Amplification of β-globin gene by PCR

The primers were designed that target amplification of 6th codon of the β-globin gene. Thus designed primer amplifies the 539bp region including 5' UTR, 1st exon, 1st intron and 2nd exon of β-globin gene as shown in Figure 3.2.

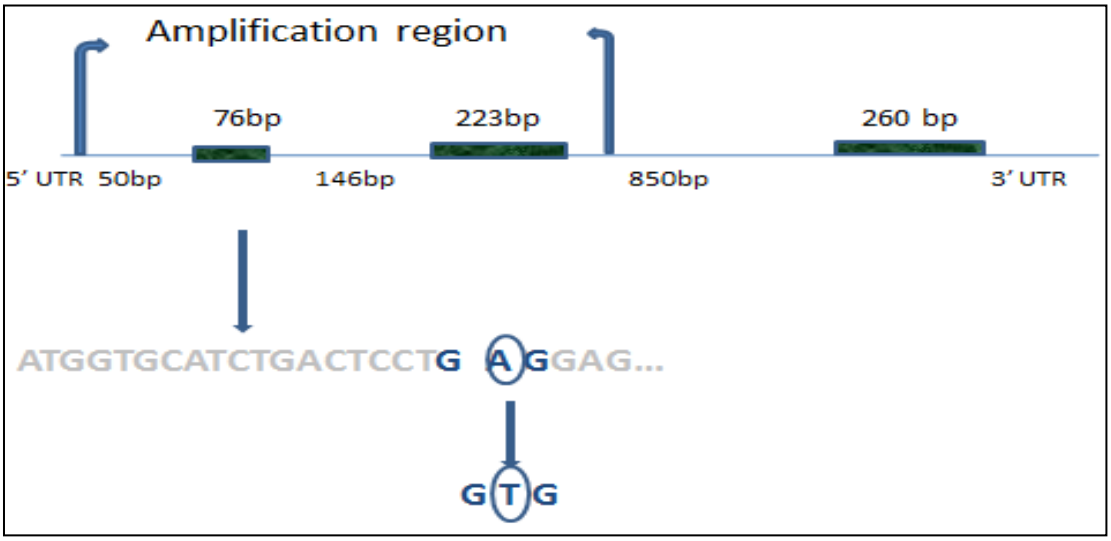

Figure3.2: Schematic representation of β-globin gene, showing 5' UTR, 3' UTR, 1st and 2nd exon and 1st introns. Three exons (dark block) and two introns (thin lines) are intervening in between exons.

The primer set used for amplification of 5' UTR, 1st exon, 1st intron and 2nd exon of β-globin gene were shown in table 3.1.

Table 3.1: The PCR primer used to amplified the targeted region of β-globin gene

Gene	**Primer**	**Nucleotide Sequence**
Human *β-globin* (HBB)	Forward Primer	5'- AGTCAGGGCAGAGCCATCTA -3'
	Reverse Primer	5'- AGGGTCCCATAGACTCACCC – 3'

Table 3.2: Composition of PCR reaction mixture

S.N.	**Reagents**	**Volume(µl)**
1.	NEB 2X Master mix	12.5
2.	MgCl2(2.5 mM)	0.7
3.	Forward primer (10 µM)	1.2
4.	Reverse primer (10µM)	1.2
5.	Template DNA (30ng/µl)	1.3
6.	Nuclease Free water	8.1
	Total	25

Table 3.3: PCR condition for the targeted region of β-globin gene

Stage	**Steps**	**No. of cycles**	**Temperature (^{0}C)**	**Time**
1	Initial denaturation	1	95	2 min
2	Denaturation	35	95	30 sec
	Annealing		55	30 sec
	Extension		72	1 min
3	Final Extension	1	72	7 min
	Hold	-	4	Infinite

3.5.1 Agarose gel electrophoresis of PCR product

Thus PCR amplified product was run in 1.5% Agarose gel. The EtBr stained DNA was visualized on UV-transilluminator and photograph was taken for record.

3.6 Restriction digestion of Amplified β-globin gene

Endonuclease Bsu 36I has three restriction site (CC↓TNAGG) on amplified products that yields four fragments of 88bp/92bp, 157bp and 202bp. On the contrary RFLP product of homozygous mutant will give three bands of 88bp, 157bp, 294bp whereas heterozygous mutant results in four bands of 88/92bp 157bp, 202bp, and 294bp.

Table3.4: Reaction mixture for Restriction digestion of amplified PCR product.

S.N.	**Components**	**Reaction volume**
1.	Cut smart buffer (10X)	1.5 µl
2.	PCR product	10 µl
3.	Nuclease free water	3 µl
4.	Bsu36I (10000U/ml)	0.5 µl
	Total reaction volume	15 µl

The reaction was mixed properly by pipetting followed by short spin. Then, it was incubated at 37^{o}c for 1 hour. The enzyme was inactivated by incubating the reaction mixture at 85^{0}C for 20 minutes.

3.7 Non-denaturing gel electrophoresis of digested products (PAGE)

3.7.1 Preparation of Polyacrylamide Gel (PAG)

The glass plates and spacers were cleaned several times with distilled water and assemble in to gel casting assembly. Then following mixture was prepared and poured into it.

Table 3.5: Composition of 10% Non-denaturing (Tris Boric EDTA) gel

S.N.	Reagents	Volume
1.	30% Acrylamide: bisacrylamide(29:1)	4 ml
2.	10X TBE buffer	1.2 ml
3.	Distilled water	6.59 ml
4.	10% APS	200 µl
5.	TEMED	10 µl
	Total	12 ml

TEMED and APS were added at last after addition of other reagents and mixed by gentle shaking. It was then immediately poured into casting tray and comb was inserted carefully by preventing insertion of air bubbles. It was then allowed to polymerize by incubating at room temperature for 40 minutes.

3.7.2 Non denaturing gel electrophoresis of restriction digestion products

After complete polymerization, polyacrylamide gel was removed from casting assembly and transferred to electrophoretic tank containing 1X TBE buffer. Comb was then carefully removed. Fifteen microliter of digested sample was mixed with three microliter of 6X Orange loading dye (Fertmentas) and loaded into well. After loading the digested sample in the gel, power pack (Bio-rad) was plugged-in and electric field was applied. It was allowed to run for 140 minutes at 60V. After completion of electrophoresis, gel was removed from electrophoretic tank and allowed to cool by immersing the gel into distilled water for 5 minutes. Then gel was removed carefully from glass plates and stained with EtBr solution (150 ml distilled water and 7.5 µl of 10 mg/ ml EtBr) by shaking at 90 rpm for 15 minutes. Finally, gel was washed with distilled water and visualized under the UV-light and photograph was taken for record.

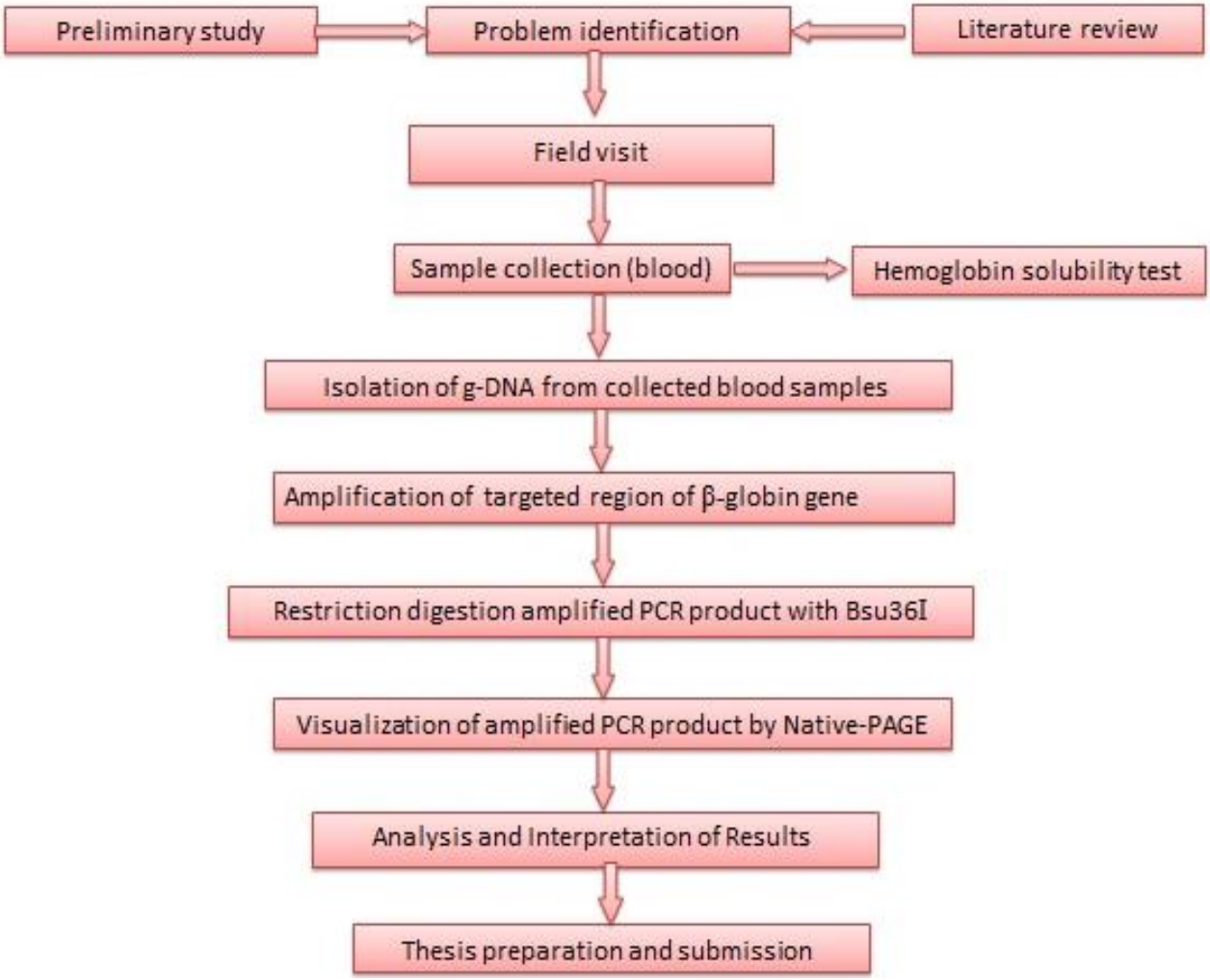

Figure 3.3: Flow diagram of overall research design

CHAPTER 4

RESULTS

This study was cross sectional study and conducted on Far-Western region of Nepal. Areas of sample collection (Kailali and Kanchanpur districts) were highly malarial endemic regions. Five sites were selected as sample collection area; four sites were from Kailali (three places of Dhangadhi and one from Attariya) district and the remaining samples from Krishnapur of Kanchanpur district. Blood samples were collected from two subgroups of Tharu viz. Rana and Chaudhary. Tharus inhabitants of these rural areas are deprived of proper health facilities, proper education and well equipped laboratory in comparison to other social group in these area. These two subgroups of Tharu have different origin. Rana Tharu are thought to be migrated from Rajput while the Dangaura Tharu are claimed to be originated in Dang valley and then migrated to Kanchanpur districts in west (Lam, 2012; Krauskopff, 1995).

4.1 Socio demographic status of sickle hemoglobin test

4.1.1 Region wise distribution of samples

Out of 116 samples were investigated for the presence of SCD, 101 (87.07%) were from Kailali district (Seti Zone) and remaining from Kanchanpur district (Mahakali zone). Among them, 73 (62.93%) samples were from Dhangadhi municipality and 28(24.14%) samples from Attariya whereas 15 (12.93%) samples were from Dangaura Tharu of Kanchanpur district.

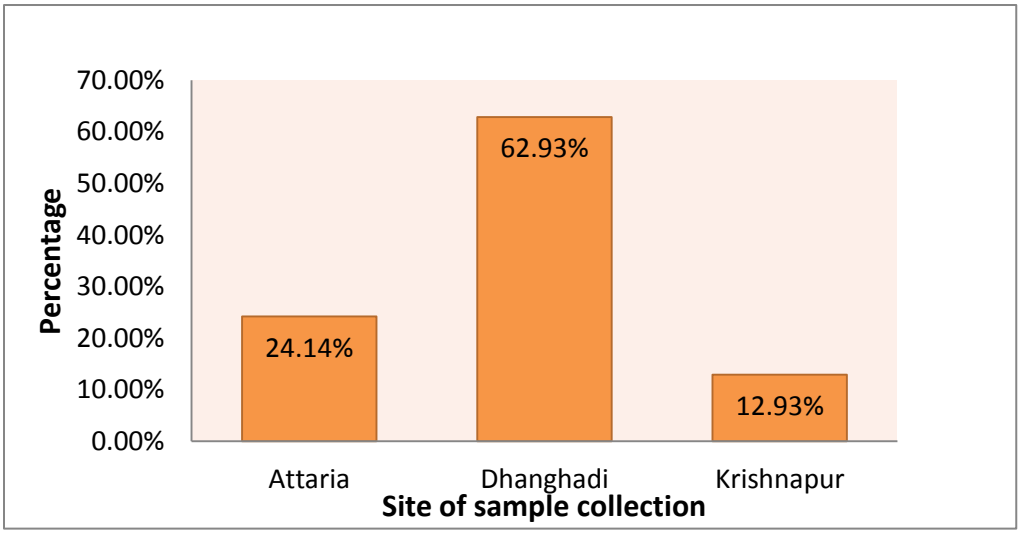

Figure 4.1: Region wise distribution of sample

4.1.2 Gender wise distribution of samples

Out of 116 samples, 60 were from male and 56 were from female. The percentage of male was 51.72% and that for female was 48.28% as depicted in Figure 4.2. The ratio of male to female was 1.107:1.

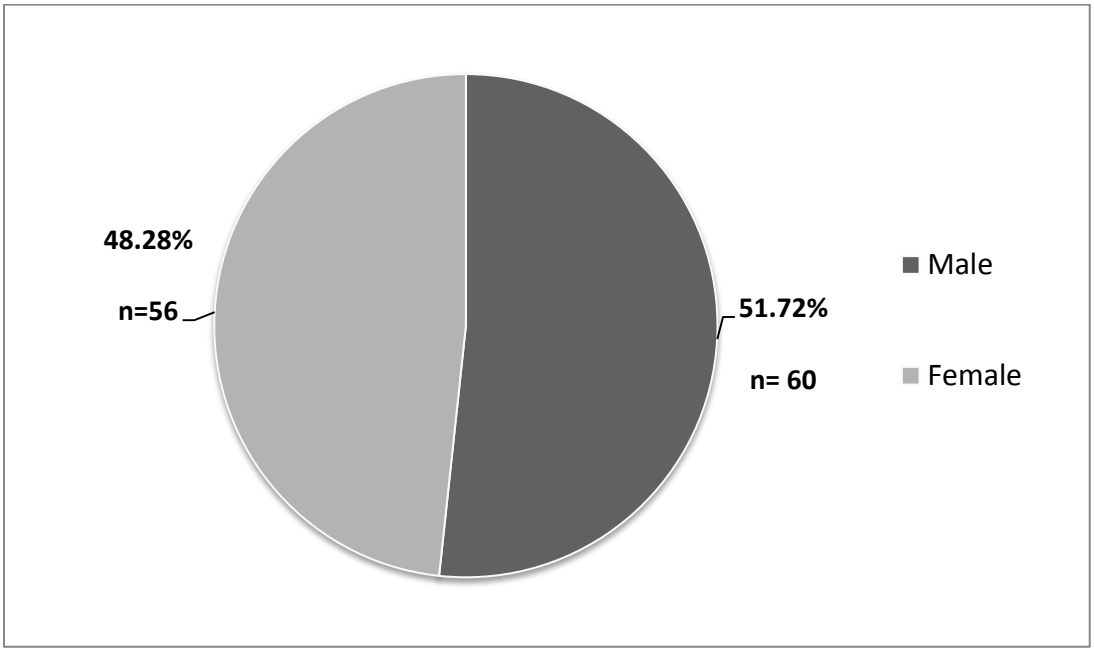

Figure 4.2: Sex wise distribution of samples for sickle cell disease test

4.1.3 Age wise distribution samples

Blood sample were taken from 18-60 years age group of Tharu population. They were categorized into three age groups; (<20 years), (20-40 years) and (40-60 years). The greatest number of the samples (64.65%) were from 20-40 years age group and the least 15.51% were from <20 age group and 19.82% cases belonged to 40-60 years age group people as shown in Figure 4.3 .

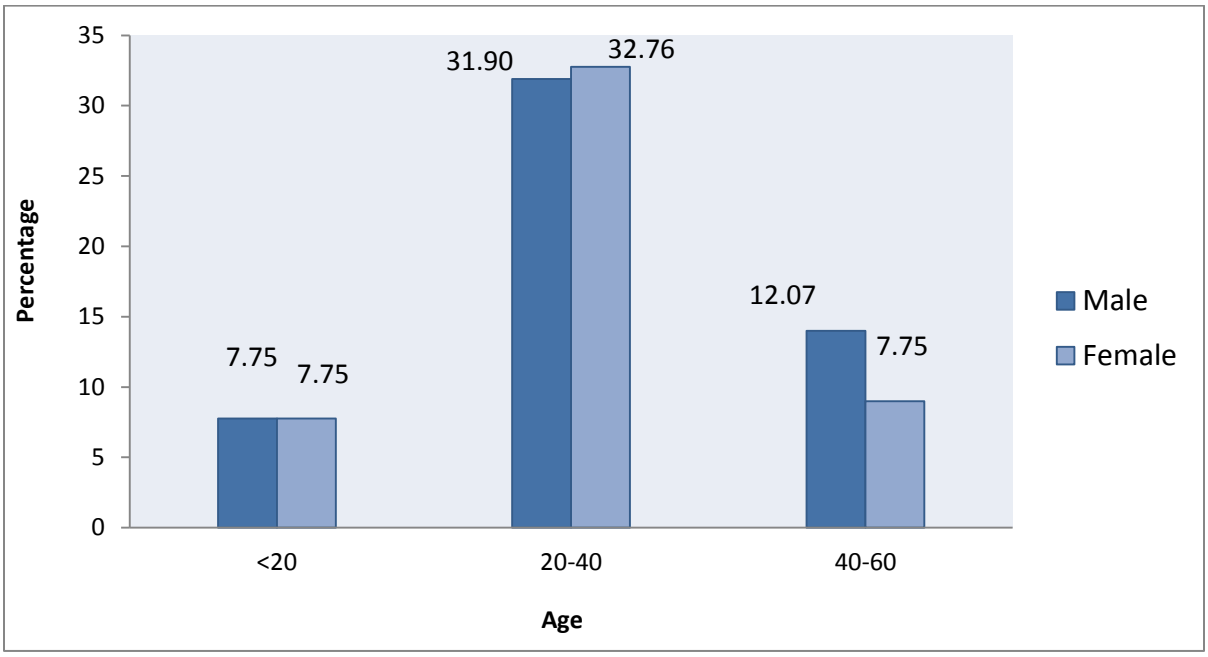

Figure 4.3: Age wise distribution of samples

4.1.4 Ethnicity wise distribution of samples

We selected two sub-groups of Tharu in our study, of them 25 (21.55%) samples were taken from Rana Tharu of Kailali districts and 91 (78.45%) sample from Dangaura Tharu (Chaudhary) of Kailali and Kanchanpur districts.

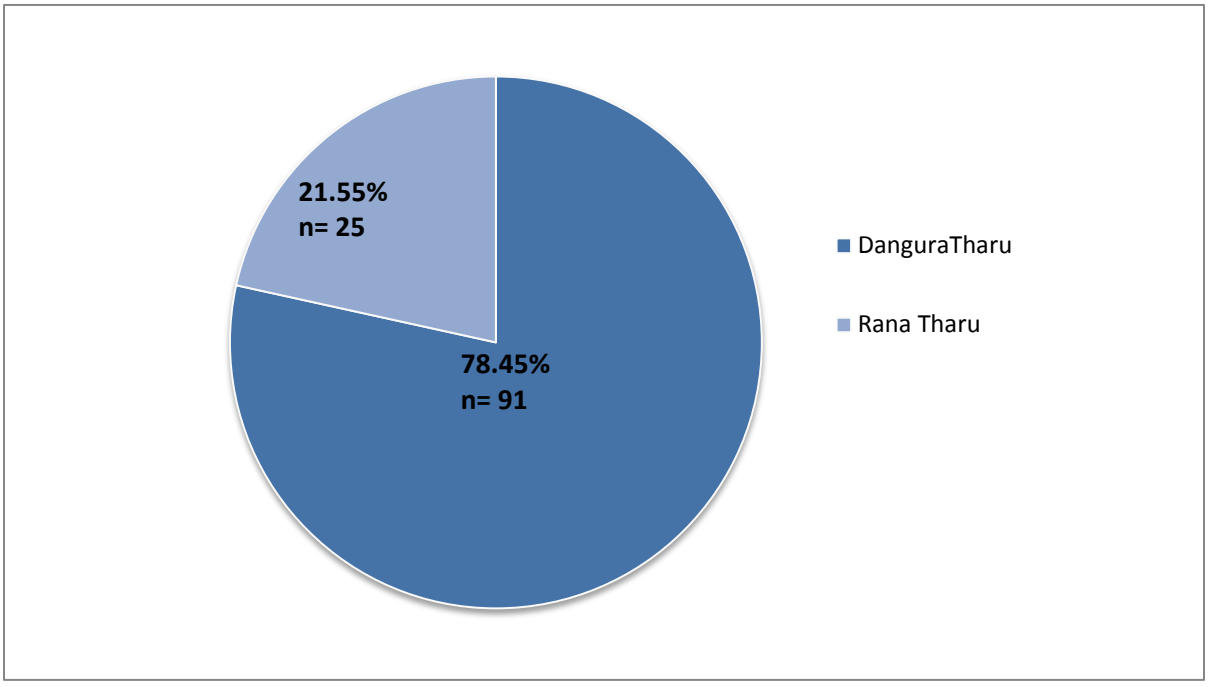

Figure 4.4: Ethnicity wise distribution of sickle cell disease test cases

4.1.5 Symptom wise distribution of samples

During sample collection period, we had taken short interview regarding the common symptoms of sickle cell disease like episodes of joint pain, fever, weakness, jaundice that occur at least two times in a year especially in winter seasons and summer seasons. Out of 116 individuals only 16(13.79%) individuals were found to have these symptoms and 100 (86.21%) individuals were asymptomatic.

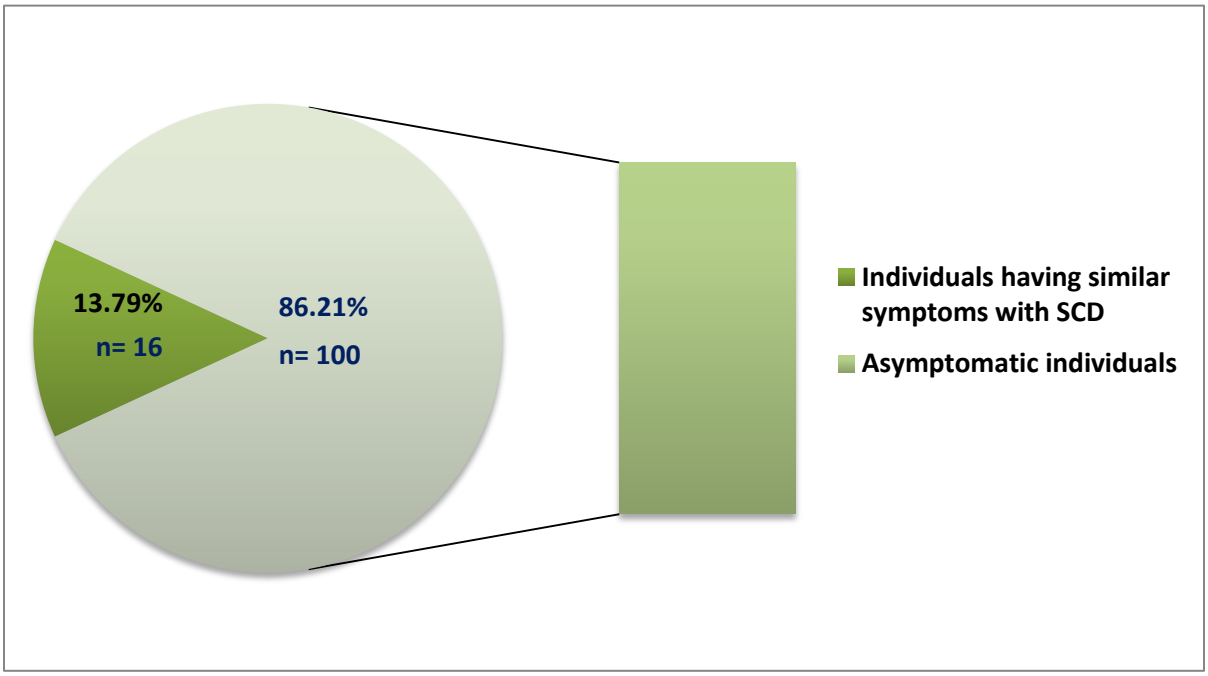

Figure 4.5: Symptom wise distribution of sickle cell disease test

4.2 Screening and confirmation of SCD

For the diagnosis of sickle cell disease both phenotypic test and genotypic test were performed individually for each sample. Phenotypic test were performed by hemoglobin solubility test as screening test. And genetic test was done to determine targeted point mutation at the 6th codon of beta globin gene for confirmation of SCD.

4.2.1 Screening of sickle hemoglobin by hemoglobin solubility test

Among 116 samples for sickle hemoglobin test cases, only 26 (22.41%) sample showed turbidity (water crystal) of hemolysate with sodium dithionate solution with high molarity phosphate buffer and no turbid solution was seen on low phosphate buffer for all samples as shown in Figure 4.6.

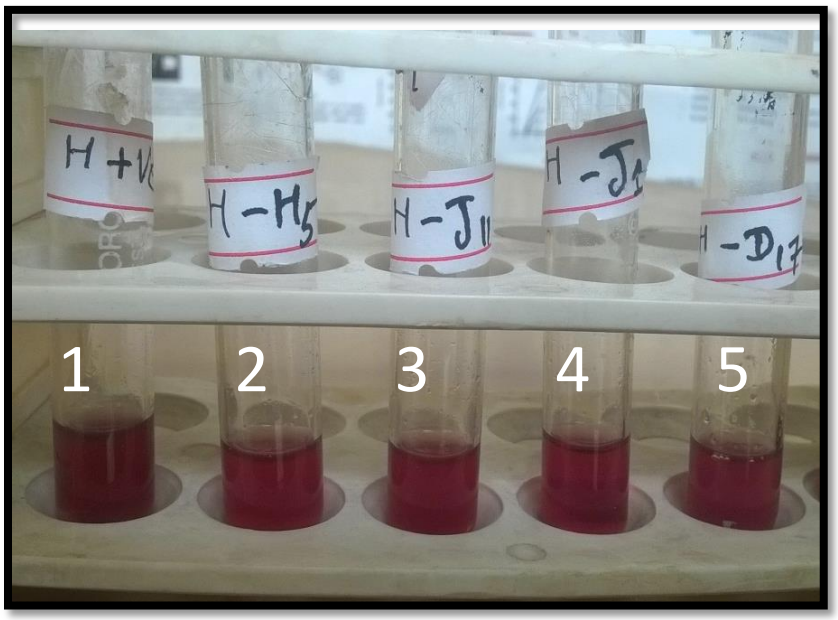

Figure 4.6: Solubility test of sample. 1- Negative control; 2- Positive control; sample 3, 4 and 5 are test samples

Hemoglobin solubility test is only performed as screening test for presence of sickle hemoglobin. Furthermore such test does not differentiate between and sickle cell disease from homozygous sickle cell anemia. Sometimes this technique gives false negative and false positive results as well. Thus, further confirmation was done by PCR- RFLP methods.

4.2.2 Confirmation of sickle cell disease by PCR- RFLP

4.2.2.1 DNA purity and quantity measurement

The quality and quantity of extracted gDNA was measured by gel electrophoresis and spectrophotometry. The concentration of extracted gDNA was found to be 10-30 µg range.

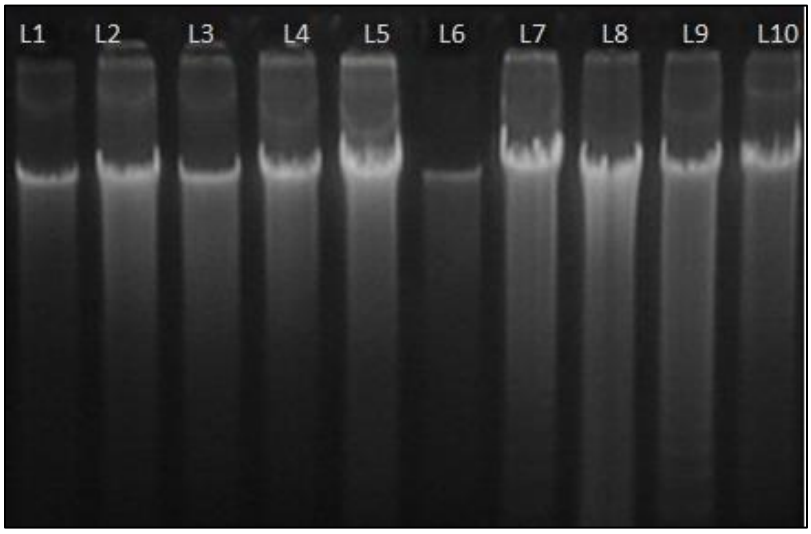

Figure 4.7: Gel image of the genomic DNA. L1- L10 Agarose gel electrophoresis (0.8%) of gDNA sample.

4.2.2.2 Confirmation of targeted region of β-globin gene

Amplification of beta globin gene that targets 6th codon that causes sickle cell disease was performed by polymerase chain reaction from genomic DNA sample. The 539 bp of desired amplicon size were confirmed by (1.5%) agarose gel electrophoresis with 100 bp DNA ladder shown in Figure 4.8.

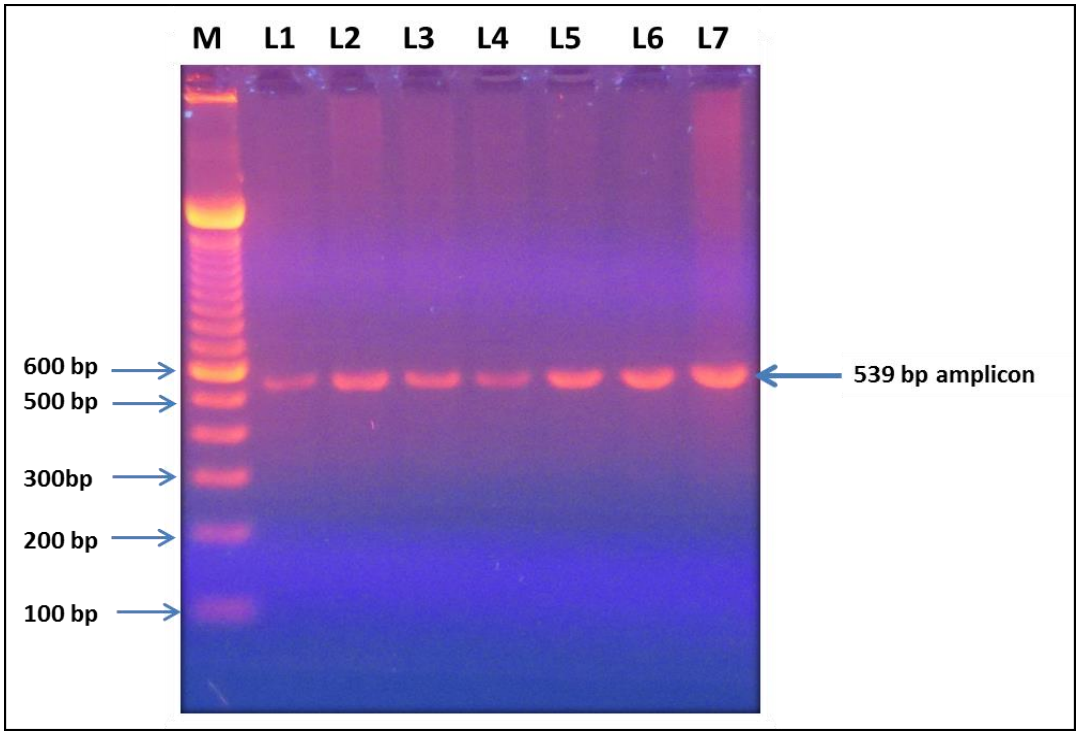

Figure 4.8: Agarose gel (1.5%) electrophoresis of PCR products of the targeted region of beta globin gene. **M** – 100 bp DNA ladder (Invitrogen); **L1 – L7** – test samples

4.2.2.3 Restriction digestion of amplified products

Thus, amplified products were subjected to restriction digestion by Bsu 36I enzyme. From restriction digestion, 25(21.55%) samples were detected as sickle cell disease carrier. This heterozygous sickle gene was confirmed by the presence of both 294 bp and 202 bp DNA fragments along with 157 bp and 88/92 bp digested products in 10 % PAGE shown in Figure 4.9.

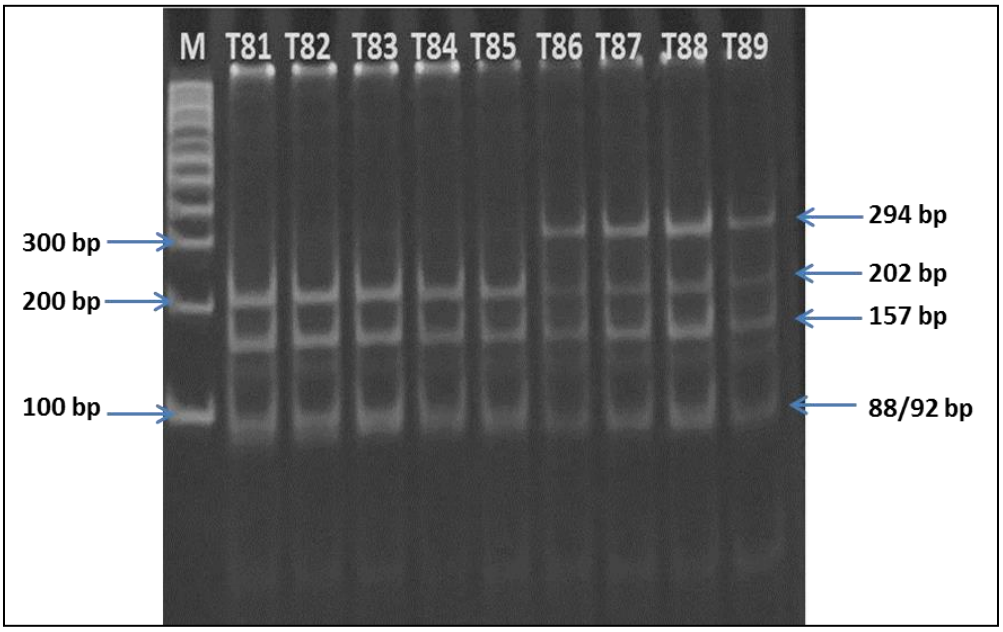

Figure 4.9: Gel image of the restriction digested products on Non-denaturing gel electrophoresis. Polyacrylamide gel electrophoresis (10%) of restriction digested products using Bsu 36I restriction enzyme for the confirmation of sickle cell disease. M – 100 bp DNA ladder NEB), T81-T85 normal digested samples. T86 sickle cell samples (carrier) and T87, T88 and T89 sample from SCD patients.

The positive controls (T87, T88 & T89) were taken from the SCD patients who were admitted to Seti Zonal hospital (shown in above Figure 4.9). The finding of sickle cell disease individuals from PCR- RFLP of this study were shown in appendix 6. All 3 patients had history of 2-3 times hospital visit in a year due to severe pain and other complications. We detected all patients to be heterozygous carrier of sickle cell disease (HbAS) as shown in Figure 4.9. Among them; one was male (T87 in Figure 4.9) who had never taken blood transfusion but his mother (T82) showed normal restriction fragment on PAGE. Another was a female (T88), who had recently taken blood transfusion therapy. Only her brother was available during sample collection, he (T86) was detected carrier for the disease. Last patient was male (T89) with the history of blood transfusion and detected carrier of sickle cell disease while normal fragment was detected on his mother (T83).

4.3 Comparison of results between hemoglobin solubility test and PCR- RFLP for detection of sickle cell disease

Twenty three (19.83%) samples showed positive results for both the tests. Three (2.59%) samples showed false positive results for hemoglobin solubility test whereas 2 (1.72%) samples showed false negative results as compared to RFLP results.

Table 4.1: Comparison Hemoglobin solubility test and PCR-RFLP results

Sickle hemoglobin solubility results				
PCR-RFLP results of HbAS		**Positive**	**Negative**	**Total**
	Positive	23 (19.83%)	2(1.72%)	25(21.55%)
	Negative	3 (2.59%)	88 (75.86%)	91(78.45%)
Total		26(22.41%)	90(77.59%)	116(100%)

4.4 Distribution of sickle cell disease

4.4.1 Region wise distribution of sickle cell carrier cases

Among the collected samples, the highest frequency of heterozygous SCD individuals were 7/28 (25%) from Attariya of Kailali districts and minimum frequency were 2/15 (13.33%) identified from Krishnapur of Kanchanpur districts. Similarly, the frequency of sickle cell carrier (heterozygous of SCD) individuals were from Rana community (Dhangadhi gaun), Chaudhary Community of Dhangadhi gaun and Jaien (Dhangadhi) were 6/25 (24%), 6/24 (22.22%) and 4/21 (19.04%) respectively as shown in Table 4.2.

Table 4.2: Region wise distribution of sickle cell carrier cases

Region	**Number of sample taken**	**Number of sickle cell carrier**	**% of total sickle cell carrier**
Attariya (Kailali)	28	7	25
Dhangadhi gaun (Rana)	25	6	24
Dhangadhi gaun Chaudhary	27	6	22.22
Jaien (Dhangadhi)	21	4	19.04

Krishnapur (Kanchanpur)	15	2	13.33
Total	116	25	21.55

4.4.2 Sex wise distribution of sickle cell carrier samples

Out of 60 samples of male, 13 (21.66%) were carrier of SCD, similarly from 56 females, 12(21.42%) were confirmed SCD carrier. The SCD carrier ratio for male to female is (1.08: 1) as shown in table 4.3.

Table 4.3: Sex wise distribution of sickle cell carrier cases

Sex	**Total number of cases**	**Number of sickle cell carrier**	**% of sickle cell carrier**
Male	60	13	21.66
Female	56	12	21.42
Total	116	25	21.55

4.4.3 Age wise distribution of sickle cell carrier samples

The highest frequency of sickle cell carriers were found in the population of age 40-60 age 6/23 (26.08%) followed by 20-40 age group 17/75 (22.66%) and least frequency of carriers were from below twenty 2/18 (11.11%) age group as shown in table 4.4.

Table 4.4: Age wise distribution of sickle cell disease cases

Age Group (Years)	Total number of test cases	Total of SCD carrier	Percentage of SCD carrier
<20	18	2	11.11
20-40	75	17	22.66
>40	23	6	26.08
Total	116	25	21.55

4.4.4 Ethnicity based distribution of SCD carrier samples

The highest frequency of sickle cell carrier 6/25(24.00%) were detected from Rana (sub group of Tharu) whereas 19/91 (20.08%) were from Dangaura sub groups as shown table 4.4.

Table 4.5: Ethnicity based distribution of sickle cell disease cases

Sub group of Tharu	Number of sample taken	No. of SCD carrier detected	% of SCD carrier
Rana	25	6	24.00
Chaudhary	91	19	20.08
Total	116	25	21.55

4.5 Association between genotype and phenotype

At time of sample collection, we had also taken the questionnaire from the individuals if they possess any symptoms similar to sickle cell disease and recorded on notebook. None of the samples were found to be homozygous mutation; even the patients (positive samples) had inherited the gene in heterozygous form. Among three patients, the female patients were presented with severe complication where as other two male patients had moderate severity of SCD. Twenty five individuals were identified as carrier of SCD among which, six of them were symptomatic and the other 19 samples had no any signs and symptoms suggestive of SCD. Thus, in our finding, we can say there is no direct observable association between genotype and phenotype. Since, sickle cell disease is multifactorial disease, we can assume other factors may also impact on phenotype.

Eighty one individuals did not describe any symptoms related to sickle cell disease; they were detected (confirmed) negative sickle hemoglobin gene by our test, as shown in table 4.6.

Table 4.6: Comparison of phenotypic symptoms and disease gene

Symptoms that matched SCD	**Symptoms but no SCD**	**Carrier but no symptoms**	**No symptoms and no SCD**
6	10	19	81

4.6 Calculation of genotype and allele frequency

Genotype frequency

Out of 116 samples, 91 samples showed no mutation at 6th codon of first exon of *β-globin*. Thus, the genotype frequency of homozygous HbAA was calculated to be 78.44%, HbAS 21.55% and none of the individual was detected as homozygous sickle cell disease (HbSS). Thus, its genotype frequency was 0 in our study.

Allele frequency

The allele frequency of major adult hemoglobin (HbA) was detected as 0.892 and allele frequency of sickle cell hemoglobin (HbS) was found to be 0.107.

CHAPTER 5

DISCUSSION

We performed sickle hemoglobin test and PCR RFLP test to detect the frequency of sickle cell disease on two subgroups of Tharu population. Hemoglobin solubility test is preliminary screening test for sickle hemoglobinopathy detection. Out of 116 total samples, 26 samples showed positive sickle hemoglobin test. Hemoglobin solubility test doesn't confirm sickle cell disease from homozygous sickle cell anemia. So to confirm the homozygous and heterozygous type of sickle cell disease, targeted region (exon 1, intron 1 and exon 2) of β-*globin* was PCR amplified. The amplicon was then digested by restriction enzyme Bsu 36I. Only 25 individuals were found to be carrier (heterozygous) for sickle cell disease and none of individuals were detected as homozygous sickle cell anemic. Considering RFLP as a standard test for SCD, hemoglobin solubility test showed false negative results for 2 samples (1.72%) and false positive results for 3 (2.59%) samples. The false positive results may be due to hyperglobulinemia, extreme leukocytosis or hyperlipidemia blood. Positive solubility test may also occur due to abnormal hemoglobin such as Hb'sI and HbC (Fabry *et al.,* 2003) but these variants are not reported in our country. False negative results may be observed in patients with severe anemia (<15% hematocrit) and individual having HbD and presence of high concentration of HbF. Since, we were unable to performed quantification of HbF and the presence of HbD, we cannot rule out these options. The HbD variants is most prevalent in Punjab region of India and Pakistan and one cases of HbD was reported in Nepali cases (Jha, 2015) but the ethnic group is not mentioned. In our study, we only focused to detect the sickle cell disease, false negative results observed only in two cases of Dangaura Tharu, the endogenous ethnic group of Dang. Thus negative results may be due to presence of greater % of HbF. Jha, 2015 reported greater % of HbF in the Nepalese sickle cell disease patients.

In normal amplified products, the enzyme has three recognition sites, so it gives 4 fragments: 92 bp, 202 bp, 157 bp and 88 bp as shown in appendix 2. However, three fragments are observed during visualization in UV, which is due to overlapping of 88 bp and 92 bp DNA fragments during migration in 10 % PAGE. For homozygous sickle cell anemia (HbSS), DNA fragment of 294 bp (additional products of 92 bp +202 bp) should be obtained due to the elimination of 1^{st} restriction site of the Bsu 36I by the point mutation (A →T) at 20^{th} bp position. But none of any our samples as well as positive controls were showed

these results. In case of carrier i.e. heterozygous forms, total 4 DNA bands of length 294 bp, 202 bp, 157 bp and 92/88 bp are expected because the carrier bears one normal and another mutant allele ($\beta\beta^S$). In heterozygous (carrier) these DNA fragments were observed because of inheritance of one normal adult hemoglobin allele (β^A) and one of sickle cell (mutant) allele (β^S) from either of their parents.

Out of samples collected, 25 (21.55%) of them were Rana Tharu and 91 (78.44%) were Chaudhary Tharu. Out of these, 6 (24%) of Rana Tharu and 19 (20.08%) Dangaura Tharus were identified to be heterozygous (carrier) for sickle cell disease. Shrestha and Karki reported sickle cell disease on 82.85% of Chaudhary and 8.57% of Rana subgroup in 35 hospital test samples of which 62.85% cases were homozygous and 2.57% were heterozygous from hemoglobin electrophoresis (Shrestha and Karki, 2013).

Similarly, Adhikari *et al.,* reported two cases of sickle cell disease in the Nepalese Tharu patients of Nawalparasi district. A one cases was homozygous and another was compound heterozygous case (Adhikari *et al.,* 2003). Jha reported sickle cell disease is second most common hemoglobinopathies after beta thalassemia and also summarized that Tharu ethnic groups have maximum hemoglobinopathies cases (Jha, 2015). She reported 21 case of sickle cell disease, out of 97 hemoglobinopathies (from hospital samples) and sickle cell beta thalassemia were also reported in 4.12% cases.

We had taken nearly equal number of samples from male (51.72%) and female (48.27%) individuals. The prevalence of sickle cell disease carrier is found to be almost similar in male i.e. 13 (21.66%) and female 12 (21.42%). However, Jha reported greater number of SCD cases in male (Jha, 2015). We had taken large number of sample 64.66% (75) from reproductive age group (20- 40 years) to check SCD with age. However, from our results, we found that the frequency of sickle cell carrier is slightly higher among the individuals of age group 40-60 compared to the age group 20-40. Jha reported maximum cases of SCD below twenty years but in our case the least number of carriers were found among the individuals of the age group below 20.

Out of three positive control samples were taken from sickle cell disease patient (from Seti-Zonal Hospital), 2 cases were male and one was female. All of these patients had history of hospitalization at least 3 times in a year. In both cases of males, their mother were detected to be normal. Out of two males, only one male case had history of blood transfusion. But in third (female patients) case, she had transfused blood recently and her younger brother was phenotypically normal but detected to be carrier of sickle cell disease by our test

results. The first two cases may be misdiagnosed by conventional hemoglobin electrophoresis results which may due to the resolution problem of electrophoresis or co-inheritance of other hemoglobin variants (HbF, HbE, and HbD) with one sickle allele or compound heterozygous sickle cell beta thalassemia might lead such symptoms. HbE is most common variants of hemoglobin in south East Asia and such variants were also reported in Nepal. The coinheritance of one sickle cell allele with these and other inherited hemoglobinopathy variants may show similar clinical phenotypic effect of sickle cell disease.

These 3 sickle cell disease patients and other 16 individuals had clinical symptoms alike to homozygous sickle cell disease, but only 6 individuals and all SCD patients were detected heterozygous for SCD. Furthermore, 19 asymptomatic individuals were detected as heterozygous SCD from RFLP results. Individual with heterozygous form of SCD are generally asymptomatic but complication may develop during performance of excessive/hard exercise and other high oxygen demanding activities like climbing on mountain of high altitude that cause excessive sickling phenomena.

The important finding of this study is neither of any patients (included positive control in this study) as well as other SCD individuals were detected as homozygous form of SCD which is the most severe form of sickle cell anemia. This result made our research more curious and raised the question of what factor that affected such condition by chance or not? The most prevalence of hemoglobinopathies has been reported on Tharu ethnic group. The clinically important variants of hemoglobin are sickle cell hemoglobin, HbE, alpha and beta thalassemia, fetal hemoglobin and HbD which are well reported in Nepal. The co-inheritance of sickle cell allele and beta thalassemia is also reported in this group.

Several study showed that PCR- RFLP test for diagnosis of sickle cell disease is superior to conventional hemoglobin electrophoresis (Abdelazim *et al.,* 2015). Hatcher *et al.,* 1992 designed an enzymatic amplification and restriction endonuclease digestion method for detection of SCD. The detection of SCD mutation at the 6^{th} codon of beta globin using PCR-RFLP is simple, rapid, sensitive and applicable for prenatal diagnosis (Haghshenas, 2004). Detection of sickle cell gene by the analysis of amplified DNA sequences was carried out in China (Huang *et al.,* 1988), Venezuela (Martínez *et al.,* 1998) and Iran (Ayatollahi *et al.,* 2005) by using the endonuclease MS II. Nanda, 2015 described RFLP analysis as a powerful technique for diagnosis of SCD because it analyses directly for a genotype without depending on expression of the gene or phenotypic expression of disease (Nadia, 2015). This research were concerned on certain malarial region of Kailali and Kanchanpur districts

and study showed the origin of Dangaura Tharu were in Dang and distributed to Kanchanpur districts, while Rana Tharu were migrated from Rajput origin of India. They have marked difference in cultural and religious rituals. The marriage and family relation between these two subgroups were prohibited before few decades (Krauskopff, 1995; Paudyal, 2012). But the incidence of such gene on both of subgroups of age even >40 age is interesting feature of our study.

Shah *et al.,* 2012 conducted a survey on six Muslim castes in Manipur, and calculated the sickle cell allele frequency. It was reported that highest sickle allele frequency was seen on Mughals (0.17%) and the least from Nagas (0.086) (Shah *et al.,* 2012). The frequency of sickle cell allele varies world widely according to geographical status as well as ethnicity. Current study presented the frequency of sickle SCD allele for Rana subgroup of Tharu (0.12) was slightly higher than Dangaura subgroup (0.104) which is similar to the study by Shah *et al.,* 2012.

Our study showed 21.55% (25/116) of the Tharu are SCD carrier. Similar research were carried out by Tamar *et al.,* 2014 in Haiti population which showed 7.22% (14/194) prevalence of SCD. This shows the high prevalence of SCD in Tharu population of our country (Tamar *et al.,* 2014). This status asks for the essence of effective programs to carry out to control the severeness of the disease.

The incidence of these genes among Tharus people might be adopted as selective pressure to protect from malarial parasite invasion. The endogamous marriage practice is more common on Tharu ethnic community which may result in greater allele frequency of SCD.

Our study identified that the sickle cell disease is distributed among Dhangadhi, Attariya, and Krishnapur. The prevalence of carrier for SCD has found to be high. If both parents happened to the carrier for SCD, there are chances of birth of homozygous SCD child, which is the most severe form of anemia. Incidences of such disease in future generation can be reduced by three methods, a) identifying carrier individual and decreasing the marriage between high risk individuals, b) prenatal diagnosis to reduce disease child birth and c) newborn screening to high risk area. Among them identifying carrier people is the best option, because prenatal diagnosis may not be available in remote areas and also may present social or religious issues. Neonatal screening approach is only applicable to prevent secondary and tertiary complications of disease. Thus, to identify frequency of sickle cell disease, premarital screening approach may be the greatest option of choice (AlHamdan *et al.,* 2007).

In our country Nepal, the actual data regarding the SCD and its carrier is unavailable. Neither any screening programs have been carried out. A rapid, reliable and high throughput technology should be applied to diagnose such disease and generate the database. Besides, it is essential to provide health education especially to the public of the disease prone area and to the health workers. The provision of free screening program may help to find out the prevalence of the disease and its carriers.

CHAPTER 6

SUMMARY

Sickle cell disease is the most common inherited disorder of hemoglobin which occurs due to the defect in β-globin subunit. Though it is more common in malarial endemic region throughout the world, due to migration it is also prevalence in other parts. Genetic cause of this disease is the point mutation at 6^{th} codon of the *β-globin* which results in substitution of glutamate to (GAG→GTG) valine. This single amino acids change leads to synthesis of structural variant of hemoglobin known as sickle hemoglobin (HbS). Heterozygous (carrier) of sickle hemoglobin provides protective effects against malarial parasites. Thus, sickle cell diseases assume to arise as selective pressure to protect malaria. However homozygous form of sickle hemoglobin HbSS, as a result of point mutation on both β-globin alleles of adult HbA is most severe if not treated.

One hundred and sixteen blood samples were collected from two Tharu subgroups (Rana and Dangaura) of Kailali and Kanchanpur districts. These samples were subjected to hemoglobin solubility test for the screening of sickle hemoglobin by observing the precipitation of HbS on phosphate buffer containing sodium dithionate as reducing agent. Twenty six samples showed positive results for hemoglobin solubility test. The genomic DNA was extracted from osmolarity methods and quantified by both gel electrophoresis and spectrophotometry. From extracted genomic DNA, exon 1, intron 1 and exon 2 of *β-globin* (539 bp); which bears the causative mutation for SCD, were PCR amplified by using specific primers. Confirmation and validation of the point mutation that causes sickle cell disease was done by restriction digestion of amplified products by using restriction enzyme Bsu 36I. Twenty five samples were confirmed to be sickle cell carrier (heterozygous) whereas none of them (even the positive samples) were found to be homozygous for the mutation. Hemoglobin solubility test is rapid and cheap for mass screening of sickle cell disease but it unable to detect homozygous and heterozygous sickle cell disease. It also gives false results, thus to confirmation and validation, reliable and cheaper technique is required. PCR- RFLP provides specific, sensitive and rapid diagnosis of sickle cell disease.

Although the origin of Rana Tharu and Dangaura were from different geographical areas and marriage practice among these groups is restricted, the distribution of HbS gene has been found on both subgroups. The frequency of heterozygous HbS allele was similar in both male 13/60(21.67%) and female 12/56(21.43%). The highest HbS carrier frequency among the Tharu of >40 age group i.e. 6/23 (26.08%) followed by 20-40 years groups (17/75 (22.66%)), and least number from <20 years groups that is 2/18 (11.11%).

This study shows the high prevalence of SCD in our country but the actual status is unknown. Lack of health awareness and rapid, easy and efficient clinical diagnostic facilities are far behind from the reach of risk populations. The treatment of disease is very expansive and hence is not affordable for poor people. The provision of reliable rapid and high throughput screening of risk individual would help to generate accurate data. Awareness to the risk population regarding the disease and its complications and encouraging them for the early treatment helps in the management of the disease. Screening at different age group like newborn screening, prenatal diagnosis of risks couple and premarital screening of risks population helps to make good strategies to elute disease number and its further management as well as reduction of risked child born in future generation.

CHAPTER 7

CONCLUSION

In this study we tried to find out the prevalence of sickle cell disease in two subgroups of Tharu ethnic community of Kailali and Kanchanpur districts of Far-western region of Nepal. These regions have been reported endemic to malaria. To find out prevalence of SCD, hemoglobin solubility test was performed and the results were confirmed by PCR-RFLP. Hemoglobin solubility showed three false positive and two false negative results. This concludes hemoglobin solubility test can only be used as preliminary test for SCD and thus the results need to be confirmed by performing a molecular test such as PCR-RFLP. Within the two subgroups of Tharu, the prevalence of SCD was found to be high on Rana Tharu compared to Dangaura Tharu. This result concludes that the prevalence of SCD is pretty common in malarial endemic regions. Also, PCR- RFLP can be used as an efficient, cost effective and convenient technique for the detection and identification of SCD patients and also the carriers. Results can assist the physicians to interpret better clinical management of sickle cell disease and application of such technique as screening programme in sickle cell disease prevalence population but asymptomatic cases would give good results that help to reduce of disease in childbirth in next generation.

Recommendations

- The present study is concerned to Rana and Dangaura (Tharu) from few areas of Kailali and Kanchanpur districts. Hence, for statistical significance, greater number of Tharu people from different subgroup should be included.
- The present analysis was done in a mutation prone in 6^{th} codon β-globin gene that causes SCD, the coinheritance of one sickle cell allele (heterozygous allele of SCD) with other variants of hemoglobin like HbC, β-thalassemia and HbD should be studied.
- Although Bsu 36I can be utilized for diagnosis of SCD, but it is unable to differentiate HbC from HbA thus sequencing could be performed.
- Technology as well as social awareness should be developed in such way as to not only detect the emergence of sickle cell disease but also prevent them.

LIMITATION OF THE STUDY

Because of limitation of time and financial constrain, our research was only focused on molecular diagnosis of SCD. We were not able to perform other phenotypic test of HbS rather than sickle solubility test. Also, the results would have been more precised if we could have been able to perform the sequencing of the samples.

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https://movietheater.co/Biogeography-and-Ecology-of-New-Guinea.pdf.

APPENDIX

Appendix 1

Composition of buffer

Nucleic Acid lysis buffer:

0.01M Tris-HCl (pH 7.6).

11.4mM Tri-sodium citrate.

1mM EDTA (pH 8).

1% SDS.

RBC lysis buffer:

0.32 M Sucrose

10mM Tris-HCl (pH 7.5)

5 mM MgCl2

1% v/v Triton X-100.

0.8% Agarose:

0.4 gram agarose + 50ml(1X) TAE buffer**.**

TE buffer: Tris-EDTA buffer

10mM Tris-HCl.

1mM EDTA (pH 8).

50X TAE buffer: Tris Acetate EDTA buffer

Tris Base – 242 gram.

Glacial Acetic Acid – 57.1ml.

0.5M EDTA (pH-8) – 100ml.

Distilled water – 1000ml.

pH of buffer – 8.5

Gel loading dye – Bromophenol Blue:

0.25% Bromophenol dye.

0.25% Xylene cyanol.

30% Glycerol in water. (Store at 4ºC).

Ethidium bromide (10mg/ml):

Add 0.5 gram of EtBr to 50ml of water. Stir on a magnetic stirrer to ensure that dye is dissolved. Wrap the container in the

Aluminium foil or transfer the solution in a dark bottle stored at room temperature.

Normal saline

8.5 gram sodium chloride in one litre distilled water.

10% Ammonium Persulphates (APS)

1g APS was dissolved in 10 ml autoclaved distilled water. 500 µl of aliquot were made in new autoclaved eppendroff tube and storage at -20ºC.

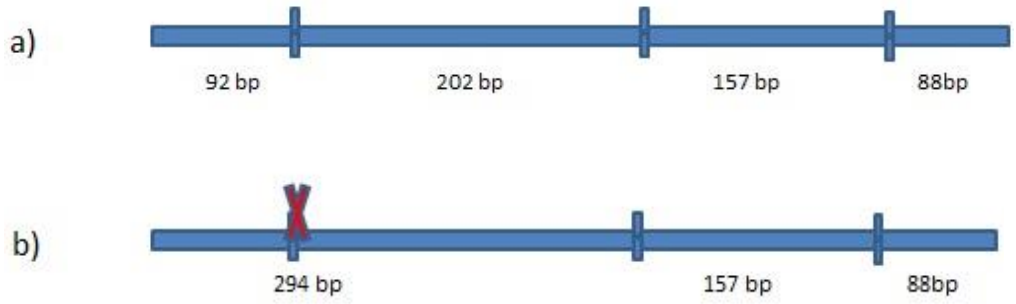
30%(W/V) acrylamide bisacrylamide solution

29g acrylamide and 1g bisacrylamide was dissolve in 100 ml double distilled water mixed completely.

10X TBE buffer

108g Tris base,55gm boric acid, and 7.44 g Na_2EDTA dissolve in distilled water using magnetic stirrer and made final volume 1l addition of water .Kept the buffer in 4ºC in freeze until use.

Appendix 2



Picture: Schematic diagram of size and number of DNA fragments generated by Bsu 36I restriction enzyme on PCR amplified (targeted) region of β-globin a) Restriction sites and its DNA fragments in healthy control b) Restriction site affected because of a point mutation in sickle cell disease.

Appendix 3

Figure: Sample collection at Krishnapur, Kanchanpur

Appendix 4

Amplified β-globin gene sequence

AGTCAGGGCAGAGCCATCTATTGACTTACATTTGCTTCTGACACAACTGTGTTCACTAGCAACCTCAAA
CAGACACCATGGTGCATCTGACTCCTGGGAGAAGTCTGCCGTTACTGCCCTGTGGGGCAAGGTGAAC
GTGGATGAAGTTGGTGGTGAGGCCCTGGGCAGGTTGGTATCAAGGTTACAAGACAGGTTTAAGGAG
ACCAATAGAAACTGGGCATGTGGAGACAGAGAAGACTCTTGGGTTTCTGATAGGCACTGACTCTCTCT
GCCTATTGGTCTATTTTCCCACCCTTAGGCTGCTGGTGGTCTACCCTTGGACCCAGAGGTTCTTTGAGT
CCTTTGGGGATCTGTCCACTCCTGATGCTGTTATGGGCAACCCTAAGGTGAAGGCTCATGGCAAGAAA
GTGCTCGGTGCCTTTAGTGATGGCCTGGCTCACCTGGACAACCTCAAGGGCACCTTTGCCACACTGAG
TGAGCTGCACTGTGACAAGCTGCACGTGGATCCTGAGAACTTCAGGGTGAGTCTATGGGACGCT

Beta globin gene sequence in chromosome 11

Genomic DNA sequence

5’UTR

ACATTTGCTTCTGACACAACTGTGTTCACTAGCAACCTCAAACAGACACCATGGTGCATCTGACT
CCTGAGGAGAAGTCTGCCGTTACTGCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGT
GAGGCCCTGGGCAGGTTGGTATCAAGGTTACAAGACAGGTTTAAGGAGACCAATAGAAACTGC
ATGTGGAGACAGAGAAGACTCTTGGGTTTCTGATAGGCACTGACTCTCTCTGCCTATTGGTCTTT
TTCCCACCCTTAGGCTGCTGGTGGTCTACCCTTGGACCCAGAGGTTCTTTGAGTCCTTTGGGGAT
CTGTCCACTCCTGATGCTGTTATGGGCAACCCTAAGGTGAAGGCTCATGGCAAGAAAGTGCTCG
GTGCCTTTAGTGATGGCCTGGCTCACCTGGACAACCTCAAGGGCACCTTTGCCACACTGAGTGA
GCTGCACTGTGACAAGCTGCACGTGGATCCTGAGAACTTCAGGGTGAGTCTATGGGACGCTTG
ATGTTTTCTTTCCCCTTCTTTTCTATGGTTAAGTTCATGTCATAGGAAGGGGATAAGTAACAGGG
TACAGTTTAGAATGGGAAACAGACGAATGATTGCATCAGTGTGGAAGTCTCAGGATCGTTTTGT
TTCTTTTATTTGCTGTTCATAACAATTGTTTTCTTTTGTTTAATTCTTGCTTTCTTTTTTTTTCTTCTC
CGCAATTTTTACTATTATACTTAATGCCTTAACATTGTGTATAACAAAAGGAAATATCTCTGAGA
TACATTAAGTAACTTAAAAAAAAACTTTACACAGTCTGCCTAGTACATTACTATTTGGAATATAG
TGTGCTTATTTGCATATTCATAATCTCCCTACTTTATTTTCTTTTATTTTTAATTGATACATAATCAT
TATACATATTTATGGGTTAAAGTGTAATGTTTTAATATGTGTACACATATTGACCAAATCGGGTA
ATTTTGCATTTGTAATTTTAAAAAATGCTTTCTTCTTTTAATATACTTTTTTGTTTATCTTATTTCTA
ATACTTTCCCTAATCTCTTTCTTTCAGGGCAATAATGATACAATGTATCATGCCTCTTTGCACCAT
TCTAAAGAATAACAGTGATAATTTCTGGGTTAAGGCAATAGCAATATCTCTGCATATAAATATTT
CTGCATATAAATTGTAACTGATGTAAGAGGTTTCATATTGCTAATAGCAGCTACAATCCAGCTAC
CATTCTGCTTTTATTTTATGGTTGGGATAAGGCTGGATTATTCTGAGTCCAAGCTAGGCCCTTTT
GCTAATCATGTTCATACCTCTTATCTTCCTCCCACAGCTCCTGGGCAACGTGCTGGTCTGTGTGCT
GGCCCATCACTTTGGCAAAGAATTCACCCCACCAGTGCAGGCTGCCTATCAGAAAGTGGTGGCT
GGTGTGGCTAATGCCCTGGCCCACAAGTATCACTAAGCTCGCTTTCTTGCTGTCCAATTTCTATT
AAAGGTTCCTTTGTTCCCTAAGTCCAACTACTAAACTGGGGGATATTATGAAGGGCCTTGAGCA
TCTGGATTCTGCCTAATAAAAAACATTTATTTTCATTG

3’ UTR

Exon 1 (50 bp 5’ UTR+ 76 bp)

ACATTTGCTTCTGACACAACTGTGTTCACTAGCAACCTCAAACAGACACCATGGTGCATCTGACT
CCTGAGGAGAAGTCTGCCGTTACTGCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTG

Intron 1 (146 bp)

GTGAGGCCCTGGGCAGGTTGGTATCAAGGTTACAAGACAGGTTTAAGGAGACCAATAGAAACT
GGGCATGTGGAGACAGAGAAGACTCTTGGGTTTCTGATAGGCACTGACTCTCTCTGCCTATTGG
TCTATTTTCCCACCCTTAG

Exon 2 (223 bp)

GCTGCTGGTGGTCTACCCTTGGACCCAGAGGTTCTTTGAGTCCTTTGGGGATCTGTCCACTCCTG
ATGCTGTTATGGGCAACCCTAAGGTGAAGGCTCATGGCAAGAAAGTGCTCGGTGCCTTTAGTA
TGGCCTGGCTCACCTGGACAACCTCAAGGGCACCTTTGCCACACTGAGTGAGCTGCACTGTGAC
AAGCTGCACGTGGATCCTGAGAACTTCAGG

Intron 2(850 bp)

GTGAGTCTATGGGACGCTTGATGTTTTCTTTCCCCTTCTTTTCTATGGTTAAGTTCATGTCATAGG
AAGGGGATAAGTAACAGGGTACAGTTTAGAATGGGAAACAGACGAATGATTGCATCAGTGTG
GAAGTCTCAGGATCGTTTTAGTTTCTTTTATTTGCTGTTCATAACAATTGTTTTCTTTTGTTTAATT
CTTGCTTTCTTTTTTTTTCTTCTCCGCAATTTTTACTATTATACTTAATGCCTTAACATTGTGTATAA
CAAAAGGAAATATCTCTGAGATACATTAAGTAACTTAAAAAAAAACTTTACACAGTCTGCCTAG
TACATTACTATTTGGAATATATGTGTGCTTATTTGCATATTCATAATCTCCCTACTTTATTTTCTTT
TATTTTTAATTGATACATAATCATTATACATATTTATGGGTTAAAGTGTAATGTTTTAATATGTGT
ACACATATTGACCAAATCAGGGTAATTTTGCATTTGTAATTTTAAAAAATGCTTTCTTCTTTTAAT
ATACTTTTTTGTTTATCTTATTTCTAATACTTTCCCTAATCTCTTTCTTTCAGGGCAATAATGATAC
AATGTATCATGCCTCTTTGCACCATTCTAAAGAATAACAGTGATAATTTCTGGGTTAAGGCAATA
GCAATATCTCTGCATATAAATATTTCTGCATATAAATTGTAACTGATGTAAGAGGTTTCATATTG
CTAATAGCAGCTACAATCCAGCTACCATTCTGCTTTTATTTTATGGTTGGGATAAGGCTGGATTA
TTCTGAGTCCAAGCTAGGCCCTTTTGCTAATCATGTTCATACCTCTTATCTTCCTCCCACAG

Exon 3 (260 bp)

CTCCTGGGCAACGTGCTGGTCTGTGTGCTGGCCCATCACTTTGGCAAAGAATTCACCCCACCAG
TGCAGGCTGCCTATCAGAAAGTGGTGGCTGGTGTGGCTAATGCCCTGGCCCACAAGTATCACT
AAGCTCGCTTTCTTGCTGTCCAATTTCTATTAAAGGTTCCTTTGTTCCCTAAGTCCAACTACTAAA
CTGGGGGATATTATGAAGGGCCTTGAGCATCTGGATTCTGCCTAATAAAAAACATTTATTTTCAT
TG

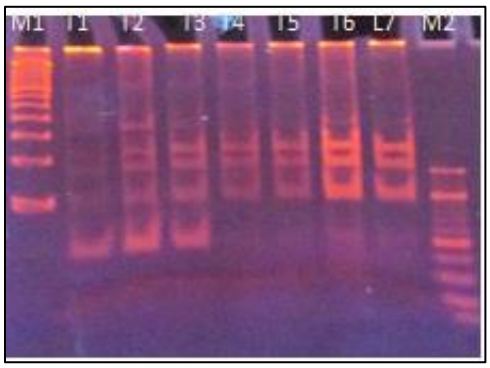
5’UTR (50 bp)

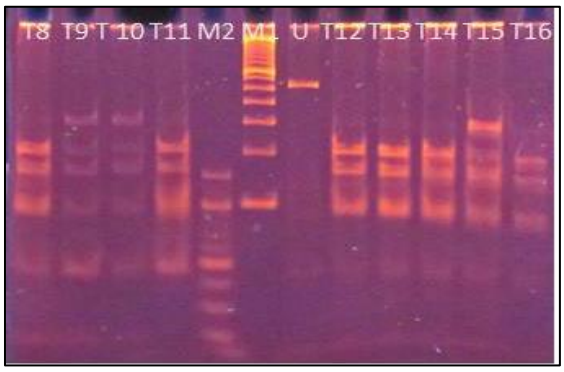
ACATTTGCTTCTGACACAACTGTGTTCACTAGCAACCTCAAACAGACACC

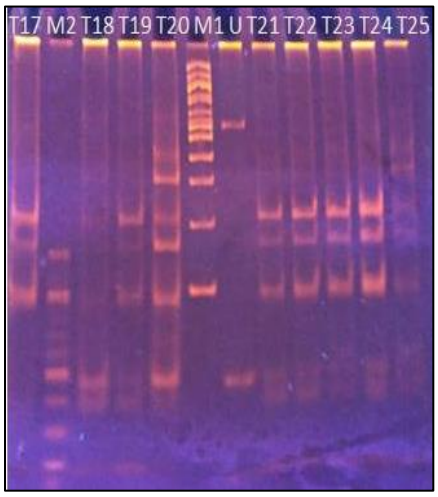
3’ UTR (391 bp)

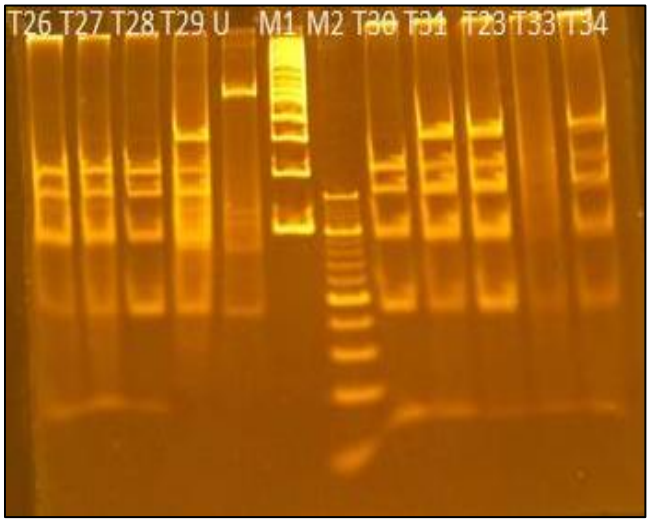
AGGCTCATGGCAAGAAAGTGCTCGGTGCCTTTAGTGATGGCCTGGCTCACCTGGACAACCTCAA
GGGCACCTTTGCCACACTGAGTGAGCTGCACTGTGACAAGCTGCACGTGGATCCTGAGAACTTC
AGGCTCCTGGGCAACGTGCTGGTCTGTGTGCTGGCCCATCACTTTGGCAAAGAATTCACCCCAC
AGTGCAGGCTGCCTATCAGAAAGTGGTGGCTGGTGTGGCTAATGCCCTGGCCCACAAGTATCA
CTAAGCTCGCTTTCTTGCTGTCCAATTTCTATTAAAGGTTCCTTTGTTCCCTAAGTCCAACTACTA
AACTGGGGGATATTATGAAGGGCCTTGAGCATCTGGATTCTGCCTAATAAAAAACATTTATTTT
CATTG

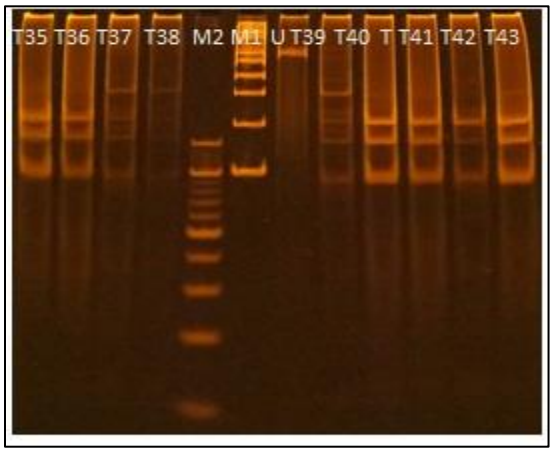
Appendix 5

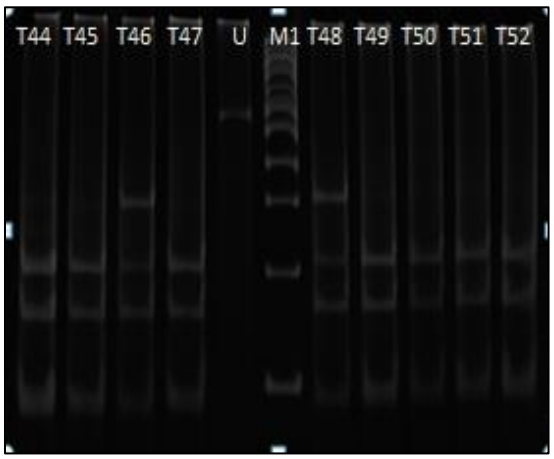

M1 T1 T2 T3 T4 T5 T6 L7 M2

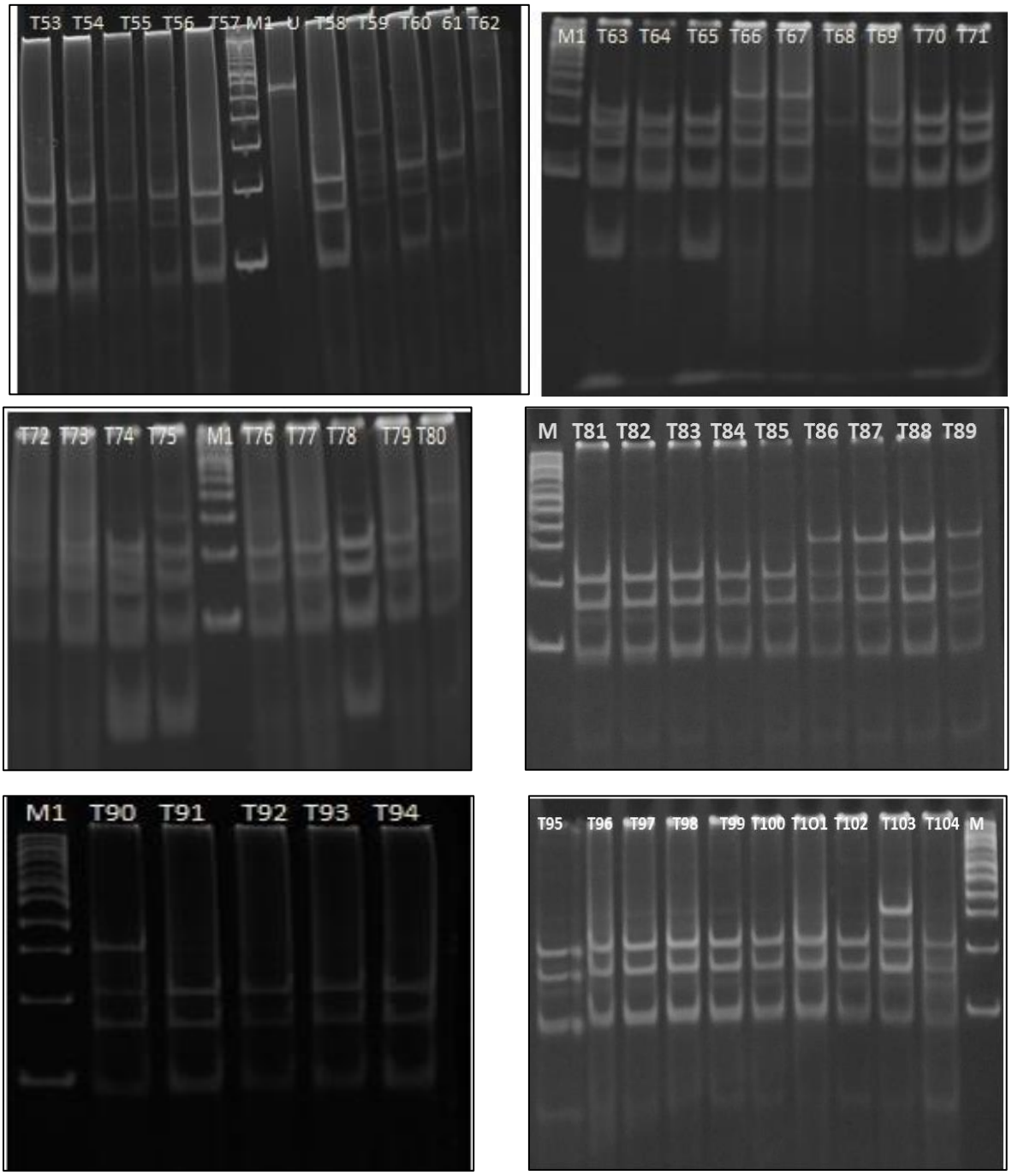

T8 T9 T 10 T11 M2 M1 U T12 T13 T14 T15 T16


T17 M2 T18 T19 T20 M1 U T21 T22 T23 T24 T25


T26 T27 T28 T29 U M1 M2 T30 T31 T23 T33 T34


T35 T36 T37 T38 M2 M1 U T39 T40 T T41 T42 T43


T44 T45 T46 T47 U M1 T48 T49 T50 T51 T52



Figures: Polyacrylamide gel electrophoresis (10%) of restriction digested products using Bsu 36I restriction enzyme for the confirmation of sickle cell disease M1- 100bp DNA marker (NEB), M2 –(10-150) bp DNA marker and other are samples.

Appendix 6

Table: Results of PCR-RFLP showing sickle cell disease (heterozygous)

S.N.	Heterozygous (HbAS) sample	Code
1.	T3	J20
2.	T9	F17
3.	T10	F16
4.	T15	F12
5.	T20	J16
6.	T25	J11
7.	T29	F24
8.	T31	DC25
9.	T32	DC21
10.	T34	G5
11.	T37	F28
12.	T38	D25
13.	T4O	G20
14.	T44	D17
15.	T48	F39
16.	T59	D23
17.	T66	D13
18.	T67	F8
19.	T75	J22
20.	T80	D1

S.N.	Heterozygous (Hb AS) sample	Code
21	T86	D13
22.	T87	H5
23.	T88	H4
24.	T89	H1
25.	T103	D6

Appendix 6: Format of consent form

Detection of the Prevalence of Sickle Cell Disease in Tharu Population of Far-western Nepal by RFLP Method

सहभागीको मन्जूरीनामा फाराम

Study No : मिति :

तपाईलाई यस अध्ययनमा सहभागी हुनलाई आह्वान गर्नुको कारण रगतको नमुना जाचँबाट थारु समुदायमा हुने वंशाणुगत रोग (Genetic disease) पत्ता लगाउनको लागि हो । यस मन्जूरीनामा पत्रको उद्देश्य तपाईलाई यस अध्ययन सम्वन्धि पर्याप्त जानकारी दिनु हो ताकी तपाई यस अध्ययनमा सहभागी हुने नहुने सहि निर्णय लिन सक्नु हुनेछ । तपाईलाई प्रष्ट नभएका कुनै पनि प्रश्नहरु सोध्न सक्नुहुने छ । तपाईबाट ३ मि.लि. रगत लिइने छ । यस प्रक्रियामा करिव ५ मिनेट समय लाग्नेछ । यी संकलित नमूनाहरुबाट **जैविक प्रविधि केन्द्रिय विभाग, त्रिभुवन विश्वविद्यालय (Central Department of Biotechnology, Tribhuvan University)** मा DNA को विश्लेशण गरिने छ । संकलित नमुनाहरुलाई गोप्य संख्यामा लेखिनेछ ताकी तपाईको व्यक्तिगत परिचय गोप्य रहन सकोस् । तथापि तपाईको व्यक्तिगत परिचयलाई गोप्य राखिने छ । रगत निकाल्नाले तपाईलाई कुनै किसिमको हानि हुने छैन ।

फाइदा :

यस अनुसन्धानमा सहभागी भएर तपाई वा तपाईंको परिवारलाई प्रत्यक्षरुपमा फाइदा हुन÷नहुन सक्छ, तर तपाईंको समुदायलाई फाइदा हुनेछ। यस अध्ययनबाट प्राप्त जानकारी मार्फत यो रोगसँग सम्बन्धित कति व्यक्तिहरु छन् भन्ने पत्ता लगाउने हो र भविष्यमा उक्त रोग लाग्नबाट कसरी निराकरण गर्ने भन्ने हो ।

गोपनीयता :

यस अनुसन्धान/अध्ययनको नतीजा प्रकाशित गर्न सकिनेछ, तर त्यसमा तपाईको नाम तथा परिचय उल्लेख हुनेछैन।

स्वेच्छिक सहभागिताको बयान :

यस अनुसन्धानमा मेरो सहभागिता स्वेच्छिक हो । मैले आफ्नो इच्छाले विना जरिवाना, डरत्रास विना तल उल्लेख गरीएका अनुसन्धानकर्ता समक्ष पूर्व सुचना बिनानै कुनै पनि बेला यस अनुसन्धानबाट सहभागिता परित्याग गर्न सक्नेछु । मैले माथि लेखिएका कुराहरु पढेको छु अथवा मलाई पढेर सुनाइएको छ , मेरो प्रश्नहरुको जवाफ दिइएको छ र आफ्नो इच्छाले यस फाराममा सही गरी मेरो शरीरबाट ३ मि.लि. रगत झिक्न अनुमति दिएको छु ।

लेखपढ गर्न नजान्नेहरुको लागि तल भर्नुहोस :

म यो प्रमाणीत गर्दछु कि मैले माथि उल्लेखित सूचना सहभागी
श्रीमान्/श्रीमती/सुश्री..... लाई सवै पढेर सुनाएको छु तथा ब्याख्या गरेको छु । मैले भनेको कुरा उहाँले बुझेको कुरा दर्शाएको छ तथा प्रश्न सोध्नको लागी पुरा मौका दिएको छ र उहाँले अनुसन्धानमा सहभागी हुन मन्जुरी जनाउनु भएको छ । यो सही/चिन्ह/औठाको छाप उहाँको हो भनि म प्रमाणीत गर्दछु ।

सहभागीको नाम :.....

उमेर :.....

ठेगाना :.....

सम्पर्क नं. :.....

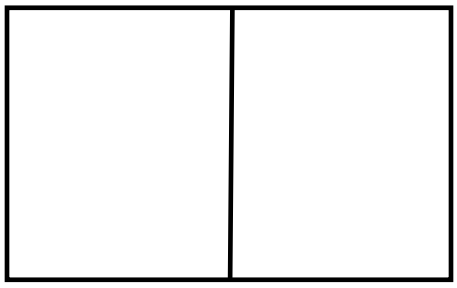
सहभागीको हस्ताक्षर **(** लेखपढ गर्न जान्नेहरुको लागि**)**:.....

फिल्डवर्करको हस्ताक्षर :.....

फिल्डवर्करको नाम :.....

मिति :.....

सहभागीको सही÷चिन्ह÷औठाको छाप



दायाँ बाँया