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DISTRIBUTION OF SICKLE CELL ALLELE IN ETHNIC SUB-GROUPS OF THARU IN NEPAL

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ABSTRACT
Sickle cell disease has been a major inherited blood disorder in several populations of the world including Tharu tribal communities of Nepal and India. This cross sectional study aims to access the prevalence of sickle cell disease in Tharu communities of Nepal by three different tests.Total 386 blood samples were collected from eight different sub-ethnic groups of Tharu communities of Rupandehi district, Nepal. The samples were tested for presence of sickle haemoglobin by sickle cell solubility test and sickling test as screening test method and those seen positive from either of these two tests were undertaken through genomic test by restriction fragment length polymorphism. Out of total 386 samples, 67 were seen positive from sickling test and 68 from solubility test. One and two samples were positive for only sickling test and solubility test respectively. 69 samples were tested by molecular method for confirmation and genotyping and out of them two were wild, 57 hetero and 10 mutant. Kanwar Tharu had highest prevalence(53.94%) and Marchaha Tharu had lowest prevalence(0%) of sickle cell allele in their gene pool. The allelic frequency of HbA and HbS were found to be 0.9 and 0.1 respectively. Prevalence of sickle cell anaemia was not found to be common in Tharu community, $\chi^2(2, N=386)=686.389, p< .00001$). The distribution of sickle cell allele was cast dependent, $\chi^2(7, N=386)=58.2823, p< 0$) and Kanwar Tharu and Dadaha Tharu are most severely affected sub-ethnic groups with about 50% occurrence of HbS in their population.

KEYWORDS: ***Blood, RFLP, Sickle cell disease, Sickle cell allele, Sickling test, sickle cell solubility test, Tharu***

1. INTRODUCTION

Sickle cell disease is a group of haemoglobinopathies including SCA, HbSC and HbSβ-thalassemia characterized by mutation in the gene encoding haemoglobin sub-unit β(G. Kato et al., 2018). The gene defect is a known SNP changing GAG codon with GTG resulting in the synthesis of valine in place of glutamic acid(A.C. Allison, 2009).

SCD is the most common hereditary disease affecting about 250,000 newborn every year worldwide(T.N. Williams and D.J. Weatherall, 2012). The primary event in the pathogenesis of SCD is HbS polymerization occurring in deoxygenated erythrocytes(P. Sundd et al., 2019). The sickled erythrocytes obstruct blood vessels and have a reduced life span, leading to haemolytic anaemia, diffuse vasculopathy and tissue damage in various target organs(F. Lionnet et al., 2012). There is a wide variability in the clinical severity of SCA, as well as in the life expectancy(M.H. Steinberg and P. Sebastiani, 2012).

The Tharu people are an ethnic group indigenous to southern foothills of Himalaya range including parts of Nepal and India(J. Mclean,1999). Most of them live in Terai region of Nepal(W. Hechler and A. Guneratne, 2003). Tharu people are found to be suffered from SCD by different researches published in Nepalese medical journals(R. Pande et al., 2019). Sickle cell anemia and β-thalasemia trait (β-TT) have been a major health threat for the Tharu living in the South-Western Terai of Nepal(N. Gautam et al., 2020). Unfortunately, health services are limited and inaccessible for Tharu individuals suffering from sickle cell disease(M. Marchand et al., 2017). We conducted a study finding the prevalence of sickle cell disease in Tharu communities of Western Nepal.

2. MATERIALS AND METHODS

The samples were collected randomly from Rupandehi district of Lumbini province, Nepal between April-July,2020. 386 individuals of eight different sub-ethnic groups of Tharu community aged four to 83 year from Tharu ethnic group participated in this survey. Approval from Tribhuvan University, Kathmandu and District Public Health Office, Rupandehi and consent from participating candidates were taken in written form.

About three ml of venous blood was collected and preserved in K2EDTA vials. Three different methods were used to detect the presence of HbS in the blood sample.
All the samples were tested for presence of sickle haemoglobin by sickling test and sickle cell solubility test. The samples diagnosed to be positive from either of these two methods were undertaken for molecular test by RFLP.

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2.1 Sickling Test

Sodium metabisulphite was used as reducing agent for sickling test and one drop of blood was mixed with one drop of 2% sodium metabisulphite solution on a glass slide. The cover slip was immediately sealed with Vaseline and allowed it to stand at room temperature for about one hour then morphology of erythrocytes was observed under compound microscope.

2.2 Sickle Cell Solubility Test

The reagent for sickle cell solubility test was prepared by following R.M. Nalbandian et al,(1971). Two ml of working solution at room temperature was put into 12X75 mm test tube followed by 0.02 ml of whole blood and mixed thoroughly. The turbidity was read after five minutes in paper board test tube holder with reading card having 18 point black type straight lines 2.5 cm behind the test tube.

2.3 RFLP Test

The samples that showed positive result from either sickling test or solubility test or both were prescribed for confirmatory RFLP test. The genomic DNA was extracted by spin column method using Quick DNA Miniprep Plus Kit 10 preps(Zymo Research Corporation, USA) following manufactures manual. The PCR primers were adopted from Adhikari, 2017 that amplifies 539 bp region including 5'UTR, 1st exon, 1st intron and 2nd exon of β-globin gene of 11th chromosome. The PCR amplified product was run in TAE 1.2% agarose gel at 100V using 100bp DNA ladder to confirm correct amplification. The product was then digested using restriction endonuclease DdeI(C↓T N A G)(Vivantech, Malaysia). Finally the digested product was run in TAE 1% agarose gel electrophoresis using 100 bp molecular ladder at 100 V for confirmation and genotyping. The goodness of fit of distribution of HbS and test of independence of distribution of HbS among different sub-ethnic groups of Tharu tribe were tested using chi-square calculator, Social Science Statistics and remaining analysis was done manually.

Table 1: PCR primer sequence

Forward primer	5'-AGTCAGGGCAGAGCCATCTA-3'
Reverse primer	5'-AGGGTCCCATAGACTCACCC-3'

3. RESULT

Out of 386 samples, 67 showed positive from sickling test and 68 from sickle cell solubility test. One sample was positive from sickling test only and two from solubility test only. Thus total 69 samples were prescribed for RFLP test where two samples were found to be homozygous dominant(HbAA), 57 heterozygous(HbAS) and 10 homozygous recessive(HbSS). The solubility test gave two false positive result. The allelic frequency were 0.9 and 0.1 for HbA and HbS respectively. Out of eight sub-ethnic groups, Kanwar Tharu showed highest prevalence(53.84%) while Marchaha Tharu had lowest(0%) prevalence of sickle cell allele. The prevalence of sickle cell anaemia was not found to be common in Tharu community $\chi^2(2, N=386)=686.389$, $p< .00001$). The distribution of sickle cell allele is cast dependent, $\chi^2(7, N=386)=58.2823$, $p< 0$).

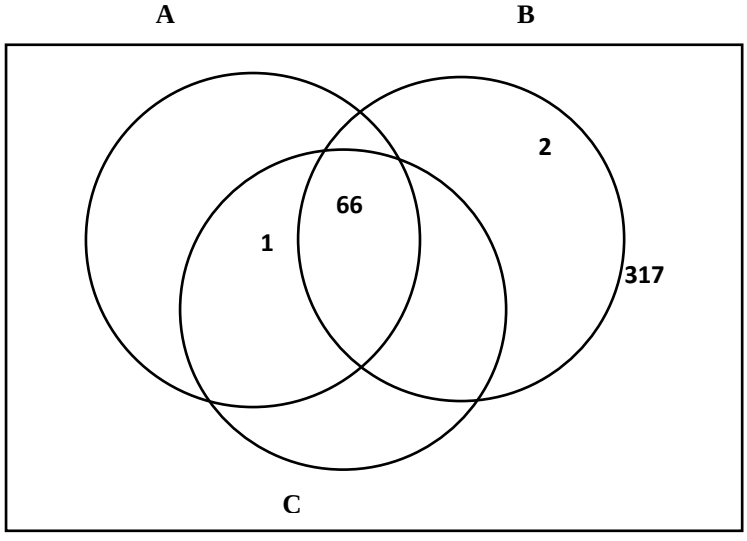

Fig. 1: Venn diagram showing comparative result of sickling, solubility and RFLP test where, A= no. of positive samples from sickling test. ; B= no. of positive samples from sickle cell solubility test. ; C= no. Of positive samples for HbS from RFLP test .

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Table 2: Table showing ethnicity wise distribution of sickle haemoglobin among different ethnic sub-groups of Tharu community of Rupandehi district, Nepal.

Sub-ethnic group	Total no. of samples	No. of positive samples	Prevalence(%)
Kanwar	26	14	53.84
Dadaha	32	15	46.87
Kathariya	125	23	18.40
Purbiya	25	3	12.00
Khausiya	21	2	9.52
Dangoriya	72	5	6.94
Baatar	82	5	6.09
Marchaha	3	0	0
Total	386	67	

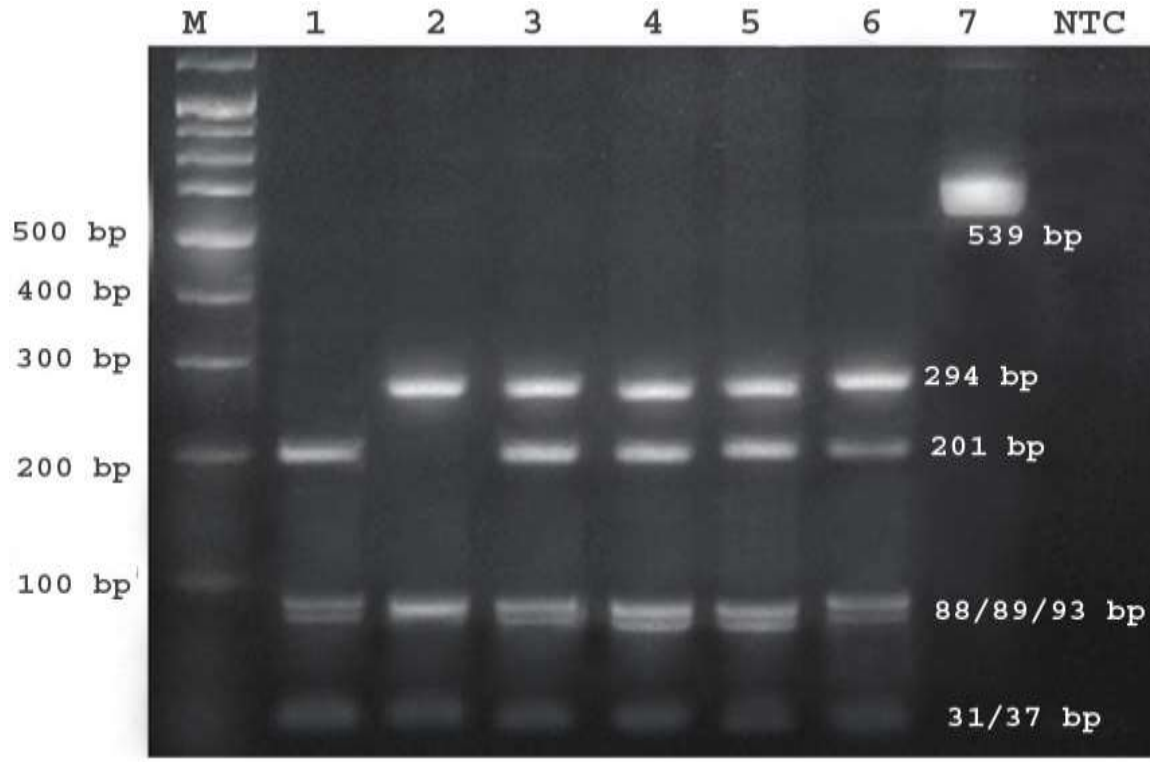

Figure 2: A photograph of TAE agarose gel 1% electrophoresis showing different bandings cut by DdeI restriction endonuclease. Lane M is the 100 bp DNA ladder, lane 1 is a negative control(AA), lane 2 is a positive control(SS), lane 3,4,5 and 6 are trait(AS), lane 7 is restriction negative control with uncut template DNA and lane NTC is a no template control.

4. DISCUSSION

This cross-sectional study was carried out in indigenous Tharu tribes of Rupandehi district of Mid-Western Terai of Nepal. A total of 386 people participated from different eight sub-ethnic groups of Tharu tribe. All the collected blood samples were undertaken through sickling test and sickle cell solubility test. There we found 67 positive from sickling test and 68 from solubility test. Out of total 69 positive samples two were positive only for solubility test and one was for only sickling test and remaining 66 were positive for both tests. When those 69 samples were undertaken through RFLP test, 57 found to be of HbAS genotype, 10 of HbSS and two of HbAA genotype. Two samples showed false positive and one false negative from solubility test while sickling test showed all true results. The prevalence of sickle haemoglobin was found to be 17.35%. Out of them Kanwar Tharu were found to have highest prevalence(53.88%) and Marchaha Tharu have lowest prevalence(0%). Dadaha Tharu had 46.87%, Kathariya 18%, Purbiya 12%, Khausiya 9.52%, Dangoriya 6.94% and Baatar 6.09% prevalence of sickle haemoglobin. The male: female ratio was 1.09:1 and the commonest age group was 11-20.

A. Shrestha and S. Karki, (2013) conducted a study in SCA in Tribhuvan University Teaching Hospital, Kathmandu, Nepal from January 2011 to January 2013. Haemoglobin electrophoresis was performed by cellulose acetate electrophoresis at alkaline pH method. Sodium dithionite was used for sickling test. A total of 35 cases were diagnosed as sickle cell disorder. The male: female ratio was 2.5:1with the commonest age group 11-20 years (42.8%). Tharu, Chaudhary

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and Tharu, Rana (91.3%) were the commonest ethnic group with both sickle cell anemia and trait. It was found that Tharu (Chaudhary; 82.8%) was the most common ethnic group with this disorder followed by Tharu (Rana; 8.5).

M. Marchand et al., (2017) screened a total of 2899 Tharu individuals aged 6 months to 40 years in the rural district of Dang in Western Nepal by using a sickling test, of whom, 271 screened positive for HbS. Those who screened positive were offered diagnostic gel electrophoresis testing. Of the 133 individuals who underwent diagnostic testing, 75.9% (n = 101) were confirmed to be Hb AS heterozygotes, 4.5% (n = 6) were confirmed to be Hb SS homozygotes and 19.5% (n = 26) were false positives.

R. Pande et al., (2019) conducted a retrospective study in sickle cell disease between 2012 to 2018 at Bheri Provincial Hospital, Nepalgunj, Nepal by using haemoglobin electrophoresis and HPLC method for diagnosis. A total of 1459 individuals of haemoglobinopathies were seen, out of which 1250 had sickle cell disease and carrier. Out of 1250patients and carriers, 608 (48.6%) were male and 642 (51.4%) were female. The mean age of patients and carriers was 24.5 ±12 yrs. Maximum number of patients 381 (30.5%) were in the age group 21-30 years. Only 156 (12.5%) patients and carriers were under the age of 11 years. Around 1221 (97.7%) of the patients and carriers belonged to the Tharu ethnic group of Nepal. Rest were non-Tharus.

Tharu tribes are indigenous to the Terai of Nepal and Northern India practicing endogamous marriage. High prevalence of sickle cell disease has been documented in Tharu ethnicity in different parts of Nepal as well as India. A detail study of its prevalence in all tribal populations of Tharu community is still lacking. Neonatal and pre-marital tests will be effective against further inheritance of SCD. So an invasive study is recommended by mass screening programs that give technical guidance to the policy makers and health workers and help in management of the burden of SCD in the Tharu community.

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