COMPARATIVE ANALYSIS OF G6PD DEFICIENCY BETWEEN DAGAURA THARU AND RANA THARU IN LALJHADI RURAL MUNICIPALITY OF KANCHANPUR DISTRICT, NEPAL

Submitted by
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A thesis submitted in partial fulfillment of the requirement for the award of the degree of Master of Science in Zoology with Special Paper Ecology

Submitted to
Central Department of Zoology
Institute of Science and Technology
Tribhuvan University
Kirtipur, Kathmandu, Nepal

September, 2019

DECLARATION

I hereby declare that the work presented in this thesis has been done by myself and has not been submitted elsewhere for the award of any degree. All sources of information have been specifically acknowledged by reference to the author (s) or institution (s).

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RECOMMENDATION

This is to recommend that the thesis entitled **"Comparative Analysis of G6PD Deficiency between Dagaura Tharu and Rana Tharu in Laljhadi Rural Municipality of Kanchanpur District in Nepal"** has been carried out by Himalaya Joshi for the partial fulfilment of master's degree of Science in Zoology with special paper Ecology. This is his original work and has been carried out under my supervision. To the best of my knowledge, this thesis work has not been submitted for any other degree in any institutions.

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LETTER OF APPROVAL

On the recommendation of supervisor "Dr. Nanda Bahadur Singh" this thesis Submitted by Himalaya Joshi **"Comparative Analysis of G6PD Deficiency between Dagaura Tharu and Rana Tharu in Laljhadi Rural Municipality of Kanchanpur District in Nepal"** is approved for the examination and submitted to the Tribhuvan University in partial fulfilment of the requirements for Master's Degree of Science in Zoology with special paper Ecology.

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CERTIFICATE OF ACCEPTANCE

This thesis work submitted by Himalaya Joshi entitled **"Comparative Analysis of G6PD Deficiency between Dagaura Tharu and Rana Tharu in Laljhadi Rural Municipality of Kanchanpur District in Nepal"** has been accepted as a partial fulfilment for the requirements of Master's Degree of Science in Zoology with special paper Ecology.

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LIST OF ABBREVIATIONS

Abbreviation	Details of abbreviations
ACT	Arteminsinin Combination Therapy
AHA	Acute Hemolytic Anemia
CDC	Centers for Disease Control
CNSHA	Chronic Non Spherocytic Anemia
DNA	De-oxy Ribonucleic Acid
EDTA	Etylenediamine Tetra acetic Acid
FRM	Formazan Ring Method
G6PD	Glucose 6-Phosphate Dehydrogenase
G6PDD	Glucose 6 Phosphate Dehydrogenase Deficiency
GSH	Glutathione (Reduced) / Reduced glutathione
GSSG	Glutathione (Oxidised) / Oxidised glutathione
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen (Reduced form)
NHRC	Nepal Health Research Council
NNJ	Neonatal Jaundice
PMS	Phenazine Methosulphate
PPP	Pentose Phosphate Pathway
UGC	University Grant Commission
WHO	World Health Organization

ABSTRACT

Glucose-6-phosphate dehydrogenase (G6PD) is a key enzyme that has a housekeeping role in all cells and is particularly critical to the functioning of red blood cells (RBCs). When G6PD activity is deficient, they have a diminished ability to withstand stress, and therefore risk (hemolysis). The large majority of G6PD deficient subjects have no clinical manifestations and the condition remains asymptomatic until they are exposed to a hemolytic trigger. The objective of this study was to compare the analysis of G6PD deficiency between Dagaura Tharu and Rana Tharu in Laljhadi Rural Municipality of Kanchanpur district in Nepal. Whole blood samples of sample size 300 were collected from those indigenous people of Laljhadi Rural Municipality of Kanchanpur district in Nepal during April 8 to May 18 2018. All collected blood samples were tested by using Formazan Ring Method. Out of 300 test cases, the G6PD deficiency by using Formazan Ring Method was found to be 24(8%). The G6PD deficiency by using Formazan Ring Method was found to be 15(10%) in Dagaura Tharu and 9(6%) in Rana Tharu. Out of 24 G6PD deficient cases, 19(79.16%) were male G6PD deficient and 5(20.83%) were female G6PD deficient. All of the deficient cases were from ethnic communities who were the inhabitants of malaria endemic areas. Primaquine is the key drug for malaria treatment. On such persons who have G6PD deficiency, this drug causes the RBC haemolysis. So Primaquine should be administered only after the testing of G6PD especially in the ethnic groups.

1. INTRODUCTION

1.1 Background

Glucose-6-phosphate dehydrogenase (G6PD) is a key enzyme that has a housekeeping role in all cells, and is particularly critical to the development and functioning of red blood cells (RBCs). Glucose-6-phosphate dehydrogenase (G6PD) is a cytoplasmic enzyme whose main metabolic rate is the production of NADPH and reduced glutathione (GSH) in the monophosphate pathway as the defence against oxidizing agents (Notaro *et al.*, 2000). NADPH is needed for the regeneration of reduced glutathione, the major antioxidant defence, which is particularly important in red blood cells (RBCs) (Beutler, 1994). G6PD enzyme activity is necessary for RBC survival as it catalyzes the only metabolic pathway capable of generating reducing power to these cells lacking mitochondria (Baird, 2011). The G6PD gene spans 18 kb on the X chromosome (Xq28), containing 13 exons and 12 introns (Chen *et al.*, 1996).

The metabolic reactions concerned are part of the Pentose Phosphate Pathway (PPP, also called the Hexose Monophosphate Shunt), the first and rate limiting step of which is catalysed by the G6PD enzyme: the oxidation of glucose-6-phosphate into 6-phosphoglucono-8-lactone, which simultaneously reduces NADP to NADPH (Greene 1993). The electron of NADPH passes to abundant glutathione dimers (GSSG) via another enzyme, glutathione reductase, reduced glutathione monomers (GSH) represent the primary defence against hydrogen peroxides, organic peroxides, and free radicals (Beutler, 2008). However, in cells that have a mutant and defective G6PD gene, it overwhelm the ability of the red blood cell to and damage may then occur (Baird *et al.*, 2001).

Glucose-6-phosphate dehydrogenase (G6PD) deficiency, or “primaquine sensitivity”, is the most common genetic disorder with an estimated 400 million people affected worldwide (Howes *et al.*, 2012). Being X-linked, G6PD deficiency occurs more frequently in males than in females (Sharma and Rajkumari, 2010). In hemizygous males and homozygous females, G6PD deficiency is fully expressed, whereas in female heterozygotes, a mixed population of normal and enzyme-deficient cells can be found, owing to random inactivation of one of the two X chromosomes early in embryonic life (Lyonization) (Davidson *et al.*, 1963).

There are more than 400 variants of G6PD gene (Ali *et al*., 2005). Most of these mutations reduce the G6PD enzyme stability and activity. As a result, G6PD deficient RBCs are more susceptible to destruction by oxidative stress such as oxidant food (fava beans) and drugs (e.g., primaquine and sulfones) (John *et al*., 2012). A host of agents like antibiotics, anti-malarials, analgesics, infections, fava beans and acute illnesses are associated with haemolysis in G6PD deficiency (Sklar, 2002). Therefore, the disease is often manifested as acute haemolytic anemia triggered by the intake of these stressors. G6PD deficiency is often quoted as an example of natural selection, with malaria being considered a major evolutionary force. The geographical distribution of G6PD deficiency variants remarkably overlaps with historically malaria-endemic areas such as Africa, Asia, and Mediterranean region (Notaro *et al*., 2000).

Among 126 Ethnic groups, Tharu is one of them which are Endemic to Terai region of Nepal (Upadhyay, 2013). Inside Ethnic group Tharu there are two sub-ethnics groups, Dagaura and Rana Tharu, which are inhibiting in Kanchanpur district. About 22.5 million Nepalese people live in malaria-prone areas with 65 of 77 districts considered endemic to the disease (MOH, 2011). Tharu ethnic group of terai region has to be known for resistance to malaria (Terrenato *et al.,* 1988).

In case of Nepal, malaria endemicity is very high due to originally hyper endemic forest, forest fringe, and foothills of outer Terai and forested inner Terai valleys, the vectors of malaria are *P vivax, P falciparum, An. fluviatilis and An. maculatus* which breed in slow running water supporting eco- system . Out of five different types of plasmodium species; namely *Plasmodium vivax, Plasmodium falciparum, Plasmodium ovalae, Plasmodium malariae, and Plasmodium konwlesi* found in the world leading to malaria disease by infecting erythrocytes. *Plasmodium vivax* and *Plasmodium falciparum* are found in Nepal (Sherchand *et al.*, 1995). This disease is seen frequently in Africa, Asia, and the countries near the Mediterranean Sea (Frank, 2005).

Primaquine has a vital and unique role in the malaria elimination fulfilling three critical functions: first, it is the only licensed radical cure of *P. vivax*; second, primaquine is the only drug active against mature, infectious *P. falciparum* gametocytes making it vital for blocking transmission; and third, in areas of emerging drug resistance, primaquine is being used in containment programmes to prevent the spread of artemisinin resistant *P. falciparum* strains. These invaluable properties make understanding the triangle of

interplaying aspects determining primaquine-induced hemolytic risk crucial: the human enzyme, the drug and the parasite (WHO 2015).

G6PD deficiency selectively affects RBCs for two reasons. First, most known mutations cause a decreased stability of the enzyme, and since these cells do not have the ability to synthesize proteins, the enzyme level decreases as cells age during their 120 days lifespan in circulation. Second, RBCs are very susceptible to oxidative stress from exogenous oxidizing agents in the blood as well as the oxygen radicals continuously generated as haemoglobin cycles between its deoxygenated and oxygenated forms (Tinley *et al*., 2010). When G6PD activity is deficient, they have a diminished ability to withstand stress, and therefore risk destruction (haemolysis) occurs. Fortunately, the large majority of G6PD deficient subjects have no clinical manifestations and the condition remains asymptomatic until they are exposed to a haemolytic trigger (Graves *et al.,* 2012). For centuries, the most common known trigger of haemolysis has been fava beans, and favism remains a public health problem in areas where these are a common food item and G6PD deficiency is prevalent (Tantular and kawamoto, 2003). However, a haemolysing trigger of great public health significance is the antimalarial primaquine, a key drug for malaria control as the only licensed treatment against (i) the relapsing liver stages of *Plasmodium vivax* hypnozoites which become dormant in infected hepatocytes and subsequently reactivate blood-stage infections, and (ii) the sexual blood stages of all species of *Plasmodium*. Since its introduction, primaquine has emerged as a major drug trigger of haemolysis in G6PD deficient individuals (Recht *et al.,* 2012)

Favism had already been recognized as a dangerous disease; and since G6PD deficiency is common in Greece, it is possible that they might had suffered from favism. In more recent times there has been a vast literature on favism. Fava beans are unique among other beans because they contain high concentrations of two glycosides, vicine and divicine; and their respective aglycones, convicine and isouramil, are powerful triggers of oxidative stress that causes the characteristic haemolytic attacks (Baird, 2001).

The most clinically serious symptom of G6PD deficiency is neonatal jaundice (NNJ), which peaks 2 to 3 days after birth and causes permanent neurological damage or death if left untreated. Not all neonates with NNJ are G6PD deficient, but this congenital condition greatly increases the risks, and in some countries is the most common cause of NNJ (Chen *et al*., 1996). Acute Hemolytic Anaemia(AHA) is the most common manifestation of the deficiency, and may be triggered by a range of exogenous agents

causing intravascular haemolysis and jaundice, and may include haemoglobinuria (dark urine), the most severe outcome of AHA is acute renal failure (Cappellini and Fiorelli, 2008).

The exceptions to G6PD deficiency being asymptomatic until triggered by certain exogenous triggers are those sporadically emerging, highly unstable variants expressing very low residual enzyme activity. These variants never reach polymorphic frequencies due to their severe pathology, which is characterized as chronic non Spherocytic Hemolytic Anemia (CNSHA). While individuals with these mutations make up only a very small minority of the population affected by G6PD deficiency (almost always males), they are the most clinically severe and may be transfusion-dependent. In addition to susceptibility to all the aforementioned triggers of AHA, the very low residual enzyme levels mean that cells cannot even protect themselves against oxygen radicals continuously generated by the on-going process of haemoglobin de-oxygenation (Au, *et al*., 2016).

Based on these pathologies, G6PD alleles can be categorized into three types: (1) those sporadic severe variants associated with chronic symptoms, (2) polymorphic types which are typically asymptomatic but susceptible to trigger-induced acute hemolytic episodes, and (3) those with normal activity. However, as suggested by Luzzatto, we distinguish only three variant types (Luzzatto, 2009) (Table 1).

Table 1: G6PD variant types and their key characteristics

Type	**Residual enzyme activity**	**Prevalence**	**Clinical significance**
Type 1	<10%	Sporadic	Severe and chronic
Type 2	1-50%	Polymorphic	Asymptomatic until risk
Type 3	Normal (>50%)	Polymorphic(wild type)	None

1.2 Significance of study

Nepal government has planned to eradicate malaria in 2025. For the elimination of malaria primaquine is the drug that should be mostly used. If such drug is administered without the testing of G6PDD, it may cause blood hemolysis in G6PD deficient persons and it will be difficult for the government to use primaquine and without the use of

primaquine it will be difficult to eradicate malaria from Nepal. So G6PD testing is one of the vital part of this study.

It is quite interesting to see the influence of G6PD gene in malaria endemic low land ethnic group. This type of mass screening G6PD genes between Dagaura Tharu and Rana Tharu has not been carried out by the government and its agencies till now in order to make the new health policy. This type of research under Central Department of Zoology will be guideline for the policy maker of Government of Nepal, its related agencies and future research.

1.3 Objectives

1.3.1 General objectives

To compare the G6PD deficiency between the Dagaura Tharu and Rana Tharu in Laljhadi Rural Municipality of Kanchanpur district in Nepal.

1.3.2 Specific objectives

a. To detect the prevalence of G6PD deficiency between Dagaura Tharu and Rana Tharu in Laljhadi Rural Municipality of Kanchanpur district in Nepal,
b. To compare age wise distribution of G6PD deficiency between Dagaura Tharu and Rana Tharu in Laljhadi Rural Municipality of Kanchanpur district in Nepal.
c. To compare sex-wise distribution of G6PD deficiency between Dagaura Tharu and Rana Tharu in Laljhadi Rural Municipality of Kanchanpur district in Nepal.

1.4 Research hypothesis

a. Null hypothesis: Significance of G6PD deficiency is not associated with ethnicity, age and sex.
b. Alternate hypothesis: Significance of G6PD deficiency is associated with ethnicity, age and sex.

1.5 Limitations

a. The chemicals were not available in local market and therefore, help from India was needed.
b. The chemicals were too costly.
c. This method only gave qualitative result, but not the quantitative result.

2. LITERATURE REVIEW

2.1 National context

The very first detection of G6PD deficiency among the four ethnic groups (Raute, Chidimar, Chepang and Munda) of Nepal using formazan ring method found that 25.5% male of Raute, 7.7% male of Munda, 3% male of Chepang and 0% male of Chidimar were G6PD deficient. The molecular study related G6PD deficiency was held in 2002. Both the G6PD Orissa and G6PD Mediterrenean were found in the Munda. The G6PD Mahidol detected by sequencing was confirmed in other twenty one G6PD deficient Raute samples indicated that the G6PD Mahidol was highly predominant in Nepalese Raute group (Singh, 2002).

Similarly, another molecular study related to G6PD deficiency in Nepal was held in 2003. During that period 300 males for G6PD activity were tested. Among them two cases were found G6PD deficient which was 0.67% which was classified as G6PD Mediterranean. The variants found in Nepal was contrast to the variants found in India and Pakistan which had no replacement at nucleotide 1311 but was similar to variants of Mediterranean and middle east countries having similar type of replacement pattern. Thus the variant found in Nepal was similar to variants of Middle East countries rather than India and Pakistan (Baral *et al.*, 2003).

Latest and profound study on G6PDD done by a group to find the correlation between G6PDd and malarial endemic regions. Using two different rapid diagnostic test kits viz. Binax-Now® and Care Start™ on total of 1341 blood samples from six districts of varying demographic representation, was done. To their surprise, it revealed the presence of vulnerable population groups for G6PDD in malaria endemic districts i.e. G6PD deficiency was higher in ethnic group Rajbanshi (11.7%) and Tharu (5.6%) (Ghimire *et al.*, 2017). Our region of interest is Kanchanpur, which is one of malarial endemic region of Nepal implying greater chances of G6PDd rates in that regions.

2.2 Global context

Screening G6PD in Dangura Tharu settled in Baharaich District of Uttar Pardesh using Bernstein method where malaria is prominent and the finding was 23.21% Tharu male were G6PD deficient whereas in female the deficiency was partial and confined to 12,5% only (Sharma and Rajkumari, 2010). G6PD deficiency was found to be absent in Jumli Thakuris living at an altitude of 12,000 feet whereas it is highest 4.50% in Rajput of India

living at both Nepal and India living at an altitude of 2000 to 3,500 feet. Brahmans and Rajputs of Nepal and India, Johari and Tolcha Bhotia show almost similar frequencies of G6PD. One of the reasons for the decreased increase of G-6-PD deficiency at high altitude could be attributed to absence of malaria (Kapoor *et al.*, 2013).

Similarly, Screening G6PD deficiency of neonates in Yogyakatra and its surrounding areas of Indonesia by Purnomo detected 145 neonates with G6PD deficiency. Among male, 6.2% had moderate and 1.4% had low enzyme activity and female had enzyme activity in normal. It was estimated that 2-6% of the populations were carriers (Purnomo, 1996). The majority of the global population at risk of *P. vivax* transmission is on the Asian continent defined as stretching from Turkey, south to Vietnam and east to the People's Republic of Korea. Out of 1044 blood samples tested using the care start G6PD test, none were positive for G6PD deficiency. It was very interesting to know that in Korean people the prevalence of G6PD deficiency was very very low (Goo *et al*., 2014).

The expectation of high prevalence of G6PD deficiency among Iranian newborns justified the results that out of 2501 newborns, the overall prevalence of G6PD deficiency was 3.2%. Frequency in male population was 5.1% and in female population was 1% (Iranpour *et al.*, 2008). However, G6PD prevalence model uncertainty was high in this region, peaking (up to 37%) in an area devoid of data across southern Pakistan. This high uncertainty emphasizes the need for additional community surveys to support mapping of G6PD deficiency in this apparently high prevalence region. The underlying G6PD mutation among these populations was reportedly the Mediterranean variant, which was identified in >70% of deficient individuals in all surveys between Turkey and Pakistan (Gething *et al*., 2012).

Further G6PD deficiency was present but at relatively low prevalence (2–5%) across much of the Asian countries like China, Japan and Indonesia. In Indonesia, out of 1126 volunteers, by Formazan Ring Method, found that 3.99% were G6pd deficient (Tantular *et al*., 1999). Similarly, among children from Yunnan with unique ethnic origin the significant difference among children from ethnic group of different region in Han Chinese was 0.75% which was lower than the majority of ethnic minorities (Yao *et al.*, 2013). G6PD deficiency screening among malaria suspected attending Gambella hospital of Southeast Ethiopia using G6PD fluorescence spot test found malaria prevalence to be 59.2% out of which 7.3% were G6PD-deficient with no significance difference between the sexes (Tseqaye *et al.*, 2014). In Nigerian children of different ethnic background the

overall prevalence of G6PD deficiency was 15.3%. Yoruba children had a higher prevalence (16.9%) than Igede (10.5%), Igbo (10.1%) and Tiv (5.0%) children (William *et al.*, 2013).

In two large case-control studies of over 2,000 African children, the common African form of G6PD deficiency (G6PD A-) is associated with a 46-58% reduction in risk of severe malaria for both female heterozygotes and male hemizygotes. (Ruwende *et al.*, 1995). The geographical correlation of its distribution with the historical endemicity of malaria suggests that G6PD deficiency has risen in frequency through natural selection by malaria (Domingo *et al.*, 2013). Attempts to confirm that G6PD deficiency is protective in field studies of malaria have yielded conflicting results, but recent results from large case control studies conducted in East and West Africa provide strong evidence that the most common African G6PD deficiency variant, G6PD A–, is associated with a significant reduction in the risk of severe malaria for both G6PD female heterozygotes and male hemizygotes (Ganczakowski, 1995).

3. MATERIALS AND METHODS

3.1 Study area

The study was conducted in Kanchanpur district of Far Western Terai region of Nepal, which is highly prone to malaria, as shown in Figure 1. Kanchanpur district a part of Far-western region in the Terai plain, is one of seventy-seven districts of Nepal. It is located in southern west of Nepal. It is bordered with Kailali district in east, Dadeldhura district in north and with modern-day India in south and west. The majority of population is occupied by ethnic Tharu community and minor groups are the peoples that have been migrated from the northern hilly region. The latitude and longitude of Laljhadi rural municipality is 28.7372° N, 80.4061° E. (Source: Google)

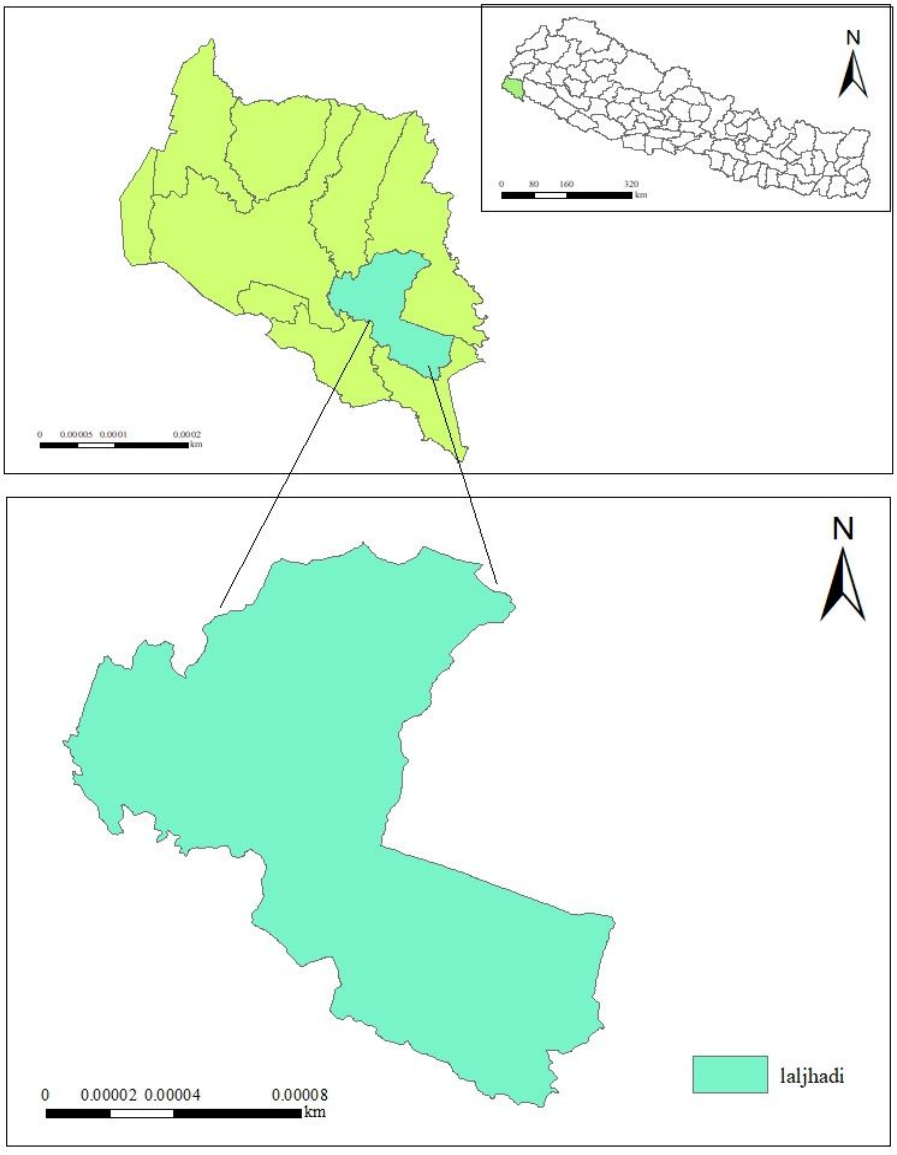


Figure 1: Map showing kanchanpur district and Laljhadi rural municipality

3.1.1 Source of population

The people were selected in malaria endemic area. All the people from ethnic community of ages 5 years to 60 years were selected randomly.

3.1.2 Selection criteria

a) Case inclusion criteria

A study was included if people were living in malaria endemic area and if the people were the inhabitants or ethnic group of Terai region.

b) Case exclusion criteria

A study was excluded if the persons were of age below 5 years or of above 60 years.

3.1.3 Sample size determination

The required sample size was estimated for the inclusiveness of large group of population for keeping sampling error as low as possible and to make the sample size more accurate, precise, and representative.

3.1.4 Ethical issues

Ethical clearance of the study was taken from Nepal Health Research Council (NHRC).The consent form is attached in **Appendix IV.**

3.2 Materials

A complete list of equipments, chemicals and other supplies used during the entire study period is given below.

3.2.1 Instruments

- Aluminium foil
- Camera
- Light illuminating box
- Filter paper
- Forceps
- Constant temperature incubator
- Forceps
- 100 ml cylinder
- Two small 100 and 50 ml sized beaker

3.2.2 Essential chemical reagents

- G-6-PD: Glucose-6-Phosphate Disodium salt oxidized form
- NADP: Nicotinamide Adenine Dinucleotide phosphate oxidized form
- PMS: Phenazine Methosulfate
- MTT: 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium Bromide
- MgCl2
- Tris HCl(PH 6.5)
- Agar powder
- Distilled water

3.3 Methods

The study was designed as a random cross-sectional study. The study was carried out from April 8, 2018 to May 18, 2018 with collaboration of Central Department of Zoology, University Grant Commission (UGC) and Nepal Health Research Council (NHRC). A total of 300 blood samples were collected from inhabitants of malaria endemic regions of Kanchanpur district, Laljhadi rural municipality of Nepal. The collected blood samples were analyzed by Formazan Ring Method (FRM) and reported as per the guidelines given in the manuals of this methods. Individual personal details were obtained by taking direct interview with tested people and noted on the excel sheet and analyzed. Individuals were selected according to the inclusion and exclusion criteria.

3.3.1 Formazan ring method (FRM)

Formazan Ring Method (FRM) was used to detect G6PD deficiency in Dagaura Tharu and Rana Tharu. Modified procedure of FRM was used by author Singh, 2002. Here, the same procedure was used, which is as follows:

a) Preparation of Agar-gel- plate

- Buffer: 10 ml of 1M Tris-HCl (PH 6.5) and 2 ml of 0.5M $MgCl_2$ were mixed together into a 100 ml volumetric flask. The distilled water was added until it reaches 100 ml.
- 100 ml buffer solution was divided into 75 ml and 25 ml and put into two beakers respectively.
- The essential reagents were weighed with the micro electric balance in the following proportions.

$G6PNa_2$: 125mg

NADP: 25mg

PMS: 25mg

MTT: 25mg

- $G6PNa_2$+NADP+PMS+MTT was completely dissolved together in 25 ml of buffer by stirring with micro spatula and then it was covered in 50 ml beaker with plastic and kept in a dark place cautiously until it was mixed with agar solution.
- 1gm of agar powder was taken out and melt completely in 75 ml of buffer by boiling for about 10 minutes.
- Two solutions (75 ml and 25 ml) combined together with a view to maintain the temperature of the combined solution to be at 55 ^{o}C.
- The combined solution was put into the plate according to the size of the plate.
- The plates were left for sometimes to get solidification the agar gel. The plates were wrapped with the aluminium foil and kept in the dark and low temperature room or stored in refrigerator.

b) Procedures

- A dish (d=3mm) from a blood stained filter paper was punched with the help of puncher. The punched blood stained filter paper was then placed and pressed onto the gel surface with the help of forceps or small tweezers.
- The agar gel plate was then wrapped with the aluminium foil and incubated at 37 ^{o}C in a incubator for about 8 hours (generally overnight).
- The incubated agar gel plate was carefully observed in the next day morning and detected small sized brown rings among big sized blue coloured formazan rings.

c) Interpretation of the test result

According to the size of the formazan ring, G6PD activity was interpreted as follows. If the size of the formazan ring was smaller than 3-5mm than the sample was assessed to be deficient if small- sized completely brown coloured formazan rings was observed. If the size of the formazan ring was bigger than 7-8mm than the sample was assessed to be normal if big- sized blue coloured formazan rings was observed. If the size of the formazan ring was in between 5-6mm than the sample was assessed to be intermediate if

light brown coloured formazan rings was observed. The test was interpreted either normal, intermediate or deficient. All G6PD deficient and intermediate samples were subjected to rescreening for the final confirmation.

d) Study Variables

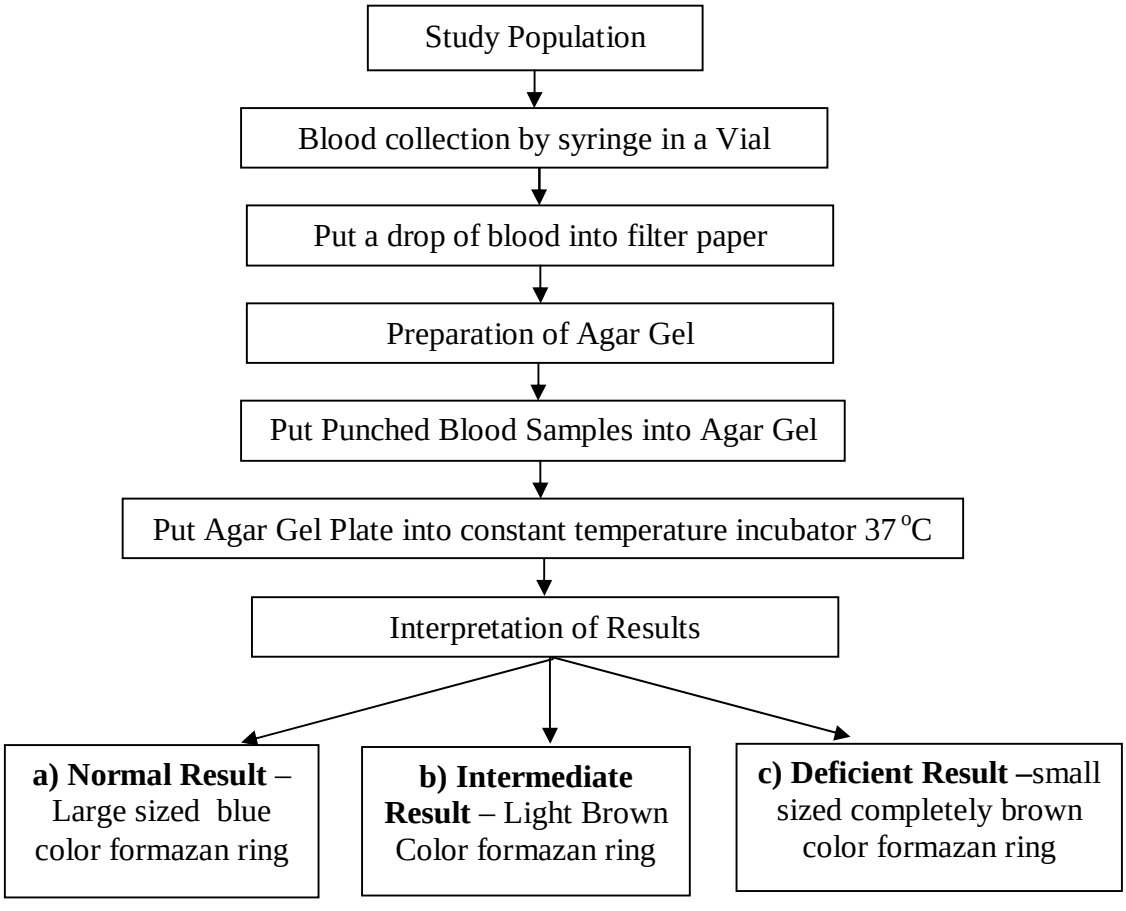
i) Dependent variables

The G6PD variants and deficient were dependent variables.

ii) Independent variables

Demographic data like age, sex, and ethnicity were independent variables of the study.

Flow chart methodology for formazan ring method



3.3.2 Data entry and analysis

The collected data were analysed by MS excel 2013. The G6PD deficiency was analyzed based on factors like sub-ethnicity, gender, age and demography to determine the significance of G6PD related to those factors.

4. RESULTS

We analyse various socio-demographic statuses of G6PD deficiency tests in the sections below:

4.1 Detection of ethnic prevalence of G6PD deficiency

4.1.1 Ethnicity based distribution of G6PD deficiency test cases

The total number of test cases were 300 individuals from Laljhadi Rural Municipality, Kanchanpur District. Out of total number of cases, 50% i.e, 150 were Dagaura Tharu and rest 50%, 150 were Rana Tharu. After the Formazan Ring Test, we came over the findings that G6PD deficiencies were quite prominent in both the enthnic group viz. Dagaura Tharu and Rana Tharu. Out of the total number of Dagaura test cases, 10% (15 out of 150) were found to have deficiency in G6PD as shown in Figure 2 whereas the percentage of G6PD deficiency was found to be 6% (9 out of 150) in Rana Tharu as shown in Figure 3**.** Nevertheless, normal and intermediate cases were seen after the successful test.

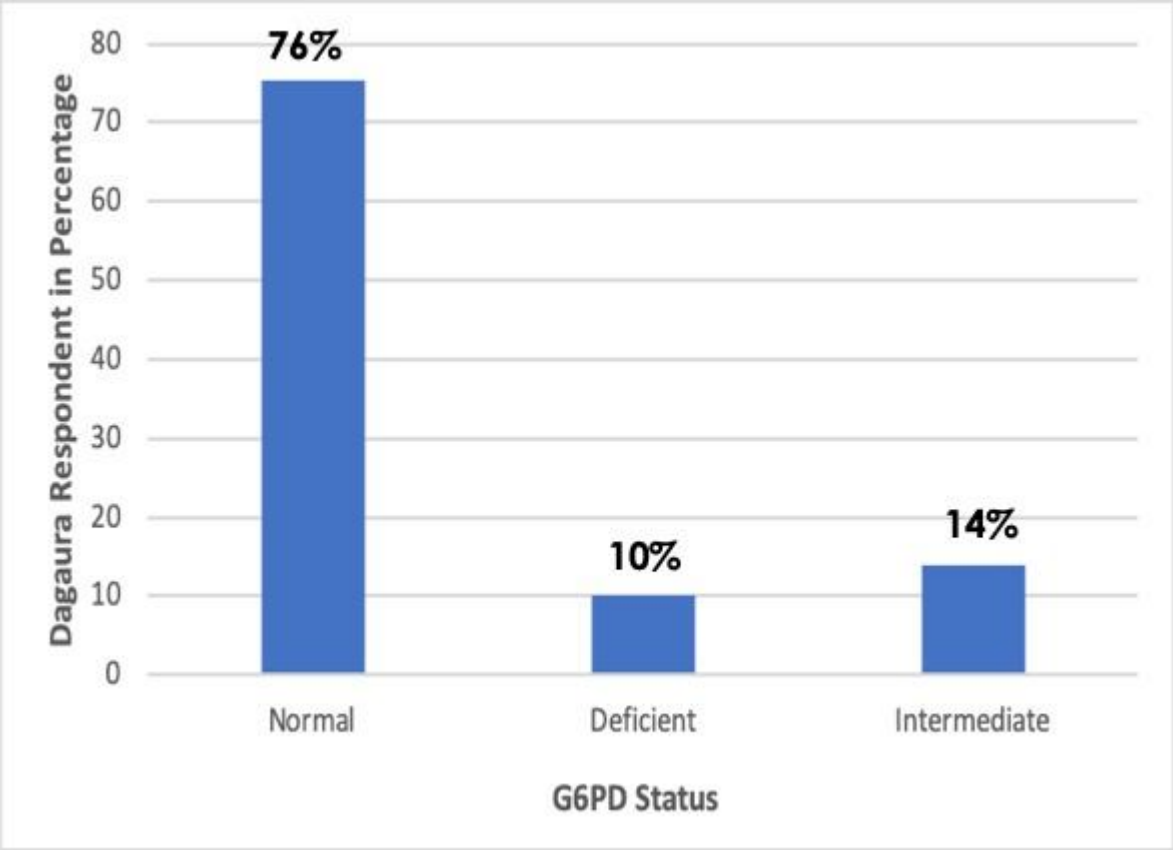


Figure 2: Ethnicity based distribution of G6PD deficiency in Dagaura

The percentage of normal G6PD was found to be 75.3% (113 out of 150 test cases) in Dagaura Tharu where as it was 81.3 % (122 out of 150 test cases) in Rana Tharu. Also, the percentage of intermediate deficiency which wa s found only in female, was 14% (21 out of 150 test cases) in Dagaura Tharu and 12.6% (19 out of 150 test cases) in Rana Tharu. This is shown graphically in Figures 2 and 3 respectively.

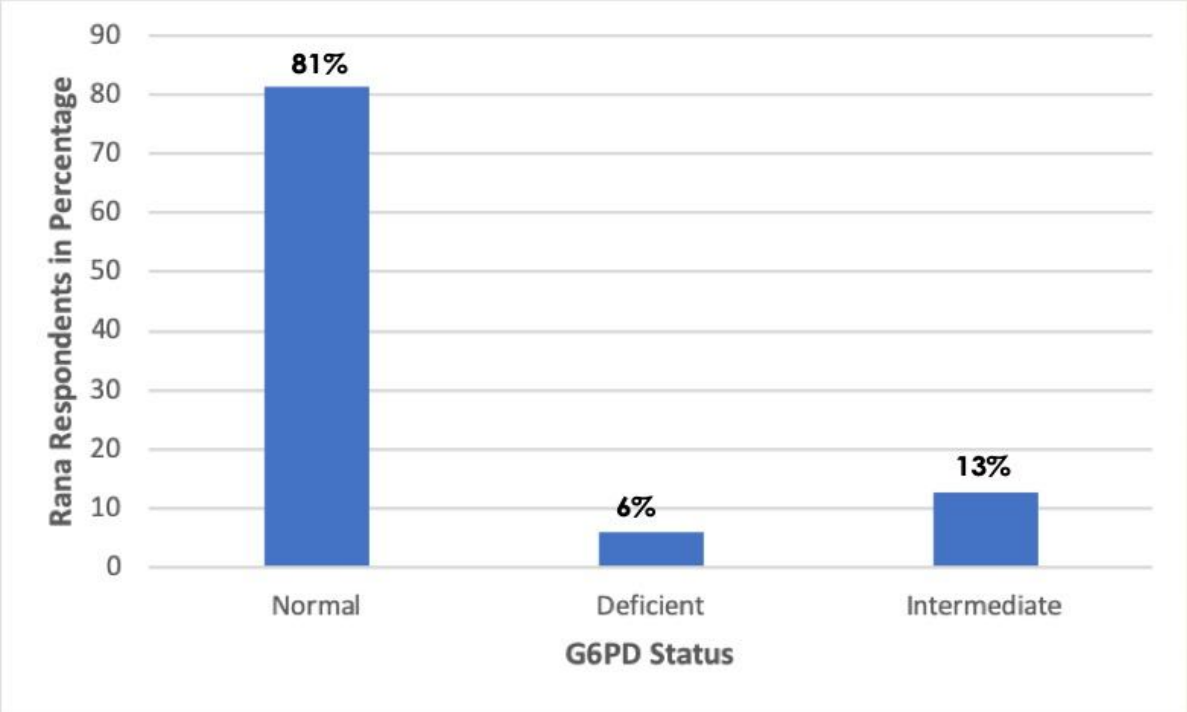


Figure 3: Ethnicity based distribution of G6PD deficiency in Rana

4.1.2 Analysis of correlation between G6PDD and ethnicity

Our null hypothesis is deficient G6PD cases are not correlated to ethnicity. Our alternative hypothesis is deficient G6PD cases are correlated to ethnicity. Our degree of freedom is 1 at 5% level of significance.

Table 2: Analysis of statistical significance between G6PDD and ethnicity

Ethnic Group	**Total number of test cases**	**Total deficient cases**	**Percentage of deficient cases**	**p-value**
Dagaura	150	15	10	**0.084**
Rana	150	9	6	

The p-value was found to be 0.084, which is greater than 0.05 and thereby accepting the null hypothesis that there is no correlation between the deficiency in G6PD and ethnicity.

4.2 Ethnic comparison of age wise distribution of G6PD deficiency

4.2.1 Age-wise based distribution of G6PD deficiency test cases

Age group of investigated 300 test cases lies between 5 years and 60 years. The percentage of individuals of age group 5-15 was 23% , age group 16-30 was 37% and age group above 30 was 40% . The age-wise distribution of investigated test cases was shown in Figure 4.

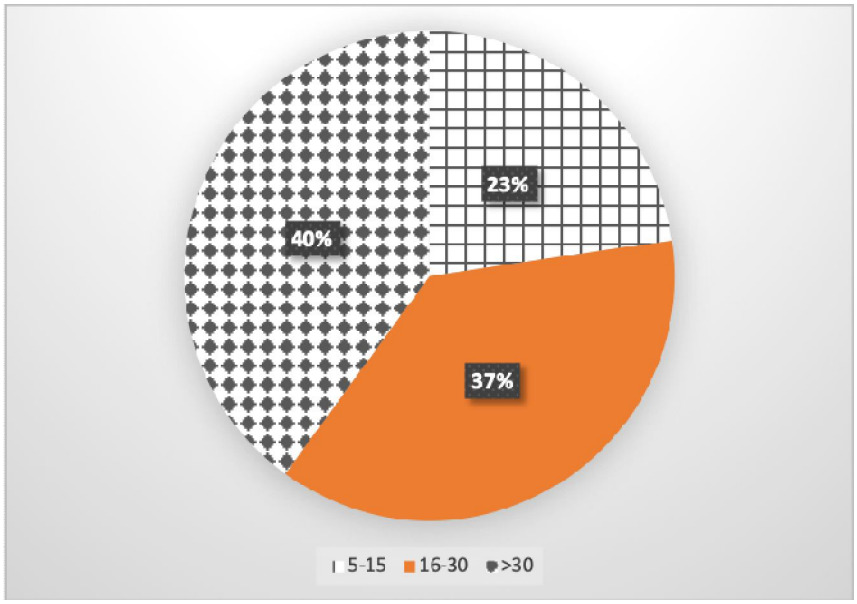


Figure 4: Age-wise distribution of test cases

We found that in the age group 5-15, 4.41% were deficient in Dagaura. However, deficiency was found slightly more in Rana i.e, 5.88%. For the age group 16-30, deficiency was found to be 5.35% in Dagaura and 0.89% in Rana. For the age group above 30 however, deficiency was seen to be 5% in Dagaura and 3.33% in Rana. On critically analysing the pattern of the graph as shown in Figure 4, the nature of deficiency of G6PD in Dagaura and Rana was found to be topsy-turvy. If one only observes the pattern of Dagaura, it is lesser in the age group 5-15, grows gradually to a peak in the age group 16-30 and then decreases gradually in the age group above 30. However, observing the pattern of Rana, it is seen that it is highest in the age group 5-15, gradually decreasing when it meets the age group 16-30 and increases when it move towards the age group above 30. However, to understand whether the deficiency in G6PD is co-related to age group or not, we need to perform p-value analysis.

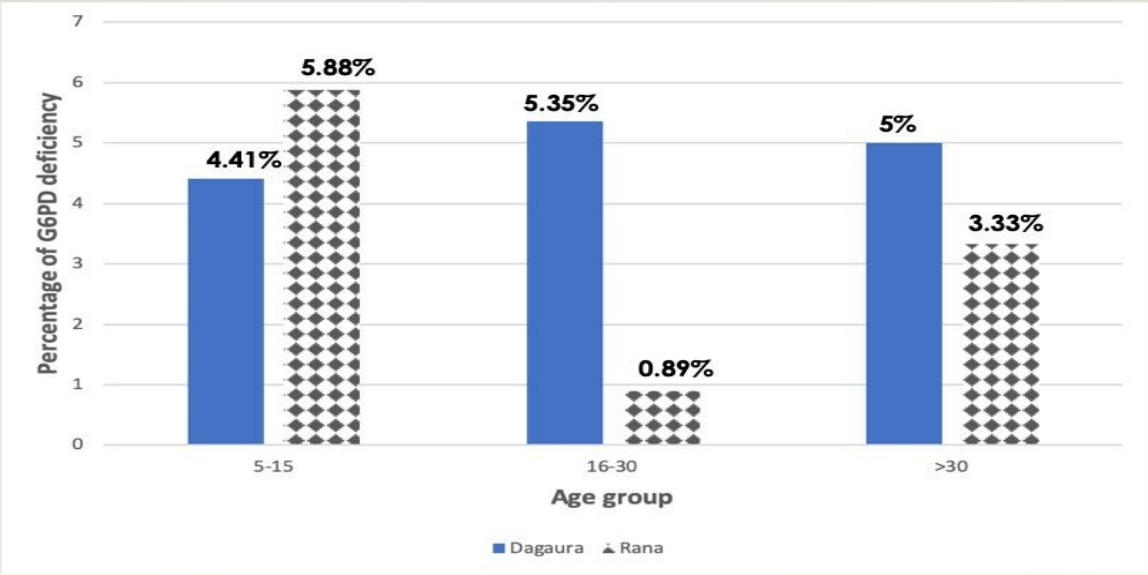


Figure 5: Age-wise based distribution of G6PD deficiency

4.2.2 Analysis of correlation between G6PDd and age-group

The highest percentage of deficient cases out of total test cases in Dagaura age-group, was found to be 5.35% in age-group 16-30. However, the highest percentage age-group in Rana was found to be 5-15. The P-value analysis was done by using Microsoft Excel 2013 which is as follows:

Table 3: Analysis of statistical significance between G6PDd and age-group

DAGAURA				
Age Group (in years)	Total number of test cases	Total deficient cases	Percentage of deficient cases	p-value
5-15	28	3	10.7	**0.186**
16-30	57	6	10.5	
Above 30	65	6	9.2	
RANA				
Age Group (in years)	Total number of test cases	Total deficient cases	Percentage of deficient cases	p-value
5-15	40	4	10	**0.993**
16-30	55	1	1.8	
Above30	45	4	8.9	

The p-value was found to be 0.186 in Dagaura Tharu and 0.993 in Rana Tharu. Both of these p-values were greater than 0.05, accepting the Null hypothesis that there wass no statistical significance between G6PD deficiency and age-group.

4.3 Ethnic comparison of Sex-wise distribution of G6PD deficiency

4.3.1 Sex-wise distribution of G6PD deficiency test cases

Out of total of 300 blood samples,150 were male and 150 were female. Therefore, the ratio became 1:1 as shown in figure 6.

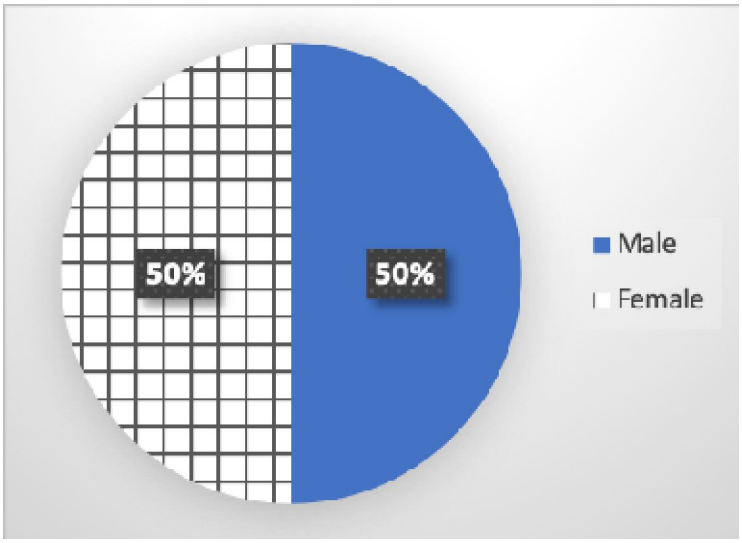


Figure 6: Sex-wise distribution of test cases

The most deficient cases were found to be 79% (19 out of 24) in male and 21%(5 out of 24) in female as shown in Figure 7.

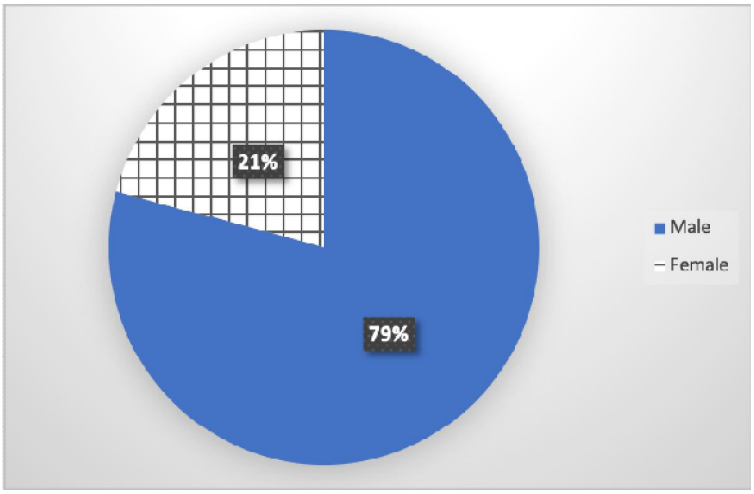


Figure 7: Sex-wise G6PD deficiency

4.3.2 Analysis of correlation between G6PDD and sex-wise distribution

Our Null hypothesis is Deficient G6PD cases are not correlated to sex. Our Alternative hypothesis is Deficient G6PD cases are correlated to sex. Our degree of freedom is 1 at 5% Level of Significance.

Table 4: Analysis of statistical significance between G6PDD and sex

Sex	**Total number of test cases**	**Total deficient cases**	**Percentage of deficient cases**	**p-value**
Male	150	19	12.7	**0.118**
Female	150	5	3.3	

The p-value was found to be 0.118, which was greater than 0.05 and thereby accepting the null hypothesis that there was no correlation between the deficiency in G6PD and sex.

5. DISCUSSION

5.1 Detection of ethnic prevalence of G6PD deficiency

This study was the random cross-sectional study. The study was conducted in malarial endemic district called Kanchanpur (specifically Laljhadi Rural Municipality). This region is bordering India and the inhabitants of this place were migrated to this place from India and other regions of Terai. Ethnic groups such as Rajbanshi, Tharu, Satar, Dhimal and Mushar are the major inhabitants in Terai Region (Upadhayay, 2013). In Nepal, status of G6PD deficiency in these ethnic groups is not clearly understood, although a few local studies have indicated some prevalence in the mentioned districts and populations. Our study basically does study between two species of Tharu viz. Dagaura and Rana. The numbers of deficient cases in Dagaura was 15 out of 150 (i.e.10%) and number of deficient cases in Rana was 9 out of 150 (i.e. 6%). The p-value was found to be 0.084, which is greater than 0.05 and thereby accepting the null hypothesis that there is no correlation between the deficiency in G6PD and ethnicity.

In India, G6PD deficiency was first reported by Kapoor *et al*. in 2013 and the prevalence rate varied from 0 to 27% in different caste, ethnic, and linguistic groups (Kapoor *et al*., 2013). The percentage of G6PD deficiency in Dagaura Tharu was found to be more than that of percentage of G6PD deficiency found in Rana Tharu. The ancestors of Dagaura Tharu arrived this present village from the Terai Region of Nepal, which has always been malarial endemic region. Due to rapid malarial infection, the chromogenic genes of these inhabitants developed resistance mutation from malarial infection, thereby increasing deficiency in G6PD (Terrenato *et al.*, 1988). The variation in the result is due to the geographical variation in sampling area, types of ethnic people, abundant of mosquito's etc.

Region wise distribution of G6PD deficient cases revealed 17 cases (34%) of total deficient cases and 2.5% of total test cases) from Kailali district, 15(30% of deficient cases and 2.2% of total test cases) from Dang district and 18(36% of deficient cases and 2.7% of total cases) were from Chitwan district. The p value was found to be (p=0.43). The highest number of G6PD deficient cases from Chitwan might be attributed to the epidemics towards dengue and malaria and also due to being the place of Tharu community from long time. Again, there are more numbers of wetland which harbors the

mosquitoes and other parasites which increases the chance of infection by malaria. By these reasons Chitwan might have the highest numbers of G6PD deficient cases (Ghimire *et al.*, 2017). The ethnic community or inhabitants of malaria endemic areas are being exposed to such parasites from thousands of years hence deficiency of ethnic community is developed and inherited as a immunity for protection against malaria and other parasitic infections (Medical dictionary).

5.2 Ethnic comparison of age-wise distribution of G6PD deficiency

The age wise distribution of the G6PDD test cases (5-60 years) revealed the highest numbers of cases 120 (40%) were from age group 31-60 years and least number of cases 68 (22.6%) were from age group 5-15 years. 112 (37.3%) cases belonged to age group 16-30. Compared to census data consistency is seen in the number of suspected cases in all the age groups.

After the Formazan Ring Method, we found that in the age group 5-15, 4.41% were deficient in Dagaura. However, deficiency was found slightly more in Rana i.e, 5.88%. For the age group 16-30, deficiency was found to be 5.35% in Dagaura and 0.89% in Rana. For the age group 31-60, deficiency was seen to be 5% in Dagaura and 3.33% in Rana. The p-value was found to be 0.186 and 0.993 in Dagaura and Rana respectively. Both of these p-values were greater than 0.05, accepting the Null hypothesis that there was no statistical significance between G6PD deficiency and age-group. In one of the recent studies done, the number of G6PD deficiency was found to increase with the age-group (Odaburhine, 2015). However, in the other investigation done with 100 individuals, the G6PD deficiency was found to be 8.3% in age-group 0-10, 36.1% in age-group 11-20, 33.3% in age-group 21-30, 11.1% in age-group 31-40 and 11.1% age-group 41-onwards (Memon, *et al.*, 2016). The pattern started with minimum in age-group 0-10, gradually increases to maximum and then again decrease over the age-group. It was supported by the pattern in Dagaura Tharu. It seems there is no significant relationship of G6PD deficiency with age-group. The highest number of G6PD deficient cases 24(3.6%) were from age group 15-30 and least numbers of G6PD deficient cases 11(1.6%) were from age group 31-60 years. Fifteen (2.2%) cases were from age group 5-15 years. Statistically there is no significant relationship between occurrence of G6PD deficiency and age group (p=0.43). The result is consistent to study in Comodia (Khim et al 2010-12) but some varies to (Chen *et al.*, 1996) in Hongkong. Nowadays the people of the age more than 13 years are being involving in unnecessary drug addictions and also in Nepal some of

young people are involved in such type of activities. G6PD becomes deficient on persistent drug users for the development of immunity (Medical dictionary). It might be the reason behind high prevalence of G6PD deficiency among age group 16-30 and also in childrens of age 5-15 years.

5.3 Ethnic comparison of sex wise distribution of G6PD deficiency

During the study, 300 whole blood samples were collected from two sub- ethnic groups i.e. Dagaura Tharu(150) and Rana Tharu(150) or G6PD test persons. Among Dagaura Tharu 150, 75(50%) were male and 75(50%) were female. Similarly, among Rana Tharu 150, 75(50%) were male and 75(50%) were female. The male to female ratio was 1:1. Although equal number of male and female test cases were taken, out of 24 deficient test cases, 19(79%) were male and 5(21%) were female. Again, for some races the G6PD deficiency is caused by one copy of defective gene in male and two copies of defective gene in female . That might be the reason of a greater number of G6PD deficient cases in male. This shows a dominancy of G6PD deficiency in males over female with ratio 4:1. The p-value was found to be 0.118, which is greater than 0.05 and thereby accepting the null hypothesis that there is no correlation between the deficiency in G6PD and sex. In the region of high incidence of G6PD deficiency Indonesia using Formazan Ring Method, it was found that 44.48% males and only 8.11% females out of total cases were G6PD deficient (Pediatrics Department Faculty of Medicine, Indonesia). Actually, G6PDD is X-linked recessive disorder. It mostly affects X chromosome. And in female there are two X chromosomes. For female to be G6PDD both the chromosomes should be defected which is rarely found. It could be the reason for the higher number of suspected cases in male.

In an investigation carried out with Dagaura Tharu of Uttar Pradesh (Sharma and Rajkumari, 2010), 23.21% males were found to have deficiency in G6PD whereas only 12.5% females had deficiency in G6PD, with surprising male to female deficiency ratio of 2:1. Our study strongly supported these previous studies.

The result was in harmony with the other studies in which number of G6PD deficient cases were more in male (Mukherjee *et al.*, 2015). Dominancy in deficiency of G6PD in males over females might be obvious because of presence of only one X-chromosome in males and two X-chromosomes in females. If one X-chromosome in male become affected in G6PD, it is said to have G6PD deficiency whereas in case of female, even if one X-chromosome gets affected, the other X-chromosome covers up over the deficiency

making it a case of intermediate deficiency (heterozygote condition). In another study done in Gadjah Mada University of Indonesia for over 140 test cases (71 males and 74 females), 9 males had deficiency in G6PD but females neither showed low nor deficiency during G6PD screening test (Purnomo, 1996). This is because for female to be deficient both the X chromosomes should be defected.

In context of Nepal most people suspected from malaria infection are being provided the drugs Primaquine, pamaquine, Chloroquine etc. The persons who are G6PD is deficient, these drugs cause haemolysis in Red Blood Cell (RBC) and also may cause the death of persons. So before providing such drugs the qualitative test for G6PD activity is needed. This study could be useful in disseminating information on the G6PD activities and the effects of anti-malarial drugs on G6PD deficient persons.

6. CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Out of 300 test cases, the total number of G6PD deficiency by Formazan Ring Method was found to be 24(8%). Among 150 blood samples of Dagaura Tharu, 15(10%) were G6PD deficient. Whereas, in 150 blood samples of Rana Tharu, 9(6%) were G6PD deficient. The p-value was found to be 0.084, which is greater than 0.05 and thereby accepting the null hypothesis that there is no correlation between the deficiency in G6PD and ethnicity. In fact, there should be the relation between ethnicity and G6PD.

The highest numbers of G6PD deficient cases were found from age group 16-30 that is 6(5.45%) and least numbers of cases were found from age group 5-15 that is 3(4.41%) in case of Dagaura Tharu. However, for Rana Tharu, the highest numbers of G6PD deficient cases were found from age group 5-15 that is 4 (5.88%) and least numbers of cases were found from age group 16-30 that is 1(0.89%). The p-value for age-wise distribution was found to be 0.186 for Dagaura Tharu and 0.993 for Rana Tharu thereby accepting the null hypothesis that there is no correlation between the G6PD deficiency and the age.

Out of total 24 deficient cases, Male deficient cases was 19 (79.16%) and female deficient cases was 5 (20.83%). G6PD deficient cases was found to be 4:1 with p value p=0.118. There was no any statistical significance of G6PD deficiency with sex. Hence, there was no any stastical significance of G6PD deficiency with the ethnicity, age and sex. The prevalence of G6PD deficiency in context of Nepal is found to be in ethnic groups of Nepal who are the inhabitants of malaria endemic areas.

6.2 Recommendations

Certain procedures if taken care would have brought better results, which are as follows:

- The screening of the G6PD deficiency prior to the Primaquine administration should be performed specially in the ethnic groups of the malaria endemic areas.
- The study of G6PD deficiency should be performed on the large population with molecular typing should be done to perform complete profile of G6PD testing.
- Anti-malarial drugs have high risk of blood haemolysis. So anti-malarial drugs should be only provided after testing and reference laboratories should be established at least in Terai region for qualitative and quantitative identification of G6PD deficiency.

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APPENDIX I

PHOTOGRAPH

Photograph 1: Collecting the blood samples From Rana Tharu

Photograph 2: After collecting the blood samples of Rana Tharu

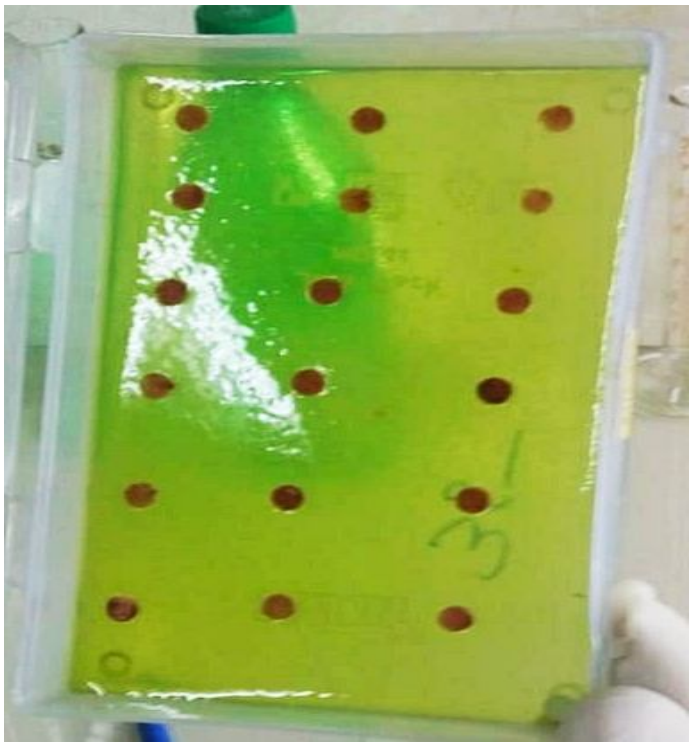
Photograph 3: Collecting blood Samples from Dagaura Tharu

Photograph 4: Collecting blood samples from Dagaura Tharu

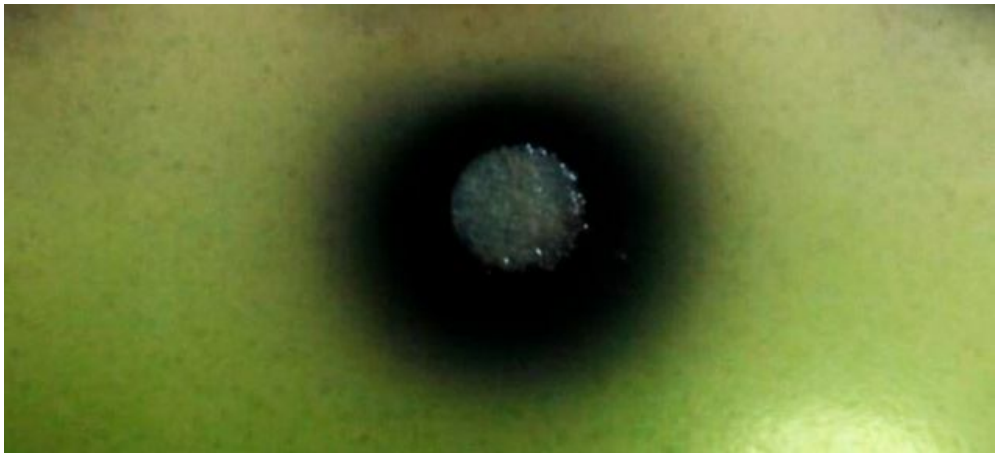
Photograph 5: Drying blood samples on filter papers

Photograph 6: Boiling the agar powder

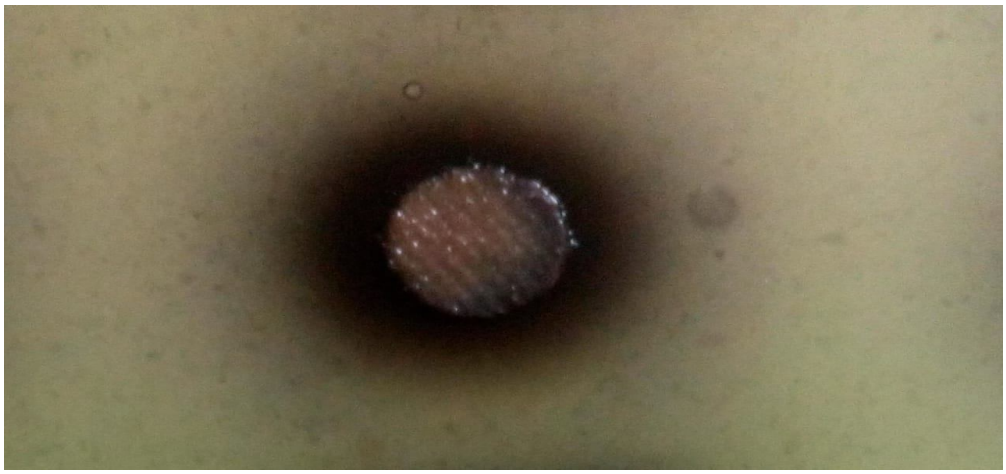
Photograph 7: Putting Dried Blood samples on agar gel plate (with Supervisor)



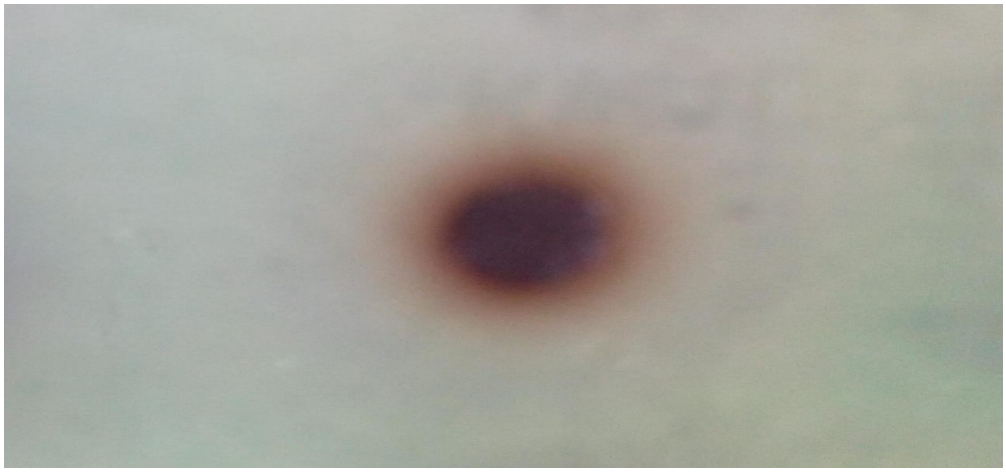
Photograph 8: After putting blood samples in agar gel plate



Normal (Blue colour)



Intermediate (Light Brown)



Deficient (Completely Brown)

Photograph 9: Results after screening for G6PD deficiency Test

APPENDIX II

COLLECTED DATA OF DAGAURA THARU (ALPHABETICALLY)

S.N.	Name	Age	Sex	G6PD Type
1	Aanita Dagaura	28	Female	Normal
2	Aarati Dagaura	13	Female	Normal
3	Aasa Dagaura	14	Female	Normal
4	Aasa Devi Dagaura	32	Female	Normal
5	Aashika Dagaura	27	Female	Normal
6	Aashis Dagaura	10	Male	Normal
7	Aashis Dagaura	16	Male	Normal
8	Agnu Dagaura	60	Male	Normal
9	Amar Dagaura	27	Male	Normal
10	Anita Dagaura	25	Female	Normal
11	Ankit Dagaura	7	Male	Normal
12	Anup Dagaura	8	Male	Normal
13	Aruna Dagaura	7	Female	Intermediate
14	Balpati Dagaura	35	Female	Intermediate
15	Basanti Dagaura	25	Female	Normal
16	Bhawana Dagaura	24	Female	Normal
17	Bibisa Dagaura	8	Female	Intermediate
18	Bikash Dagaura	15	Male	**Deficient**
19	Bikram Dagaura	44	Male	Normal
20	Bimal Dagaura	24	Male	Normal
21	Bimla Dagaura	19	Female	Intermediate
22	Bindu Dagaura	27	Male	Normal
23	Bindu Devi Dagaura	21	Female	Intermediate
24	Bishna Dagaura	40	Female	Intermediate
25	Bishna Dagaura	22	Female	Normal
26	Bishnu Dagaura	13	Male	Normal
27	Biswojit Dagaura	16	Male	Normal
28	Budhani Dagaura	50	Female	Normal
29	Chachiya Devi Dagaura	55	Female	Intermediate

30	Chuliya Dagaura	15	Female	Normal
31	Daani Dagaura	60	Male	Normal
32	Deep Bahadur Dagaura	60	Male	Normal
33	Dev Ram Dagaura	56	Male	Normal
34	Dhan Bahadur Dagaura	14	Male	Normal
35	Dhan Bahadur Dagaura	21	Male	**Deficient**
36	Dhaniram Dagaura	40	Male	**Deficient**
37	Dilliraj Dagaura	40	Male	Normal
38	Dinesh Dagaura	18	Male	Normal
39	Dinesh Dagaura	20	Male	Normal
40	Dipendra Dagaura	14	Male	**Deficient**
41	Dippu Dagaura	16	Male	Normal
42	Dukhani Dagaura	40	Female	Normal
43	Faguram Dagaura	39	Male	Normal
44	Faguram Dagaura	47	Male	Normal
45	Fudiya Devi Dagaura	35	Female	Normal
46	Fulpati Dagaura	48	Female	Intermediate
47	Fulpati Dagaura	25	Female	Normal
48	Gangi Dagaura	32	Female	Intermediate
49	Ghappu Dagaura	63	Male	**Deficient**
50	Gholu Prasad Dagaura	52	Male	**Deficient**
51	Ghotaktiya Dagaura	45	Female	Normal
52	Govinda Dagaura	25	Male	Normal
53	Gyani Dagaura	15	Female	Intermediate
54	Harina Devi Dagaura	30	Female	Normal
55	Hariram Dagaura	50	Male	Normal
56	Hikmat Dagaura	45	Male	Normal
57	Ishwari Dagaura	20	Female	Normal
58	Janaklal Dagaura	58	Male	Normal
59	Jit Bahadur Dagaura	20	Male	Normal
60	Jiva Dhan Dagaura	17	Male	Normal
61	Jugan Dagaura	34	Female	Normal

62	Juguni Dagaura	60	Female	Normal
63	Kalawati Dagaura	24	Female	Intermediate
64	Kalpana Dagaura	14	Female	Normal
65	Kamala Dagaura	20	Female	Normal
66	Khusi Dagaura	57	Male	Normal
67	Kishani Dagaura	18	Female	Normal
68	Kishor Dagaura	28	Male	**Deficient**
69	Kumari Dagaura	56	Female	normal
70	Lahanu Dagaura	53	Male	**Deficient**
71	Lal Bahadur Dagaura	19	Male	Normal
72	Lal Bahadur Dagaura	45	Male	Normal
73	Laxmi Dagaura	22	Female	Intermediate
74	Maghchin Dagaura	45	Male	Normal
75	Man Bahadur Dagaura	40	Male	Normal
76	Man Bahadur Dagaura	39	Male	Normal
77	Man Singh Dagaura	18	Male	Normal
78	Manaku Dagaura	60	Male	Normal
79	Manju Dagaura	26	Female	**Deficient**
80	Maya Dagaura	28	Female	Normal
81	Milan Dagaura	8	Male	**Deficient**
82	Mira Dagaura	40	Female	Intermediate
83	Mira Dagaura	30	Female	Normal
84	Nageshwor Dagaura	20	Male	**Deficient**
85	Naina Dagaura	20	Female	Normal
86	Narendra Dagaura	32	Male	Normal
87	Naresh Dagaura	24	Male	**Deficient**
88	Niraj Dagaura	10	Male	Normal
89	Pardesi Dagaura	30	Male	Normal
90	Parwati Dagaura	30	Female	**Deficient**
91	Pendu Ram Dagaura	53	Male	Normal
92	prabin Dagaura	28	Male	Normal
93	Pradip Dagaura	8	Male	Normal

94	Punam Dagaura	24	Female	Intermediate
95	Purati Dagaura	57	Female	Normal
96	Pyari Dagaura	38	Female	Normal
97	Pyari Dagaura	45	Female	Intermediate
98	Rabin Dagaura	18	Male	Normal
99	Raimola Dagaura	52	Female	Normal
100	Raj Bahadur Dagaura	38	Male	Normal
101	Rajeshwori Dagaura	21	Female	Normal
102	Rajkumari Dagaura	22	Female	Normal
103	Ram Bahadur Dagaura	55	Male	Normal
104	Ram Bahadur Dagaura	54	Male	Normal
105	Ram kishan Dagaura	31	Male	Normal
106	Ram Lal Dagaura	26	Male	Normal
107	Ram Pati Dagaura	38	Female	Normal
108	Ram Prasad Dagaura	55	Male	Normal
109	Ram Pyari Dagaura	55	Female	Normal
110	Ramjiu Dagaura	58	Male	Normal
111	Rampati Dagaura	39	Female	Intermediate
112	Rampati Dagaura	28	Female	Intermediate
113	Rampati Dagaura	13	Female	Normal
114	Rima Dagaura	37	Female	Normal
115	Rinu Dagaura	26	Female	Normal
116	Runchi Dagaura	49	Female	Normal
117	Sagar Dagaura	13	Male	Normal
118	Salina Dagaura	13	Female	Normal
120	Sanju Dagaura	30	Female	Normal
121	Sankar Dagaura	12	Male	Normal
122	Sapana Dagaura	12	Female	Normal
123	Sarisma Dagaura	13	Female	Normal
124	Setiya Dagaura	43	Female	Intermediate
125	Shekhar Dagaura	6	Male	Normal
126	Sher Bahadur Dagaura	22	Male	Normal

127	Sita Dagaura	48	Female	Normal
128	Sita Dagaura	35	Female	Intermediate
129	Sita Ram Dagaura	49	Male	Normal
130	Sita Ram Dagaura	57	Male	Normal
131	Sonia Dagaura	59	Female	**Deficient**
132	Srijana Dagaura	11	Female	Intermediate
133	Subash Dagaura	9	Male	Normal
134	Sudip Dagaura	10	Male	Normal
135	Sudiya Dagaura	55	Female	Normal
136	Sumita Dagaura	21	Female	Normal
137	Sundar Prasad Dagaura	60	Male	Normal
138	Sunil Dagaura	19	Male	Normal
139	Sunita Dagaura	22	Female	Normal
140	Sunita Dagaura	30	Female	Normal
141	Surat Dagaura	12	Male	Normal
142	Suresh Dagaura	15	Male	Normal
143	Sushila Dagaura	34	Female	**Deficient**
144	Sushila Dagaura	22	Female	Normal
145	Tara Dagaura	30	Female	Intermediate
146	Tikaram Dagaura	28	Male	Normal
147	Tikaram Dagaura	28	Male	Normal
148	Tiku Devi Dagaura	45	Female	Normal
149	Ujayali Dagaura	39	Female	Normal
150	Vangi Dagaura	40	Female	Normal

APPENDIX III

COLLECTED DATA OF RANA THARU (ALPHABETICALLY)

S.N.	Name	Age	Sex	G6PD Type
1	Aarati Rana	12	Female	Intermediate
2	Aasika Rana	24	Female	Normal
3	Aasu Rana	12	Female	Normal
4	Aman Rana	10	Male	Normal
5	Amaru Rana	22	Male	Normal
6	Anarkali Rana	40	Female	Normal
7	Anarkali Rana	29	Female	Normal
8	Arjun Rana	18	Male	Normal
9	Avishek Rana	11	Male	**Deficient**
10	Balmati Rana	24	Female	Normal
11	Barfi Rana	48	Female	Normal
12	Basant Rana	17	Male	Normal
13	Battulla Rana	38	Female	Normal
14	Baula Rana	50	Female	Intermediate
15	Bhagaula Rana	45	Female	Normal
16	Bhangu Rana	56	Female	Normal
17	Biddhu Rana	50	Male	Normal
18	Bijaya Rana	17	Male	Normal
19	Bimala Rana	20	Female	Normal
20	Binnu Rana	40	Female	Normal
21	Bipana Rana	15	Female	Intermediate
22	Bipana Rana	15	Female	Normal
23	Bipin Rana	16	Male	Normal
24	Bir Bahadur Rana	15	Male	Normal
25	Birma Rana	54	Female	Normal
26	Bishal Rana	14	Male	**Deficient**
27	Bishni Rana	11	Female	Normal
28	Chetram Rana	47	Male	Normal
29	Deepesh Rana	14	Male	**Deficient**

30	Deepa Rana	14	Female	Normal
31	Deepak Rana	18	Male	Normal
32	Deepak Rana	23	Male	Normal
33	Dhanendra Rana	14	Male	Normal
34	Dhirendra Rana	18	Male	Normal
35	Dubaichandra Rana	15	Male	Normal
36	Fulchandra Rana	52	Male	Normal
37	Fulmati Rana	40	Female	Normal
38	Fulmati Rana	55	Female	Normal
39	Fulsahattar Rana	45	Female	Normal
40	Geet Bahadur Rana	40	Male	Normal
41	Govinda Rana	27	Male	Normal
42	Govinda Rana	23	Male	Normal
43	Hari Rana	70	Male	Normal
44	Hemanti Rana	24	Female	Normal
45	Hira Rana	50	Male	Normal
46	Hira Rana	54	Male	Normal
47	Indersingh Rana	60	Male	Normal
48	Jai Devi Rana	30	Female	Intermediate
49	Janaki Rana	23	Female	Normal
50	Janntarwati Rana	22	Female	Normal
51	Janntiya Rana	50	Female	Normal
52	Januka Rana	27	Female	Normal
53	Jara Rana	18	Female	Normal
54	Jitendra Rana	16	Male	Normal
55	Jitendra Rana	25	Male	Normal
56	Josebh Rana	9	Male	Normal
57	Kabbutar Rana	50	Female	Normal
58	Kailasho Devi Rana	40	Female	Intermediate
59	Kaillash Rana	60	Female	Normal
60	Kamal Rana	26	Male	Normal
61	Kandi Rana	50	Female	Normal

62	Khauki Rana	14	Female	Normal
63	Krishna Rana	21	Male	Normal
64	Kumbhakarl Rana	35	Male	Normal
65	Kushma Rana	48	Female	Normal
66	Labari Rana	50	Female	Normal
67	Labra Rana	60	Male	**Deficient**
68	Labru Rana	54	Male	Normal
69	Lajjawati Rana	23	Female	Normal
70	Lajju Rana	50	Female	Normal
71	Laxmi Rana	15	Female	Normal
72	Madhuri Rana	16	Female	Intermediate
73	Manish Rana	10	Male	Normal
74	Mansa Rana	33	Male	Normal
75	Maule Rana	15	Male	Normal
76	Mukesh Rana	12	Male	Normal
77	Neelam Rana	16	Female	Normal
78	Nembatti Rana	38	Female	Normal
79	Niraj Rana	11	Male	Normal
80	Nirmala Rana	26	Female	Normal
81	Nirmala Rana	21	Female	Intermediate
82	Pandey Rana	27	Male	Normal
83	Poonam Rana	8	Female	Normal
84	pPashupati Rana	20	Male	Normal
85	Pradip Rana	15	Male	Normal
86	Prakash Rana	27	Male	Normal
87	Premlal Rana	26	Male	**Deficient**
88	Premwati Rana	45	Female	Normal
89	Pritam Rana	13	Male	Normal
90	Punam Rana	16	Female	Normal
91	Puran Rana	17	Male	Normal
92	Puran Rana	28	Male	Normal
93	Puspa Rana	15	Female	Normal

94	Rabita Rana	14	Female	Normal
95	Radha Rana	30	Female	Intermediate
96	Radhika Rana	21	Female	Intermediate
97	Rajani Rana	24	Female	Normal
98	Rajaram Rana	15	Male	Normal
99	Rajendra Rana	21	Male	Normal
100	Rajkumar Rana	15	Male	Normal
101	Rajmati Rana	20	Female	Normal
102	Rakesh Rana	12	Male	Normal
103	Ram Bahadur Rana	59	Male	Normal
104	Ram Bhajan Rana	14	Male	Normal
105	Ram Dayal Rana	36	Male	Normal
106	Ramchandra Rana	30	Male	Normal
107	Ramdas Rana	21	Male	Normal
108	Ramdhiraj Rana	40	Male	Normal
109	Ramesh Rana	17	Male	Normal
110	Ramkali Rana	36	Female	Normal
111	Ramkali Rana	50	Female	Normal
112	Ramkumari Rana	36	Female	**Deficient**
113	Ramsingh Rana	53	Male	**Deficient**
114	Ramwati Rana	32	Female	Normal
115	Revati Kanta Rana	32	Male	Normal
116	Roshan Rana	36	Male	Normal
117	Rupesh Rana	15	Male	Normal
118	Sabada Rana	35	Female	Normal
119	Sabitri Rana	25	Female	Normal
120	Sahiuba Rana	60	Female	Normal
121	Samjhana Rana	15	Female	Intermediate
122	Sandeep Rana	10	Male	Normal
123	Sangeeta Rana	17	Female	Intermediate
124	Sanidev Rana	15	Male	Normal
125	Santariya Rana	35	Female	Intermediate

126	Santosh Rana	18	Male	Normal
127	Sapana Rana	22	Female	Intermediate
128	Sarita Rana	45	Female	Normal
129	Sarita Rana	16	Female	Intermediate
130	Sarswati Rana	15	Female	Intermediate
131	Sarvan Rana	10	Male	**Deficient**
132	Sevi Chandra Rana	35	Male	Normal
133	Sewa Rana	32	Male	Normal
134	Shyam Rana	23	Male	Normal
135	Sibiya Rana	30	Female	Normal
136	Siddhu Rana	35	Male	Normal
137	Sima Rana	16	Female	Intermediate
138	Sohan Rana	13	Male	Normal
139	Sriram Rana	59	Male	Normal
140	Suchma Rana	5	Female	Normal
141	Sukamaya Rana	5	Female	Normal
142	Sumitra Rana	35	Female	Normal
143	Sundar Rana	55	Female	Normal
144	Sunita Rana	25	Female	Intermediate
145	Surendra Rana	24	Male	Normal
146	Suryansa Rana	5	Male	Normal
147	Taaul Rana	60	Male	Normal
148	Tirthalal Rana	45	Male	**Deficient**
149	Urmila Rana	15	Female	Intermediate
150	Usha Rana	17	Female	Intermediate

APPENDIX IV

ETHICAL CONSENT FORM

Government of Nepal

Nepal Health Research Council (NHRC)

Estd. 1991

Ref. No.: 1762

11 February 2018

Mr. Himalaya Joshi
Principal Investigator
Central Department of Zoology
TU, Kirtipur, Kathmandu

Ref: **Approval of Thesis Proposal** entitled **Analysis of glucose-6-phosphate dehydrogenase (G6PD) deficiency in Dagaura Tharu and Rana Tharu of Dekhatbhuli VDC-9, Kanchhanpur, Nepal**

Dear Mr. Joshi,

It is my pleasure to inform you that the above-mentioned proposal submitted on **18 November 2017** (**Reg. no. 467/2017)** please use this Reg. No. during further correspondence) has been approved by Nepal Health Research Council (NHRC) Ethical Review Board on **7 February 2018**.

As per NHRC rules and regulations, the investigator has to strictly follow the protocol stipulated in the proposal. Any change in objective(s), problem statement, research question or hypothesis, methodology, implementation procedure, data management and budget that may be necessary in course of the implementation of the research proposal can only be made so and implemented after prior approval from this council. Thus, it is compulsory to submit the detail of such changes intended or desired with justification prior to actual change in the protocol. Expiration date of this proposal is **April 2018.**

If the researcher requires transfer of the bio samples to other countries, the investigator should apply to the NHRC for the permission. The researchers will not be allowed to ship any raw/crude human biomaterial outside the country; only extracted and amplified samples can be taken to labs outside of Nepal for further study, as per the protocol submitted and approved by the NHRC. The remaining samples of the lab should be destroyed as per standard operating procedure, the process documented, and the NHRC informed.

Further, the researchers are directed to strictly abide by the National Ethical Guidelines published by NHRC during the implementation of their project proposal and **submit progress report in between and full or summary report upon completion.**

As per your project proposal, the total research amount is **NRs. 71,000.00** and accordingly the processing fee amounts to **NRs. 1,000.00.** It is acknowledged that the above-mentioned processing fee has been received at NHRC.

If you have any questions, please contact the Ethical Review M & E Section at NHRC.

Thanking you,

Prof. Dr. Anjani Kumar Jha
Executive Chairperson

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