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MOLECULAR DIAGNOSIS OF SICKLE CELL ANEMIA IN CHOLANAICKAN TRIBALS

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ABSTRACT OF THE PROJECT PROPOSAL

1.	Project No.	KFRI RP 626/2011
2.	Title	Molecular diagnosis of sickle cell anemia in Cholanaickan tribals
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5.	Research Fellow	Mr. Saju K. Michael
6.	Objectives	1. DNA based molecular diagnosis of the sickle cell anemia through restriction enzyme analysis in the Cholanaickan tribal community 2. To conduct genetic counselling programs with the help of tribal community health workers in case of possible occurrence of the disease
7.	Duration	3 Years
8.	Funding Agency	KSCSTE Plan funds

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CONTENTS

1. INTRODUCTION

1.1. Haemoglobin disorders

Haemoglobin disorders refer to a diverse group of disorders caused by an imbalance in the normal pattern of globin gene expression (Weatherall and Clegg, 2001). Haemoglobinopathies are characterized by qualitative and quantitative abnormalities in the synthesis of haemoglobin (Hb)[1]. They are single gene disorder and more than 1000 mutations have been documented starting from rearrangements, deletions of genes to single base pair substitutions altering aminoacid sequence (Hardison et al., 2002). Prevalent haemoglobinopathic disorders are sickle cell anaemia, thalassemia, G6PDH deficiency and variant haemoglobins. These disorders are manifested owing to a reduced synthesis of one or more globin chains (thalassemia) or the synthesis of a structurally abnormal Hb variant (sickle cell disease). The World Health Organization (WHO) had estimated 5 to 7 per cent of the world's populations as susceptible to haemoglobinopathies (WHO, 2006). Most of these inherited single gene heamoglobin disorders are transmitted from one generation to another affecting millions of people around the globe (Makani et al., 2007).

Haemoglobin disorders are generally prevalent in the tropical countries. The evidence for the occurrence of the haemoglobinopathies in the Indian subcontinent has been indicated in the findings of Indus Valley civilization from about 200 –5000 BC. In India, sickle cell disease is the second most common haemoglobinopathy next to thalassemia. Dr.James Henrick was the first to observe peculiar elongated sickle shaped red blood cells' in the blood of an African medical student and the genetic basis for the sickle cell disease was first suggested by Dr. Emmel. In 1949, Linus A Pauling identified defective haemoglobin molecule in the blood as the basis of sickle cell disease (Pauling, 1949). In India, the sickle

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[1]Haemoglobin (Hb) molecules are a set of proteins formed by pairing of two alpha (α) and two beta (β) globin polypeptide chains into a tetromeric unit. Beta globin gene encodes four different globin molecules: embryonic ε-globin, fetal γ-globin, adult δ-globin and adult β-globin, all expressed differently during specific times of development. The co-ordinated expression of these globin chains maintains a balanced and high concentration of haemoglobin within red blood cells throughout (Clark and Thein, 2004).

cell disease was detected for the first time in an Irula boy of Nilgiris in 1952 (Lehmann and Cutbush, 1952) and then in Eastern India (Dunlop and Mazumdar, 1952). Subsequently, it has been detected in many other regions of India and of the world (Urade, 2012).

1.2. Sickle Cell Disease (SCD)

Sickle cell disease (SCD) is an autosomal recessive genetic blood disorder. The gene defect is a single nucleotide mutation (single nucleotide polymorphism - SNP) involving G**A**G>G**T**G transversion, which leads to an amino acid substitution at position 6 in the β-globin chain (glutamic acid to valine) on the surface of the haemoglobin molecule. This amino acid change results in altered characteristics which causes HbS (sickle haemoglobin) to become less soluble compared to HbA (normal adult haemoglobin), and when HbS becomes deoxygenated, polymerization of HbS occurs. Consequently, the elongated rigid fibers damage the cytoskeleton of the red blood cell, causing the shape of the cell to change from the original doughnut shape to sickle shape. As the red blood cell becomes re-oxygenated the HbS fibers dissociate and revert back to the original shape. The repeated cycles of sickling/ unsickling events lower the life span of the red blood cell from 120 days to less than 20 days. These sickled cells then form homotypic aggregations and results in obstruction of blood flow to vital organs and are associated with substantial morbidity, clinical manifestations and premature mortality. The polymerization events are comparatively less for people who are sickle cell carriers and they suffer only from mild anemia as the normal allele is able to produce over 50 per cent of the haemoglobin (Steinberg, 2008).

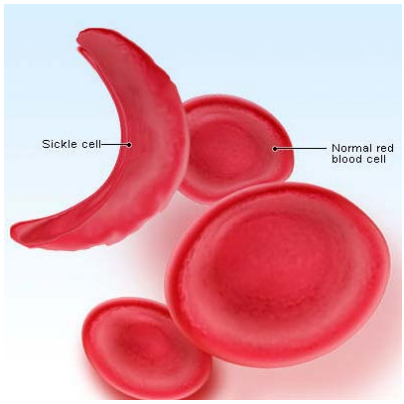


Figure 1. Normal Red blood cells (RBCs) and Sickle shaped RBC' due to the disease

1.3. Inheritance

The single nucleotide point mutation leading to sickle cell anemia, was reported to originate as a result of three independent mutational events (two in Africa and one in either Saudi Arabia or central India) that occurred between 3,000 and 6,000 generations ago (approximately 70,000 –150,000 years) (Desai and Dhanani, 2004). The transmission of the SCD follows the simple Mendelian principle and thus, expression of disease requires both aberrant copies of defective hemoglobin gene. Heterozygotes are denoted as carriers of the disease and they sustain normal life with much milder symptoms. If an individual inherits one abnormal hemoglobin 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 there is a 25 per cent chance in each pregnancy of having a child who will inherit both abnormal hemoglobin genes and have the SCD.

1.4. Clinical features

Sickle cell anemia patients suffer a wide spectrum of illness. The most important of these is the chronic anemia with an extremely low haemoglobin concentration. The sickle shaped red blood cells tend to aggregate and block capillaries under low oxygen tension. For children below 7 years, when the sickle red blood cells are trapped in the spleen, severe anemia along with rapid splenic enlargement occurs. For children between 6-18 months, painful swelling of hands and/ or feet happens. SCD leads to common symptoms, such as, anemia, recurrent severe painful crisis, acute chest syndrome, bone necrosis, renal failure, osteomyelitis, splenic sequestration crises, recurrent jaundice, bone and joint pains, leg ulcers, growth retardation, hepato-splenomegaly, as well as it affects the general health of the individual (Feroze and Aravindan, 2001; Serjeant and Serjeant, 2001; Balgir, 2007).

1.5. Diagnosis

In India, the commonly practiced diagnostic tests for haemoglobinopathies include red cell indices, red cell morphology, reticulocyte count, sickling test, estimation of foetal haemoglobin (F) and adult A2 fraction, estimation of serum ferritin, iron binding capacity, haemoglobin electrophoresis (both alkaline and acidic), haemoglobin variant analysis or high pressure liquid chromatography (HPLC) and DNA mutations analysis, among others

(Balgir, 2000). Majority of the haemoglobinopathies are autosomal recessive, therefore heterozygous individuals are generally fit and well for this autosomal recessive haemoglobin disorder. DNA diagnostics are mainly performed for carrier screening and for the detection of the single nucleotide point (SNP) mutation detection leading to haemoglobin disorders. Preliminary screening and confirmation for the sickle cell disease are generally carried out by sickle cell preparation test as well as haemoglobin electrophoresis test. Further information on the genetic status responsible for this haematological disorder is through the molecular diagnostic techniques (Clark and Thein, 2004).

Most of the DNA based diagnostic techniques of the haemoglobin disorders are based on the polymerase chain reaction (Saiki et al., 1985). Variability is attributed to the difference in the specific sets of primers used depending on whether a mutation is a deletion, a rearrangement or a point mutation. A plethora of PCR based techniques are available for the diagnostics of haemoglobin disorders. They include allele-specific oligonucleotide (ASO) hybridization or dot-blot analysis, reverse dot blot analysis, allele-specific priming or amplification refractory mutation system (ARMS), restriction enzyme analysis, amplification created restriction analysis, mutagenically separated PCR and gap-PCR. More precise and rapid method PCR amplification and subsequent sequencing of the amplified products has become a routine tool for mutation identification. The compact size of globin genes (1.2kb-1.6kb) are amenable to direct sequencing analysis by which most of the point mutations within the gene can be identified (Clark and Thein, 2004).

Restriction enzyme analysis is a simple, relatively, cheap and robust technique and is the most precise molecular diagnostic tool for haemoglobin disorders like SCD, alpha thalassemia and beta thalassemia which naturally create or abolish restriction enzyme cutting sites (Weatherall and Clegg, 2001). Restriction fragment length polymorphisms (RFLPs) arise because mutations can create or destroy the sites recognized by specific restriction enzymes, leading to variations between individuals in the length of restriction fragments produced from identical regions of the genome. Restriction enzymes such as MstII, MsII, Eco811, Dde1, among others are generally used for the restriction analysis of beta globin gene for detecting the genetic status of sickle cell anemia (Hatcher et al., 1992).

With the availability of bioinformatic tools, the sequences can also be screened for the putative recognition sites for restriction enzymes. The target DNA containing the specific sequence mutation responsible for a globin disorder is amplified by PCR and the product is then digested by the restriction enzyme and the resulting DNA fragment separated on gels. The presence or absence of the recognition site is determined from the pattern of the RFLP analysis. SCD is generally caused by homozygosity for point mutation (70 %) happening in the beta globin chain at the 6^{th} codon position wherein aminoacid glutamic acid is replaced by valine and 29 per cent is by compound heterozygosity. The occurrence of sickle point mutation (A >T) in the 6^{th} codon of the beta globin chain removes cleavage sites for Mnl I, Mst II, Eco811, Dde I, among others. Dde I restriction analysis is the most commonly practiced molecular diagnostic tool for sickle cell disease (Karkun and Bhagat, 2011).

1.6. Malarial hypothesis

SCD usually occurs in African and American origins. The commonly accepted hypothesis says that the SCD occurs in the hyper endemic regions of malaria caused by *Plasmodium falciparum* and is responsible for the maintenance of high frequencies of this trait (Allison, 1954). The distribution of sickle cell trait in hyper endemic malarial regions of Africa supports this hypothesis which also hold true for India in some instances (Negi, 1976). A correlation between sickle cell trait and malarial endemicity favouring the hypothesis has been reported among some populations of central India and Maharashtra (Mukherjee et al., 1982). Sickle cell trait in India is mostly seen in regions with an earlier history of malaria or was present until recent times. The heterozygous sickle cell carrier trait (HbAS) is reported to provide protection against mortality against malaria which has attained high frequency in many parts of the tropical world (Sutaone, 2013). Lower mortality risk is associated with HbAS due to its protection against malaria related mortality (Aidoo et al., 2002). Since the spread of sickle cell gene is not uniform in Indian context, further acceptance of this hypothesis warrants detailed investigation (Sutaone, 2013).

1.7. Prevalence of Sickle cell anemia

Of the 300 million SCD people worldwide, 50 per cent are residing in India (Kaur et al., 2013). Inherited diseases of genetic origin such as sickle cell anemia, alpha and beta

thalassemia, glucose-6-phosphate dehydrogenase (G6PD) deficiency, among others, are reported to be prevalent in the Indian tribal populations (Balgir, 2004; Balgir and Sharma, 1988; Balgir, 1996). In India, tribal populations from nine States depict a very high percentage of incidences of HbS gene upto an alarming rate 48.5 per cent (Urade, 2012). SCD is highly prevalent in the Western, Central, and Eastern regions and in pockets of the South in the States of Maharashtra, Madhya Pradesh, Orissa, Andhra Pradesh, Gujarat, Chattisgarh, Tamil Nadu, and Kerala (Balgir, 1996). Such high incidences of SCD can be attributed to the consanguineous marriages happening within each tribal community (Mukherjee and Das, 1990; Balgir, 2014). Of the 36 tribal communities in the Kerala State, highest rate of sickle cell disease has been reported in the Irula tribe (14.2 %) while it was only 4.1 per cent in Mullakurumba, 3 per cent in Paniya tribe, and 1.6 per cent in Kattunaickans. The incidence of sickle cell carriers among the tribals of Kerala vary from 9.4 per cent in Kattunaickan, 17.1 per cent in Irula, 21.1 per cent in Paniya and 29 per cent in Mullakurumba (Sahani and Nandy, 2013).

1.8. Cholanaickans and their health status

Among the various tribal communities in Kerala, Cholanaickans, particularly vulnerable tribal group, are the numerically small (124 numbers in 42 households), semi-nomadic hunter-gatherer community forming only 0.08 per cent of the total tribal population in the State (Census of India, 2011). Their life is totally confined to the interior evergreen forests of the upper Ghat section (chola) of the Nilambur valley, mostly in caves and is known as ‘cavemen of Kerala’ (Bhanu, 1989). Cholanaickans are considered to be an offshoot of Kattunaickans (Misra, 1977; Mathur, 1977b; Viswanathan, 1985; Somasekharan Nair, 1981). Etymologically, Cholanaickans are known as the king of evergreen shola forests. They generally live in distinct territories called ‘chemmam’ and the eldest male member of each ‘chemmam’ is known as ‘chemmakkaran’(Kakkoth, 2005). The Cholanaickan was included under the scheduled tribes list of the Kerala State only in 2002. Their endogamous population with specific social system lives in close association with the forest ecosystem, shows clear geographical isolation and have only limited interaction with the mainstream society.

Various studies had been carried out previously on the ethnographic and developmental aspects of these hunter-gatherer communities in Kerala (Bhanu, 1982a, 1982b, 1988, 1989, 1991a, 1991b; Mathur, 1977a, 1977b; Misra, 1977; Sinha and Sharma, 1997; Viswanthan, 1985; Kakkoth, 2005, 2008, 2012). The population of Cholanaickans was 281 in 1971 (Mathur, 1977b) and has consequently reduced in the following years. In 1977, it was 229, which further reduced to 205 in 1979 (Bhanu, 1989). In 1998, the population reported was 365 (KIRTADS, 1998), 347 in 2007 (ITDP, 2007), 416 in 2011 (KILA, 2011) but as per the 2011 Census report, the total number of the community is only 124 comprising 42 households. The settlement details provided in the KILA report (2011) depicts a population number of 119 housed exclusively in the Alakkal settlement. This probably could be due to the presence of Kattunaickans who have been treated as Cholaniackans. Highly skewed sex ratio has also been reported for the Cholanaickans (722 females per 1000 males) with a literacy rate of only 12.5 % (Census of India, 2011).

Although efforts have been taken by the Government to move them to a more settled lifestyle they prefer their nomadic way of life and currently reside in rock shelters or in open camp-sites, some have a more settled system as they are close to mainstream. Although, approximately 50 per cent of the families have been provided with houses, they continue to lead a semi-nomadic life. They subsist on food gathering-hunting, collection of NTFPs (non-timber forest products) and forest labour. They are largely dependent on NTFP and other forest related activities. Food gathering is a family activity. Hunting and fishing are undertaken mainly during the summer months. With low literacy levels, wage labour is being seen as an emerging trend for alternate employment.

Latest survey statistics projects a poor demographic profile with low female population, indicative of poor health status. Very few studies have been undertaken to analyze the health status of Cholanaickan tribal community leading to this population decline and health care facilities were totally absent in the past. Actions are being taken for providing healthcare facilities to the community thereafter. Recently, Health Department has started organizing medical camps on a regular basis in the settlement and also holding awareness programs along with other NGOs, involving the active participation of tribal

health promoters from the settlements. The general health profile of the community is much below the average and anemia is highly prevalent in the community. Since their collection of wild food from the natural sources is also on the decline, to satisfy the nutritional requirements, the public distribution system supplies them with rice, sugar, dhal and salt. Along with the Tribal and Health Departments, many NGOs are also actively involved in creating awareness on the nutrition front and health aspects by conducting focal group discussions and periodic awareness camps in the community (Keystone Foundation, 2013). Along with the decline of population size years from 282 in 1977 to 124 in 2011 in the particularly vulnerable Cholanaickan community, consanguineous marriages happening continuously may further augment the risk factor associated with the genetic disorders. Till date, no published information is available regarding the genetic status of this community with respect to the sickle cell haemoglobin disorder.

1.9. Objectives of the study

In this context, the present study envisages to carry out the following objectives,

1. DNA based molecular diagnosis of the sickle cell anemia in the Cholanaickan tribal community through restriction enzyme analysis
2. To conduct genetic counselling programs with the help of tribal community health workers in case of possible occurrence of the disease

2. MATERIALS AND METHODS

2.1. Settlement details

As per 2011 census, there only 124 members in the Cholanaickan tribal community. 11 settlements of Cholanaickan community are situated in Karulai and Vazhikadavu ranges of Nilambur Forest Division. Of which, 7 are in Karulai range (*viz*. Achanallai (in Amarambalam panchayathu), Kuppamalai, Myladipotti colony, Enicol, Poochapara, Makkaberiallai, Mannallai and DT colony) and 4 in Vazhikadavu range (*viz.* Thannikadavu, Allakkal, Punchakolli, Poovathipoyil). In 2011, the number of settlements in Karulai has reduced from 8 to 7 (KILA Report, 2011).

2.2. Sample collection

A total of 33 random blood samples were collected by a medical team from the Government Medical College, Kozhikode, as part of All India Sickle Cell Anemia Screening Program (Appendix 1). Blood samples were collected from the members of the community with informed consent. Blood was collected by a deep finger prick using a disposable lancet. The finger was lightly squeezed so as to elicit four to five large drops of blood, which is collected directly into a 4 ml test tube containing normal saline. Sickle solubility test was carried out to identify the presence of sickle haemoglobin (Daland and Castle, 1948). One drop of blood was used to prepare a peripheral smear. One or two drops of blood sample were applied on a Whatman 903 filter membrane (Guthrie card) which was allowed to dry at room temperature. Paper cards are a popular method of storing blood due to their ease of use and long term stability at room temperature. The dried Guthrie cards were stored at room temperature before analysis. This research work was approved by the Institutional Ethics Committee constituted by the Kerala Forest Research Institute, Peechi, Thrissur, Kerala.

Sickle cell solubility test

The solubility test is the most common screening test for sickle cell or presence of HbS. It is based on the relative insolubility of HbS when combined with a reducing agent (sodium dithionite). When anticoagulated blood is mixed with reducing agent, the red cells will lyse due to the presence of saponin and the hemoglobin in the red cells will be released. If HbS

is present, it will form liquid crystals and give a cloudy or turbid appearance to the solution. If HbS is not present, the solution will appear transparent, with rare exceptions. The solubility test cannot be used to differentiate sickle cell disease (homozygous for HbS) from sickle cell trait (heterozygous for HbS).

Peripheal smear analysis

The peripheral smear was prepared, stained with Leishman's stain and screened in all the subjects particularly for hypochromic microcytic anemia. In all cases of sickle cell anemia the following were specifically looked for: presence or absence of irreversibly sickled cells, target cells, anisopoikilocytosis, polychromasia and nucleated red cells. Irreversibly sickled cells and target cells were assigned a grading of 3+ when they were present in almost all fields, 2+ when seen frequently and 1+ when only occasional cells were present.

2.3. DNA extraction protocol

Extraction of blood from spotted filter paper was performed by using Harris UNI-CORE Punch (GE Health Care, UK) and QIAcard FTA purification Reagent (GE Health Care, UK), according to the manufacturer's protocol and the resultant samples were used for direct PCR amplification. Three pieces of 2 mm diameter circle were punched out from the dried blood spot samples into a 1.5 ml microcentrifuge tube. Rehydration of the dried blood spot discs is performed by adding 200 µl of FTA purification reagent. The paper discs were washed repeatedly in the FTA reagent through inverted mixing for 5 minutes for a total of three washes to purify the DNA. Two repeated washes with Tris EDTA buffer (10 mM Tris Hcl, 0.1 mM EDTA, pH 8.0) were given with 5 minutes each for every wash. The washed disc was allowed to dry at room temperature for about an hour. Direct PCR was performed with the dried filter paper disc containing DNA.

2.4. PCR amplification

The quality of genomic DNA extracted by this method was determined by PCR amplification of the 376 bp beta globin gene fragment with the upstream primer 5´ACCTCACCTGTGGAGCCAC3' and downstream primer 5' ´GAGTGCACAGATCCCCAAAGGACTCAAA3'. PCR reactions were performed on a PTC 200

thermal cycler (MJ Research) in 0.2 ml thin walled tubes. The PCR was performed using KAPA Blood PCR Kit (Kapa Biosystems Inc. USA) according to the manufacturer's protocol. A PCR mixture of 20 µl solution contain 2x Kappa buffer Blood mix (10 µl), 0.5 µM of each primer, 3 units of Taq DNA polymerase and the filter paper disc containing DNA. Cycling conditions consisted of an initial denaturation step at 95°C for 5 minutes followed by 35 cycles each of 94°C for 1 minute, 63°C for 1 minute, and 72°C for 30 seconds, with a final extension at 72°C for 7minutes. After the PCR amplification, the amplified DNA was analyzed on 1 per cent agarose gel.

2.5. Restriction Enzyme Digestion

After gel extraction, the restriction digestion was performed with the help of Dde1 restriction enzyme. 15 µl of the amplified product was treated with 0.5 µL of Dde1 restriction enzyme solution (Boehringer, Mannheim, Germany), and digested at 37^{o}C for 45 minutes in 1.5 µl cut smart buffer (10X). This was followed by an enzyme inactivation at 65°C for 20 minutes. Subsequently, the digestion products were separated according to size on a 2 % agarose gel and visualized by ethidium bromide staining under ultraviolet light and compared with a 100 bp DNA ladder. The Dde1 enzyme has a recognition site at codon 6 in the normal beta globin gene, and cleaved the normal amplified beta globin DNA into two fragments (201bp and 175bp), while the fragment amplified from DNA of sickle cell mutation remained uncleaved (376bp) due to the absence of the recognition site. The agarose gel electrophoresis of the amplified DNA along with the Dde1 digested products, can detect the homozygous, heterozygous states and normal controls.

3. RESULTS AND DISCUSSION

PCR has been successfully employed for the diagnosis of infectious diseases, genetic disorders, cancer diagnostics and to study evolutionary pattern (Gibbs, 1990). For the diagnosis of sickle cell anemia in the Cholanaickan tribal community, a 376 bp fragment of the beta globin gene containing codon 6 was PCR amplified using the beta globin specific primers (Figure 1).

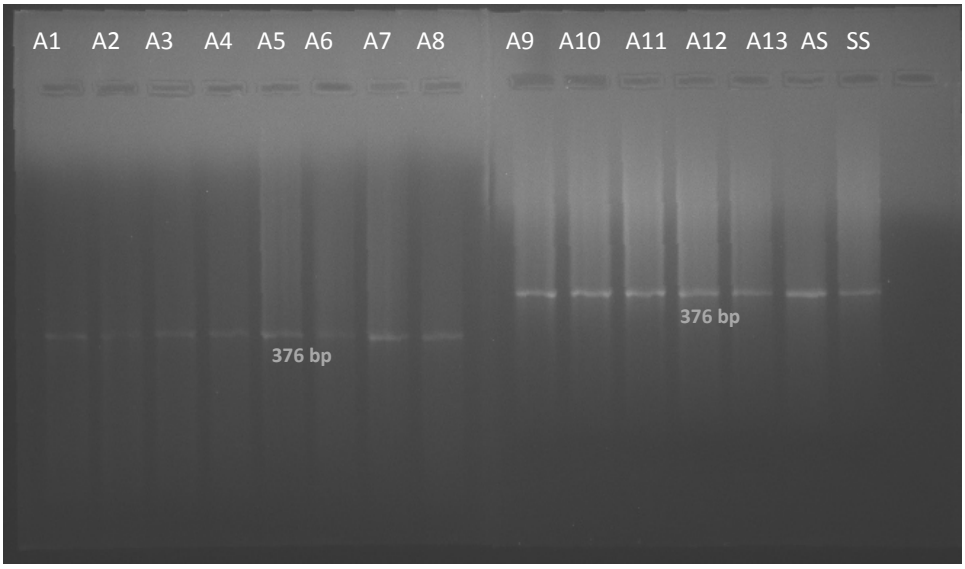


Figure 1: PCR amplified fragment of beta globin gene (376 bp) for samples A1 - A13; AS - control heterozygous sickle trait sample; SS - control sickle cell diseased sample.

The restriction enzyme digestion of the PCR amplified beta globin produced specific RFLP banding pattern to detect the presence of SCD/ carrier status in the Cholanaickan tribal community (Figures 2 - 5). Dde1 restriction enzyme has a recognition site (CTNAG) at codon 6 in the normal beta-globin gene. Point mutation (A is substituted by T) takes place either in one of the beta globin alleles or in both the alleles in the case of sickle cell trait or SCD

Figure 2. Dde 1 based RFLP of beta globin gene (A1 to A12 - normal; A13 - heterozygous carrier; AS – heterozygous carrier (control sample); SS – homozygous sickle diseased (control sample)

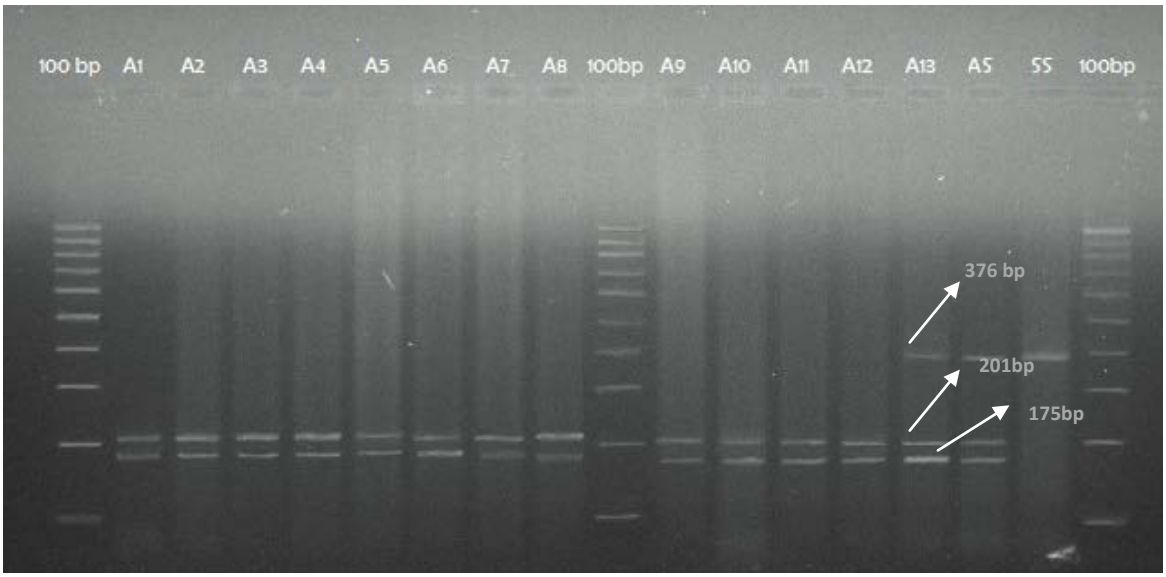


Figure 3. Dde 1 based RFLP of beta globin gene (B1 to B6 - normal; C1 to C5 - normal; heterozygous carrier (control sample); SS – homozygous sickle diseased (control sample)

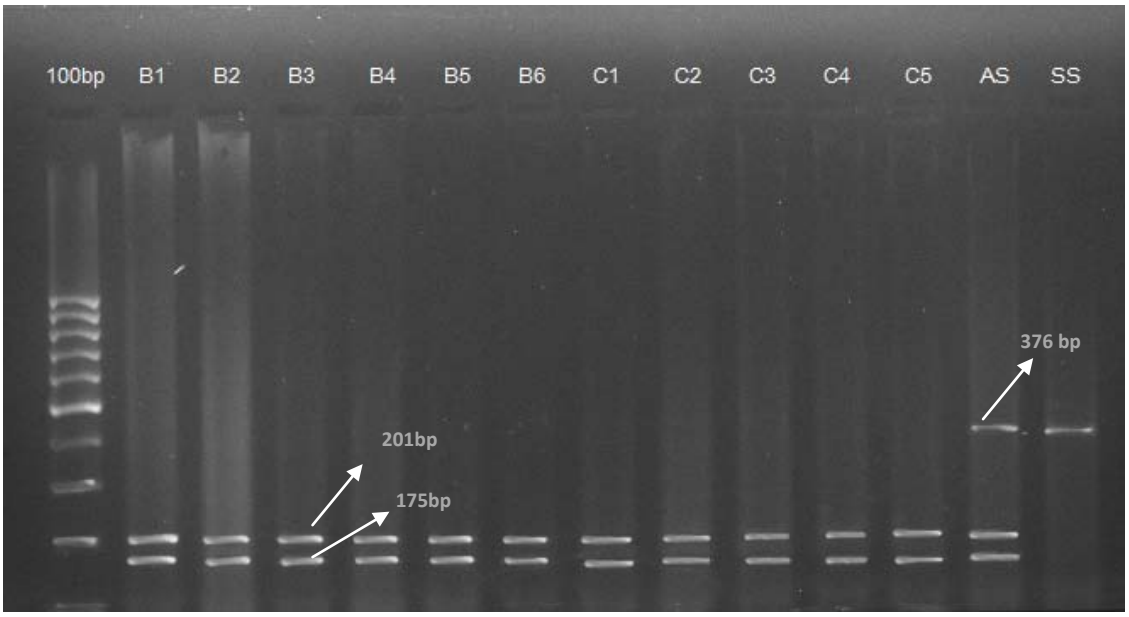


Figure 4. Dde 1 based RFLP of beta globin gene (C6 to C8 - normal; D1 to D2 - normal; SS – homozygous sickle diseased (control sample)

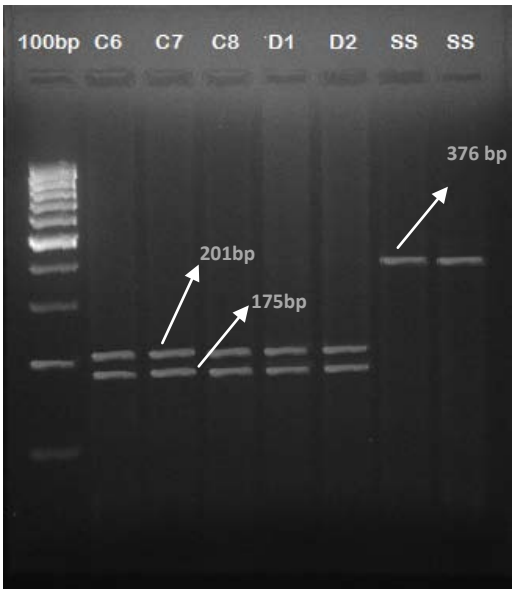
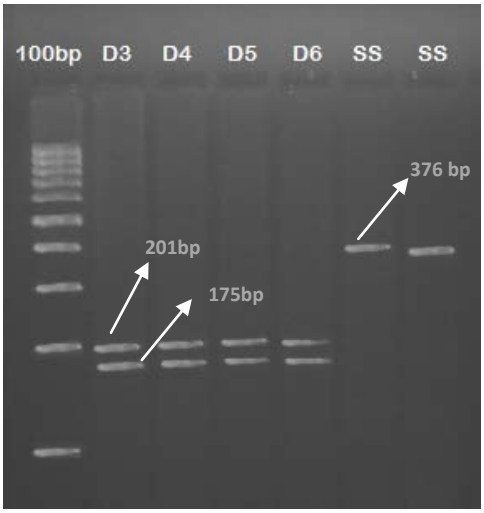


Figure 5. Dde 1 based RFLP of beta globin gene (D3 to D6 - normal; SS - control (diseased)



respectively. The normal amplified beta globin DNA fragment will be restricted into two fragments of 201 bp and 175 bp which had been visualized by agarose gel electrophoresis. If there is a sickle cell point mutation in either one of the beta globin alleles at the 6^{th} codon position (heterozygous sickle cell carrier state), the normal beta globin allele will be restricted into fragments of 201 bp and 175 bp whereas the mutated allele (376 bp) cannot be restricted by Dde1 enzyme due to the nucleotide change (A > T) in its restriction site. Thus, there will be three fragments in the sickle cell carrier state, 376 bp, 201 bp and 175 bp. The sickle cell mutation in the homozygous state, both the beta globin alleles will be mutated at the 6^{th} codon position and the restriction enzyme (Dde1) fails to recognize the restriction site. Thus, there will be only one band at 376 bp (Hatcher et al., 1992).

Out of the analyzed 33 samples, one sample (A13) had bands at three positions 376 bp, 201 bp and 175 bp, indicating the heterozygous nature of the sickle cell gene (Fig. 2). The rest of the 32 samples were having normal beta globin gene and displayed two restricted bands at 201 bp and 175 bp. None of the samples had SCD with two mutated sickle cell alleles with the presence of an undigested single band at 376 bp. In the case of sickle cell disease, mutation at the Dde1 specific restriction site eliminates the Dde1 restriction site (Karkun and Bhagat, 2011). The results illustrate the usefulness of RFLPs for the precise identification of the sickle cell trait with only one beta globin mutated allele. Since the sickle cell carrier person detected in the community is of 70 years of age and unmarried, there is least chance for the transfer of this mutant beta globin allele to the next generation. Hence there is absolutely no need for the genetic counseling programs within the community as envisaged in the second objective.

Mutant sickle cell hemoglobin has valine substituted for the glutamic acid at the sixth aminoacid position of the beta globin chain. Two hypotheses exist with respect to the origin of beta globin mutation. This novel mutation is claimed to occur as a result of migration and acclimatization from Arabian Peninsula to India, Eastern Saudi Arabia and Equitorial Africa. The second theory was the multiple mutation hypotheses due to several independent mutational events which resulted in the evolution of several divergent haplotypes (Abdelrahim and Bekhieta, 2006). Independent origin of sickle haemoglobin

mutation in Senegal and Western Africa has also been suggested (Mears et al., 1981). Five distinct haplotypes of HbS gene have been described according to the occurrence in specific geographical locations. Benin, Senegal, Bantu and Cameroon are the four haplotypes mainly found in Africa while the 5th one is the Saudi Arabia/ Indian haplotype. Less clinical severity is often associated with the 5th haplotypes because of the higher levels of HbF in their blood (Sarnaik, 2005; Ghosh and Colah, 2011).

Under low oxygen tension, the sickle haemoglobin polymerizes and becomes poorly soluble and the red cell with polymerized sickle haemoglobin become distorted and rigid (Beutler, 2002). Sickling of red cells containing mutant haemoglobin microscopically under a coverslip is the rapid screening method for sickle cell anemia. The diagnosis of sickle cell anemia using haemolysates prepared from the peripheral blood followed by electrophoresis on cellulose acetate is often practiced for the screening of sickle cell disease. However, electrophoretic mobility pattern of several other variant forms of haemoglobin is identical to that of sickle cell haemogloin which is a serious setback of the approach (Winfred and John, 1999). High performance liquid chromatography and isoelectric focusing have also been recommended as a diagnostic method for sickle cell disease (Mario et al., 1997).

Advances in molecular techniques have allowed elucidation of the molecular basis of most of the genetic diseases that affect people. Polymerase chain reaction coupled with restriction enzyme digestion has been recommended as the most accurate and rapid method for sickle cell diagnosis (Saiki et al., 1985). One of the advantages of this method is that it does not require any radioisotope or external probe like the routine RFLP analysis. Previously, many researchers have reported the effectiveness of restriction analysis for the diagnosis of sickle cell anemia. Prenatal diagnosis of sickle cell haemoglobinopathy has been carried out by restriction endonuclease analysis (Driscoll et al., 1987; Bankar et al., 1993). Antenatal diagnosis of sickle cell anemia has also been reported (Chang and Kan, 1981; Ramsay et al., 1984). Detection of sickle cell gene by the analysis of amplified DNA sequences was carried out in China (Huang et al., 1989) and Venezuela by using the endonuclease MS II (Martinez et al., 1998). Driscoll et al. (1987) reported an evaluation of 55 pregnancies at risk for a sickle hemoglobinopathy using prenatal diagnosis by restriction

endonuclease analysis, with MstII and HpaI. The efficiency of MS II restriction analysis of the beta globin gene for the detection of SCD and heterozygous carriers were also demonstrated (Ayatollahi and Haghshenas, 2004). Podhorodecka et al. (2011) analysed the frequency of HbS mutation using Eco811 restriction analysis in Shagia and Manazir Arab tribes inhabiting in Sudan.

SCD is a burning health problem among different tribal groups in India, which varies from 5 to 34 per cent (Kaur et al., 2013) with an average frequency of the sickle gene 4.3 per cent (range 0 - 44 %) (Mohanty and Mukherjee, 2002). Consequent to the migration and admixture of tribals and non-tribals, sickle cell disease has also encroached into the non-tribal communities living in close proximity with tribal populaions (Feroze and Aravindan, 2001; Kaur et al., 2013). In the heterozygous state, this mutation confers resistance against the malarial parasite *Plasmodium falciparum* without otherwise affecting the fitness of the individual (Allison, 1954). Due to the adaptive advantage of the heterozygote, the disease is still prevalent, especially among people with recent ancestry in malaria stricken areas, such as; Africa, the Mediterranean, India and the Middle East (Kwiatkowski, 2005). In addition to the tribal communities, sickle cell trait is reported to be present among scheduled castes and other communities, who are living in close proximity with tribal populations. Therefore, the trait has been transmitted among these groups due to admixture with tribal groups (Kaur et al., 2013).

Lehmann and Cutbush (1952) first reported the geographical distribution of sickle cell haemoglobin disorder in India. Estimates indicate that the sickle cell trait or the carrier state occurs in 10 - 30 per cent of many, predominantly tribal populations throughout central India (Balgir and Sharma, 1988). In India, maximum numbers of homozygous sicklers are found in the states of Madhya Pradesh and Chhattisgarh followed by Gujarat, Maharashtra and Andhra Pradesh. The sickling allele is, therefore, spread over the Central Belt from Gujarat through Madhya Pradesh to Orissa in East and over the Western tract from Gujarat through Maharashtra to South of Kerala. Gene flow for HbS showed a decline from South to North (Bhasin, 2006). According to National Family Health survey (NFHS-3),

socio-economic status of the community has an influence on the incidence of sickle cell anemia in India.

In Kerala, a high frequency of sickle cell gene has been reported among the tribal communities of Wayanad and non-tribal community, Wyanadan Chettis (Feroze and Aravindan, 2001; Kaur et al., 1997; ASWHINI, 2010). A population based study of sickle gene frequencies and disease characteristics were carried out in the scheduled tribes, Paniyas, Kurumbas, Adiyas, Kattunaickans, Ooralis and the non-tribal community of Wayanadan Chettis (Feroze and Aravindan, 2001). The sickle cell gene frequency in Attappady region has been reported in different communities *viz.* Irula – 0.133, Kurumba – 0.108, Muduga – 0.133 (Feroze and Aravindan, 2004). The gene frequency of haemoglobin S ranged from 0.019 in Kattunaickan to 0.196 in Wayanadan Chettis. The survival of patients with sickle cell anemia seems to be higher in Kerala due to wide and efficient health care delivery system in Kerala. So far, sickle cell disease has not been reported from the particularly vulnerable Cholanaickan community. Molecular diagnosis for sickle cell carrier trait in the present study could detect one carrier individual in the community. Cholanaickans are supposed to be originated from Kattunaickans (Mathur, 1977b), where the SCD was earlier reported with a frequency of HbS as 0.016 (Feroze and Aravindan, 2004). Of late, inter-marriages are quite common among Kattunaickans and Cholanaickans, which may be a causative for more incidences of the SCD within this dwindling population in near future. The limited number of individuals in the community and consequent high occurrence of consanguineous marriages may lead to a serious situation which demands awareness creation and regular health monitoring.

Primary health care system (PHC) can play a major role at the community level so as to create public awareness of the problem, increase the life span and survival of the affected individuals. The facility and technical know-how for the diagnosis of the disorder can be made available at the PHC so as to monitor the disease progression within the community. Early diagnosis before the appearance of the symptoms can enhance the life expectancy of the individuals considerably through a comprehensive medical care and management approach. The most important aspect of comprehensive care for managing

SCD would be awareness creation on the disease, intervention before the appearance of symptoms through administration of hydroxyurea, antibiotics, nutrition and folic acid supplementation. To improve the general health of the patients, cost effective procedures such as, the use of penicillin to prevent further infections is advocated. Over the last decade, significant progress has been made in the early diagnosis and management of the sickle disease through prolonged treatment with hydroxy urea which eventually reduce the occurrence of painful crisis and life threatening complications (Balgir, 1999; Steinberg et al., 2009).

Basic facilities to manage the SCD are inadequate in most of the developing countries where it is a major health problem. Eventhough, simple screening protocols are available, often by the time a patient is diagnosed for the sickle disease, the disease must have progressed beyond the ability to contain it. The birth of affected babies can also be reduced to a greater extent through prenatal screening. But because of the uncertainties associated with the prognosis of these techniques, they are always least preferred for the management of SCD. Genetic counseling coupled with neonatal diagnosis with reliable blood tests can reduce the number of sickle disease affected infants to a larger extent. Neonatal diagnosis followed by the administration of prophylactic antibiotics or vaccines against SCD proved to reduce the number of deaths. Availability and accessibility to health care services can significantly enhance the life span of the affected individuals and improve the quality of lives of sickle cell patients in developing countries. General improvement in health care services in the developing countries through national control programmes can further strengthen the existing facilities and can improve the quality of life of sickle cell patients in developing countries as well. In view of the scale of the public health problem, a comprehensive approach to prevention and management of sickle cell anaemia is essential (Weatherall, 2010).

4. SUMMARY AND CONCLUSIONS

Cholanaickans are numerically small (124 as per 2011 Census), semi-nomadic hunter-gatherer tribal community inhabiting in the deep forests of Nilambur valley, Malappuram Disrict of Kerala State. Over the past few decades, the demographic size of Cholanaickans has been decreased from 282 in 1977 to 124 in 2011 (Census of India, 2011). In India, the incidences of sickle cell anemia have been reported more among the tribal communites (73 %) when compared to scheduled castes or other classes. Consanguineous marriages occurring frequently among this demographically small and diminishing tribal community may further augment the risk factor associated with genetic disorders. Till date, no published information is available regarding the genetic status of this community with respect to the sickle cell haemoglobin disorder. SCD had been reported previously among the Kattunaickan tribal community (Feroze and Aravindan, 2004), of whom the Cholanaickans are reported to have originated (Mathur, 1977b). The present project envisages identifying the genetic status of SCD through restriction enzyme analysis of PCR amplified beta globin gene.

The study could demonstrate the usefulness of molecular diagnosis through PCR-RFLP for the identification sickle cell haemoglobin genetic disorder among the most vulnerable Cholanaickan tribal community. Blood samples were collected from 33 individuals of the community as part of the All India Sickle Cell Anemia Screening Program by the medical team of Kozhikode Medical College. Sickle cell solubility test was carried out in all the analyzed individuals. DNA was isolated from dried blood spot on filter paper disc purified by FTA agent (GE Healthcare, UK). Direct PCR was performed using beta globin specific primers to amplify 376 bp beta globin gene fragment. The PCR amplified beta globin fragment was subjected to Dde1 restriction enzyme digestion since the presence of sickle cell mutation eliminates the Dde1 restriction site.

Among the analyzed 33 samples, one sample had bands at three positions 376 bp, 201 bp and 175 bp respectively, indicating the presence of one mutated allele leading to a heterozygous carrier state. The rest of the 32 samples were normal for the beta globin gene

which produced two restricted bands of 201 bp and 175 bp. None of the samples were detected positive for the SCD, caused due to mutations in both the beta globin alleles resulting in an undigested single band at 376 bp. The method could detect one sickle cell carrier trait which is heterozygous for the mutated sickle globin gene (HbS). The sickle cell carrier trait is not generally regarded as a disease state because of the mild complications associated with it. However, it is important to be aware of the presence of carrier gene as they are more prone to complications at high altitudes or during exhaustive labour. Since the sickle cell carrier person detected in the community is of 70 years of age and unmarried, there is least chance for the transfer of this mutant beta globin allele to the next generation. Now, intermarriages among the Kattunaickans and Cholanaickans are quite a common practice, which could be a causative for more incidences of SCD within this most vulnerable community in near future.

Unlike in the past, health care facilities are reaching the far fledged settlements of Cholanaickans nowadays. Medical camps have been conducted on a monthly basis in their settlements mediated by medical practitioners of the PHCs and their health issues are regularly being taken care of. In view of the probable risk factor for the occurrence of SCD in immediate future within the community, preventive strategies based on creating awareness about the SCD, genetic literacy and access to primary health care facilities are critical. Further strengthening of the existing health care system through additional facilities for proper screening, diagnosis and management of SCD can contribute to enhanced lifespan, survival and overall welfare of this particularly vulnerable tribal community.

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Appendix 1. Details of samples collected for sickle cell anaemia

Serial No.	Name	Settlement	Sample code
1	Pannapuzha Veeran	Kuttankalllu	A-1
2	Karimpuzha Vijayan	Makivari Allai	A-2
3	Kuppamala Kaallan	Varachil Mala	A-3
4	Ashna allai Joy (shanthan)	Ashna allai	A-4
5	Ashna allai Chathan	Ashna allai	A-5
6	Poochapara Kariyan	Poochapara	A-6
7	Poochapara Krishnan	Poochapara	A-7
8	Vinayan (Karimpuzha Vijayan)	Makivari allai	A-8
9	Mani Poochapara	Poocha para	A-9
10	Manesh (Son of Paannapuzha Revi)	Paannapuzha	A-10
11	Ravindran (Son of Paannapuzha Revi)	Paannapuzha	A-11
12	Poocha para Joy	Poochapara	A-12
*13	Karimpuzha Mathan	Paanapuzha	A-13
14	Mathi	Ashna allai	B1
15	Vella	Ashna allai	B2
16	Beera	Ashna allai	B3
17	Mathi	Ashna allai	B4
18	Eninja	Ashna allai	B5
19	Karikka	Ashna allai	B6
20	Thali Chathan	PunchaKolli	C-1
21	Swapana	Puchakolli	C-2
22	Mari (Wife of Poochapara Kariyan)	Poochapaara	C-3
23	Ayyappan (Son of Poochapara Kariyan)	Poochapaara	C-4
24	Vinod C (Son of Mannallai Chellan)	Maancheri	C-5
25	Vinu (Son of Mannallai Chellan)	Maancheri	C-6
26	Kuppamalai Haridas (Son of Thadi Mathan)	Naagamala	C-7
27	Chathi (Wife of Thadi Mathan)	Naagamala	C-8
28	Poochapara Madavan	Poochapara	D-1
29	Poochapara Cheriya Kannan	Poochapara	D-2
30	Kuppamala Chathan	Valkettu mala	D-3
31	Paannapuzha Ravi	Pannapuzha	D-4
32	Sindu (Wife of Paanna Puzha Revi)	Paannapuzha	D-5
33	Nandu (son of Paanna puzha Revi)	Paannapuzha	D-6

*person with sickle cell carrier trait